

## MODIFICATIONS OF ERYTHROPOIESIS IN MYELODYSPLASTIC SYNDROMES TREATED WITH RECOMBINANT ERYTHROPOIETIN AS EVALUATED BY SOLUBLE TRANSFERRIN RECEPTOR, HIGH FLUORESCENCE RETICULOCYTES AND HYPOCHROMIC ERYTHROCYTES

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### ABSTRACT

**Background.** The aim of this study was to evaluate the erythropoietic modifications induced *in vivo* by recombinant erythropoietin (r-EPO) in myelodysplastic syndromes (MDS), by means of some new, non invasive laboratory parameters.

**Patients and Methods.** The serum levels of soluble transferrin receptor (STR), a marker of total marrow erythroid activity, in combination with the automatized detection of the high fluorescence reticulocytes (HFR) and the hypochromic erythrocytes (HE), respectively as indexes of effective erythropoiesis and functional iron deficiency, were longitudinally measured in 25 patients affected by MDS treated with r-EPO and correlated to conventional clinical and laboratory features.

**Results.** A stimulation of erythropoiesis was documented in 8 patients, whose serum levels of STR showed a significant, early (within 16 days) increase during the treatment with r-EPO. However, only 3 of these patients evidenced a concomitant increase of HFR and these were the only subjects who experienced a significant clinical response. Two of these patients also developed a functional iron deficiency while on treatment, as documented by an increase of HE, despite normal serum iron, transferrin saturation and even very high levels of ferritin. They needed iron supplementation to maintain the response to r-EPO. No modification of STR, HFR and HE occurred in the remaining unresponsive 17 patients during at least two months of treatment. Serum levels of thymidine kinase, an aspecific marker of cellular proliferative activity, paralleled those of STR. No correlation was found between STR, HFR or HE and serum levels of endogenous EPO, hemoglobin or transfusion needs in MDS patients.

**Conclusions.** These findings suggest that an heterogeneous and complex pattern of erythroid response exists in MDS treated with r-EPO. In addition, our results indicate that STR, HFR and HE may provide some useful informations for the clinical management of these patients.

*Key words: myelodysplastic syndromes, erythropoietin, soluble transferrin receptor, high fluorescence eiculocytes, hypochromic erythrocytes, thymidine kinase*

Several clinical studies have indicated that about 20 to 25% of patients affected by myelodysplastic syndromes (MDS) improve anemia and reduce the need of red cell transfusions following treatment with recombinant ery-

thropoietin (r-EPO).<sup>1-23</sup> However, the biological basis of r-EPO effect in MDS is still unclear. In particular, it is unknown if this drug is effective on the clonal, dysplastic hemopoiesis or rather acts on the normal residual erythroid lineage.

Table 1. Clinical and laboratory characteristics of MDS patients analysed before r-EPO treatment. RA: refractory anemia; RAEB: RA with blast excess; RARs: RA with ring sideroblasts.

N. of patients	25 (12 males, 13 females)
Mean age	63.3 years (range 18-78 years)
FAB subtype	11 RA, 6 RAEB, 8 RARs
Mean time from diagnosis	20 months (range 1-51 months)
Hemoglobin	Mean 7.2 g/dL, range 5.9-7.9 g/dL
Karyotype abnormalities	In 10 out of 22 tested patients
Serum erythropoietin	Mean 377 miU/mL, range 28-2420 miU/mL
Serum ferritin	Mean 762 mg/mL, range 150-3300 mg/mL
Serum thymidine kinase	Mean 8.8 U/L, range 3-41 U/L
N. of red cells transfusions	Mean 3.5/month, range 1-8/month
Fetal hemoglobin	< 1% in 24 patients, 5% in one patient

Erythrokinetic, cytogenetic, molecular and cultural studies could well elucidate this issue, but they are quite complex and expensive, and also need repeated bone marrow samples.

In order to overcome these difficulties, we analyzed the modifications of erythropoiesis in 25 MDS patients treated with r-EPO using three recently developed non invasive techniques: the measurement of serum levels of the soluble transferrin receptor (STR) and the automatized evaluation of high fluorescence reticulocytes (HFR) and hypochromic erythrocytes (HE).<sup>24-26</sup>

STR is a truncated form of the entire cell membrane transferrin receptor molecule. The circulating levels of STR are related to the erythroid activity in normal subjects, as well as in different types of anemia.<sup>27,28</sup> Erythrokinetic studies have confirmed also that serum STR assay is a simple and reliable method for quantitating total erythropoiesis *in vivo*.<sup>29</sup> HFR are usually detected by a cytofluorimetric assay and considered a measure of effective erythropoiesis. In fact, HFR are recently released *young* reticulocytes, with a high RNA content.<sup>30,31</sup> HE are the automatically measured proportion of erythrocytes with an hemoglobin concentration below 28 g/dL. HE are a marker of early, functional iron deficiency, also in the absence of significant modifications of other parameters of iron metabolism, including serum iron, transferrin and ferritin.<sup>32,33</sup>

The present study suggests that the combined use of these parameters is valuable for detecting erythropoietic modifications and for improving

the management of MDS patients treated with r-EPO.

#### Patients and Methods

Twenty-five transfusion dependent patients diagnosed as having a MDS, according to the FAB criteria,<sup>34</sup> were included in this study. Main clinical and laboratory features before r-EPO therapy are illustrated in Table 1.

r-EPO (Eprex, Cilag; Globuren, Dompè Biotec; Epoxitin, Jansen) was given subcutaneously, at the initial dose of 450 U/kg/wk, subdivided in three administrations per week. The dose was increased to 900-1050 U/kg/wk (300 U/kg three times a week or 150 U/kg/d), when no response was observed. All patients underwent not less than two months of treatment. The response was defined as the complete interruption of red-cell transfusions, with stable hemoglobin levels above 8 g/dL.

Several hematological parameters were evaluated, including fetal hemoglobin (HbF), serum iron, transferrin, ferritin, erythropoietin (ELISA method), and thymidine kinase (s-TK), as a marker of marrow proliferative activity. Bone marrow examination and cytogenetic analysis (available in 22 patients) were carried out at the beginning and at the end of the study. STR, HFR and HE were monitored every week for two months at least during the r-EPO treatment. Serum levels of STR were measured using a commercially available enzyme immunoassay (Clinigen TM, Amgen Diagnostics), employing

a polyclonal antibody in a sandwich EIA format. HFR were determined with the automatized analyser Sysmex-Toa R-1000, equipped with a laser system which evaluates the amount of RNA in the reticulocytes on a whole blood sample treated with auramin. HE were measured using a Technicon-H2 Automatized System program on samples which were also evaluated for other routine hematological parameters.

Predicted s-EPO and STR values were determined for each hematocrit from the regression equation between  $\log(\text{s-EPO})$  or  $\log(\text{STR})$  and hematocrit in a reference group including 42 normal subjects and 48 anemic patients with adequate erythropoietic and erythropoietic response. Ratios of observed to predicted (O/P) s-EPO and STR values were then calculated.<sup>24</sup>

The Mann-Whitney U test and Pearson's coefficient were used for statistical analysis.

## Results

### Clinical response

Response to r-EPO was observed in 3 of 25 patients (12%). Two patients had sideroblastic and one refractory anemia. Their hemoglobin levels increased, respectively, from 7.8 to 10.3, 6.5 to 12.9, and 7.3 to 11.2 g/dL. One of the responders also exhibited an increased number of platelets (from  $30 \times 10^9/\text{L}$  to  $120 \times 10^9/\text{L}$ ). These responses were achieved at the r-EPO doses of 450 U/kg/wk in one patient and 1050 U/kg/wk in the other. In all responders the diagnosis of MDS had been posed within the 7 months before therapy onset.

### Laboratory parameters

Responders had normal karyotype and inadequate baseline levels of s-EPO (28, 69, and 106 miU/mL, respectively; mean 67.6 miU/mL; mean normal values 18.5 miU/mL, range 5-30), as shown by O/P ratio  $<0.8$ . They also increased their HbF levels up to 5, 9.2 and 11%, respectively. Baseline mean s-EPO value in non-responders was 388.6 miU/mL (range 60-2420 miU/mL). In unresponsive patients, inadequate levels of s-EPO for the degree of their anemia (absolute values below 130 miU/mL,

O/P ratio  $<0.8$ ) were measured in 5/22 cases. No substantial modification of circulating iron, ferritin, or transferrin saturation occurred during the treatment. A significant increase of the peripheral and bone marrow myeloid blasts, leading to interruption of r-EPO, and the reduction from 70% to 15% of marrow sideroblasts were respectively observed in two of the responders. No further significant morphological changes were found in the bone marrow of both responsive and unresponsive patients after two months of r-EPO treatment. Karyotypes remained unchanged throughout the study. The appearance of circulating erythroblasts was observed in two of the responders.

### STR, HFR, HE and s-TK

Table 2 reports STR, HFR and HE values in controls and in MDS patients before the r-EPO treatment. Baseline HFR and HE were not significantly different between responders and non-responders (data not shown). Normal STR absolute levels, although unadequate to the degree of anemia, based on the O/P ratio, were found before treatment in all responsive patients (1620, 1920 and 2810  $\mu\text{g}/\text{L}$ , respectively) and in 17 non-responders (range: 1800-2800  $\mu\text{g}/\text{L}$ ), including 5 patients who increased their STR levels under r-EPO, without a concomitant increase of hemoglobin (see below). The remaining 5 patients who did not benefit by r-EPO had high absolute STR levels (range: 3200-5320  $\mu\text{g}/\text{L}$ ). However, the difference in the

Table 2. Levels of STR, HFR and HE measured in MDS patients before r-EPO treatment and in normal controls.

	MDS (25)		normal controls (42)	
	Mean±SD	Range	Mean±SD	Range
STR $\mu\text{g}/\text{L}$	2910±1870	1620-5320	1600±540	710-3030
HFR %	1.1±0.9	0.2-3.8	0.7±0.8	0.2-2.4
HFR $\mu\text{L}$	480±210	150-1100	440±180	220-1190
HE %	4.9±4.8	1.2-18.7	3.8±2.1	0.9-6.5

Table 3. Modifications of STR, HFR, HE and s-TK observed in MDS patients under r-EPO treatment: relationship to response.

	patients	STR	HFR	HE	s-TK
NO RESPONSE	17	-	-	-	-
NO RESPONSE	5	+	-	-	+
RESPONSE	1	+	+	-	+
RESPONSE	2	+	+	+	+

(+ = increase; - = no change)

mean of STR levels between responders (2110  $\mu\text{g/L}$ ) and non-responders (2350  $\mu\text{g/L}$ ) was not significant. No correlation was found between each of the three parameters (STR, HFR, HE) and s-EPO, hemoglobin, s-TK or transfusion needings (data not shown).

Table 3 schematizes the STR, HFR, HE and s-TK modifications during the treatment with r-EPO, according to response. An increase of STR was observed in 8 patients after 10-16 days, with a peak after 35-47 days. The raised STR values were ranging between 60 and 280% in responsive patients and between 55 and 120% in non-responders. HFR increased only in responsive patients, with a maximum level in the range of  $8 \times 10^9/\text{L}$  to  $11 \times 10^9/\text{L}$  (10.5 to 14%) on days 38 to 45. HE reached 22 and 40% in two of responders, after 4-6 weeks. In these latter patients a decrease of hemoglobin levels after an initial response to r-EPO therapy was promptly corrected by the administration of iron per os. This resulted in a rapid recovery of normal values of HE. Representative examples of STR, HFR and HE modifications are illustrated in Figure 1.

An early increase of s-TK (up to 61 U/L, normal values < 5U/L), within the first 10 days of treatment, was observed in all patients who also showed increased STR.

### Discussion

Although the best method for quantitating erythropoiesis is still the ferrokinetic study, other approaches are being available, allowing analysis of erythroid activity in both normal and pathologic conditions.<sup>35</sup> In this respect,

MDS patients, particularly those treated with r-EPO, have a specific interest.<sup>5,36</sup>

The results of the present study indicate that the combined evaluation of some simple and non-invasive parameters, including STR, HFR and HE, is valuable for assessing the effect of r-EPO on erythropoiesis in MDS patients. These parameters, in fact, allow to sub-classify the r-EPO treated MDS patients in different groups.

In the first group, that enclosed patients in which r-EPO had not any clinical effect on erythropoiesis, RST, HFR and HE did not change during therapy. Therefore, in this large subpopulation of MDS patients, r-EPO is not effective both on biological and clinical parameters.

A second group of unresponsive patients was characterized by a significant increase of STR and s-TK, without production of HFR, as expression of a possible stimulating effect of r-EPO on the ineffective erythropoiesis.<sup>25,26,37</sup> This phenomenon, already reported in a single patient with MDS,<sup>5</sup> suggests that in a proportion of MDS subjects erythropoiesis probably maintains the capacity of responding to EPO, although the response is inadequate to improve clinical outcome. Whether these patients can benefit of treatments with r-EPO in association with other cytokines and/or differentiating agents warrants to be investigated. The group of responsive patients presented an increase of STR, HFR and s-TK. In absence of erythroid cells proper cytogenetic and molecular markers, it is not possible to establish whether in these patients r-EPO stimulates residual, normal erythroid progenitors, or the clonal, dysplastic erythropoiesis, or even both. However, in this setting, the induction of HbF producing red cells, a finding also observed in thalassemic patients treated with r-EPO,<sup>38</sup> has to be considered.

As recently observed,<sup>35,36</sup> a consistent proportion of our MDS patients had levels of STR lower than expected on the basis of anemia severity, although normal or even increased in absolute values. This pattern, in combination with inadequate production of s-EPO, low hematocrit and reduced production of reticulocytes, is classified as *intrinsic marrow hypoproliferation*.<sup>35</sup> In agreement with Bowen andco-

workers,<sup>36</sup> we did not find any correlation between STR and s-EPO. These findings further suggest for an alteration of mechanisms regulating erythroid marrow response to EPO in MDS. This contrast with other non clonal conditions, including congenital or acquired hemolytic anemias,<sup>35,39</sup> where a well-defined correlation does exist between STR and s-EPO levels. Thus, in some MDS, pharmacological doses of r-EPO could be effective by overcoming this dysregulation. However, a not negligible number of MDS patients, in particular those who are dependent on red cell transfusions, may also have a blunted

production of s-EPO, as observed in the present and in other studies.<sup>40,41</sup> In these cases, the possible role of cytokines with inhibitory activity on erythropoiesis and, particularly, on erythropoietin production, such as tumor necrosis factor and interleukin-1, is under investigation.<sup>42,43</sup> Furthermore, low STR levels well correlate with a good response to r-EPO in patients with renal failure,<sup>44</sup> who have a *defective s-EPO production*, according to a recent functional classification of anemias.<sup>35</sup> Interestingly, all our responsive patients also had inappropriately low levels of both STR and s-EPO. An ongoing Italian, double blinded prospective study (r-EPO vs placebo) is now also attempting to address the question of whether STR and EPO levels may have a role in predicting response to r-EPO in MDS.

In the present study, s-TK levels increased very early in all patients in which also STR increased. TK is a scavenger enzyme mainly present in dividing cells, which is usually considered an indirect marker of cellular proliferative activity in several hematologic disorders.<sup>45</sup> s-TK may have prognostic relevance in MDS,<sup>46,47</sup> where probably also reflects the cell intramedullary destruction.<sup>47</sup> However, s-TK is not specific for erythroid proliferative activity in MDS.<sup>46,48</sup> Therefore, this marker is of limited value in evaluation of MDS patients treated with r-EPO. Iron deficiency is common in anemic patients treated with r-EPO with normal or reduced iron stores. However, it is often difficult to prove this defect and to decide whether to treat with iron MDS subjects presenting with high levels of ferritin. Our findings provide evidence that the increase of HE in some of MDS responding to r-EPO is a useful and reliable approach for identifying patients who rapidly develop a functional iron deficiency. Likely, this results from a failure to delivery iron from stores and the transferrin pool to the rapidly proliferating erythroblasts.<sup>32,33</sup> These patients require iron supplementation, although in the presence of high ferritin levels resulting from previous transfusions. This also prevents of considering unresponsive to r-EPO those patients with an impaired iron supply to the erythroid marrow.<sup>37,49</sup>

In conclusion, our results demonstrate that the modifications of erythropoiesis in MDS

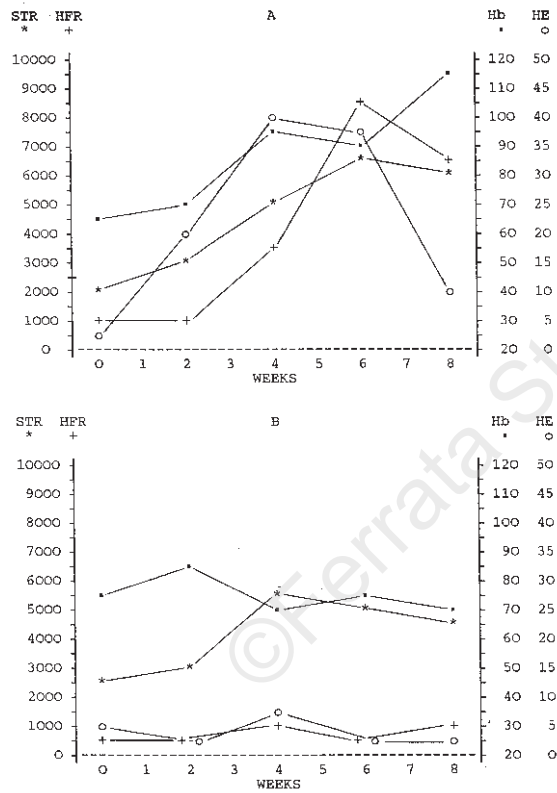


Figure 1. Longitudinal evaluation of STR, HFR and HE in two representative MDS patients treated with r-EPO. Patient A was a responder who stopped his transfusions during the treatment, but needed oral iron supplementation after one month to maintain the response. Patient B did not show any significant modification of hemoglobin and continued to be transfused, despite the relevant increase of STR. STR levels are expressed as  $\mu\text{g/L}$ , HFR as absolute number/ $\mu\text{L}$ , hemoglobin (Hb) as  $\text{g/L}$ , and HE as percent.

patients treated with r-EPO are quite heterogeneous and that the evaluation of STR, HFR and HE contributes to their understanding. Furthermore, STR, HFR and HE also provide useful informations in delivering some clinical decisions for the management of MDS treated with r-EPO, i.e. the suspension of the treatment in absence of an early increase of both STR and HFR, or the identification of patients developing functional iron deficiency by means of HE. This may be of particular relevance in subjects treated with an expensive drug such as r-EPO.

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