Red Cell Disorders

Mature erythrocyte indices: new markers of iron availability

This study was aimed at evaluating mature erythrocyte indices as new markers of iron status. Contrarily to those in the whole red blood cell (RBC) population, mature erythrocyte parameters are valid markers of iron status that remain independent of erythropoietic activity. When reticulocytosis is low, these parameters are similar to whole RBC parameters.

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The major cause of resistance to recombinant human erythropoietin (rHuEPO) is iron deficiency. The percentage of hypochromic red blood cells (%HYPO) has been demonstrated to be the most sensitive and specific parameter of functional iron deficiency.[1] However, %HYPO is also dependent on erythropoietic activity.[2,3] An ideal marker of functional iron deficiency should be independent of erythropoietic activity, although the influence of this could be minimized by measuring mature erythrocyte parameters, excluding the confounding effect of reticulocytes. The aim of this study was to evaluate mature erythrocyte parameters as markers of iron deficiency independently of erythropoietic activity.

Normal values were determined in 57 members of the nursing staff of the University Hospital of Liège. The influence of erythropoietic activity was studied in 14 patients with autoimmune hemolytic anemia at the peak of reticulocytosis. Mature erythrocyte parameters were validated in 20 patients with iron deficient anemia (IDA), 21 with genetic hemochromatosis, 6 with megaloblastic anemia due to Biermer's disease and 11 with heterozygous β-thalassemia.

Red cell parameters were measured with an Advia 120 cell counter (Bayer Diagnostics, Tarrytown, NY, USA). Parameters are expressed as mean ± standard deviation. The normal range for all parameters has been determined as the 95 central percentiles of the distribution (Table 1). Comparisons between groups were performed using Student's t tests. Correlations were calculated with Spearman's or Pearson's coefficient of correlation, as appropriate.

The mean values and reference ranges established for the mature erythrocyte population were very similar to those of the whole red blood cell (RBC) population since the influence of reticulocytes was negligible (1.0±0.3% reticulocytes) (Table 1). Compared to the mature erythrocyte population, reticulocytes had a 20.1±2.9% greater mean cell volume (MCV), an 8.7±2.3% greater hemoglobin (Hb) content but a 9.6±1.5% lower Hb concentration. Similar results have already been published.[4] Patients with AIHA were characterized by high reticulocytosis (11.6 %) (Table 1). Parameters of the whole RBC population showed macrocytic anemia and high %HYPO (8.1%). Paradoxically, mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were high and 60% of the cells had a high Hb content. In the reticulocyte population, 41.6% of the cells were hypochromic and MCHCr was decreased. However, reticulocytes were macrocytic and MCHr was high. Only 3.7% of the reticulocytes had a low Hb content. In the mature erythrocyte population, MCVm was increased to a lower extent than

Table 1. Comparisons between normal subjects and AIHA patients for the whole RBC population, reticulocytes and mature erythrocytes.

	Whole population		*Reticulocytes*		*Mature erythrocytes*	
	Normal (n=57)	*AIHA (n=14)*	*Normal (n=57)*	*AIHA (n=14)*	*Normal (n=57)*	*AIHA (n=14)*
MCV (fL)	87.0±4.3	99.3±7.2°	106.6±3.3	125.6±12.6°	88.8±4.1	95.5±5.9°
MCH (pg)	29.1±1.6	33.8±3.2°	32.5±0.3	36.4±2.8°	29.9±1.5	33.5±3.3°
MCHC (pg/mL)	33.5±0.9	34.7±2.5*	30.6±0.9	29.2±2.1°	33.8±0.8	35.4±2.6°
%MICRO (%)	0.7±0.5	0.9±0.8	0.1±0.3	0.1±0.2	0.4±0.3	1.0±0.8°
%MACRO (%)	0.7±1.4	13.1±9.6°	6.6±6.4	53.8±27.2°	0.6±1.3	7.4±6.4°
%HYPO (%)	0.9±0.9	8.1±5.5°	15.7±9.0	41.6±22.8°	1.1±0.9	3.4±2.4
%HYPER (%)	0.8±0.7	11.1±11.1°	0.1±0.2	0.4±0.4°	1.3±1.1	12.2±12.3°
%low CH (%)	23.9±10.6	11.1±10.1°	7.6±4.9	3.7±2.8†	24.1±10.6	11.9±10.7°
%high CH (%)	29.6±12.5	60.1±24.1°	55.9±13.3	78.4±16.0°	29.3±12.5	58.0±25.0°
Ferritin (ng/mL)	89.7±73.6	644.6±438.2°	–	–	–	–
SI (μmol/L)	18.9±4.3	21.8±10.5	–	–	–	–
RBC (10^9/μL)	5.03±0.43	2.53±0.62°	–	–	–	–
Hb (g/dL)	14.4±1.0	8.6±2.4°	–	–	–	–
Retic Hb (mg)	16.8±4.4	102.6±43.2°	–	–	–	–
Hct (%)	43.6±2.9	25.4±6.00°	–	–	–	–
Retic (106/μL)	51.7±13.4	280.4±111.0°	–	–	–	–
Retic (%)	1.0±0.3	11.6±4.6°	–	–	–	–
Retic prod index	1.0±0.3	3.4±1.5°	–	–	–	–

**p < 0.01; °p < 0.001. Reticulocyte production index (Retic prod index) = [%retic * (Hct patient / Hct reference)] / maturation index. Maturation index is considered as constant and = 2.*

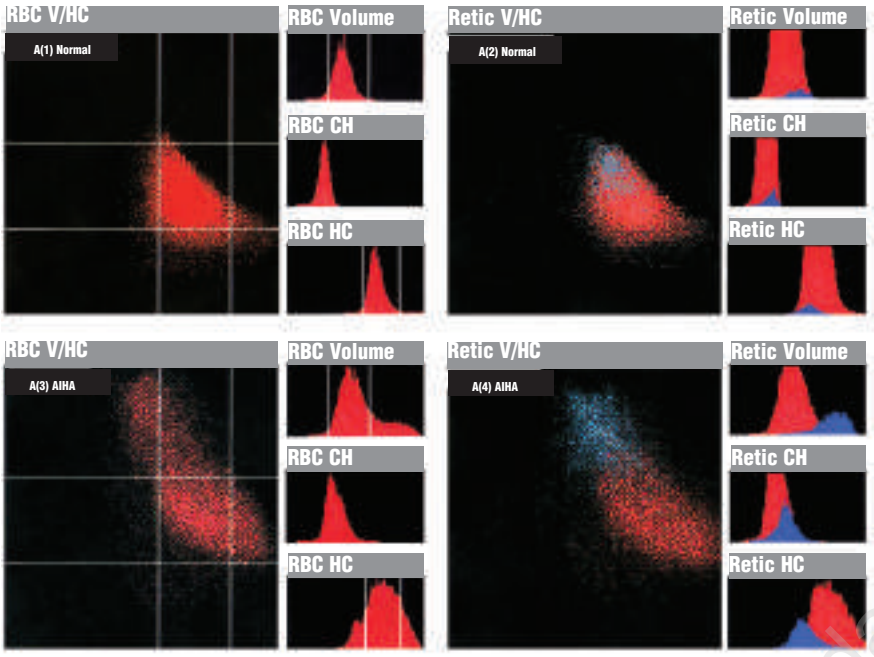
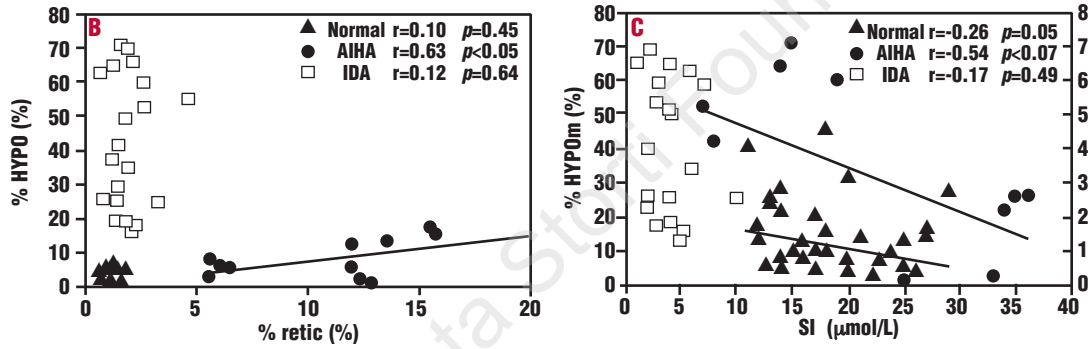


Figure 1. A. Comparison of a healthy subject (Normal) and a representative patient with AIHA. The whole RBC population is shown in the left panel. An upper left extension of the V/MCH graph is observed in AIHA. In the right panel, the indentification of reticulocytes (in blue) demonstrates that the hypochromic cells in AIHA are reticulocytes. B. Correlation between %HYPO and %reticulocytes. There is a significant positive correlation in the case of stress reticulocytosis (AIHA). C. Correlation between %HYPOm and serum iron (SI). %HYPOm correlated negatively with serum iron in AIHA and the normal population. This correlation was not significant in IDA probably because of the small range of SI values in this disorder.



in the whole or reticulocyte populations, MCHCm was more increased and %HYPOm was only slightly elevated (3.4%). Figure 1A shows mature RBC and reticulocyte parameters in a representative normal subject and an AIHA patient, respectively. In the AIHA patient, the whole cell population V/MCH graph shows an upper-left extension of cell distribution, these abnormal cells thus being macrocytic and hypochromic. The reticulocyte V/MCH graph shows that these hypochromic cells are reticulocytes. The histograms demonstrate that, compared to the reticulocytes from the normal subject, the AIHA reticulocytes, considered as hypochromic, have an increased Hb content and an increased volume.

In all other pathological conditions, mature erythrocyte parameters were similar to those in the whole erythroid population because of the small proportion of reticulocytes.

We also examined the relationship between %HYPO on the one hand and reticulocytes or iron availability on the other hand, both in the normal population and in IDA and AIHA. In AIHA, %HYPO was positively correlated with the reticulocyte count (Figure 1B). Moreover, while %HYPO was high in patients with low serum iron, it did not correlate with this parameter in any pathology. Conversely, %HYPOm was not significantly correlated with the reticulocyte count, even in AIHA. However, %HYPOm was significantly correlated with serum iron in the normal population and in AIHA, but not in IDA (Figure 1C). In conclusion, this is the first study investigating the possible role of mature erythrocyte parameters for monitoring iron status. Reticulocytes had a larger volume and a lower Hb concentration than did mature erythrocytes. These differences are responsible for bias in parameter determination in the whole RBC population in case of stress reticulocytosis. Mature erythrocyte measurements are capable of excluding such bias. Moreover, %HYPO was directly influenced by the reticulocyte count but not by serum iron whereas %HYPOm was independent of erythropoietic activity and correlated well with functional iron deficiency. This makes %HYPOm a good candidate for iron monitoring in hemodialysis patients treated with rHuEPO in whom both functional iron deficiency and increased erythropoietic activity are encountered, and even more so at initiation of treatment than in the maintenance phase.

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Red Cell Disorders

A new polyadenylation site mutation associated with a mild β-thalassemia phenotype

At least 180,000 autochthonous and allochthonous people are carriers of a large spectrum of hemoglobinopathies in The Netherlands.[1] We describe two cases, the first, a 33-year old Surinamese Creole woman, studied because of an intermediate hemolytic anemia; the second, a couple requesting analysis because of a previously diagnosed carrier state in the male partner, while the carrier state in the pregnant female was uncertain.

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Hematologic parameters were measured on fresh EDTA blood on a semiautomatic counter and lysates were examined on alkaline starch gel electrophoresis. The HbA2 and HbF levels were estimated by automatic high performance liquid chromatographic (HPLC) analysis (Variant I and Variant II, Bio-Rad Laboratories, Hercules, CA, USA). Biochemical and molecular analyses were done according to previously described procedures.[2-4] Samples were analyzed on an ABI Prism™ 377 sequencing apparatus (Applied Biosystems, Perkin Elmer Corporation, Foster City, Ca, USA). The haplotype of the β-genes was determined using the following 7 polymorphic sites and restriction enzymes: 1; 5'-ε (Hinc II), 2; Gγ (Hind III), 3; Aγ (Hind III), 4; Ψβ (Hinc II), 5; 3'- Ψβ (Hinc II), 6; 5'-β, (Hinf I), 7; 3'-β (Hinf I) according to Varawalla *et al.*[5] and Sutton *et al.*[6]

Family #1. A 33-year old Surinamese woman (II-3), her mother (I-2) and two older sisters were examined because of a chronic hypochromic microcytic hemolytic anemia. The proband (II-3) presented with the parameters of an intermediate β-thalassemia. Her mother was microcytic but not anemic with a strongly elevated HbF level (58%).

Table 1. Hematologic, biochemical and molecular data in family 1.

Parameters	*I-1*	*I-2*	*II-1*	*II-2*	*II-3**
Age/gender	?/M	69/F	50/F	48/F	33/F
Hb (g/dL)	n.d.	13.5	13.5	12.6	10.8
PCV (l/L)	n.d.	0.40	0.41	0.40	0.34
RBC (10^{12}/L)	n.d.	6.25	5.8	4.9	5.7
MCV (fL)	n.d.	64	71	80	60
MCH (pg)	n.d.	21.6	22.9	25.3	19.0
Hb A_2 (%)	n.d.	4.6	5.7	2.1	8.56
Hb F (%)	n.d.	58	1.2	12	3.6
Osmotic fragility	n.d.	↓↓↓	↓↓↓	↓↓↓	↓↓↓
Haptoglobin (mg/dL)	n.d.	30	265	76	44
Ferritin (μg/L)	n.d.	Normal	Normal	Normal	Normal
Inclusion bodies	n.d.	n.d.	n.d.	n.d.	n.d.
Erythr. morphology	n.d.	++	+	+	+++
ZPP (μmoL/mol heme)	n.d.	n.d.	n.d.	n.d.	n.d.
β/α synth. ratio	n.d.	n.d.	0.7	0.6	0.22
Gγ (%)	n.d.	54	n.d.	54	n.d.
Aγ (%)	n.d.	46	n.d.	46	n.d.
β defect 1	→AATATA	δβdel	-88 (C→T)	δβdel	→AATATA
β defect 2		-88 (C→T)			-88 (C→T)
β-cluster haplotype					
Fragment					
1	–/–#	+/–	+/–	–/–	+/–
2	–/–	–/–	–/–	–/–	–/–
3	–/–	–/–	–/–	–/–	–/–
4	–/–	–/–	–/–	–/–	–/–
5	+/+	+/+	+/+	+/+	+/+
6	+/+	+/+	+/+	+/+	+/+
7	+/–	+/+	+/+	+/+	+/–

**proband; #: deduced; n.d.: not done; +,++,+++: degree of abnormality.*

The proband's two older sisters had the phenotypes of heterozygous β-thalassemia (II-1) and of a mild δβ-thalassemia trait with increased HbF level (II-2). The father (I-1) was not available for examination. Direct sequencing revealed the –88 (C→T) β^+ thalassemia mutation[7] and the new poly A mutation AATAAA→AATATA. These data are summarized in Table 1.

Family #2. A Surinamese couple was referred for risk assessment after the male partner had been diagnosed as a thalassemia carrier. The carrier state of the male was easily established and confirmed at the molecular level as a –29 (A→G) mutation in the presence of α^+ thalassemia heterozygosity. His pregnant partner presented non-thalassemic indices and a normal or a slightly elevated HbA_2 level depending on the HPLC system used for measurements. After sequencing the woman was found to be a carrier of the same poly A mutation previously found in family #1. The DNA of the fetus showed no thalassemia mutations. Haplotype analysis revealed that the poly A mutation was associated with the same haplotype (– – – – + + –) in both families (Tables 1 and 2). These data are summarized in Table 2.

The proband (II-3) in family #1 had an intermediate phenotype caused by the β^+ -88 C→T and the new polyadenylation site mutation, which destabilizes an otherwise normal mRNA product. The proband's mother (I-2) showed a false homozygous pattern for the -88 mutation, and a very high HbF expression (58%), without anemia or hemolysis. A coexisting $G\gamma A\gamma(\delta\beta)^0$ deletion, called HPFH-2, was confirmed (Table 1).

The routine analysis of the couple (family #2) confirmed a typical β thalassemia minor phenotype in the man and a