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3 **Clinical Significance of TFR2 and EPOR Expression in Bone Marrow Cells in**
4 **Myelodysplastic Syndromes.**
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8 *Running title: TFR2 and EPOR expression in MDS*
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Abstract

Myelodysplastic syndromes (MDS) are heterogeneous hematopoietic disorders characterized by bone marrow failure, cytopenias and a tendency to transform into acute myeloid leukemia. Anemia and transfusional need can be major problems for patients with MDS. Erythropoiesis stimulating agents (ESAs) therapy is a standard treatment for the anemia of most patients with MDS; however not all MDS patients respond to ESAs and a majority of patients eventually relapse after an initial response. This study aimed to identify the clinical impact of TFR2 and EPOR expression in bone marrow cells at diagnosis in MDS patients. We report the expression pattern of TFR2 α and TFR2 β in various subtype of MDS in comparison to that of EPOR. We provide evidence that TFR2 expression could be a potential molecular marker associated with response to erythropoietin (Epo) treatments in MDS patients and that lower TRF2 expression is associated with the poorest survival in MDS.

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3 Myelodysplastic syndromes (MDS) are a group of clonal disorders characterized by
4 ineffective bone marrow hematopoiesis, peripheral blood cytopenias and substantial risk
5 for transformation into acute myeloid leukemia (Tefferi *et al*, 2009).
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8 Although scoring systems are used to predict the prognosis of MDS (Greenberg *et al*,
9 1997; Greenberg *et al*, 2012), they provides information that unfortunately do not always
10 coincide with clinical outcome. Percentage of blasts (>10%) and unfavorable cytogenetic
11 abnormalities are the strongest predictors for poor outcome and are associated with high
12 risk or disease progression to acute leukemia. Those patients, if possible, should undergo
13 to allogenic stem cell transplantation or, if not eligible, to hypomethylating agents
14 treatment.
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19 In lower-risk MDS patients, the main clinical problem is chronic anemia. Anemia responds
20 in 30–50% of the cases to erythropoiesis-stimulating agents (ESAs). Some prognostic
21 factors of erythroid response to ESAs have been well identified, with better response rates
22 in patients with no or limited red blood cell (RBC) transfusion requirement, low baseline
23 serum EPO level and no-aberrant myeloid blast at flow cytometry (Park *et al*, 2008). For all
24 these reason, the clinical care of MDS patients is still challenging, mainly due to MDS
25 phenotypic heterogeneity and lack of well-established markers that effectively monitor
26 MDS natural history. Therefore, in the lower-risk MDS subset, predicting at diagnosis
27 patients with risk of treatment failure are pivotal to personalizing treatments in order to
28 improve the quality of life and to prolong survival.
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36 Transferrin receptor 2 (TFR2), homologous to TFR1, is a protein mutated in
37 hemochromatosis type 3 and contributes to regulate hepcidin in the liver (Ramos *et al*,
38 2011). It is also expressed in erythroid cells (declining as the erythroid progenitors mature)
39 and in myeloid malignant disorders (Kawabata *et al*, 2001). TFR2 associates with
40 erythropoietin receptor (EPOR) (Forejtnikova *et al*, 2010) and is required for efficient
41 erythropoiesis (Nai *et al*, 2015; Nai *et al*, 2014). Unlike TFR1, the TFR2 gene gives rise to
42 two isoforms referred to as TFR2 α (full-length) and a shorter TFR2 β (Kawabata *et al*,
43 1999).
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49 This retrospective study aimed to investigate whether TFR2 isoforms are differentially
50 expressed in patients with MDS, and if so, whether TFR2 is associated with patient's
51 clinical outcomes. Moreover, in the same cohort of patients, we focused on EPOR
52 expression level since it was previously reported that TFR2 could act as an escort protein
53 for this receptor, being required for EPOR efficient cell surface expression (Forejtnikova *et*
54 *al*, 2010). Bone marrow (BM) aspirates were obtained from 6 individuals with non-
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3 malignant hematological disorders (4 males, 2 females, median age of 57 years ranging
4 from 45 to 73) and from 42 treatment-naive patients at the diagnosis of MDS. Diagnosis of
5 MDS was made according to the World Health Organization (WHO) criteria (Vardiman *et*
6 *al*, 2009). The patient group consisted in 28 male and 14 female with a median age of 71
7 years (range 49-85) and a WHO 2008 distribution as follows: RA n= 19, RARS n=2,
8 RCMD n=6, RAEB-1 n=8, RAEB-2 n=7.
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13 After informed consent and ethical approval (EMATO/TFR2-RE, code 92/2015) were
14 obtained, RNA was extracted from total bone marrow cells collected at diagnosis and
15 TFR2 α , TFR2 β and EPOR expression were quantified by quantitative Real-Time PCR, in
16 comparison to that of RNA Universal (Stratagene), normalized for ABL1. Primer
17 sequences were: a) 5'TTTCCACCAGGGCAGACTCT3', b) 5'TCCCGAAGGCTGGTTTG3'
18 for TFR2 α ; c) 5'AGTCCCCACCTCTCCCCGCT3', d) 5'GTGTTGGGGTGAGCCGGATC3'
19 for TFR2 β ; e) 5'GAGCGTACAGAGGGTGGAGA3', f) 5'AGGATGACCACGAGGATGAG3'
20 for EPOR. The relative gene expression was calculated using the equation, $2^{-\Delta\Delta Ct}$.
21 Statistical analyses were performed using GraphPad Prism software. Comparisons
22 between groups were performed by means of Mann–Whitney U-test (nonparametric
23 analysis), and P values <0.05 indicated a significant difference.
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32 In MDS patients TFR2 α and TFR2 β showed higher variability in expression (TFR2 α
33 6.08 \pm 5.58; TFR2 β 3.15 \pm 1.16) than in non-malignant BM cells (TFR2 α 8.23 \pm 2.97; TFR2 β
34 3.51 \pm 0.47). Due to MDS morphological and clinical heterogeneity, we evaluated TFR2 α
35 and TFR2 β expression in the different WHO subgroups (Figure 1A-B). Among the different
36 MDS subtypes, the expression of TFR2 α and TFR2 β was significantly lower in RAEB2
37 (TFR2 α 4.44 \pm 2.11; TFR2 β 2.15 \pm 0.59) when compared with controls (p<0.05 and p<0.005,
38 respectively). A similar expression level was also seen in RARS but the limited number of
39 patients with this condition precludes any statistical analysis. TFR2 α and TFR2 β
40 expression was not correlated with total white blood cells, neutrophils and platelets counts,
41 age at diagnosis and no significant differences were observed between sex (not shown).
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50 We next compared TFR2 expression with that of EPOR. Similarly to TFR2, EPOR
51 expression in bone marrow cells varied more widely in MDS patients (18.00 \pm 2.90) than in
52 non-malignant individuals (17.31 \pm 1.62) and was statistically lower in RAEB2 (8.18 \pm 0.99,
53 p<0.005) (Figure 1C). In addition, conducting a Spearman's correlation analysis on the
54 mRNA expression of both TRF2 isoforms and EPOR, we found that TFR2 α and TFR2 β
55 expression is positively correlated with EPOR expression (Figure 1D-E).
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3 To assess the clinical implication of TFR2 and EPOR expression in bone marrow cells, we
4 categorized MDS patients into four expression sub-groups according to TFR2 and EPOR
5 expression relative to the mean of the non-malignant BM and we analyzed the erythroid
6 response in the cohort of patients that underwent to Epo treatment. We noticed that only
7 patients with at least one of the TFR2 isoforms and EPOR levels comparable to normal
8 controls reached an increase in hemoglobin level of ≥ 1.5 g/dl after 12 weeks of treatment.
9 Instead, all non-responders, with the exception of 2 patients high-expressing TFR2/EPOR,
10 had low levels of TFR2 or EPOR mRNA (Figure 2A).

11 We finally tested the effects of TFR2 α , TFR2 β and EPOR expression on survival in
12 RAEB1-2 and RCMD (Figure 2B-C-D). Comparisons between Kaplan-Meier curves were
13 carried out by log-rank test. In the first year of follow-up, patients with very low/low TFR2 α
14 or TFR2 β expression levels had a significantly worse overall survival (OS) than those with
15 normal/high TFR2 expression ($p < 0.05$ and $p < 0.01$ respectively). No significant difference
16 was noted between patients with low or normal/high EPOR expression, although there was
17 a tendency for poorer survival in the very low/low EPOR expression group.

18 Ever since it was demonstrated that TFR2 is a component of EPOR complex (Forejtnikova
19 *et al*, 2010), there has been interest on understanding its extra-hepatic function. The TFR2
20 erythroid function has been recently described in normal erythropoiesis in mouse models
21 lacking systemic or hematopoietic TFR2 (Nai *et al*, 2015; Nai *et al*, 2014). Considering the
22 natural history of the disease, MDS are suitable models for studying how TFR2 expression
23 change in clonal hematologic disorders and how it is related to EPOR. Moreover, as iron
24 overload can have an adverse effect on hematopoietic precursors, knowing how the
25 expression of an iron-related gene is modulated is important to understand how iron
26 metabolism and hematopoiesis interact. We showed that TFR2 α , TFR2 β and EPOR have
27 a much more variable pattern of expression in MDS compared to normal and that,
28 differently compared to what previously reported (Kawabata *et al*, 2001), their expression
29 are significantly lower in high risk MDS like RAEB2. Therefore, the lack of TFR2 could
30 impair the entire myeloid lineage differentiation, as it does in human erythroid progenitors
31 delaying erythroid terminal differentiation (Forejtnikova *et al*, 2010).

32 The data presented here demonstrate that the reduction of TFR2 isoforms mRNA is
33 associated with poorer survival in patients with high grade MDS. This is in agreement with
34 the work of Nakamaki *et al*. (Nakamaki *et al*, 2004) that found that *de-novo* acute myeloid
35 leukemia patients with high levels of both TFR2 isoforms survived significantly longer.
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3 Furthermore, we established a positive correlation between TFR2 and EPOR mRNA
4 expression implying a possible co-regulation or interplay at transcriptional level of these
5 two interacting proteins. Finally, we reported that TFR2 and EPOR expression could
6 provide information on Epo treatment response since this was achieved only in individuals
7 with both TFR2 and EPOR mRNA levels similar to normal. High level of expression seems
8 to be also deleterious, probably due to an altered receptor signalling.
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11 Limitations of our study include the relatively small number of patients and hence limited
12 amount of cases used for the treatment-response analysis. Nevertheless, the level of bone
13 marrow TFR2 mRNA seems to offer additional information on predicting Epo treatment
14 response. However, before considering TFR2 and EPOR expression levels in clinical
15 decision making, additional validation studies, are needed, also to define optimal cut-off
16 levels.
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19 In conclusion, our data provide evidence suggesting that TFR2 and EPOR expression
20 could be used as potential molecular markers for a better management of MDS patients
21 clinical course. To our knowledge, this is the first study to analyze TFR2 clinical value in
22 MDS patients.
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Peer Review

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FIGURES LEGENDS.

Figure 1. TFR2 and EPOR gene expression in newly diagnosed MDS BM samples.

(A-C) Real time analysis of TFR2 α , TFR2 β and EPOR transcripts in subtypes of MDS according to WHO classification, compared to non-malignant BM samples.

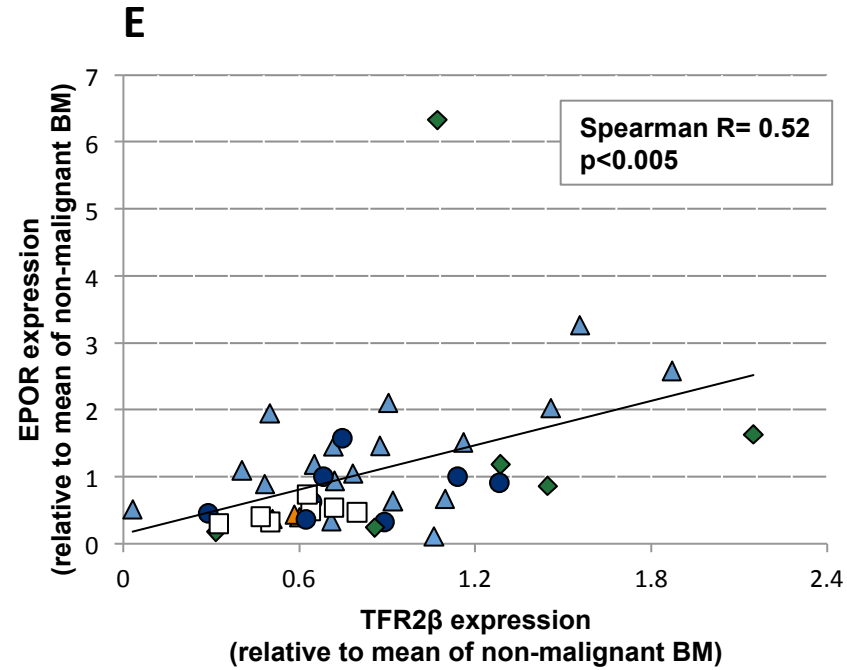
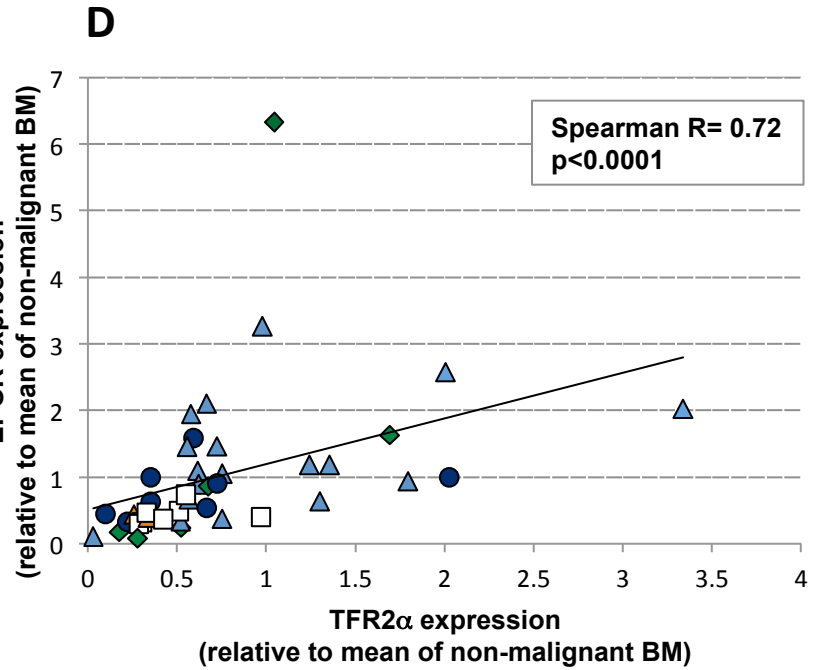
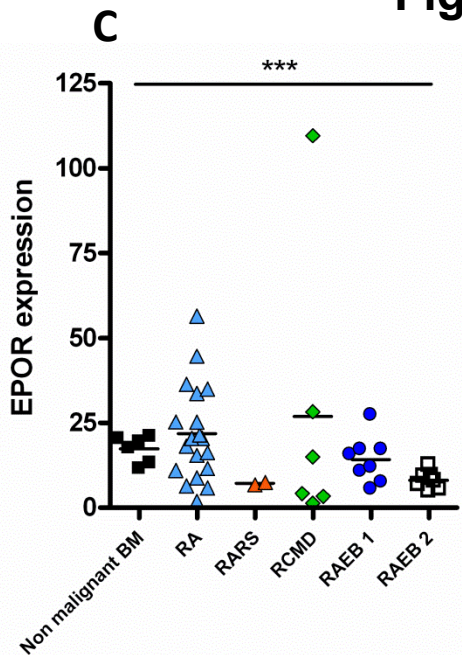
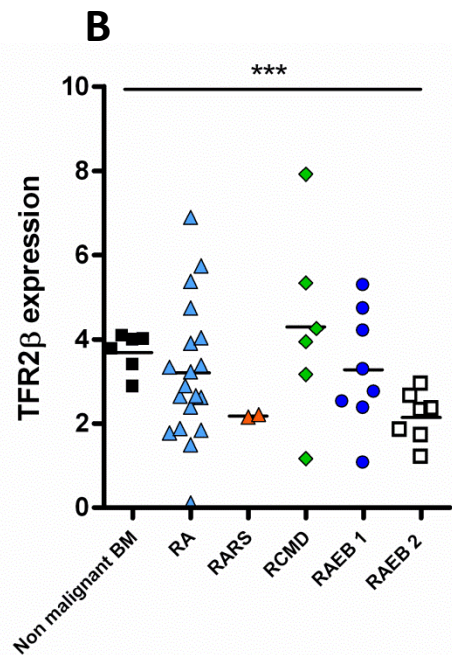
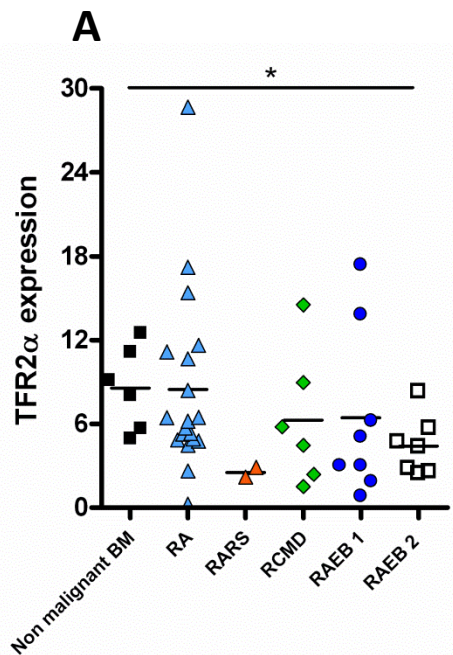
**p-value<0.01. The lines indicate mean value of each group.

(D-E) Spearman's correlation analysis on the mRNA expression of TFR2 α , TFR2 β and EPOR in our cohort of MDS patients. R and p values are indicated. Color and shape code of each individual point in the graphs are the same reported in figure 1 A-C.

Figure 2. Potential value of TFR2 in the clinical diagnostic application of MDS patients.

(A) Clinical and laboratory parameters of myelodysplastic patients treated with erythropoietin. WHO = World Health Organization classification of myelodysplastic syndromes, Dosage = erythropoietin dosage, RA = refractory anemia, RCMD = refractory cytopenia with multilineage dysplasia, RAEB-1 = refractory anemia with excess of blasts type 1, RAEB-2 = refractory anemia with excess of blasts type 2, RARS = refractory anemia with ringed sideroblasts, Hb = hemoglobin, Δ Hb = hemoglobin variation after 12 weeks of treatment. Patients that responded to Epo treatment are highlighted in red. MDS patients were categorized into four expression sub-groups according to TFR2 and EPOR expression relative to the mean of the non-malignant BM cells, as follows: "very low" group when TFR2 and EPOR levels were less than minus two standard deviations (SD) from the mean of non-malignant BM; "low" between minus two and minus one SD; "normal" between minus and plus one SD; "high" more than one SD. (B-C-D) Kaplan-Meier survival curves for MDS RAEB1-2/RCMD patients based on TFR2 α , TFR2 β or EPOR expression level. *p-value<0.05 analyzed by log-rank test.

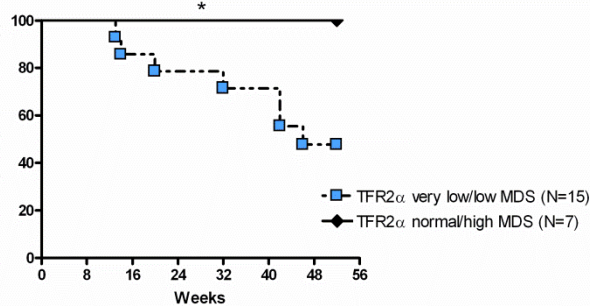
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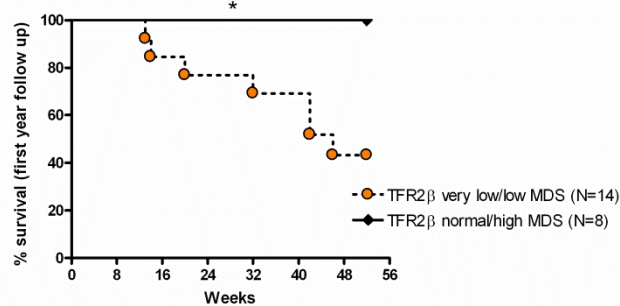
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Case #	WHO	Dosage	Transfusion need	Hb (g/dL)	ΔHb (g/dL)	TFR2α	TFR2β	EPOR
1	RA	40000 U/week	no	10,5 -> 12,4	1,9	low	normal	normal
6	RA	40000 U/week	no	9,3 -> 9,9	0,6	high	high	high
12	RA	40000 U/week	no	8,8 -> 9,2	0,4	very low	very low	very low
13	RARS	40000 U/week	3RCB units/month	8,2 -> 8,8	0,6	low	very low	very low
17	RA	40000 U/week	no	9,3 -> 12,4	3,1	normal	very low	normal
25	RCMD	40000 U/week	no	9,7 -> 10	0,3	very low	very low	very low
41	RA	40000 U/week	no	9,2 -> 10,5	1,3	high	high	high
2	RAEB 1	80000 U/week	no	9,7 -> 10,6	0,9	low	very low	low
10	RAEB 1	80000 U/week	3RCB units/month	6,8-> 6,7	-0,1	very low	low	very low
11	RA	80000 U/week	3RCB units/month	7,5 -> 8,1	0,6	low	normal	low
14	RAEB 1	80000 U/week	4RCB units/month	7,3 -> 6,8	-0,5	very low	very low	very low
26	RCMD	80000 U/week	no	9,1 -> 13,3	4,2	normal	high	normal
29	RAEB 2	80000 U/week	2RCB units/month	7,9 -> 8,3	0,4	very low	very low	very low
34	RA	80000 U/week	no	8,3 -> 11,2	2,9	normal	high	normal
37	RCMD	80000 U/week	4 RCB units/month	7-> 7,2	0,2	very low	normal	very low
39	RAEB 1	80000 U/week	no	10,1 -> 12,2	2,1	high	normal	normal
40	RA	80000 U/week	2 RCB units/month	8,5-> 7	-1,5	normal	normal	low

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