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Iron Deficiency in Hemodialysis Patients – Evaluation of a Combined Treatment with Iron Sucrose and Erythropoietin-Alpha: Predictors of Response, Efficacy and Safety

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1. Introduction

Chronic kidney disease is a public health problem and one of the outstanding causes of death in the industrialized world. The most serious condition is advanced chronic renal failure requiring replacement therapy by dialysis or kidney transplantation. In recent years the incidence is stabilizing but the prevalence is increasing probably due to the progressive aging of the population and increased comorbidity with other chronic disorders such as diabetes mellitus, hypertension and obesity (de Francisco et al. 2007). It is also a major cause of anemia in developing countries (Maiz, Abderrahim, and Zouaghi 2002) . The long-term survival and good quality of life of patients with chronic renal failure depends, among other factors, on hemoglobin, iron status and bone marrow response to erythropoiesis stimulating agents (ESA). Anemia is an almost constant complication of advanced renal failure which may contribute to worsen preexisting heart disease and, as a consequence, accelerate the progression of renal dysfunction (Kuwahara et al. 2011), (Silverberg et al. 2009). The administration of erythropoietin to patients with kidney and heart failure improves both processes, not only increasing hemoglobin but also by a direct effect of erythropoietin on cardiac function (Belonje, de Boer, and Voors 2008). In general, anemia is normocytic, normochromic and usually well tolerated until the advanced stages of kidney disease. It is usually a complication of stage 3 chronic kidney disease (KDOQI and National Kidney Foundation 2006). Its etiology is multifactorial: shortening of life span of erythrocytes, presence of inhibitors of erythropoiesis in plasma, inadequate production of endogenous erythropoietin (EPO) for the degree of anemia, blood loss, and iron and vitamin deficiency(Tsubakihara et al. 2010) (Belonje, de Boer, and Voors 2008), (Chamney et al. 2010). The outstanding cause is impaired secretion of erythropoietin due to renal disease, while other factors may contribute to its establishment, maintenance or aggravation.

The specific treatment of choice for anemia of chronic renal failure is recombinant human erythropoietin (rHuEPO), a drug considered a historic milestone since the time of its clinical use in 1986 (Winearls et al. 1986), and the major therapeutic advance in anemia of chronic renal failure. Erythropoietin remains vital in the treatment of anemia of renal failure, but in the coming years could be replaced by new erythropoiesis-stimulating agents under investigation (Schmid and Schiffl 2010). The use of erythropoietin led to a drastic reduction of transfusions and androgen therapy with the consequence of decreasing the complications of these treatments. Suitable doses of the drug, as well as the correction of other contributing factors to anemia, are necessary to maintain adequate levels of hemoglobin in the range of 11-12 g / dl (KDOQI 2007).

Iron is an essential factor to achieve and maintain effective erythropoiesis in patients with chronic renal failure treated with rHuEPO, due to frequent losses of blood in the hemodialyzer, overstimulation of erythropoiesis induced by erythropoietin and possible gastrointestinal bleeding. Moreover, the renal disease itself and chronic inflammation can sequester iron in the mononuclear phagocytic system preventing its use for erythropoiesis. These factors as a whole significantly increase the demand for iron. Capacity of intestinal iron absorption in these patients is not enough to maintain adequate levels of iron stores. On the other hand, the supply of iron improves response to rHuEPO and aids to lower its dose up to 30%. However, iron administration must be handled with caution, as an excess is not safe. The body has mechanisms to regulate iron absorption in the gastro-duodenal tract, but lacks a physiological mechanism to remove excess iron. Therefore treatment with intravenous iron should be monitored carefully.

Other causes of anemia in dialysis patients such as: hemolysis, aluminum intoxication, infection, chronic inflammation and hyperparathyroidism should not be overlooked; on the contrary the anemia of renal failure is not generally characterized by a lack of folic acid or vitamin B12 (Bravo, Galindo, and Bienchy 1994).

For all these reasons, patients need iron administration (usually intravenous) and regular monitoring of iron body stores. The diagnosis and treatment protocols should be optimized to adjust the cost, improve quality of life and prolong survival, as it has recently been reported that patients with hemoglobin above 12 g / dl have a higher survival rate than those with lower levels (Pollak et al. 2009).

The evaluation of anemia and iron status in chronic renal failure has peculiarities that make it different from other processes. Therefore we find it useful to include a brief description of the normal values in adults, the diagnosis of iron deficiency in general and the peculiarities of the diagnosis of iron status in patients with chronic renal failure. The World Health Organization specifies the normal range of hemoglobin and defines anemia as a hemoglobin decrease below the normal lower limit (Nutritional anaemias (Anonymous1968). Hematology analyzers measure the hemoglobin directly, with great precision and accuracy, while the hematocrit is a calculated value. Moreover hemoglobin remains constant from the extraction of blood until it is analyzed, while the hematocrit increases with time due to changes in erythrocyte volume (Tsubakihara et al. 2010). However some publications rely on hematocrit as an indicator of anemia, thus we consider both parameters. Red cell indices are very useful in the diagnosis of anemia whatever the etiology, and parameters of iron metabolism are essential for the diagnosis of iron deficiency anemia. Here we only state the normal range for adults of both sexes, excluding infants, children and pregnant women.

Below we present laboratory data useful for the study of iron deficiency anemia in general and the specific laboratory tests to evaluate iron status in chronic renal failure and hemodialysis patients.

1.1 Red blood cells: Normal values in adults

Hemoglobin: ♂: 14-17 g / dl; ♀: 12-14 g / dl
Hematocrit: ♂: 42-52% ♀: 36-46%
Red cell indices: mean corpuscular volume (MCV) 81-99 fl;
mean corpuscular hemoglobin (MCH), 27-31 pg; mean corpuscular hemoglobin concentration (MCHC) 34 ± 2 g/ dl
Reticulocytes: ♀: 0.4 to 2.4%, ♂: 0.6 to 2.6%; in absolute numbers: 40 - 100 x 10^9 / l

1.2 Assessment of normal iron status in adults

Serum iron: ♂ and ♀ postmenopausal 50-150 mg/dl; ♀ reproductive age 35-140 mg/ dl
Transferrin: 200-350 mg/dl
Transferrin saturation (serum iron x 100/ transferrin): 30%
Serum ferritin: ♂ and ♀ postmenopausal 30-400 ng/ml; ♀ reproductive age 15-150 ng/ml
Absence of iron stores in patients not on dialysis <10-15 ng/ml

Iron overload > 800 ng/ml. High levels of serum ferritin must be interpreted with caution in the clinical setting of tumors, inflammation and chronic conditions because ferritin may be elevated even if iron stores are normal or low. In these cases the soluble transferrin receptor is useful in estimating iron deposits. Normal levels of soluble transferrin receptor 0.8 to 1.8 ng /l.

1.3 Diagnosis of iron deficiency anemia

In patients without renal impairment, the diagnosis of iron deficiency anemia is based on the assessment of Hb (♂ <13 g/ dl and ♀ <12 g/ dl) and hematocrit (♂ <42% ♀ <36%) along with a decrease in mean corpuscular volume (MCV <80fl) mean corpuscular hemoglobin (MCH <25 pg) and mean corpuscular hemoglobin concentration (MCHC <32g/dl). The diagnosis of iron deficiency anemia also requires a reduction of serum iron below the following levels: (♂ and ♀ postmenopausal (<50 mg / dl) and ♀ reproductive age (<30 mg / dl), increased transferrin (> 360 mg / dl) decrease saturation of transferrin (<20%) and decreased serum ferritin (♂ and ♀ postmenopausal <30 ng / ml and ♀ reproductive age <15 ng / ml), all of them may be complemented by an increase in soluble transferrin receptor (> 2 ng /l).

1.4 Diagnosis of iron deficiency in chronic renal failure

In chronic kidney disease 60-80% of cases have absolute or functional deficiency of iron, especially in haemodialysed patients.

The European Best Practice Guidelines (EBPG) and the Kidney Disease Outcomes Quality Initiative (EBPG K / DOQI) have specified slightly different criteria for diagnosis of anemia in patients with chronic renal failure (Tsubakihara et al. 2010).

With respect to hemoglobin (Hb):

♂ Hb <13.5 g / dl (EBPG) and Hb <13.5 g / dl (K / DOQI)
♀ Hb <11.5 g / dl (EBPG) and Hb < 12.0 g / dl (K / DOQI)
> 70 years old Hb <12 g / dl (EBPG)

With respect to hematocrit, values differ by sex, race and age, decreasing values with increasing age, like hemoglobin. The reference values to define anemia by hematocrit in Japanese adult males are <40% and in adult women ♀ <35%(Tsubakihara et al. 2010)

If MCV is decreased or normal the most probable diagnosis is iron deficiency anemia, nevertheless other less frequent diagnoses must be ruled out such as: anemia of chronic disease, sideroblastic anemia, thalassemia, hemolytic anemia, aplastic anemia, myelodysplastic syndrome and pure red cell aplasia. The latter, though extremely rare has been associated with erythropoietin therapy, mainly if administered subcutaneously. On the contrary if the MCV is elevated macrocytic anemia should be considered due to deficiency of vitamin B12 and / or folic acid, liver disease, hypothyroidism, aplastic anemia, myelodysplastic syndrome or drugs that interfere with DNA synthesis.

The traditional parameters of iron metabolism, which are used for the diagnosis of iron deficiency in chronic kidney disease, are: serum iron <40 mg / dl, transferrin saturation < or = 20%, ferritin < or = 100 ng / ml and Hb <11 g / dl.(KDOQI and National Kidney Foundation 2006).

1.5 New red cell indices and iron status markers – Advantages over traditional markers

The anemia of chronic renal failure is complex and multifactorial, hence the analysis of individual parameters is neither accurate nor does it provide a predictive value to determine which patients will respond to therapy. This has led in recent years to the publication of numerous studies to find new laboratory tests that can identify patients with absolute or functional iron deficiency, in order to individualize and improve treatment. These parameters as well as others not related to the diagnosis of anemia have also been used to try to predict response to treatment with iron and erythropoietin.

Serum ferritin is not a good index for estimating iron deposits in the course of chronic kidney disease ((Kalantar-Zadeh, Kalantar-Zadeh, and Lee 2006). However on the other hands serum ferritin is useful for monitoring iron therapy in chronic kidney disease ((Nakanishi et al. 2010). To overcome drawbacks of ferritin as an estimator of iron stores several authors have studied the usefulness of soluble transferrin receptor with discrepant results.(Chang et al. 2007) have found a good correlation between the increase in soluble transferrin receptor and decreased iron stores. However (Gupta, Uppal, and Pawar 2009, 96-100) have not found any utility in transferrin soluble receptor as a marker of iron deficiency in patients with renal failure. In this context (Chen, Hung, and Tarng 2006a) studied the *TfR-F index* calculated by the ratio of transferrin receptor and the logarithm of serum ferritin, concluding that it is more sensitive than transferrin receptor to assess iron stores and may guide the IV iron therapy in hemodialysis patients better.

For over a decade reticulocyte parameters have been used to monitor erythropoiesis in various diseases, among which are patients with chronic renal failure and anemia (Remacha

et al. 1997). (Agarwal, Davis, and Smith 2008) show that the fraction of immature reticulocytes behaves as an indicator of response in the anemia of chronic renal failure. (C.H. Fourcade, L. Jary, and and H. Belaouni 1999) have studied the reticulocyte profile under both regenerative and hypo regenerative bone marrow conditions. (Maconi et al. 2009) have made comparative studies of reticulocyte and erythrocyte parameters in the clinical setting of patients with anemia. (Brugnara, Schiller, and Moran 2006) have shown the usefulness of hemoglobin content of reticulocytes and red cells: Ret-He and RBC-He (Sysmex XE 2100) and CH and CHr (Bayer ADVIA 2120), in the identification of different stages of iron deficiency in hemodialysis patients. The correlation of both parameters is good and the performance of Ret-He is good for absolute iron deficiency, with an AUC of 0.913 a sensitivity of 93.3%, and a specificity of 83.2%. The diagnostic performance of Ret-He is less favorable for funcional iron deficiency, as the AUC is low (0.657).

Others such as (Kalantar-Zadeh et al. 2009), have studied iron metabolism markers and parameters of renal osteodystrophy looking for predictors of response to erythropoiesis-stimulating agents in hemodialysis patients and found that in the long term, low iron stores in patients with hyperparathyroidism and high bone marrow turnover are associated with hypo-responsiveness to ESA.

The sensitivity and specificity of some other parameters such as transferrin saturation, serum ferritin, hypochromic red cells (% Hypo), reticulocyte hemoglobin content (CHr) and soluble transferrin receptor (sTfR) have been published by (Tsubakihara et al. 2010). According these authors the most sensitive parameters to detect functional iron deficiency are serum ferritin and hypochromic red blood cells while the CHr is more specific. On the other hand CHr is the most sensitive parameter to detect iron overload, whereas ferritin (> 800ng/mL) and hypochromic red blood (<10%) cells are more specific (Tsubakihara et al. 2010).

In a multicenter study with participation of 9 hospitals in Europe, (Zini et al 2006) studied the utility of a new analytical parameter, low density hemoglobin, (LDH% Beckman-Coulter), compared with HCM, in hemodialysis patients with functional iron deficiency and concluded that they are useful parameters. The percentage of hypochromic red cells (%Hypo) has been incorporated to National Kidney Foundation KDOQI guidelines for monitoring recombinant human erythropoietin therapy. (Urrechaga 2010) (Urrechaga 2008) find a good correlation between (% Hypo) and low-density hemoglobin (LDH%), Both behave as good markers of iron deficiency in different types of anemia (anemia of kidney disease, iron deficiency anemia, anemia of chronic disease and beta thalassemia) and are equivalent. LDH% is a parameter calculated by a mathematical function based on mean cell hemoglobin concentration (MCHC) (Urrechaga 2010)

The anemia of chronic renal failure is also a chronic disease anemia, and therefore parameters such as IL6, TNF-alfa and other proteins such as neopterin, hepcidin and hemojuvelin should be evaluated. In this context (van der Putten et al. 2010 recently set out the clinical role of changes in hepcidin levels and response to treatment with EPO in patients with inflammation and renal and cardiac damage. Previously (Zaritsky et al. 2009) propose hepcidin as a new biomarker of iron status in chronic kidney disease. Moreover, pro-hepcidin has been proposed as a useful parameter in the evaluation of iron status in chronic kidney disease (Barrios, Espinoza, and Baron 2010; Arabul et al. 2009; Shinzato et al. 2008)

2. Objectives

This trial evaluates a treatment protocol for patients with anemia of chronic kidney disease on hemodialysis

It has two objectives:

a. To find new predictors of response
b. To evaluate a combined treatment regimen with EPO and iron in terms of safety, stability and efficacy.

Although planned in advance, this work meets the recommendation of Coyne (Coyne 2010) (2010) which specifies: "rather than focus on individual products, we should perform trials comparing anemia management strategies to assess safety, efficacy, and cost".

Hematological parameters	Hb	Coulter LH750®
	RBC, WBC	Coulter LH750®
	HCT, MCV, MCH, MRV, MCHC, MPV, MSCV	Coulter LH750®
	RET %, RET#, HLR%, HLR#	Coulter LH750®
	IRF	Coulter LH750®
	NE %, NE#	Coulter LH750®
Iron metabolism	Iron	COBAS-Integra 400 Roche® Diagnostic
	Transferrin	COBAS-Integra 400 Roche® Diagnostic
	TSAT	COBAS-Integra 400 Roche® Diagnostic
	Ferritin	COBAS-Integra 400 Roche® Diagnostic
	TfR-F index, sTfR	Roche® Diagnostic
	RBC Ferritin,	COBAS-Integra 400 Roche® Diagnostic
	EPO	Access 2 Beckman Coulter
	ESR	Alifax Beckman Coulter
Inflammation	Fibrinogen	ACL TOP IL
	CRP	High-sensitivity nephelometry. Beckman
	IL6	Access 2 Beckman Coulter
Vitamins	Vit B12, Folate, RBC Folate	Access 2 Beckman Coulter
Platelets	Plt	Coulter LH750®
	PPV	Coulter LH750®
	PDW	Coulter LH750®
Liver	ALT, GGT, AT III	Roche® Diagnostic
Calculated parameters	RPI	calculated : (RET% x HCT)/45
	RSf®	calculated : $\sqrt{MCV \cdot MRV}/1000$
	Maf®	calculated : Hb x MCV/100
	VHDWf®	(MCV x Hgb)/(RDW x 10)

Table 1. Column 2 shows the panel of laboratory tests. These are grouped by category in column 1. Column 3 shows the instrumentation used. The tests were done with reagents supplied by the manufacturers of the devices, strictly following the operating instructions

manual. (CRP= C- reactive protein, EPO= Erythropoietin, ESR = Erythrocyte Sedimentation rate) Hb=Hemoglobin, HCT =Hematocrit ,HLR#=High light scatter reticulocytes (absolute), HLR%=High light scatter reticulocytes (relative to Hb), IRF=Immature reticulocyte fraction, Maf®=Microcytic anemia factor, IL6= Interleukin 6, MCH=Mean corpuscular Hemoglobin, MCHC=Mean corpuscular hemoglobin content, MCV=Mean corpuscular volume, MPV=Mean platelet volume, MRV=Mean reticulocyte volume, MSCV=Variance of MCV measurement, NE#=Neutrophils count (absolute), NE%=Neutrophils %, RBC=Red blood cell count, Plt= Platelets count, PPV=Platelet Packed Volume, RET#=Reticulocytes count (absolute), RET% = Reticulocytes %, RBC Folate= Intra-Red Blood Cells Folate), RPI= Reticulocyte production index , sTfR=soluble Transferrin Receptor, TSAT= Transferrin saturation, RBC Ferritin = Intra-Red Blood Cell Ferritin, TfR-F index =Transferrin Receptor-Ferritin Index (Soluble Transferrin Receptor/log Ferritin), VHDWf= Volumen Hemoglobin Distriburion Wide factor, WBC=White Blood Cells count.

To achieve these goals we used the laboratory data recommended in the 2006 KDOQI guide in order to evaluate anemia and iron status: "*Complete blood count (CBC) including red blood cell indices (mean corpuscular hemoglobin [MCH], mean corpuscular volume [MCV], mean corpuscular hemoglobin concentration [MCHC]), white blood cell count, differential and platelet count, absolute reticulocyte count, serum ferritin to assess iron stores and serum ferritin and the transferrin saturation (TSAT) or content of Hb in reticulocytes (CHr) to assess adequacy of iron for erythropoiesis*"; as well as other parameters that we feel could be useful in predicting response, according to publications mentioned above (see table 1)

3. Materials, patients and methods

3.1 Study groups and therapy

This is a prospective, open, nonrandomized trial. Forty-two patients from the dialysis department of the "Hospital Clinico Universitario de Zaragoza" (Spain) have been included. Patients were treated by hemodialysis (HD), EPO and Iron. Patients were not transfused during the study.

All patients gave informed consent. We analyzed the parameters specified in Table 1, monthly for 6 months.

In addition hemoglobin, reticulocyte and iron status were evaluated at the 7th month to assess response to therapy received during the sixth month.

3.2 EPO doses

3.2.1 Initial

- Pre-dialysis, 40 U / kg once a week
- Dialysis: 40 U / kg three times a week

3.2.2 Adjustment

- Evaluate four weeks later. If Hb increase exceeds 5%: do not change dose, otherwise increase 20 U / kg.

- Reassess every 4 weeks. Increase 20 U if the rise in hemoglobin is less than 5%
- Maximum 200 U / kg.
- Do not abruptly discontinue administration of erythropoietin, unless there is life threatening risk.

The maintenance phase begins when the target is reached (hemoglobin 12g/dl)

3.2.3 Maintenance

If the hemoglobin is 12 g / dl or greater decrease the dose of erythropoietin as follows:

- If the last dose was greater than 80 u / kg, reduced by half.
- If the last dose was less than 80 U / kg, left as maintenance 30 U / kg.

3.3 Iron therapy

3.3.1 Pre-dialysis

Iron (oral ferrous sulfate) is given to all patients with chronic renal failure non dialyzed with hemoglobin less than 11 g / dl or ferritin below 100 ng / mL and transferrin saturation below 20%.

Patients with an inadequate response to oral iron or gastrointestinal intolerance have been treated with 200 mg of Intravenous iron sucrose before starting the dialysis program.

3.3.2 Dialysis

All patients on dialysis included in this assay were treated with iron sucrose: 100 mg of iron sucrose in 100 cc saline in slowly perfusion at the end of each hemodialysis session (three times per week)

3.3.3 Target

Ferritin levels between 200 – 400 ng/ml and transferrin saturation > 20%

3.3.4 Adjustment of iron dose

As shown in table 2

Conditions	Adjustment of iron doses
If ferritin < 200 ng /ml	100 mg weekly
If ferritin 200-350 ng / ml	100 mg every 15 days
If ferritin 350 – 500 ng / ml	100 mg monthly.
If ferritin > 500 ng /ml	Stop iron therapy
If TfSat > 50%	Stop iron therapy regardless of serum ferritin

Table 2. Schedule for dose adjustment of Iron

With the exception of 7 patients, 6 laboratory panels per patient (one panel per month) are available. Of the 7 patients with missing data 3 died and 4 were transplanted. None of the

deaths were related to the treatment with iron or erythropoietin. The laboratory data of these patients until death or transplantation were included in the statistical analysis.

Hemoglobin and iron status were also analyzed one month after the last (6th) panel in order to classify patients as responders or non-responders. Finally 224 laboratory panels were analyzed.

4. Definitions and criteria of response

Most authors only use hemoglobin as a criterion of response. This is correct when evaluating a long period of time, but for short periods the response may have started before there was an increase in hemoglobin. Therefore we have chosen a target compound in which the main data is the increase or maintenance hemoglobin, but giving the option to evaluate earlier data of erythropoiesis (reticulocytes and sTfR (Soluble transferrin receptor)) and the rise in hemoglobin over two months, instead of evaluating only the change in hemoglobin from one month to the next.

4.1 Criteria

Response was assumed if one of the following four conditions was fulfilled.

1. Increase of Hb ≥ 0.5 g/dl in the next control and/or increase of Hb for the following two controls > 0.8 g/dL, under condition that both changes are positive.
2. Achieve or maintain minimum levels of hemoglobin of 11 g/dl in women and 12 g/dl in men with the following two conditions:
3. Hb increase
4. Ret# >50 x 10^9/L
5. Increase of sTfR > 20%, provided that Hb also increases
6. Increase of Ret#>40 x 10^9/L provided that Hb also increases.

As can be seen to increase or maintain hemoglobin at normal levels is a prerequisite. However, we reduce the quantitative criteria for hemoglobin if the increase in reticulocytes or soluble transferrin receptor suggests an early recovery. Others consider, like us, the increase of reticulocytes as one of the criteria of response to erythropoietin (Brugnara, 2003)

4.2 Definition and classification of iron deficiency status

Applying the criteria of (Ng et al. 2009, c247-52) patients were divided into 4 groups, whose definitions and frequencies are as follows:

- Group 1. Absolute Iron deficiency. (Ferritin < 100ng/ml and Transferrin Saturation < 20%): 5
- Group 2. Functional Iron deficiency. (Ferritin ≥ 100ng/ml and Transferrin Saturation < 20%): 33
- Group 3. Ferritin < 100ng/ml and Transferrin Saturation ≥ 20%: 9
- Group 4. Iron repletion (Ferritin > 100ng/ml and Transferrin Saturation ≥ 20%): 198

To avoid confusion between functional iron deficiency and iron deficiency, hereinafter the latter will be referred to as absolute iron deficiency.

5. Statistical analyses

5.1 Directed at objective A

All statistical analyses were performed with SPSS.

All parameters were tested regarding departures from normal distribution by KS-test (Kolmogorov-Smirnov). In case of normal distributed parameters, group-wise means and standard deviations were calculated. In case of non-normality log transformation or description by non-parametric approaches (median, rank-correlation) were used to avoid biases related to not normally distributed parameters. All comparisons were performed by U-test. For parameters with significant group-wise differences the area under ROC curve (AUC) was calculated.

A correlation matrix (Pearson-correlation, rank-Spearman correlation) of all parameters was calculated and evaluated by factor analysis in order to gain information about relationship between the parameters and to reach a reduction of dimensions (variables) in subsequent statistical analysis (Härdle and Simar 2003) Parameters that were found associated using the statistical program were regarded as a parameter-class.

Bonferroni correction was used to avoid false positive results of significance due to multiple testing. The number of components N_C resulting from factor analysis was used for correction. Starting from a significance level of α=0.05, α_{comp}=0.05/N_C was used for group-wise comparisons.

In order to calculate models describing dependence of binary outcome variable (response) on parameter values, logistic regression was performed. Parameters c_i determined in the model can be used in equations of the following type (n-dimensional model for logistic regression), where p_{resp} is probability of response, X_i is value of blood parameter and c_i is coefficient for blood parameter X_i within the model:

$$p_{resp} = \frac{1}{1 + \exp(-(c_0 + c_1 X_1 + .. + c_n X_n))} \tag{1}$$

In practice, ranges of probability of response between 0 (prediction of no response) and 1 (prediction of response) can be calculated by putting all parameter values X_i into the formula.

Resulting models were evaluated by ROC analysis and calculating AUC, as mentioned above. Furthermore, practical issues like robustness of parameters and ability of a simple data management (e.g. avoiding usage of mean differences) were considered.

In addition cases were separated according to iron status classification and compared by univariate two-factor ANOVA (factors: iron status group, response) with post-hoc-comparisons

5.2 Statistical analyses directed to objective B

The descriptive statistics were carried out as follows.

The first step was to study the evolution of hemoglobin each month using parametric (Student) and nonparametric (comparing the overall monthly evolution of hemoglobin by

the Kruskall-Wallis test) methods followed by bivariate comparisons of each month with the next(Mann-Whitney). Although hemoglobin fits a normal distribution we also used nonparametric tests to avoid false positive results due to sample size. In this section we choose hemoglobin as the dependent variable, not including any other criteria of response. This is because the purpose of this section to compare the parameters with hemoglobin, the therapeutic target that really determines the quality of life of patients.

In a second step we use multiple linear regression to obtain a mathematical model that relates the levels of hemoglobin with selected parameters.

The graphic presentation of hemoglobin evolution over time has been made by Box Plot to display the median, interquartile distribution, outliers and overlapping. We have also represented the means on a bar graph with confidence interval of 95%.

Finally we applied the same statistical model to the parameters that quantify the iron status: serum iron, transferrin, transferrin saturation, ferritin and soluble transferrin receptor. This statistical treatment is aimed to check whether iron treatment regimen is optimal, or requires changes.

6. Results

6.1 Descriptive statistics and groups

6.1.1 Demography

42 patients; gender F: 15, M: 27; age: median: 73 years (range: 22 – 88)

6.1.2 Responses

79 responses, 97 non responses, 88 not available (no EPO therapy, transplanted, death or missing data. (Table 3).

		Transferrin Saturation	
		< 20%	>20%
Ferritin ng/ml	<100	Group 1(ID) 5 (2%)	Group 3 9 (3.7%)
	>100	Group 2 (FID) 33 (13.5%)	Group 4 (IR) 198 (80.8%)

Table 3. Distribution of cases according to groups defined by Young and Chun (2009) (see text)

6.1.3 Iron status

Applying the criteria of Young and Chun (2009) frequencies of the four groups of iron status are:

- Group 1. - Absolute Iron deficiency (ID) (Ferritin < 100ng/ml and Transferrin Saturation < 20%): 5
- Group 2. - Functional Iron deficiency (FID) (Ferritin ≥ 100ng/ml and Transferrin Saturation < 20%): 33

- Group 3. - Ferritin < 100ng/ml and Transferrin Saturation ≥ 20% (Iron status is not well defined in this group). Transferrin saturation is enough for erythropoiesis, but iron stores may be low: 9
- Group 4. - Iron repletion (IR) (Ferritin > 100ng/ml and Transferrin Saturation ≥ 20%): 198

6.1.4 Descriptive statistics of all parameters

The descriptive statistics and comparison with normal distribution is shown in table 4.

	N	Mean	SD	Median	Minimum	Maximum	Comparison with normal distribution (Kolmogorov-Smirnov)
HLR (%)	189	0.825	0.36	0.836	0.062	2.24	n.s.
HLR# (n/mm^3)	189	28.7	12.5	28.6	1.88	66.7	n.s.
RPI	189	1.55	0.534	1.59	0.12	3.14	n.s.
RET (%)	192	2.08	0.735	2.13	0.149	5.05	n.s.
RET# (n/mm^3)	192	72.1	24.5	73.2	9.15	150	n.s.
IRF	189	0.387	0.0757	0.393	0.16	0.56	n.s.
Hb (g/dL)	224	11.1	1.44	11.1	7.18	14.8	n.s.
HCT (%)	192	34	4.33	33.9	21.7	45	n.s.
RBC 10^3 / mm^3)	195	3490	444	3470	2130	4690	n.s.
Maf	224	10.8	1.6	11	6.54	14.7	n.s.
RSf	189	0.11	0.00692	0.11	0.09	0.13	n.s.
MCV (fL)	224	97.3	4.93	97.6	83.8	113	n.s.
MSCV (fL)	189	95.9	6.04	95.5	83.5	116	n.s.
MRV (fL)	189	124	11	124	96.3	165	n.s.
MCH (g/L)	195	31.8	1.83	32.1	26.8	35.8	n.s.
EPO (IU/L)	238	21.3	39.6	12	1.75	465	p<0.00001
sTfRFindex	221	0.755	0.247	0.724	0.282	2.02	n.s.
sTfR (mg/L)	222	1.82	0.498	1.8	0.8	3.2	p=0.046
Fibrinogen (mg/dl)	222	521	121	518	246	993	n.s.
ESR (mm/ first hour)	222	43.2	28.2	35.5	1	142	p=0.0038
CRP (mg/L)	223	1.73	4.78	0.64	0	38.6	p<0.00001
IL6 (ng/L)	238	10.5	15.3	6.43	0.6	160	p<0.00001
Ferritin (ng/ml)	223	376	275	326	24.3	2290	p<0.00001
Plt (103/mm^3)	192	213	56	216	92.5	368	n.s.
PPV (%)	192	0.178	0.0412	0.175	0.0713	0.288	n.s.
TfSat (%)	223	33.2	12.3	32.2	9.8	82.8	n.s.
Iron (µg/dL)	223	56.7	22.4	53.5	9.87	198	n.s.
MCHC (g/dl)	192	32.6	0.723	32.6	31	34.8	n.s.
MPV (fl)	191	8.46	1.07	8.34	6.13	11.4	n.s.

RDW (%)	192	15.8	1.47	15.7	13.2	20.9	n.s.
@NE	192	66.8	8.09	66.6	46.6	83.9	n.s.
NE#	192	5	1.61	4.79	1.16	9.74	n.s.
WBC (103/ mm^3)	192	7.33	2.2	7.46	0	14.9	n.s.
VitB12 (pg/ml)	224	534	218	484	180	1180	p=0.025
RBC Folate	223	723	796	421	107	3670	p<0.00001
PDW	192	16.7	0.554	16.6	15.6	18.5	n.s.
ALT (U/L)	222	14.8	12.2	12	1	73	p<0.00001
GGt (U/L)	222	31.5	38.8	17	4	211	p<0.00001
RBCFerritin (ng/ml of RBC)	224	731	345	638	223	2160	p=0.00023
Transferrin mg/dl	223	175	35.7	169	73.8	351	n.s.
ATIII (% of normal control)	220	101	16.9	101	62	149	n.s.

Table 4. Descriptive statistics and Kolmogorov-Smirnoff test (n.s. = not significant difference from normal distribution)

All parameters are normally distributed, with the exceptions of Ferritin, EPO, ESR, CRP, IL6, Vit B12, RBC, Folate, RBC ferritin, ALT and GGT. In all of these differences with the normal distribution are avoided by logarithmic transformation.

6.1.5 Factorial analysis

The coefficients given in Table 5 refer to the association of each parameter to one of the classes determined by factor analysis. The row-wise maximum relates each parameter to one of the 12 classes. Some of the parameters contribute to several components. As an example, Maf, which is a product of MCV and Hb, is associated to class 3 (contains Hb) as well as class 2 (contains MCV). In a similar manner, WBC is not only associated to Plt and Pct, but also to neutrophil-linked parameters NE# and NE%.

(See Table 5)

		Component											
		1	2	3	4	5	6	7	8	9	10	11	12
1	HLR %	0.97											
	HLR #	0.97											
	RPI	0.94											
	RET #	0.94											
	RET %	0.93											
	IRF	0.65		0.35									
2	Hb		0.97										
	HCT		0.97										
	RBC		0.92										
	Maf		0.88	0.40									

	VHDW		0.75						-0.37				
3	RSf			0.95									
	MCV			0.90									
	MRV			0.84									
	MSCV			0.84									
	MCH			0.81					-0.47				
4	EPO (log)				0.81								
	TfR-F index				0.51			-0.50				-0.42	
	sTfR				0.42			-0.38				-0.46	
5	Fibrinogen					0.83							
	ESR (log)		-0.33			0.78							
	CRP (log)					0.74							
	IL6 (log)					0.59						0.43	
	Ferritin (log)				-0.39	0.43		0.40					-0.40
6	Plt						0.94						
	PPV						0.88						
7	TfSat%							0.84					
	Iron							0.73					
8	MCHC								-0.79				
	MPV						-0.33		0.60				
	RDW				0.41				0.55				
9	NE #						0.35			0.80			
	NE %									0.75			
	WBC						0.40			0.63			
10	Vit. B12 (log)										0.77		
	RBC Folate (log)										0.75		
	PDW						-0.45				-0.57		
11	ALT (log)											0.78	
	GGT (log)											0.66	
	RBC Ferr. log)								-0.35			0.49	-0.35
12	Transferrin												0.84
	ATIII										0.37		0.53

Table 5. Factor analysis and correlation matrix.

Parameters with column maximum-wise (HLR% in case of class 1, Hb in class 2, Rsf® in class 3 and so on) are representative of class in the best manner.

6.1.6 Functional description of classes

It is worth noting that each of the classes (selected by factor analysis) contain a group of parameters that have a common physiological or pathological role.

- Class 1 is associated with erythropoietic activity: reticulocytes and reticulocyte indices
- Class 2 is related to parameters that provide information about the absence, presence or severity of anemia
- Class 3 is related to red cell indices and their derivatives obtained by mathematical formulas. They report on the type of anemia.
- Class 4 is associated with stimulation of erythropoiesis
- Class 5 is associated with chronic inflammatory disease
- Class 6 reports on platelets
- Class 7 is associated with iron status
- Class 8 is associated with microcytosis and anisocytosis.
- Class 9 reports on neutrophils.
- Class 10 reports on vitamin deficiency.
- Class 11 reports on liver disease.
- Class 12 reports of acute phase reactants not included in class 5.

6.1.7 Weekly erythropoietin dose and response to treatment

(See table 6)

			Response		Total	
EPO doses			No response	Response		Significance
	2000	Count	3	2	5	n.s.
		%	3.1%	2.5%	2.8%	
	3000	Count	3	2	5	n.s.
		%	3.1%	2.5%	2.8%	
	4000	Count	10	4	14	n.s.
		%	10.3%	5.1%	8.0%	
	6000	Count	14	11	25	n.s.
		%	14.4%	13.9%	14.2%	
	8000	Count	20	18	38	n.s.
		%	20.6%	22.8%	21.6%	
	12000	Count	18	15	33	n.s.
		%	18.6%	19.0%	18.8%	
	16000	Count	29	27	56	n.s.
		%	29.9%	34.2%	31.8%	
Total		Count	97	79	176	n.s.
		%	100.0%	100.0%	100.0%	

Table 6. No significant differences were found between the different doses of erythropoietin.

6.1.8 Univariate analysis of selected parameters comparing the groups of iron status and split by responders and no-responders

Table 7 shows statistical analyses of all parameters and their relations with the Iron Status classification and State of Response. In this analysis the iron status groups: ID, FID and IR are only taken into account. The 9 patients in group 3 have been excluded. ANOVA results have been compared with those of the Mann-Whitney test because the number of cases in each group is unbalanced. The Mann-Whitney, being nonparametric, is less affected by the imbalance. Parameters of the same class of those selected by factor analysis, behave similarly. Hb, RET# and sTfR show significant differences because they enter into the definition of response.

Parameters with column maximum are those that best represent their class as detailed above (material and methods).

Parameter	Iron Status	Response	Effect		Response via Mann Whitney U-Test
			Iron Status	Response status	
Rsf	**n.s.**	n.s.			
MCV	**n.s.**	n.s.			
MCH	**n.s.**	n.s.			
MSCV	n.s.	n.s.			
MRV	n.s.	n.s.			
HLR%	n.s.	n.s.			
HLR#	p=0.033	n.s.			
RET%	n.s.	n.s.			
RET#	n.s.	n.s.			
RPI	p=0.045	n.s.			
IRF	p=0.045	n.s.			
Hb	**p=0.000**	p=0.025	ID, FID < IR	Resp<nonresp	**p=0.0028**
HCt	**p=0.001**	p=0.029	ID < IR	Resp<nonresp	p=0.07
RBC	**p=0.002**	p=0.033	ID<IR	Resp<nonresp	**p=0.044**
Maf	**p=0.000**	p=0.039	ID < IR	Resp<nonresp	p=0.014
Plt	n.s.	n.s.			
PPV	n.s.	n.s.			
WBC	n.s.	n.s.			
TfRFindex	n.s.	n.s.			
sTfR	p=0.085	n.s.	ID<IR		
EPO	n.s.	p=0.016	FID: Resp<non resp IR: Resp>= non resp		**p=0.011**
ESR	n.s.	n.s.			
CRP	**p=0.000**	n.s.	FID > IR, ID		
IL6	**p=0.000**	n.s.	FID > IR,		
MCHC	n.s.	n.s.			**p=0.0007**
MPV	n.s.	n.s.			
RDW	p=0.031	n.s.	FID, ID <IR		

Ferritin	**p=0.000**	n.s.	trivial result	
Iron	**p=0.000**	n.s.	trivial result	
TSAT	**p=0.000**	n.s.	trivial result	
Vit. B12	n.s.	n.s.		
RBC Folate	n.s.	n.s.		
PDW	n.s.	n.s.		
ALT	n.s.	n.s.		
GGT	p=0.043	n.s.	ID, (FID) > IR	
RBC Ferr	n.s.	n.s.		
NE#	n.s.	n.s.	ID<IR	
NE%	n.s.	n.s.	ID<IR	
Transferrin	**p=0.000**	n.s.	trivial result	
AT III	n.s.	n.s.		

Table 7. Univariate two-factor ANOVA (Iron Status, Response) and Mann-Witney test. Significant differences for iron status are shown in column 3, for response status in column 4 and their effect in column 5. Parameters that enter into the definition of iron stages are logically associated with themselves; therefore we specify "trivial result".

6.1.9 Description of responses to therapy

Hereafter "case" means each result (6 per patient) for the referred parameter.

From 42 patients, 83 cases with EPO-treatment were considered as responses (38.6%). 132 cases (61.4%) were not responding. Among the responses, 40 (18.6% of 215) fulfilled 1 criterion of response, 36 cases (16.7%) 2 criteria, 5 cases (2.2%) 3 criteria and 2 cases (0.9%) 4 criteria. The distribution of responses according to the criteria is as follows:

- Criterion 1. - 63 cases. (Increase of Hb ≥ 0.5 g/dl in the next control and/or increased of Hb in the whole of the following two controls > 0.8 g/dL, under condition that both changes are positive)
- Criterion 2. - 48 cases (Achieve or maintain normal Hb for hemodialysis patients (male: 12 g/dl, female: 11 g/dl) under two conditions: Hb increase and Reticulocytes >50 x 10^9/L)
- Criterion 3. - 17 cases (Increase of sTfR > 20%, provided that Hb also increases)
- Criterion 4. -7 cases. (Increase of Ret#>40 x 10^9/L provided that Hb also increases)

6.1.10 Parameters predicting response

Table 8 shows the descriptive statistics and the response to combination therapy with iron and erythropoietin. We selected three variables (MCHC, EPO and Hb) with the highest levels of significance to determine their predictive value on response to therapy.

AUC of related ROC for these three parameters are respectively: 0.631 (95% CI: 0.54 - 0.70) for Hb, 0.612 (95% CI: 0.58 - 0.73) for EPO, and 0.662 (95% CI 0.57 – 0.73) for MCHC as shown in Figure 1, 2 and 3.

AUC of related ROC curves

	Non Response						Response						
	N	Mean	SD	Med	Min	Max	N	Mean	SD	Med	Min	Max	p
HLR %	78	0.795	0.372	0.803	0.117	2.24	66	0.897	0.332	0.895	0.256	1.57	0.076
HLR #	78	27.7	12.2	28.4	4.55	66.7	66	30.8	12.4	31.3	7.7	62	0.143
RPI	78	1.52	0.517	1.62	0.121	3.14	66	1.65	0.535	1.7	0.589	2.84	0.161
RET %	80	2.05	0.781	2.13	0.149	5.05	67	2.24	0.66	2.31	0.963	3.71	0.067
RET #	80	71.6	23.4	74.5	19.8	150	67	76.3	24.8	76.2	29	134	0.287
IRF	78	0.376	0.078	0.388	0.156	0.52	66	0.395	0.0622	0.396	0.225	0.52	0.226
Hb	**97**	**11.4**	**1.32**	**11.3**	**8.62**	**14.8**	**79**	**10.7**	**1.54**	**10.7**	**7.18**	**14.5**	**0.0028**
HCT	79	34.6	3.93	34.3	26.6	45	68	33.3	4.89	33	21.7	44.8	0.0752
RBC	**81**	**3540**	**385**	**3480**	**2750**	**4620**	**69**	**3410**	**502**	**3340**	**2130**	**4690**	**0.0442**
Maf	**97**	**11.1**	**1.51**	**11.3**	**7.86**	**14.4**	**79**	**10.4**	**1.69**	**10.5**	**6.54**	**13.8**	**0.0137**
VHDW	79	7.08	1.32	7.4	4.26	9.69	68	6.7	1.28	6.85	4.04	9.61	0.0505
RSf	78	0.11	0.006	0.11	0.0967	0.12	66	0.11	0.0076	0.11	0.094	0.134	0.744
VCM	97	97.4	4.63	97.5	83.8	109	79	97.4	5.11	98.1	85.8	109	0.891
MSCV	78	95.6	5.76	95.3	83.5	116	66	96.2	6.47	95.8	83.9	111	0.506
MRV	78	124	8.74	124	105	143	66	125	12.3	123	96.3	165	0.785
MCH	81	31.9	1.77	32.1	26.8	34.8	69	31.5	1.85	32	27.5	35.1	0.261
EPO	**97**	**11**	**11.9**	**8**	**1.8**	**78.6**	**79**	**18.4**	**27.6**	**10.1**	**1.1**	**200**	**0.0107**
TfR-F index	95	0.761	0.249	0.728	0.366	2.02	79	0.759	0.245	0.725	0.282	1.36	0.885
sTfR	95	1.85	0.495	1.8	1	3.2	79	1.82	0.512	1.8	0.8	3.1	0.752
Fibri-nogen	**96**	**539**	**128**	**526**	**284**	**993**	**79**	**498**	**110**	**487**	**264**	**836**	**0.0296**
ESR	97	44.2	29	35	8	125	78	40	27.9	32	3	142	0.309
CRP	97	1.89	5.5	0.69	0	38.6	79	1.33	2.93	0.64	0.11	24.3	0.552
IL-6	97	10.7	20.6	6.15	0.75	160	79	8.14	7.48	6.3	0.6	48.5	0.772
Ferritin	97	379	250	348	24.3	1940	79	344	211	320	33.2	1160	0.296
Plt	79	214	57	222	114	327	68	212	57.6	210	114	368	0.892
PPV	79	0.176	0.042	0.173	0.0919	0.27	68	0.178	0.0397	0.177	0.088	0.288	0.718
TfSat%	97	33.8	12.3	32.3	11.4	82.8	78	32.5	10.9	32.3	12.6	68.5	0.611
Iron	97	59.6	25.3	54.1	15.7	198	79	54.1	17.7	53.5	12.2	91.9	0.218
MCHC	**79**	**32.7**	**0.662**	**32.7**	**31.1**	**34.8**	**68**	**32.3**	**0.665**	**32.3**	**31**	**34**	**0.0007**
MPV	78	8.29	1.1	8.3	6.13	11.4	68	8.54	1.02	8.3	6.4	11	0.302
RDW	79	15.8	1.48	15.6	13.3	20.9	68	15.8	1.47	15.7	13.2	20.8	0.864
NE %	79	65.9	8.37	66.6	46.6	82.4	68	67.1	8.11	65.1	53.6	83.9	0.503
NE #	79	4.86	1.59	4.63	2.42	9.74	68	5	1.61	4.89	1.16	8.93	0.424
WBC	79	7.25	2.13	7.03	0	14.9	68	7.21	2.37	7.62	0	14.1	0.546
Vit B12	97	525	210	482	184	1160	79	527	199	498	191	1180	0.839
RBC Folate	96	691	804	318	107	3670	79	787	920	388	109	3500	0.38
PDW	79	16.7	0.612	16.7	15.6	18.5	68	16.6	0.52	16.6	15.6	17.9	0.516
ALT	96	13.5	9.99	12	1	56	79	15.8	13.5	13	1	73	0.22

GGt	96	30	38.5	16	4	201	79	33.1	39.1	19	5	210	0.286
RBC Ferr.	97	704	310	632	281	1950	79	698	309	583	230	2010	0.735
Transfer-rin	97	174	31.4	169	79.1	280	79	176	41.5	169	73.8	351	0.811
ATIII	95	102	15.6	103	68	149	78	103	19	104	65	149	0.724

Table 8. Descriptive statistics of all variables split by response o no response (from month to month). Significant differences have been highlighted.

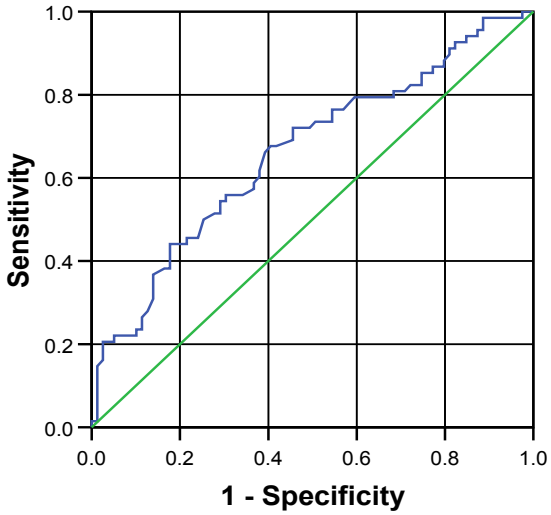

Fig. 1. MCHC AUC: 0.662 (0.573 – 0.75)

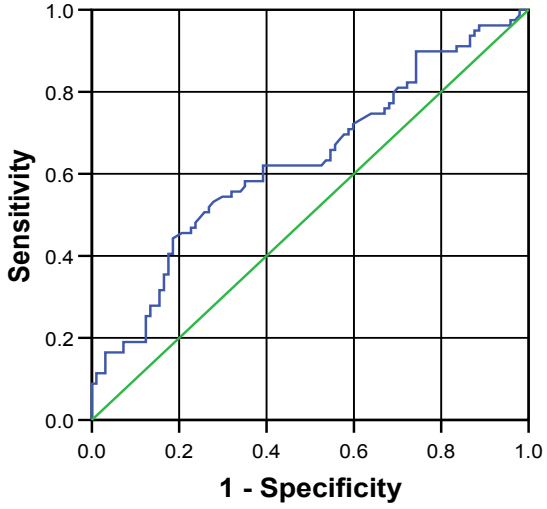

Fig. 2. HB AUC: 0.631 (0.548 – 0.714)

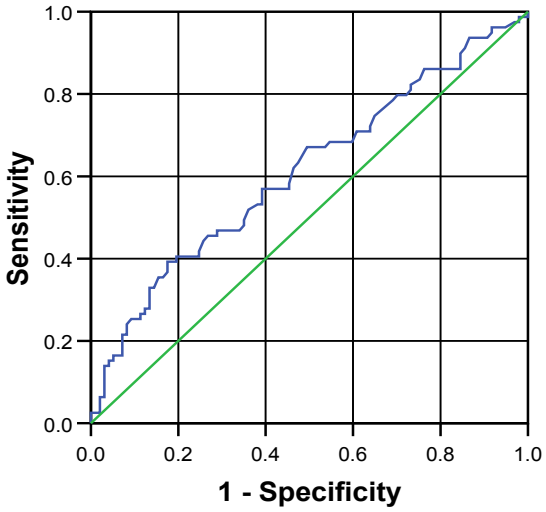

Fig. 3. EPO AUC: 0.612 (0.528 – 0.696)

6.1.11 ROC curves and AUC

The area under the curve of each of the three variables is too low to be practical in predicting response to treatment. So we tried to make a composite model with more prediction power.

6.1.12 Prediction model

Based on these results, several models to predict the response on the basis of parameters evaluated in this study have been calculated by logistic regression. The optimal model was chosen by achieving maximal AUC with a minimum of parameters and taking into account practical issues. The best three-parameter combination was RBC, EPO and MCHC (AUC=0.72). RBC, belonging to the same class as hemoglobin, slightly improves the AUC (+0.1). For this reason the model includes RBC, EPO and MCHC, instead of HB, EPO and MCHC. We could not find any other parameter able to increase the power of the predictive model. Figure 4 shows the ROC curve of this composite three parameter model. The AUC is 0.72 (%95 CI: 0.62 – 0.80). (Fig.4.)

The related formula is:

$$p_{resp} = \frac{1}{1 + exp(-(23.39 + 1.327 \cdot log(EPO) - 0.838 \cdot MCHC - 0.787 * \frac{RBC}{1000}))} \quad (2)$$

This model, being the best, has relative clinical utility because the AUC is not as high as would be desirable.

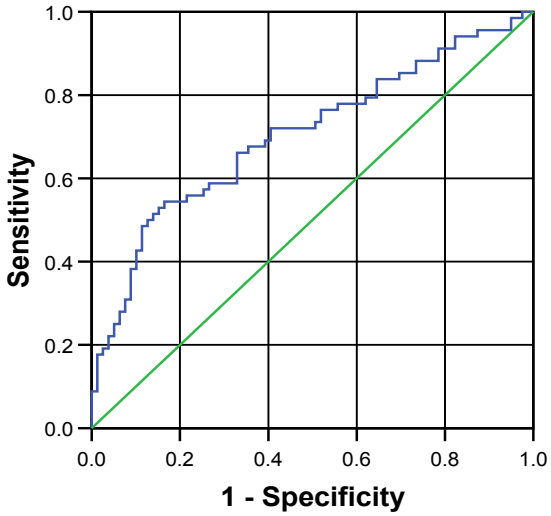

Fig. 4. ROC curve of the combination: RBC, EPO and MCHC (AUC: 0.72 (0.619 – 0.791)

7. Efficacy and safety

This section not only tries to verify the effectiveness and safety of treatment but also looks for correlations between hemoglobin and other variables. These correlations should not be interpreted as predictors of response, since hemoglobin is considered as a whole rather than month to month as in the previous section. Therefore we try to find a mathematical model to predict hemoglobin levels considering all the time of observation as a whole. For this purpose we have selected hemoglobin as a dependent variable. We have also selected explicative variables that measure the erythropoietic activity (reticulocytes and soluble transferrin receptor), indicators of iron status (serum iron, ferritin, transferrin and transferrin saturation), other nutritional factors (vitamin B12 and folic acid) and some indicators of acute or chronic inflammation (IL-6 and CRP) that can interfere with iron metabolism and erythropoiesis. (Table 9)

Statistical planning of this section is as follows:

- Descriptive statistics: means and frequencies
- Comparative evolution of hemoglobin in the 6 months: ANOVA and nonparametric
- Statistical comparison of patients with hemoglobin <11g/dl versus≥11g/dl: ANOVA, equality of variances and robust tests.
- Multiple linear regression stepwise forward (hemoglobin as dependent variable and all others as explicatory).
- Variables that did not fit the normal distribution have been replaced by their respective logarithms in parametric tests.

7.1 Descriptive statistics

Descriptive statistics are shown in table 9

	N	Min	Max	Mean	S.D.
EPO	245	1.75	465.12	23.5789	42.74901
IL-6	245	.60	375.39	15.3838	39.48357
CRP	243	.05	38.60	2.0386	4.86809
Hb	245	7.20	14,8	11.0665	1.54600
HCT	245	21.40	59.50	33.7563	4.62126
RETICULOCYTE	244	9.10	150.40	73.8156	24.98430
Fe	244	9.87	197.61	54.8609	22.59088
TRANSFERRIN	244	73.79	350.58	174.2232	34.89244
FERRITINE	244	33.20	2290.00	399.6719	292.31897
sTfR	244	.70	6.61	1.8598	.60920
B12	245	180.00	1455.00	541.3102	226.92288
FOLIC	245	1.60	200.00	27.5331	54.87474
FOLIC-INTRAE	244	85.50	3667.70	700.0311	770.10104
TSAT	244	8.54	87.09	31.9443	12.69501

Table 9. Descriptive statistics of variables used in this section

7.2 Hemoglobin stability

As shown in table 10, the means of hemoglobin vary from 10.9 to 11.3 during the six months follow-up period and remain always within the desired range. The minima of each month correspond to different patients, indicating at least a partial recovery of those who have held the minimum in any of the previous months. None of the patients exceeded 15 g / dl of hemoglobin as can be seen in the Box-Plot (Graph A1). This graph also shows the extreme values, which show only 6 out of a total of 245 samples. The evolution of individual patients who have had these outliers can be seen in Graph A-2.

MONTH	Mean (g/dl)	N	Standard deviation	Minimum	Maximum
1	10.9689	42	1.53798	7.2	14.3
2	11.1318	42	1.60113	7.2	14.2
3	10.9167	42	1.57231	7.2	14.9
4	10.9350	40	1.38408	7.4	14.6
5	11.1921	38	1.25383	8.2	13.5
6	11.2972	36	1.92628	8.1	14.8
Total	11.0665	245	1.54600	7.2	14.8

Table 10. Means of hemoglobin for each month during the six month follow-ups

7.3 Comparative statistics

7.3.1 Evolution of hemoglobin

Since hemoglobin fits normal distribution we compared the six month results globally using ANOVA with the result that no significant difference could be found ($F=0.394$, $p=0.853$).

To avoid possible errors due to sample size we have also used a nonparametric test (Kruskall-Wallis) with the same result: no significant difference (Chi square 1.473, p = 0.920). Median of hemoglobin and dispersion are represented as box plot on Fig. 5.

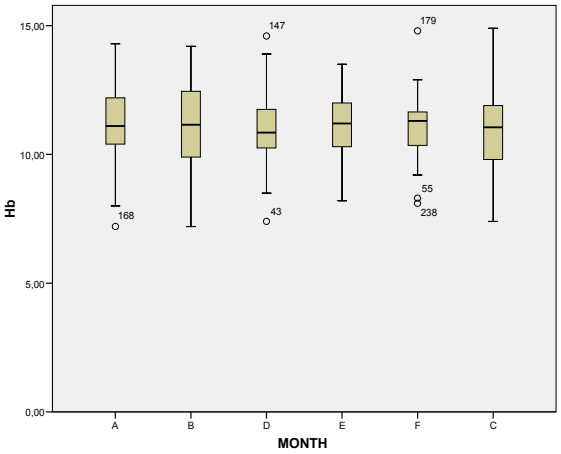

Fig. 5. Box-Plot of the evolution of Hb during the 6 months of dialysis. Outliers are identified with the sample number. Checking the table data we observe that all these extreme values come from different patients.

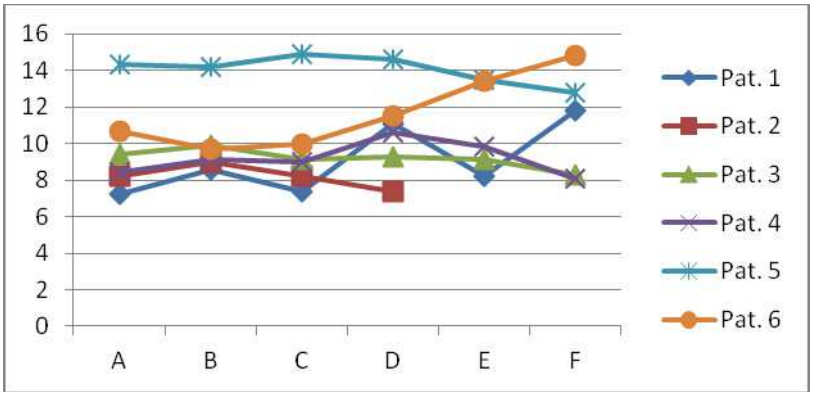

Fig. 6. Individual evolution of the six patients with extreme values. The vertical axis shows the values of hemoglobin (g / dl). The X axis shows the months of observation from A (first) to F (sixth). Patient 2 was excluded from the trial in the fourth month when the patient had a transplant. Patients 3 and 4 remained below 10g/dl of hemoglobin for most of the observation period. None of the patients exceeded 15 g/dl of hemoglobin.

Fig. 7 shows the mean hemoglobin concentration month by month and the error bars for 95% interval of confidence. The means remain in the range 10.9-11.3 g/dl. No significant differences with parametric methods (ANOVA, Student) and nonparametric (Kruskall – Wallis and Mann-Witney)

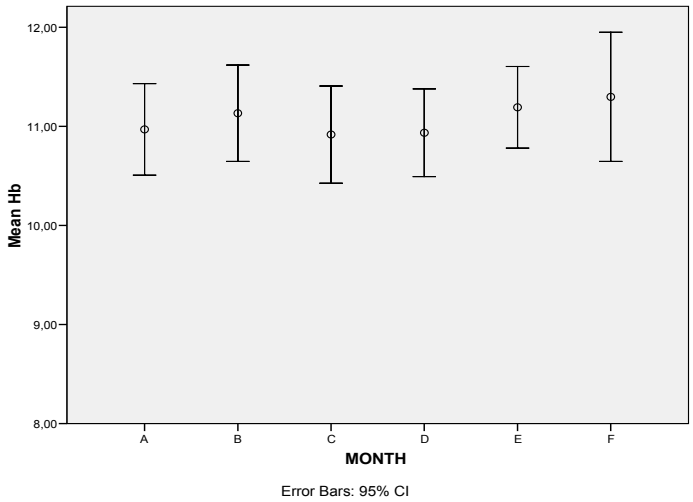

Fig. 7. Mean hemoglobin month by month. Error bars indicate confidence intervals of 95%

7.3.2 Patients with hemoglobin less than 11g/dl compared with those with hemoglobin ≥ 11g/dl

In this section we look for differences between the variables using a cutoff of 11 g/ dl of hemoglobin. Cases above and below this cutoff are almost balanced (n =117 (47.8%) <11g/dl) and n= 128 (52.2%≥ 11g/dl))

The homogenization of variances tested detected 5 variables with significant differences; therefore we applied robust tests to avoid false results due to bias. The table 11 shows the results.

	Statistic F	Sig.	Statistic Welch	Sig.
CRP	**4.629**	**.032**	**4.416**	**.037**
RET	1.597	.208	1.584	.209
Fe	**22.626**	**.000**	**23.323**	**.000**
TRANSF	.760	.384	.732	.393
FOLIC-INTRA	.441	.507	.435	.510
TSAT	**24.433**	**.000**	**24.665**	**.000**
Log EPO	**14.840**	**.000**	**14.479**	**.000**
Log IL-6	**9.970**	**.002**	**9.813**	**.002**
Log FERRITINE	.862	.354	.834	.362
Log sTfR	1.316	.252	1.296	.256
Log B12	.124	.725	.122	.727
Log FOLIC	.013	.908	.013	.909
SRTf/logFERR	.179	.672	.102	.751

Table 11. Homohenization of variances test using a hemoglobin cutoff of 11g/dl.

Table 12 shows the means and standard error of the variables split by an 11g/dl hemoglobin cut-off.

	Mean	Std. Error	Count	p (Welch)
EPO, Hb<11g/dl	32,316	5,245	117	
EPO, Hb>11	15,563	1,841	128	.000
iIL-6, Hb<11g/dl	21,233	4,972	117	
iIL-6, Hb>11	10,037	1,512	128	.002
RCP, Hb<11g/dl	2,742	,547	115	
RCP, Hb>11	1,406	,324	128	.037
Hb, Hb<11g/dl	9,850	,089	117	
Hb, Hb>11	12,178	,094	128	.000
RETIC, Hb<11g/dl	71,695	2,413	116	
RETIC, Hb>11	75,737	2,119	128	.209
Fe, Hb<11g/dl	47,936	1,661	116	
Fe, Hb>11	61,136	2,171	128	.000
TRANSFERRIN, Hb<11g/dl	176,270	3,843	116	
TRANSFERRIN, Hb>11	172,369	2,454	128	.393
FERRITINE, Hb<11g/dl	412,477	32,283	116	
FERRITINE, Hb>11	388,068	20,490	128	.362
sRTf, Hb<11Hb/dl	1,834	,065	116	
sRTf, Hb>11	1,883	,045	128	.256
B12, Hb<11g/dl	543,487	23,179	117	
B12, Hb>11	539,320	18,008	128	.727
FOLIC, Hb<11g/dl	30,862	5,583	117	
FOLIC, Hb>11	24,491	4,362	128	.909
FOLIC-INTRA, Hb<11g/dl	734,179	77,436	117	
FOLIC-INTRA, Hb>11	668,572	62,481	127	.510
sRTf:logF, Hb<11g/dl	,755	,028	116	
sRTf:logF, Hb>11	,761	,021	128	.751
TSAT, Hb<11g/dl	27,914	1,068	116	
TSAT, Hb>11	35,596	1,119	128	.000

Table 12. Means and standard error of the variables split by an 11g/dl hemoglobin cut-off.

7.3.3 Multiple linear regression

Since there is no significant difference in monthly hemoglobin we handle all the data together. The dependent variable is hemoglobin and the rest explicative. The hematocrit was excluded because it is obviously closely related to hemoglobin. For variables that do not fit the normal distribution we made a logarithmic transformation. The method used is forward stepwise multiple linear regression. In the resulting model only three variables remain: serum iron, log soluble transferrin receptor and log EPO. Note that the correlation coefficient of the latter is negative.

Model		Not standardized coefficients		Standardized coefficients	t	Sig.
Steps		B	Standard error	Beta	zero-order	Partial
1	(constant)	10.109	.251		40.321	.000
	Fe	.018	.004	.259	4.179	.000
2	(constant)	9.267	.334		27.765	.000
	Fe	.021	.004	.307	4.960	.000
	Log sRTf	2.672	.721	.229	3.705	.000
3	(constant) (Intercept)	10.396	.413		25.167	.000
	Fe	.018	.004	.261	4.301	.000
	Log sRTf	3.603	.728	.309	4.952	.000
	Log EPO	-1.053	.241	-.274	-4.363	.000

Dependent variable: Hb

Table 13. Forward step-wise multiple regression model. (Dependent variable hemoglobin)

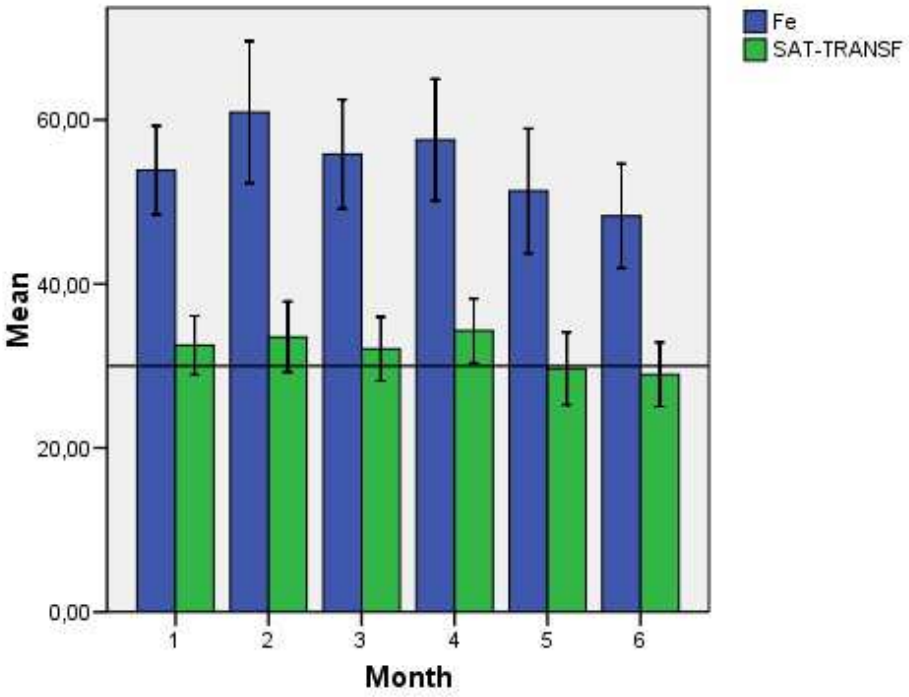

Fig. 8. Serum iron (mg/dl) and transferrin saturation (%) (TSAT). The central horizontal line plots the normal transferrin saturation of 30%. All means are above this line except for the sixth month. Nevertheless the latter is 28.6%, well above 20%. Error bars show the confidence interval 95%.

8. Iron, transferrin and saturation

Fig. 8. shows the serum iron and transferrin saturation for each of the six months of observation. The error bars show confidence intervals of 95%. All media, with their respective confidence intervals are in the field of 30% saturation of transferrin, enough for an adequate iron supply to erythroblasts. However, some individual values are below those desirable. Specifically, 33 cases (14.2%) have transferrin saturation less than 20%.

8.1 Ferritin

Descriptive statistic for ferritin split by months is shown in table 14.

	Mean	Std. Dev.	Std. Error	Count	Minimum	Maximum
FERRITIN. Total	399.672	292.319	18.714	244	33.200	2290.000
FERRITIN. A	425.107	325.013	48.450	45	39.900	1952.000
FERRITIN. B	378.284	249.318	37.586	44	33.200	1314.900
FERRITIN. C	416.134	247.962	38.725	41	42.600	1159.100
FERRITIN. D	376.029	317.108	50.139	40	49.400	1940.000
FERRITIN. E	371.563	219.388	35.589	38	40.000	972.400
FERRITIN. F	431.210	382.965	63.827	36	109.700	2290.000

Table 14. Evolution of ferritin during the observation period. Consecutive months are represented by A to F.

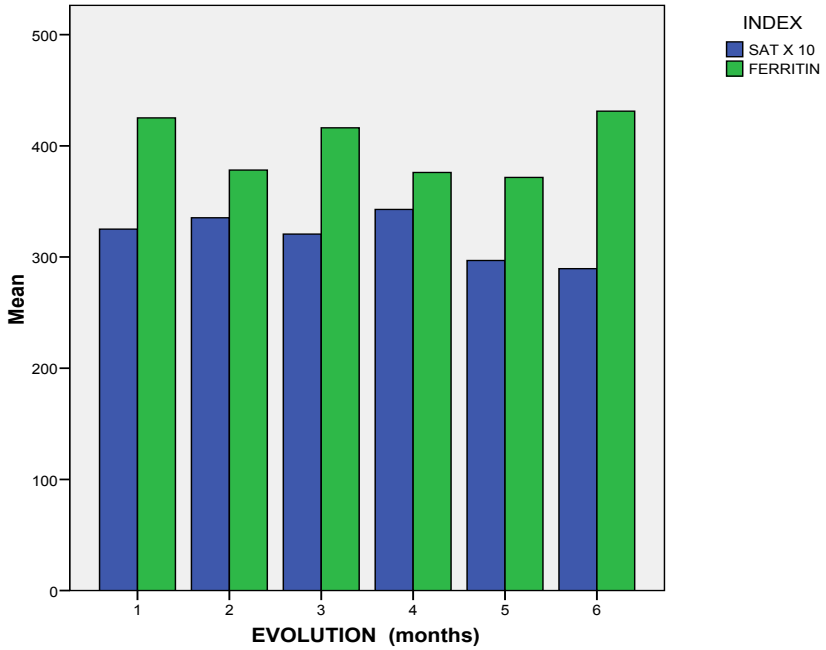

Fig. 9. Mean ferritin (green bars) and transferrin saturation (TSAT) (blue bars). The latter has been multiplied by 10 to fit in the graph better.

Mean ferritin is above 300 ng/ml in all cases. This does not necessarily mean that iron stores are adequate, as in renal failure and in other chronic diseases ferritin does not measure iron stores accurately. In 40 cases, the ferritin is less than 100 ng/ ml. In one case ferritin is above 2.000 ng/ dl and in six cases over 1.000. The standard deviation is high because ferritin does not fit a normal distribution.

8.2 Soluble transferrin receptor and soluble transferrin receptor/log ferritin index

Fig. 10. shows the evolution of soluble Transferrin Receptor

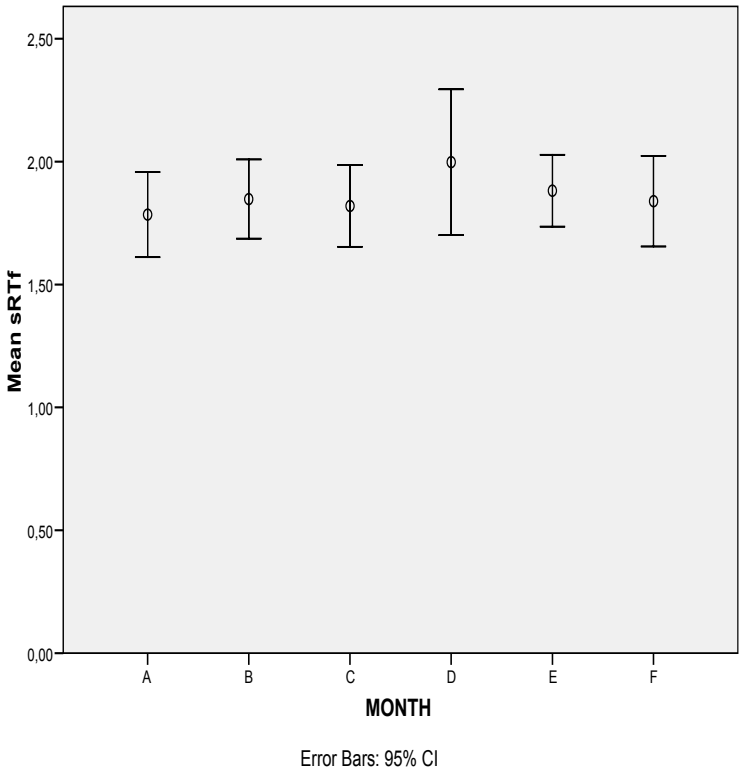

Fig. 10. Evolution of soluble Receptor of Transferrin

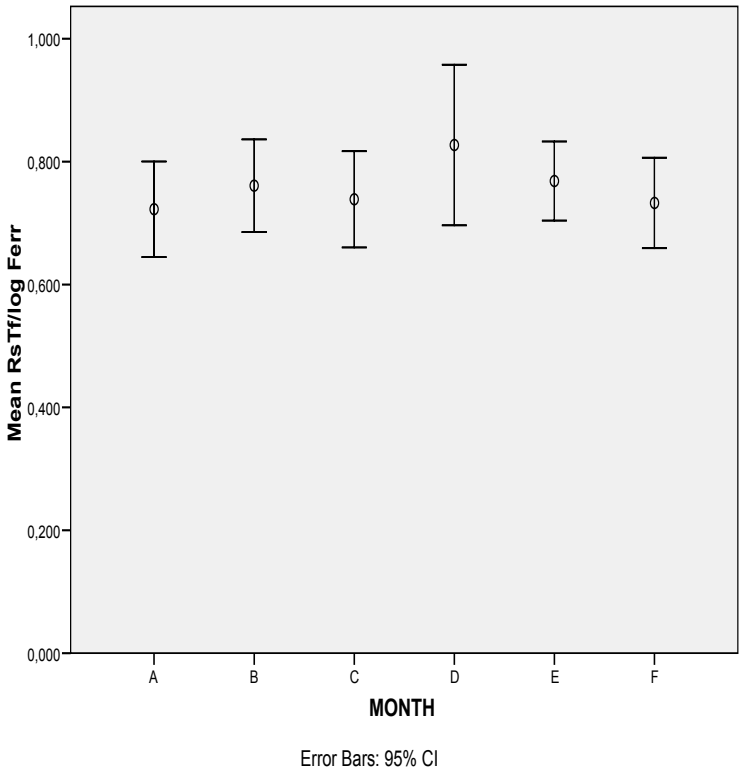

Fig. 11. Evolution of sTfR / Log Ferritin index.

9. Conclusions

9.1 Predictors of response to therapy

ANOVA shows significant differences between responders and non-responders for hemoglobin, hematocrit, red blood cells, erythropoietin, MCHC and Maf). Nevertheless only Hemoglobin (or red blood cells), EPO and MCHC were found to be the best predictors of response by multiple regression. However, the AUC of the ROC curves of each of these parameters separately is low and therefore its clinical utility is weak. The combination of red blood cells, erythropoietin and MCHC results in a mathematical model that significantly improves the predictive value of each of these variables, with an AUC of 0.72. (95% Confidence Interval: 0.62-0.80). The mathematical model is:

$$p_{resp} = \frac{1}{1 + \exp(-(23.39 + 1.327 \cdot \log(EPO) - 0.838 \cdot MCHC - 0.787 * \frac{RBC}{1000}))} \quad (3)$$

This model does not improve with the inclusion of additional variables and has the advantage that it only includes routine analytical data, without sophisticated tests. The statistical procedure that we have described (factorial analysis, data reduction, logistic regression and ROC curves) may be a useful tool for future studies with more patients and longer observation which may improve the accuracy of prediction of response.

9.2 Efficacy and safety

9.2.1 Hemoglobin

The mean hemoglobin level for the whole period of observation is X = 11.07g/dl; (SD 1.546; n = 245). When comparing the mean hemoglobin of each month, there are no significant differences and the small differences that are shown have no clinical significance. Therefore, the hemoglobin remains stable throughout the observation period with a mean within the desired range. Only six cases have been detected with extreme values (Box-Plot). None of the patients exceeded hemoglobin 15g/dl, and only two patients had hemoglobin levels persistently below 10g/dl. The cases above and below 11g/dl of hemoglobin are almost balanced (n =117 (47.8%) <11g/dl) and n= 128 (52.2%≥ 11g/dl)). These data confirm that this protocol maintains stable hemoglobin levels in the planned target. Achieved hemoglobin levels are somewhat lower than those recommended in the latest revision of K-DOKI clinical guidelines (KDOQI 2007), but conform to those recommended by others (Coyne 2010)(Akizawa et al. 2008).

When applying a cutoff of 11g/dl of hemoglobin there are significant differences above and below this value for the following parameters: C-reactive protein, serum iron, transferrin saturation, erythropoietin, and interleukin 6 (univariate analysis).

The stepwise multiple regression (with hemoglobin as the dependent variable), allowed us to develop a model in which, in the final step, only three parameters remain: serum iron, transferrin soluble receptor and erythropoietin (the latter with a negative beta coefficient). In this model the constant is 10.396 and the standardized beta coefficients: iron (0.261), log soluble transferrin receptor (0.309) and log erythropoietin (-0.274).

9.2.2 Iron

Means and standard deviations of the variables that establish the status of iron are as follows: serum iron 54.86 ± 22.59, transferrin 174.22 ± 34.89, transferrin saturation 31.94 % ± 12.70, ferritin 399.67 ± 292.31 and soluble transferrin receptor 1.86 ± 0.61. No significant differences were detected for these variables when comparing different months of observation.

Of these variables only the mean serum iron is significantly different ($p < 0.001$) in cases with hemoglobin less than 11g/dl (x = 47.93) than in cases with more than 11g/dl hemoglobin (x = 61.14).

Although the mean transferrin saturation of each month is in the desired range, 33 cases (14.2%) had a transferrin saturation of less than 20%.

The mean index of transferrin soluble receptor / log ferritin is 0.7579 (above the 0.6 cut-off for iron deficiency) and the frequency distribution shows that 73.8% of the cases are above 0.6. These cases are likely to have iron deficiency, according to.(Chen, Hung, and Tarng 2006b)

Although the mean transferrin soluble receptor is 1.856 mg/L (less than 2 mg/L cut-off for iron deficiency), 37.2% had levels above 2 mg/L. These cases are likely to have iron deficiency, according to (Chang et al. 2007)

Therefore some patients have had iron deficiency at least once during the observation period in an amount ranging between 14.2% and 73.8%, depending on whether we use a less or more sensitive indicator.

Therefore, the iron dose should be increased slightly to maintain deposits and availability of iron in adequate quantity for normal erythropoiesis.

10. Discussion

Predictors of response reported so far are : albumin (Agarwal, Davis, and Smith 2008), clinical factors (Bamgbola, Kaskel, and Coco 2009) , (Bistrian and Carey 2000), protocol adherence(Chan et al. 2009), soluble receptor of transferrin (Chang et al. 2007; Chen, Hung, and Tarng 2006a), low iron stores (low ferritin and low transferrin saturation), malnutrition, hyperparathyroidism, and high-turnover bone disease (Kalantar-Zadeh et al. 2009).

In our model hypo-response to combination therapy with erythropoietin and iron occurs in patients with higher hemoglobin (11.4 in non-responders, versus 10.7 in responders), lower serum erythropoietin (11 in non-responders versus 18.4 in responders), higher MCHC (32.7 in non-responders versus 32.3 in responders). To our knowledge this is the first time that MCHC is included in a model to predict response to combination therapy with erythropoietin and iron in anemia in hemodialysis patients.

The difference in mean MCHC between responders and non-responders is marginal, but statistically significant.

It may seem odd that a higher level of hemoglobin is a predictor of no response, but can be explained by the fact that a level equal to or greater than 12g/dl hemoglobin involves a reduction of 50% of the dose of erythropoietin, according to our protocol. In a patient with chronic renal failure on hemodialysis serum erythropoietin level depends largely on that which is administered as a drug because the endogenous secretion is strongly suppressed. Of the three variables in the model, the serum erythropoietin is the most easily adjustable in the short term, since it can be achieved by increasing the dose. Although our protocol has been shown effective in maintaining a stable mean hemoglobin level of 11g/dl, it is insufficient to achieve the target of 12 g/ dl recommended in the latest revision of the K-DOKI guideline (KDOQI 2007). This trial had been designed before the amendment was published. Taking this into account it would be necessary to review the criteria for erythropoietin dose reduction conditioned by the level of hemoglobin. The importance of maintaining stable hemoglobin levels has been previously highlighted (Zaoui et al. 2011)

(Mathieu et al. 2008). The last authors describe a cohort of patients in Switzerland with stable hemoglobin levels, although slightly higher (mean hemoglobin 11.9 g/dl), than those presented in this paper.

Hemoglobin levels above 13g/dl, although recommended in some instances, have been associated with increased mortality. Recently, this increased mortality has been attributed to thrombocytosis induced by iron deficiency and therefore appropriate doses of intravenous iron could counteract the adverse effect of high doses of erythropoietin (Kalantar-Zadeh et al. 2009). However thrombocytosis associated with erythropoietin treatment to achieve high levels of hemoglobin can be attributed not only to iron deficiency but also to high doses of erythropoietin (Vaziri 2009).These data should be confirmed before raising the target hemoglobin level.

In the last decade hematological cell counters provide new erythrocyte and reticulocyte cell indices that are useful for the differential diagnosis of anemia. Some of these indices could be useful to establish the status of iron metabolism in the anemia of patients with advanced renal disease on hemodialysis and to predict response to treatment. If confirmed, the CBC would provide data that would spare more expensive laboratory tests. Unexpectedly, the best predictor in terms of this work is MCHC, an erythrocyte index of routine, instead of the most innovative. In general the red cell indices were used to assess the iron status rather than as predictors of response. Each author recommends the ones they have examined, but there is no consensus because the results are variable and generally not confirmed. Some of these indices are: reticulocyte hemoglobin equivalent (Ret He) (Brugnara, Schiller, and Moran 2006), serum soluble transferrin receptor (Chang et al. 2007), transferrin receptor-ferritin index (Chen, Hung, and Tarng 2006a), Soluble transferrin receptor and sTfR-F index (Choi 2008), soluble transferrin receptor and mean corpuscular hemoglobin (Choi 2007), as we have discussed in more detail in the introduction.

The serum iron is an infrequently used index of iron deficiency. In general, low serum iron is unreliable as a marker of iron deficiency because of the wide variation throughout the day. Transferrin saturation is generally preferred because in the case of iron deficiency, serum transferrin increases while serum iron decreased; but this is not the case in chronic renal failure in which both values decrease in parallel and thus the transferrin saturation shows little variation. In our patients the standard deviation of serum iron, expressed in percentage is 41%, while the same variation for transferrin is only 20%. Despite such a high dispersion, serum iron enters the multiple regression model, while transferrin saturation does not. Moreover serum iron is the only indicator of iron status with significant mean difference when applying a cutoff of 11g/dl hemoglobin. Therefore, although the serum iron is not a good indicator of iron status at the individual level, it may be useful in clinical trials.

Therefore we believe that serum iron should be taken into account in the case of anemia of chronic kidney disease.

It is not easy to compare our results with others because the treatment protocol and response criteria are different. Moreover there are no randomized clinical trials designed to look for safety of treatment with intravenous iron in the long term and it is unclear which of the two trends are correct: Increasing the dose of iron to save erythropoietin, or increasing

the dose of erythropoietin to diminish the possible adverse effects of iron (Coyne 2010). The dilemma is important because high doses of erythropoietin also produce adverse effects. The most clear demonstration that increasing the number of patients treated with iron results in a decrease in erythropoietin dose in daily clinical practice (not only in clinical trials) are shown in the data published by (Hasegawa et al. 2011) . Although the balance between iron and erythropoietin therapy is still subject to discussion, it is clear that the combination therapy with both drugs reduce morbidity, mortality, hospital admissions and costs (Knight et al. 2010).

In the present trial initial iron dose is 100 mg three times a week intravenously at the end of dialysis. The initial dose of erythropoietin is 40 U / K (2,800 U for a person of average weight 70K) three times a week.

In a longitudinal study with data collected in Japan between 1999 and 2006 weekly doses of iron (160 mg / month) and erythropoietin (5531 U / week) are lower, but it must also be considered that the mean hemoglobin level was lower (10.4 g / dl)(Akizawa et al. 2008).

(Charytan et al. 2001) using a schedule similar to ours (iron sucrose injection administered as 1,000 mg in 10 divided doses by IV push) concludes that this therapy is safe and effective for the treatment of iron deficiency in patients with dialysis-associated anemia.

Iron Sucrose is the most widely iron used in patients with chronic kidney disease, but iron dextran of low molecular weight is equally effective (Atalay et al. 2011).

Several trials compare the costs and effectiveness of treatment of classical formulations of iron and erythropoietin with the new ones (some not yet approved for kidney patients) (Bhandari 2011)(Cuesta Grueso et al. 2010). Although the efficacy of these new drugs is similar to the former ones, there is no conclusive evidence to justify the increased cost of new formulations of iron and erythropoietin. In one study ferric carboxymaltose was effective in increasing the mean level of hemoglobin from 9.1 to 10.3 g / dl, but at the expense of 7.4% of serious adverse effects (Covic and Mircescu 2010). Of the new iron preparations only isomaltose iron at doses between 600 and 1,000 mg is cost effective compared with iron dextran (Bhandari 2011).

A comparative study was carried out: one branch with intravenous iron sucrose and the other with oral iron succinate. Both showed a positive response but the first one demonstrated better results. Although erythropoietin dose was lower in the intravenous iron branch, the total cost of both drugs together was higher. Gastrointestinal adverse events were higher in the oral branch (Li and Wang 2008).

Several reports have found that the detection and early treatment of anemia of chronic kidney disease with erythropoiesis-stimulating agents improves the quality of life, including cognitive and physical performances: memory, sustain attention, energy level, work capacity, aerobic capacity, and immune function (Fishbane 2010).

The alternative to transfusions most often used in patients with anemia of chronic kidney disease is erythropoietin and intravenous iron. Combination therapy is even more important in patients with anemia and cardio renal disease (Silverberg et al. 2009; Silverberg 2011; Silverberg 2010; Silverberg et al. 2010).

More than 90 % of end renal disease patients maintained on dialysis respond to traditional recombinant human erythropoietin (rHU EPO) or to EPO analogues (biosimilars, biogenerics) (Schmid and Schiffl 2010).

The therapeutic dose of EPO are higher than normal endogenous rates of EPO production in healthy subjects, and the amount of IV-iron given may be greater than the amount needed for normal erythropoiesis. This increase in erythropoietin and iron needs may be due to chronic inflammation and vitamin C deficiency, present in many patients with chronic kidney disease (Handelman 2007). The comparison between clinical trials is difficult not only for the selection of patients and the different doses of erythropoietin and iron, but also for the management of chronic inflammation and replacement of folic acid and vitamins B12 and C, data that are not always specified.

The doses of erythropoietin should be considered together with hemoglobin levels and resistance to treatment and comorbidities. In patients with chronic renal failure, without mention of heart disease, adverse effects are associated with low levels of hemoglobin (<9.5 g/ dl) despite high doses of erythropoietin (Bradbury et al. 2009), or with therapeutic targets of high hemoglobin levels (13.5 to 15 g / dl)(Belonje, de Boer, and Voors 2008) . In patients with simultaneous kidney and heart failure, erythropoietin improves cardiac function, even if it fails to raise the level of hemoglobin (Belonje, de Boer, and Voors 2008) (Pappas et al. 2008). To understand the role of erythropoietin it should be noted that its physiological effects are beyond the stimulation of erythropoiesis (Diskin et al. 2008).

Darbepoetin is as effective as epoetin alpha, but at a higher cost (Cuesta Grueso et al. 2010).

A possible way to save costs would be to give biosimilar erythropoietin rather than original. A clinical trial has demonstrated the equivalence of Eprex with GerEpo (biogeneric) in the short term, but the short duration of the trial (12 weeks) did not provide any conclusions about safety (Goh et al. 2007).

The current status of new anti-anemic drugs, including their indications and license in Europe and USA can be found in (Macdougall and Ashenden 2009). New intravenous iron preparations seem promising (Ferumoxytol), as well as erythropoietin with prolonged effect (CERA) and peptides that stimulate erythropoiesis by a mechanism different from erythropoietin (Hematide). Clinical trials are needed to establish the usefulness of these new drugs in patients with chronic kidney disease.

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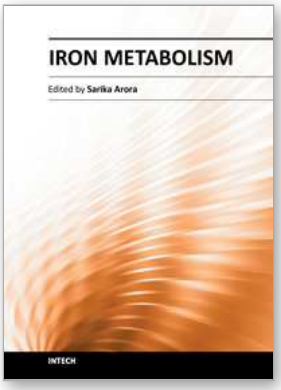

Iron Metabolism

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Iron has various functions in the body, including the metabolism of oxygen in a variety of biochemical processes. Iron, as either heme or in its "nonheme" form, plays an important role in key reactions of DNA synthesis and energy production. However, low solubility of iron in body fluids and the ability to form toxic hydroxyl radicals in presence of oxygen make iron uptake, use and storage a serious challenge. The discovery of new metal transporters, receptors and peptides and as well as the discovery of new cross-interactions between known proteins are now leading to a breakthrough in the understanding of systemic iron metabolism. The objective of this book is to review and summarize recent developments in our understanding of iron transport and storage in living systems.

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