Resistance to recombinant human erythropoietin therapy in haemodialysis patients*

*Resistência à terapêutica com eritropoietina humana recombinante em doentes hemodializados**

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Abstract

To better clarify the mechanism of resistance to recombinant human erythropoietin (rhEPO) therapy in haemodialysis patients, we studied systemic changes associated with this resistence in haemodialysis patients under rhEPO therapies, with particular interest on inflammation, leukocyte activation, iron status, oxidative stress and erythrocyte damage. We studied 63 chronic kidney disease (CKD) patients under haemodialysis and rhEPO therapies (32 responders and 31 non-responders to rhEPO therapy) and 26 healthy volunteers. In 20 of the CKD patients (10 responders and 10 non-responders to rhEPO therapy), blood samples were also collected immediately after dialysis to study the effect of the haemodialysis procedure.

When compared to controls, haemodialysis patients presented lymphopenia, which results, at least in part, from a decrease in total circulating CD3+ T-lymphocytes and affect both the CD4+ and the CD8+ T-cell subsets. These lymphocytes presented markers of enhanced continuous activation state and enhanced ability to produce Th1 related cytokines. Furthermore, haemodialysis patients presented raised markers of an inflammatory process, and of an enhanced neutrophil activation, as showed by the high serum levels of elastase. Concerning to iron status, patients showed increased ferritin and prohepcidin serum levels, and a decrease in transferrin. Furthermore, some changes were observed in erythrocyte membrane protein composition and in band 3 profile, being the decrease in spectrin the most significant change. Higher plasma levels of total antioxidant status (TAS), lipidic peroxidation (TBA) and TBA/TAS ratio were also found.

Resumo

Com o objectivo de clarificar o mecanismo de resistência à terapêutica com eritropoietina humana recombinante (EPOhr) em doentes hemodializados, estudamos alterações a ela associada, com particular interesse na inflamação, activação leucocitária, ciclo do ferro, *stress* oxidativo e lesão eritrocitária. Foram estudados 63 doentes renais crónicos (DRC) em hemodiálise e terapêutica com EPOhr (32 respondedores e 31 não respondedores à terapêutica com EPOhr) e 26 indivíduos controlo. Em 20 dos DRC (10 respondedores e 10 não respondedores à terapêutica com EPOhr), foram também colhidas amostras de sangue imediatamente após a hemodiálise para estudar os efeitos deste procedimento.

Quando comparados com os controlos, os DRC em hemodiálise apresentaram linfocitopenia, resultante de uma diminuição da contagem dos linfócitos CD3+ e em que ambos os subtipos de linfócitos T CD4+ e CD8+ se encontravam diminuídos. Estes linfócitos apresentavam marcadores celulares de estimulação continuada aumentados e capacidade aumentada de produzir citoquinas associadas com a resposta imune do tipo Th1. Adicionalmente, estes doentes apresentavam marcadores inflamatórios, e aumento na activação dos neutrófilos. No que se refere ao estudo do ciclo do ferro, os DRC apresentavam aumento dos níveis séricos de ferritina e prohepcidina, e uma diminuição na transferrina. Adicionalmente, foram também encontradas alterações na composição proteica da membrana dos eritrócitos e no perfil da banda 3, sendo a diminuição da espectrina a alteração mais significativa. Aumento na capacidade

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When comparing the two groups of patients, we found that non-responders presented a significant decrease in total lymphocyte and CD4+ T-cell counts, a more accentuated inflammatory process and indicators of enhanced neutrophil activation. No significant differences were found in serum iron status markers between the two groups of patients, except for the soluble transferrin receptor, which was higher among non-responders. Prohepcidin serum levels were significantly lower in non-responders, but were higher than those in the control group. An accentuated decrease in erythrocyte membrane spectrin, alterations in band 3 profile [decrease in band 3 proteolytic fragments (Pfrag) and in Pfrag/band 3 monomer ratio], and a trend to higher values of membrane bound haemoglobin were also found in non-responders patients.

In conclusion, although the etiology of resistance to rhEPO therapy is still unknown, our work confirms that inflammation seems to have an important role in its pathophysiology. We also showed that resistance to rhEPO therapy is associated with "functional" iron deficiency, lymphopenia and CD4+ lymphopenia, higher elastase plasma levels, increased interleukin-7 serum levels, and alterations in erythrocyte membrane protein structure and in band 3 profile. Further studies are needed to understand the rise in inflammation with the associated need in higher doses of rhEPO and the reduced iron availability.

Key-Words: *Resistance to recombinant human erythropoietin, haemodialysis, erythropoietin, Inflammation, iron status, leukocyte activation.* ⏮

Quando comparamos os dois grupos de DRC, verificamos que os não respondedores à terapêutica com EPOhr apresentavam diminuição no número total de linfócitos e nos linfócitos T CD4+, e aumento nos marcadores inflamatórios e na activação dos neutrófilos. Não encontramos diferenças significativas nos parâmetros relacionados com o ciclo do ferro, com excepção do receptor solúvel da transferrina, que se encontrava aumentado nos não respondedores. Os níveis séricos de prohepcidina encontravam-se diminuídos nos não respondedores; no entanto, encontravam-se mais elevados que no grupo controlo. Diminuição acentuada no conteúdo em espectrina, alterações no perfil de banda 3 [diminuição fragmentos proteolíticos da banda 3 (Pfrag) e na razão Pfrag/monómero de banda 3], e uma tendência para valores aumentados de hemoglobina ligada à membrana foram também encontrados nos DRC não respondedores à terapêutica com EPOhr.

Em conclusão, apesar da etiologia à resistência à terapêutica com EPOhr não estar ainda completamente esclarecida os nossos resultados confirmam que a inflamação parece ter um papel muito importante. Encontramos também relação entre resistência è terapêutica com EPOhr com défice funcional em ferro, linfocitopenia e linfocitopenia T CD4+, níveis plasmáticos aumentados de elastase, níveis séricos aumentados de interleucina-7, e alterações na estrutura das proteínas de membrana do eritrócito e no perfil de banda 3. Mais estudos serão necessários para se entender a associação entre a inflamação, e resistência à terapêutica com EPOhr e diminuição na disponibilidade em ferro.

Palavras-chave: Eritropoietina humana recombinante, hemodiálise, inflamação, ciclo do ferro, activação leucocitária. ⏮

Introduction

Anaemia is a common complication that contributes to the burden of chronic kidney disease (CKD) under haemodialysis. It has also a negative impact on cardiovascular system, cognitive function, exercise capacity and quality of life, resulting in a significant mortality and morbidity in these patients (1-3).

This anaemia can result from a decreased bone marrow production of red blood cells (RBC), due to the inability of the failing kidneys to secrete erythropoietin (EPO) (4); to the accumulation of uremic toxins, or to excessive toxic storage of aluminum in the bone marrow (5). Blood loss and premature RBC destruction are also frequently associated with anaemia in CKD stage 5 patients (6-9).

Until 18 years ago, the treatment of anaemia of CKD was blood transfusion; since there, the management of this anaemia has been improved by the introduction of rhEPO therapy. This therapy allowed a significant reduction in the associated adverse effects of anaemia and improved patient's quality of life.

Until recently, only two forms of rhEPO were commercially available for clinical use, epoetin alfa (Eprex®) and epoetin beta (NeoRecormon®), which are structurally very similar. Subcutaneous or intravenous administration of these epoietins can be used once daily, two or three times a week, depending on the clinical status of the patient. The rhEPO dose is adjusted to achieve target haemoglobin levels between 11 and 12 g/dL (10).

Several different types of erythropoietic agents have been developed recently, namely darbopoietin alfa (Aranesp®), which contains five N-linked glycosylation sites (only three for EPO), resulting in a longer plasma half-life. Treatment of anaemia of

CKD with darbopoietin alfa allows a less frequent administration, as compared to the others epoetins (11).

In spite of the benefits to CKD patients, we must refer that the use of rhEPO has been associated with some complications, namely, hypertension, thrombosis, allergic reactions and pure red cell aplasia (12,13).

The introduction of rhEPO therapy in the early 1990s for treatment of anaemia of CKD has led to a significant reduction in anaemia and to an improvement in patients' quality of life (14-17). There is, however, a marked variability in the sensitivity to rhEPO, with up to 10-fold variability in dose requirements to achieve correction of the anaemia. Furthermore, around 5-10% of the patients show a marked resistance to rhEPO therapy (15, 17-20). The European Best Practice Guidelines define resistance to rhEPO therapy as a failure to achieve target haemoglobin levels (between 11 and 12 g/dL) with maintained doses of rhEPO higher than 300 IU/Kg/week of epoetin or of higher doses than 1.5 mg/Kg/week of darbopoietin-alfa (10).

The reasons for this variability in rhEPO response are unclear (17, 20-23). There are a lot of conditions reported as associated with rhEPO resistance, namely, inflammation, oxidative stress and iron deficiency, as major causes (20-23), and blood loss, hyperparathyroidism, aluminium toxicity and vitamin B12 or folate deficiency, as minor causes. However, exclusion of these factors does not eliminate the marked variability in sensitivity to rhEPO (24). Furthermore, most of the published studies investigated only, a few number of parameters and included a very limited number of patients. Therapeutic and haemodialysis procedure have changed in the last few years. The value of those changes concerning the haemodialysis membrane biocompatibility and vascular access, need also to be clarified in what concerns to resistance to rhEPO therapy.

The aim of this study is to better clarify the mechanisms of resistance to rhEPO therapy. We studied systemic changes associated with resistance to rhEPO therapy in haemodialysis patients (responders and non-responders) with particular interest on inflammation, leukocyte activation, iron status, oxidative stress and erythrocyte damage.

Materials and Methods

Subjects

We performed a cross-sectional study by evaluation 63 CKD patients under hemodialysis (36 males, 27 females; mean age ± SD of 62.1 ± 15.7 years) and rhEPO treatment. The CKD patients included 32 responders and 31 non-responders to rhEPO therapy. Classification of the patients, as responders or non-responders, was performed in accordance with the European Best Practice Guidelines (10), which defines resistance to rhEPO as a failure to achieve target hemoglobin levels (between 11 and 12 g/dL) with maintained doses of rhEPO higher than 300 IU/Kg/week of epoetin or 1.5 mg/Kg/week of darbopoietin-a. The rhEPO maintenance dose for responder's patients was 89.65 ± 57.62 U/Kg/week and for non-responders was 572.99 ± 193.84 U/Kg/week. The two groups of patients were matched for age, gender, weight, body mass index, mean time under hemodialysis, urea reduction ratio, urea Ktv and parathyroid hormone serum levels.

Twenty of the CKD patients (10 responders and 10 non-responders to rhEPO therapy) were also evaluated before and immediately after hemodialysis.

CKD patients were under therapeutic hemodialysis three times per week, for 3 to 5 h, for a median period of time of 36 months. All patients used the high-flux polysulfone FX-class dialysers of Fresenius, 34 with FX60, 27 with FX80 and 2 with FX100 dialyser type. The causes of renal failure in patient's population were as follows: diabetic nephropathy (n=19), chronic glomerulonephritis (n=11), polycystic kidney disease (n=3), hypertensive nephrosclerosis (n=3), obstructive nephropathy (n=3), pyelonephritis associated with neurogenic bladder (n=1), nephrolithiasis (n=1), chronic interstitial nephritis (n=1), Alport syndrome (n=1), renal vascular disease due to polyarteritis (n=1) and chronic renal failure of uncertain aetiology (n=19). Patients with autoimmune disease, malignancy, hematological disorders, and acute or chronic infection were excluded. Intravenous iron supplementation was based on the European Best Practice Guidelines for the management of anaemia in patients with hemodialysis (10). All patients gave their informed consent to participate in this study.

Healthy volunteer's controls were selected based on normal hematological and biochemical values, and no history of kidney or inflammatory disease. They were also matched as far as possible for age

and gender with CKD patients (8 males, 17 females; mean ± SD age 47.81 ± 14.69).

Assays

Blood samples

Blood samples were collected from CKD patients before starting haemodialysis. In 20 of the CKD patients (10 responders and 10 non-responders to rhEPO therapy), blood samples were also collected immediately after the dialysis session.

Haematological data

Hemoglobin concentration and white blood cell count were measured using an automatic counter (Sysmex K1000, Hamburg, Germany) and leukocyte differential counts were evaluated in Wright-stained blood films. Reticulocyte count was made by microscopic counting on blood smears after vital staining with New methylene blue (reticulocyte stain; Sigma, St Louis, MO, USA). The reticulocyte production index (RPI) was calculated as an appropriate way to measure the effective RBC production, by correcting for both changes in haematocrit (degree of anaemia) and for premature reticulocyte releasing from the bone marrow (25).

Iron status

Serum iron concentration was determined using a colorimetric method (Iron, Randox Laboratories Ltd., North Ireland, UK), whereas serum ferritin and serum transferrin were measured by immunoturbidimetry (Ferritin, Laboratories Ltd., North Ireland, UK; Transferrin, Laboratories Ltd., North Ireland, UK). Transferrin saturation (TS) was calculated by the formula: TS (%) = 70.9 x serum iron concentration in (µg/dL) / serum transferrin concentration in (mg/dL). Enzyme-linked immunosorbent assays were used for measurement of plasma soluble transferrin receptor (s-TfR) (Human sTfR immunoassay, R&D systems, MN, USA), and serum prohepcidin concentrations (Hepcidin Prohormone ELISA, IBL, Hamburg, Germany).

Inflammatory markers

Interleukin 2 receptor (s-IL2R) (Human IL-2 SRa, R&D systems, MN, USA), IL-7 (R & D Systems, Minneapolic, Minnesota, USA) and C-reactive protein (CRP) (N high sensitivity CRP, Dade Behring) were determined according to the manufacturer's recommendations. Serum albumin levels were measured using a colorimetric assay end-point method (Albumin Plus; Roche GmbH, Mannheim, Germany). IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α and interferon (INF)-γ serum levels were quantified using the BDTM Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (BDB).

Leukocyte activation

T-cell cytokine production was performed in heparinised blood cells were cultured for 4 hours in RPMI 1640 medium at 37°C in a 5% CO2 and 95% humidity sterile environment in the presence of 25 ng/ml of phorbol-12 myristate 13-acetate, 1 µg/ml of ionomycin, and 10 µg/ml of brefeldin A (stimulated samples) or only with brefeldin A (unstimulated samples). Immediately after the incubation period, cells were stained with allophycocyanin (APC) conjugated anti-CD3 and fluorescein isothiocyanate (FITC) conjugated anti-CD8 mouse anti-human monoclonal antibodies (MAbs) (Becton Dickinson, Biosciences, San Jose, CA – BDB) for 15 min in the dark at room temperature (r.t.). After incubation, cells were washed once in 2 ml of phosphate-buffered saline (PBS). After discarding the supernatant, cells were fixed, permeabilized, and stained with phycoerythrin (PE) conjugated MAbs directed against human cytokines (IL-2, TNF-α and INF-γ) or PE-conjugated isotype-matched MAbs reagents (negative controls) (Pharmingen, San Diego, CA). For this purpose, the Fix & Perm reagent (Caltag, San Francisco, CA) was used, strictly following the recommendations of the manufacturer. Once stained, cells were washed once in 2 ml of PBS, suspended in 0.5 ml of PBS and analyzed in the flow cytometer.

T-cell phenotype was evaluated by flow cytometry with a whole blood stain-lyse-and-then-wash method, using the FACS lysing solution (BDB). Cells were stained with anti-CD8 APC or anti-CD4 APC, anti-CD28 PE-cyanin 5 (PC5), anti-HLA-DR PE and anti-CD57 FITC MAbs (BDB). HLA-DR and CD57 expression were used to evaluate early and late T-cell activation and absence of CD28 expression on T lymphocytes was used to quantify the fraction of memory effector T-cells / large granular lymphocytes (22-25). Data acquisition was performed in a FACSCalibur flow cytometer, equipped with a 488 nm argon ion and a 635 nm red diode lasers, using the CellQUESTTM software program (BDB). For T-cell phenotype and T-cell cytokine production analysis the Paint-A-Gate PRO software program (BDB) was used. In order to quantify the expression of costimulatory molecules and activation related

markers on T-cells, the percentage of HLA-DR+, CD28+ and CD57+ cells were recorded after gating for CD8+ and CD4+ T- lymphocytes. Evaluation of cytokine production by CD8+ and CD4+ T-cells was based on the percentage of cytokine+ cells within CD3+CD8+ and CD3+CD8- lymphocytes, respectively. Serum cytokine levels analysis was performed using the BDTM CBA Software.

Neutrophil activation was performed by determinate plasma levels of elastase and lactoferrin by enzyme immunoassays (human PMN Elastase ELISA, Bender MedSystems; Lactoferrin ELISA Kit, Calbiochem, respectively).

Erythrocyte damage

To prepare the RBC membranes, plasma and leukocytes were isolated and discarded after centrifugation on a double density gradient (Histopaque 1.077 and 1.119, Sigma). RBCs were washed in saline solution and then lysed by hypotonic lysis, according to Dodge et al. (26). The obtained membrane suspensions were washed in Dodge buffer, adding phenylmethylsulphonyl fluoride as a protease inhibitor in the first two washes, with a final concentration of 0.1mM. The protein concentration of the RBC membrane suspension was determined by Bradford's method (27).

The electrophoretic analysis of the RBC membrane proteins was carried out on a discontinuous system of polyacrylamide in the presence of sodium dodecylsulfate (SDS-PAGE), using a 5-15% linear acrylamide gradient gel and a 3.5-17% exponential acrylamide gradient gel, according to the Laemmli and Fairbanks methods, respectively (28,29). The proteins were stained with Coomassie brilliant blue and scanned (Darkroom CN UV/wl, BioCaptMW version 99; Vilber Lourmat, Marne-La-Vallée, France). The relative amount of each major RBC membrane protein (spectrin, ankyrin, band 3, 4.1, 4.2, 5, 6 and 7 proteins) was quantified by densitometry (Bio1D++ version 99; Vilber Lourmat, France). The electrophoretic analysis for each RBC membrane sample was performed in duplicate gels, and, in each gel, duplicates of each sample were loaded. Moreover, some ratios between membrane protein values, for patients and controls, were evaluated to further analyse membrane protein interaction.

Band 3 profile was performed in RBC membranes treated with an equal volume of a solubilisation buffer containing 0.125 M Tris HCl pH 6.8, 4% sodium dodecil sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol, heat-denatured and submitted to polyacrylamide gel electrophoresis (SDS-PAGE) (20 µg protein/lane), using the discontinuous Laemmli system (28) (a 9% separating gel and a 4.5% stacking gel). Membrane proteins were electrophoretically transferred from gels to a nitrocellulose sheet with a porosity of 0.2 µm (Sigma) (30). Additional reactive sites on the nitrocellulose were blocked by incubation in 5% of low fat dry milk and 0.1% Triton-X 100 in PBS (phosphate-buffered saline), pH 7.0, for 1 h at room temperature and under gentle rotation. Band 3 immunoblot was performed (31); monoclonal antibodies antihuman band 3, produced in mouse, recognising an epitope located in the cytoplasmic pole of the band 3 molecule (32) (Sigma), were added (dilution 1:3000) and incubated for 4 h; the washing of the nitrocellulose was followed by the addition and incubation with antimouse Ig peroxidase linked (Sigma) for 1 h (dilution 1:4000). The incubations were carried out at room temperature; the dilutions of the antibodies were prepared with PBS pH 7.0 containing 0.1% Triton-X 100 and 5% of low fat dry milk. In the washes, the same buffer without dry milk was used. Hydrogen peroxide and α-cloronaphtol were used to develop the immunoblot. The band 3 profile was quantified by densitometry (Bio1D++ version 99; Vilber Lourmat, France). We evaluated the percentage of HMWAg, of band 3 monomer and of Pfrag.

Oxidative stress

Glutathione peroxidase (GPx) and superoxide dismutase (SOD) erythrocyte activities were evaluated by using commercially available kits (RANSEL and RANSOD, Randox, UK, respectively). Total antioxidant status (TAS) was evaluated in serum by a colorimetric assay (TAS, Randox) Plasma lipidic peroxidation was estimated by TBA (TAS, Randox). assay (26).

Membrane bound haemoglobin (MBH) was measured spectrophotometrically at 415 nm, after membrane protein dissociation with Triton X-100 (5% in Dodge buffer). The background was read at 700 nm to correct the absorbance at 415 nm; the obtained value and the protein concentration were used to estimate the percentage of MBH.

Data analysis

For statistical analysis, we used the Statistical Package for Social Sciences (SPSS; SPSS, version 15.0 for Windows, Inc. Chicago, IL, USA). Kolmogorov Smirnov statistics were used to evaluate sample normality distribution. Multiple comparisons between groups were performed by one-way ANOVA supple-

mented with Turkey's HSD *post-hoc* test. For single comparisons, we used the Students *t*-test whenever the parameters presented a Gaussian distribution and the Mann-Whitney *U*-test in the case of a non-Gaussian distribution. To compare data before and after haemodialysis, we used paired-samples T-test or Wilcoxon test. Spearman's rank correlation coefficient was used to evaluate relationships between sets of data. Significance was accepted at p less than 0.05.

Results and Discussion

We summarise the most important findings observed for the haemodialysis patients (all patients' *vs* controls) and for non-responders patients (non-responders *vs* responders) in table I.

Haemodialysis patients

To compensate the anaemia of CKD patients under haemodialysis, mainly due to renal failure in EPO production, therapeutic administration of rhEPO is nowadays included in the therapeutic protocols for these patients. However, the dose chosen to be used should only targets to partially correct the anaemia. In fact, pre-dialysis levels of haemoglobin higher than the target levels (11-12 g/dL) are not desirable, due to the risk associated with the effects arising from post-dialysis haemoconcentration and its related complications (33). In our patients, we found an increase of about 11% in haemoglobin concentration after haemodialysis. The increase in haemoglobin levels post-haemodialysis is associated to a translocation of erythrocytes from the splanchnic circulation (and possibly from the splenic circulation), in order to compensate the hypovolemic stress during dialysis ultrafiltration (34,35).

Our CKD patients under haemodialysis and rhEPO therapies presented lower haemoglobin concentration, haematocrit and erythrocyte count when compared with controls. These reductions are regenerative, as showed by the increase in reticulocyte count and in reticulocyte production index (RPI), reflecting the erythropoietic stimulus triggered by rhEPO therapy; these changes are also associated with anisocytosis, as suggested by the high red blood cell distribution width (RDW). This anisocytosis could result from the observed reticulocytosis and/or from an erythrocyte damage induced by the haemodialysis procedure or even from changes induced by plasma metabolites and/or leukocyte activation products. This last hypothesis was later tested by evaluating the erythrocyte changes and leukocyte activation induced by the haemodialysis procedure.

The haemodialysis patients also presented lymphopenia, which results, at least in part, from a decrease in total circulating CD3+ T-lymphocytes and affect both the CD4+ and the CD8+ T-cell subsets. Furthermore, the lymphocytes presented markers of enhanced continuous activation state [increased proportions of both early (HLA-DR) and late-activation (CD57) markers on both CD4+ and CD8+ T-cell subsets]. This, probably, results from persistent antigen stimulation/chronic inflammation associated with haemodialysis and/or chronic renal failure, which probably also justifies the increase of the fraction of memory effectors T-cells / large granular lymphocytes, as shown by the higher percentage of CD4+/CD28- and CD8+/CD28- T-cells observed. Moreover, T-cells from haemodialysis

Table I – Most important findings in haemodialysis patients (as compared to control) and in non-responders patients (as compared to responders).

	All patients *vs* controls	**Non-responders *vs* responders**
Anaemia	+	+
Hypochromia	-	+
Anisocytosis	+	+
Reticulocytosis	+	-
Lymphopenia	+	+
T-cell activation	+	-
Production of Th1 cytokines	+	-
Neutrophil activation	+	+
Inflammation	+	+
Iron metabolism disturbance	+	+
Alterations in erythrocyte membrane protein composition	+	+
Band 3 profile changes	+	+

(-): no differences; (+): statistically significant differences.

patients show an enhanced ability to produce Th1 related cytokines (IL-2, INF-γ and TNF-α) after short term *in vitro* stimulation. This increased capacity to produce Th1 cytokines could justify, at least in part, the anaemia found in these patients. In fact these cytokines are described to be associated to an inhibitory effect on erythropoiesis.

The presence of an inflammatory process (higher neutrophil count, increased neutrophil/lymphocyte ratio, higher levels of IL-6, soluble IL-2 receptor and CRP, and decreased albumin serum levels) was a consistent finding in our haemodialysis patients. The causes for this inflammatory response are not well clarified. There are several potential sources, including bacterial contamination of the dialyser, incompatibility with the dialyser membrane and the type of the vascular access.

Besides the higher neutrophil count, as compared to controls, haemodialysis patients also presented increased plasma levels of neutrophilic elastase. However, the elastase/neutrophil ratio was similar to that found in controls, suggesting that the rise in elastase levels could be related to the increased neutrophil count and be part of the inflammatory process. It was reported (36) that elastase modulates cytokine expression at epithelial and endothelial surfaces, up-regulating cytokines such as the pro-inflammatory IL-6, and, therefore, elastase may be one of the major factors involved in the development of several chronic inflammatory diseases, although it is difficult to conclude if increased elastase plasma levels are the cause or the consequence of an inflammatory process. We figured, therefore, that the potential role for the high levels of elastase observed in CKD patients should be considered and clarified its origin.

During the haemodialysis procedure a neutrophilic activation process occurs, as shown by the increase in elastase and lactoferrin concentrations observed immediately after the haemodialysis procedure (and in their ratios per neutrophil). However, in contrast to elastase, plasma levels of lactoferrin were not increased before the haemodialysis procedure, probably because lactoferrin could be used or cleared from circulation in a pronounced way. Indeed, lactoferrin is involved in iron metabolism, and could be involved in the decreased iron availability for erythropoiesis. Moreover, elastase, as a serinoprotease, may modulate pathogenic changes in plasmatic constituents and in cells, namely, in erythrocytes.

The inflammatory stimulus has an important impact in iron metabolism, by mobilizing iron from erythropoiesis traffic to storage sites within the reticuloendothelial system, inhibiting erythroid progenitor proliferation and differentiation (37). This change in iron mobilization leads to an iron depleted erythropoiesis. In our haemodialysis patients, we found an increase in ferritin serum levels and a decrease in transferrin. These results suggest an increase of iron storage and a reduction in iron mobilization for erythropoiesis. An increased level of prohepcidin, the precursor molecule of hepcidin, was also found in haemodialysis patients. IL-6 strongly induces hepcidin mRNA expression (38,39), indicating that the high levels of prohepcidin found in haemodialysis patients could be related to the underlying chronic inflammation. The increased hepcidin expression during inflammation and infection explains sequestration of iron in the macrophages and inhibition of intestinal iron absorption, the two hallmarks of the anaemia of inflammation, which is normocytic or microcytic and hipocromic (40). However, no correlations between prohepcidin and the studied inflammatory markers were found, suggesting that other factors than just inflammation regulates prohepcidin serum levels. We identified the haemodialysis procedure, which removes approximately 10% of prohepcidin from circulation, as of one of these factors.

As previously referred, the high RDW (anisocytosis) could result from reticulocyosis or erythrocyte damage. Actually, some changes were observed in erythrocyte membrane protein composition of our haemodialysis patients, being the decrease in spectrin the most significant change. This spectrin deficiency may account for a poor linkage of the cytoskeleton to the membrane, favouring membrane vesiculation, and, probably, a reduction in the erythrocyte lifespan of haemodialysis patients (41). Other alterations were also found, namely, increases of protein bands 6 and 7, and in band 4.1/spectrin and spectrin/band 3 ratios which may further reflect an altered membrane protein interaction and destabilization of membrane structure.

A primary protein deficiency, as occurs in Hereditary Spherocytosis, may occur isolated or impose secondary deficiencies in one or more proteins (42), being the type and amount of the secondary protein deficiencies involved in the haematological and clinical outcome of the disease (43). Moreover, "minor" erythrocyte membrane proteins may play a role in membrane vesiculation, especially when associated to deficiencies in major erythrocyte membrane proteins (43). The erythrocyte membrane protein alterations that we observed could be related

to the interaction with higher concentrations of several circulating metabolites, and/or with leukocyte activation products (44). The increased neutrophil activation products that we found prior and after the haemodialysis procedure are able to induce oxidative and proteolytic changes in erythrocytes, as reported elsewhere (45). Indeed, we found a different erythrocyte membrane band 3 profile in haemodialysis patients, a decrease in HMWAg, Pfrag and in Pfrag/band 3 monomer and HMWAg/band 3 monomer ratios. This profile presents changes reflecting the presence of younger erythrocytes, but also of damaged erythrocytes. Indeed, a decrease in HMWAg, accompanied by an increase in Pfrag, is found in younger erythrocytes; however, the latter change, in HMWAg, was not observed in haemodialysis patients.

Inflammatory conditions have been associated with oxidative stress, leukocyte activation and oxidative changes in erythrocyte, namely in band 3 profile (45,46).

Haemodialysis patients also exhibited higher plasma levels of TAS, TBA and TBA/TAS ratio (higher rise in TBA than in TAS), reflecting an increased oxidative stress in haemodialysis patients. We also showed that the haemodialysis procedure *per se* does not lead to an increase in the studied markers of oxidative erythrocyte damage. Actually, no differences were found after haemodialysis, in band 3 profile and, besides that, a decrease in MBH, TBA and TAS were observed, probably reflecting the improvement in the plasmatic environment and/or in the removal of damaged erythrocytes.

Alterations associated with resistance to rhEPO therapy

CKD patient's non-responders to rhEPO therapy presented a mild to moderate anaemia, even with the administration of higher rhEPO doses. This anaemia is hypochromic [decreased mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC)], and is associated with a more accentuated anisocytosis than in responder patients. Considering that all patients were under iron and folate prophylactic therapies, and, that iron status and vitamin B12 serum levels were normal, these changes cannot be attributable to a deficiency in these erythropoietic nutrients.

Actually, the CKD patient's non-responders to rhEPO therapy seem to present a "functional" iron deficiency, characterized by the presence of adequate iron stores as defined by conventional criteria, but apparently with an inability to sufficiently mobilize iron to adequately support erythropoiesis. In fact, no statistically significant differences were found in serum iron status markers between the groups of patients (responders and non-responders), except for the soluble transferrin receptor (s-TfR), which was higher among non-responders. This "functional" iron deficiency is reflected by a decrease in MCH and in MCHC, and by a more accentuated anisocytosis (high RDW) in non-responders patients. Furthermore, the inverse correlation found between CRP and mean cell volume, MCH, serum iron and transferrin saturation, are in agreement with the observations that the chronic inflammation enhanced in non-responders, may lead to trapping of iron within the macrophages and to a reduction in serum iron levels. Additionally, CKD patient's non-responders to rhEPO therapy presented lower prohepcidin serum levels when compared with responders. As previously referred, hepcidin is upregulated by inflammation; however, erythropoietin downregulates liver hepcidin expression (47). As non-responders patients were treated with much higher doses of rhEPO compared with responders, the lower prohepcidin levels found in those patients could be explained by this mechanism.

A more accentuated decrease in erythrocyte membrane spectrin, though without statistically significance, was found in non-responders CKD patients. This spectrin deficiency was accompanied by the production of hypocromic and anisocytic erythrocytes, and this anisocytosis could reflect an even more enhanced change in membrane protein interactions in non-responders (as referred, a significant reduction in spectrin was observed when compared to control values). However, a large overlapping of spectrin values was found in the two groups of patients (responders and non-responders) suggesting that the disease itself and/or the interaction with haemodialysis membranes could be involved in the observed erythrocyte membrane protein alterations (48). Non-responders patients also showed a decrease in Pfrag and in Pfrag/band 3 monomer ratio and a trend to higher values of MBH, suggesting that they present a higher erythrocyte damage that may result from an even more adverse plasmatic microenvironment. When studying the differences in membrane protein composition before and after the haemodialysis procedure, we found that this spectrin reduction was enhanced in the non-responder group; moreover, we observed that

this could be associated to an enhanced inflammation process and/or to the interaction with proteases released by neutrophils as we also found significant rises in elastase and lactoferrin, both products of leukocyte activation. We should not exclude the hypothesis that the much higher doses of rhEPO used in non-responders, can be involved in erythrocyte membrane protein alterations.

Resistance to rhEPO therapy in haemodialysis patients seems, therefore, to be associated to an increase in erythrocyte damage.

The previous studies on erythrocytes strongly suggest an important role for the inflammatory process. Indeed, non-responders patients presented an enhanced inflammatory process with higher CRP and lower albumin, as compared to responders. This enhanced inflammatory stimulus induces the release of cytokines, including pro-inflammatory cytokines that can have an erythropoiesis-suppressing effect. Moreover, CRP serum levels were positively correlated with weekly rhEPO/Kg dose, suggesting that CRP should be further studied as biomarker of resistance to rhEPO therapy in haemodialysis patients. Furthermore, it confirmed that resistance to rhEPO therapy is associated with an enhanced inflammatory state. To further clarify the role of inflammation we studied the potential contribution of lymphocytes and neutrophils.

Statistically significant decreases in total lymphocytes and CD4+ T-cell counts were found in non-responder patients. There are some possible explanations for this lymphocyte depletion, namely, increased turnover, disturbances of lymphocyte homeostasis due to uraemia, and increased peripheral lymphocyte apoptosis associated with the activation stimulus (49,50). The exact mechanism of this T-cell depletion is still unclear. As IL-7 emerged recently as a key cytokine involved in controlling the homeostatic turnover and the survival of peripheral resting memory CD4+ T cells, we have hypothesized that the serum levels of IL-7 could be related with this decreased number of total lymphocytes and CD4+ T-cells. In fact, our results showed a relationship between increased levels of IL-7 and decreased number of total lymphocytes and CD4+ T-cell count, suggesting that the increased IL-7 serum levels could be related with ongoing T-cell depletion. However, further studies are needed to understand the mechanism of lymphocyte loss and its consequences and/or involvement in the resistance to rhEPO therapy, as a higher IL-7 concentration seems to predict a poorer response to rhEPO therapy.

Concerning neutrophils we found slightly higher values for non-responders. Plasma levels of elastase were enhanced in non-responders. The elastase/neutrophil ratio was similar to that found in controls, suggesting that the rise in elastase levels could be related to increased neutrophil count and be part of the inflammatory process. Furthermore, a statistically significant positive correlation was found between elastase levels and CRP, and the with weekly rhEPO doses, corroborating that hypothesis. However, no differences were found between responders and non-responders patients in neutrophilic activation during haemodialysis procedure, suggesting that the higher elastase levels found in non-responders is likely to result from a chronic and not acute stimulus triggered by the haemodialysis process, or to the increased number of neutrophils found in these patients. Our results show that elastase, but not lactoferrin, may prove to be a good marker of resistance to rhEPO therapy in haemodialysis patients.

Conclusion

Although the etiology of resistance to rhEPO therapy is still unknown, our work confirms that inflammation seems to have an important role in its pathophysiology. We also demonstrated that resistance to rhEPO therapy is associated with "functional" iron deficiency, lymphopenia and CD4+ lymphopenia, higher elastase plasma levels, increased IL-7 serum levels, and alterations in erythrocyte membrane protein structure and in band 3 profile.

The exact origins of the inflammatory process remain unclear. We speculate that the release of elastase during the haemodialysis procedure could have an important role in amplifying the inflammatory process in haemodialysis patients, particularly in non-responders. Furthermore, this elastase release could also have an important role in the alterations found in erythrocyte membrane protein structure and in band 3 profile, further contributing to worsening of anaemia. The inflammatory process, the rhEPO doses administrated and the lactoferrin release during haemodialysis could have an important role in iron uptake from the small intestine, in the release of iron from macrophages and, finally, in the availability of iron for erythropoiesis, as proposed in Fig. 1.

In conclusion our data suggest that further studies are needed to understand the rise in inflammation with the associated need in higher doses of rhEPO and reduced iron availability.

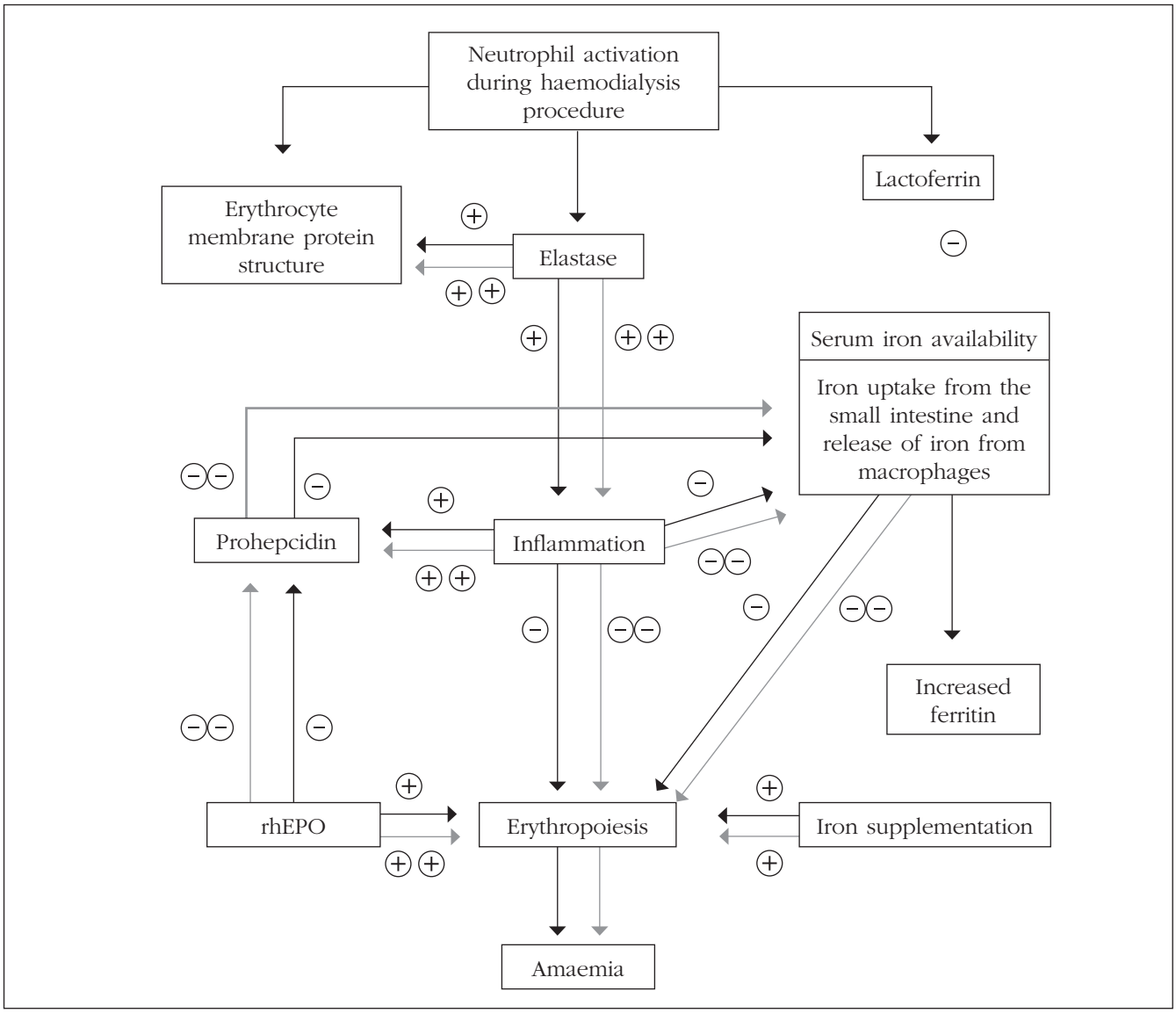

Fig. 1 – Potential mechanisms involved in the pathophysiology of resistance to rhEPO therapy. Black lines represents responders patients and gray lines non-responders patients; (+) represents activation and (-) inhibition.

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