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Resistance to Recombinant Human Erythropoietin Therapy in Haemodialysis Patients

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1. Introduction

The involvement of a humoral factor (named as haemopoietin) in the regulation of haematopoiesis, was firstly described in literature in 1906 (Carnot & Deflandre, 1906). However, only 40 years later a linkage between erythropoietin (EPO) and erythropoiesis was described (Bondsdorff & Jalavisto, 1948), and only in the 1950s was established that the kidney is the main site of production of EPO (Jacobson, 1957). In 1977, EPO was purified from urine collected from patients suffering from aplastic anaemia (Miyake, 1977). The nucleotide sequence of human EPO gene was determined in 1985 and the cloning and expression of the gene led to the production of recombinant human EPO (rhEPO) (Lin, 1985; Jacobs, 1985).

EPO is an endogenous cytokine that is essential in erythropoiesis regulation. This glycoprotein has a molecular mass of 30-35 kDa, 165 amino acids and is heavily glycosylated, with the carbohydrate moiety comprising approximately 40% of its weight. There are three N-terminal glycosylation sites at aspartate residues 24, 38 and 83, and one O-linked acidic oligonucleotide side-chain at serine 126. Human EPO has two disulphide bridges, between cysteines 7 and 161, and between cysteines 29 and 33, which are important in maintaining its *in vivo* bioactivity and the correct shape for binding to the EPO receptor (EPOR) (Lai, 1986).

The regulation of EPO gene expression occurs essentially at the transcriptional level by DNA-dependent mRNA synthesis and gene activation. In kidneys, hypoxia gives rise to increased EPO expression, stimulated by the DNA binding protein, hypoxia inducible factor, which binds to the 3´ flanking region of EPO gene (Wang & Semenza, 1993). EPO is secreted into the plasma and, within the bone marrow, binds to EPOR in the surface of erythroid progenitor cells. EPOR activation follows a sequential dimerization activation mechanism involving the Janus kinase 2 (JAK2), and phosphorylation and nuclear translocation of signal transducer and activator of transcription 5 (STAT5) pathways (Fig. 1).

The first clinical trial using rhEPO in the treatment of the anaemia of end-stage renal failure was published in 1987 (Eschbach, 1987), and, nowadays, rhEPO is currently used for the treatment of that anaemia in haemodialysis (HD) patients (Kimel, 2008; Obladen, 2000), as well as for a variety of other clinical situations associated with anaemia.

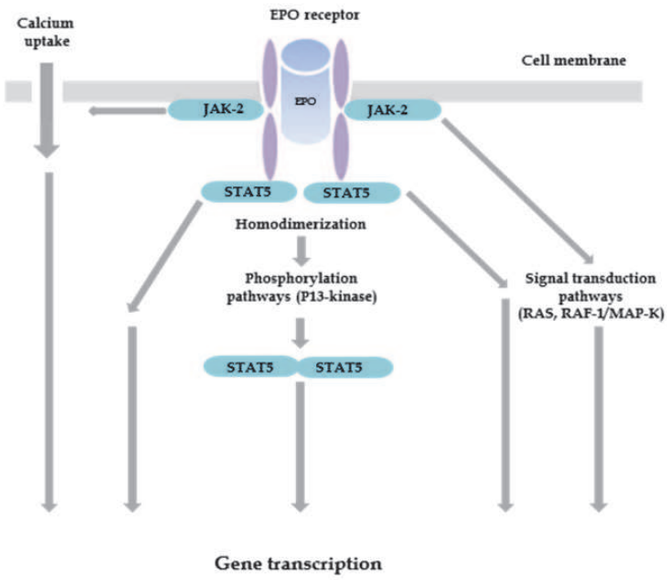

Fig. 1. Schematic diagram showing signalling pathways activated by EPO receptors (adapted from Marsden, 2006).

Anaemia is a common complication that contributes to the burden of HD patients. It has also a negative impact on cardiovascular system, cognitive function, exercise capacity and quality of life, resulting in a significant morbidity and mortality in these patients. The introduction of rhEPO therapy for treatment of anaemia of HD patients led to a significant reduction in anaemia and to an improvement in patients' quality of life (Locatti, 1998; Bárány, 2001; Locatelli, 2004a; Smrzova, 2005). 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 (Bárány, 2001; Macdougall, 2002a; Schindler, 2002; Smrzova, 2005). 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 µg/Kg/week of darbopoietin-alfa (Locatelli, 2004b).

Resistance to rhEPO has been reported as an independent risk factor for mortality in HD patients, due to both the inability to achieve the target haemoglobin levels and to the administration of high rhEPO doses, which have been associated with increased risk of myocardial infarction, congestive heart failure and stroke.

The reasons for the variability in rhEPO response are unclear (Foley, 1996; Spittle, 2001; Drueke, 2002; Cooper, 2003; Himmelfard, 2004; Smrzova, 2005). There are several conditions reported as associated with rhEPO resistance, namely, inflammation, oxidative stress and iron deficiency, as major causes (Foley, 1996; Gunnell, 1999; Spittle, 2001; Drueke, 2002; Cooper, 2003; Himmelfard, 2004; Pupim, 2004; Smrzova, 2005), and blood loss, hyperparathyroidism, aluminium toxicity and vitamin B12 or folate deficiencies, as minor causes. However, exclusion of these factors does not eliminate the marked variability in sensitivity to rhEPO (Macdougall, 2002b). In this chapter, a revision of the mechanisms proposed to underlie the resistance to rhEPO therapy will be performed, with particular emphasis on the role of inflammatory cytokines, neutrophil activation, iron status, and erythrocyte damage.

2. Inflammatory cytokines

Inflammation is the physiological response to a variety of noxious stimuli, such as tissue injury caused by infection or physical damage. It is a complex process that involves the participation of several cells and molecules, and may present different intensities and duration.

Inflammation usually refers to a localised process. However, if the noxious stimulus is severe enough, distant systemic changes may also occur, and these changes are referred as "acute phase response", which is accompanied by signs and symptoms such as fever, anorexia, and somnolence. This acute phase response may include neuroendocrine, metabolic and haematopoietic changes, as well as changes in non-protein plasma constituents (Ceciliani, 2002). The haematopoietic response includes leukocytosis and leukocyte activation, thrombocytosis, and anaemia secondary to erythrocyte damage and/or decreased erythropoiesis (Trey & Kushner, 1995).

Inflammatory stimuli induces the release of cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL) -1, IL-6, and interferon (IFN)-γ, which may be produced by several cells, including leukocytes, fibroblasts and endothelial cells (Kushner, 1999). This release of cytokines causes many systemic changes, including increased synthesis and release of positive acute-phase proteins, such as C-reactive protein (CRP) and fibrinogen, as well as the suppression of negative acute-phase proteins, such as albumin and transferrin (Mcdougall, 1995; Cooper, 2003; Smrzova, 2005).

The causes for the inflammatory response in HD patients are not well clarified. There are several potential sources, including bacterial contamination of the dialyser, incompatibility with the dialyser membrane and infection of the vascular access. However, the dialysis procedure may only be partially responsible for the inflammatory response, because even patients with renal insufficiency who are not yet on dialysis present raised inflammatory markers, which rise further after starting regular HD treatment, suggesting that the disease *per se* triggers an inflammatory response (Gunnel, 1999; Schindler, 2002; Macdougall, 2002b).

The exact mechanisms by which the effects of inflammation on erythropoiesis occur are still to be determined. However, along an inflammatory response (Fig. 2), the iron from the erythropoiesis traffic is mobilised to storage sites within the reticuloendothelial system, inhibiting erythroid progenitor proliferation and differentiation, and blunting, therefore, the response to EPO (endogenous and/or exogenous). An erythropoiesis-suppressing effect has been also attributed to increased activity of pro-inflammatory cytokines reported in association with inflammatory conditions, and this relationship has been proposed as a potential factor associated to rhEPO therapy resistance (Gunnel, 1999; Schindler, 2002; Macdougall, 2002b; cooper, 2003). Actually, some studies have shown that genetic variations in some pro-inflammatory cytokines leading to increased levels of the cytokines, may play an important role in the pathogenesis of the anaemia (Maury, 2004). Recently, it was proposed that the c.511C>T polymorphism in the gene encoding for IL-1β, which is associated with increased serum levels of IL-1β, is linked to increased needs of rhEPO to correct anemia (Jeong, 2008).

It was reported that pro-inflammatory cytokines, such as IL-1, IL-2, IL-4, IL-6, TNF-α and INF-γ diminish BFU-E and CFU-E cells, resulting in suppression of erythropoiesis (Macdougall, 2002a). In fact, some of these cytokines, such as IL-1, IL-6, TNF-α, IFN-γ, and C-reactive protein (CRP), have been reported to play an important role in rhEPO resistance (Panichi, 2000; Pecoits-Filho, 2003; Cooper, 2003). Moreover, it was reported (Macdougall,

2002a) that serum derived from HD patients suppresses erythroid colony-forming response to rhEPO, in a manner that can be inhibited by antibodies against TNF-α and INF-γ. These data also strongly suggest a key role in the rhEPO response for these inflammatory mediators (Waltzer, 1984; Foley, 1996; Meier, 2002; Cooper, 2003).

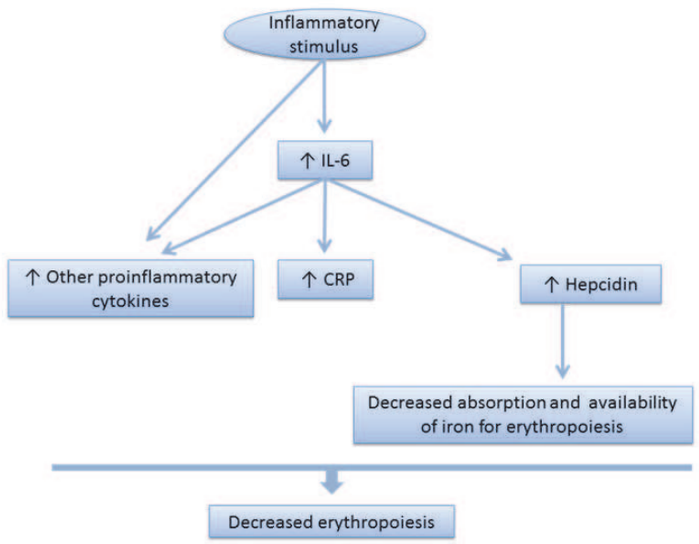

Fig. 2. Inflammatory stimulus is associated to a decrease in erythropoiesis. Interleukin-6 (IL-6) and hepcidin have a critical role in the association between inflammation and erythropoiesis.

Recently, our group demonstrated that non-responders patients, as compared to responders, presented higher CRP and neutrophil/lymphocyte ratio, and lower albumin serum levels (Costa, 2008a), suggesting a relationship between resistance to rhEPO therapy and the inflammatory response. Moreover, we observed a CD4+ lymphopenia associated with increased IL-7 serum levels (Costa, 2008b), an activation stage of T-cells and an enhanced ability of these cells to produce Th1 related cytokines (IL-2, INF-γ and TNF-α) after short term *in vitro* stimulation. This increased capacity of T-cells to produce Th1 cytokines could justify, at least in part, the anaemia found in HD patients.
These results, published by our group and by others, show that raised inflammatory cytokines are a consistent finding associated with resistance to rhEPO therapy, by acting directly in erythropoiesis and/or indirectly, by decreasing iron availability for erythropoiesis.

3. Neutrophil activation

Leukocytosis and recruitment of circulating leukocytes into the affected areas are hallmarks of inflammation. Leukocytes are chimio-attracted to inflammatory regions and their transmigration from blood to the injured tissue is primarily mediated by the expression of cell-adhesion molecules in the endothelium, which interact with surface receptors on leukocytes (Muller, 1999; Sullivan, 2000). This leukocyte-endothelial interaction is regulated by a cascade of molecular steps that correspond to the morphological changes that accompany adhesion. At the inflammatory site, leukocytes release their granular content and may exert their phagocytic capacities.

In acute inflammation, the leukocyte infiltration is predominantly of neutrophils, whereas in chronic inflammation a mononuclear cell infiltration (predominantly macrophages and lymphocytes) is observed. Although leukocyte-endothelial cell interaction is important for leukocyte extravasation and trafficking in physiological situations, there is increasing evidence that altered leukocyte-endothelial interactions are implicated in the pathogenesis of diseases associated with inflammation, possibly by damaging the endothelium or altering endothelial function (Harlan, 1985; Ley, 2007).

Leukocytosis is essential as the primary host defence, and neutrophils, the major leukocyte population of blood in adults, play a primordial role. It is well known that neutrophils have mechanisms that are used to destroy invading microorganisms. These cells use an extraordinary array of oxygen-dependent and oxygen-independent microbicidal weapons to destroy and remove infectious agents (Witko-Sarsat, 2000). Oxygen-dependent mechanisms involve the production of reactive oxygen species (ROS), which can be microbicidal (Roos, 2003), and lead to the development of oxidative stress. Oxygen-independent mechanisms include chemotaxis, phagocytosis and degranulation. The generation of microbicidal oxidants by neutrophils results from the activation of a multiprotein enzyme complex known as the reduced nicotinamide adenine dinucleotide phosphate oxidase, which catalyzes the formation of superoxide anion ($O_2^{\cdot-}$). Activated neutrophils also undergo degranulation, with the release of several components, namely, proteases (such as elastase) and cationic proteins (such as lactoferrin).

Elastase is a member of the chymotrypsin superfamily of serine proteinases, expressed in monocytes and mast cells, but mainly expressed by neutrophils, where it is compartmentalized in the primary azurophil granules. The intracellular function of this enzyme is the degradation of foreign microorganisms that are phagocytosed by the neutrophil (Brinkmann, 2004). Elastase can also degrade local extracellular matrix proteins (such as elastin), remodel damaged tissue, and facilitate neutrophil migration into or through tissues. Moreover, elastase also modulates cytokine expression at epithelial and endothelial surfaces, up-regulating the production of cytokines, such as IL-6, IL-8, transforming growth factor β (TGF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF); it also promotes the degradation of cytokines, such as IL-1, TNF-α and IL-2. There is evidence in literature that high levels of elastase are one of the major pathological factors in the development of several chronic inflammatory lung conditions (Fitch, 2006).

Few data exists in literature about a possible correlation between leukocyte activation, particularly with neutrophil activation, and resistance to rhEPO therapy. In a recent study, we found that patients under HD, particularly those who were non-responders to rh-EPO therapy, presented a decreased expression of the CXCR1 neutrophil surface markers (Pereira, 2010) and higher elastase plasma levels (tables 1 and 2).

	Controls (n=18)	**Responders (n=26)**	**Non-Responders (n=8)**
CXCR1 (MFI)	308.40 ± 76.3	261.30 ± 45.74*	222.85 ± 29.01*§
CD11b (MFI)	236.3±81.9	223.33 ± 73.99	207.96 ± 86.50

Table 1. Neutrophil activation markers for controls and for responders and non-responders HD patients.*p<0.05 *vs* controls; § p<0.05 *vs* Responders. MFI: mean fluorescence intensity. * Data are presented as the mean fluorescence intensity of each cell marker (MFI) ± two standard deviations. Adapted from Pereira, 2010.

	Controls (n=26)	Responders (n=32)	Non-responders (n=31)
Hb (g/dL)	13.90 (13.2-15.00)	11.70 (10.83-12.68)*	10.4 (9.00-11.30) *§
White cell counts (x 10^9/L)	5.78 ± 1.59	6.42 ± 1.96	6.04 ± 2.26
Lymphocytes (x 10^9/L)	2.35 ± 0.75	1.58 ± 0.49*	1.36 ± 0.69 *§
Monocytes (x 10^9/L)	0.25 ± 0.08	0.40 ± 0.13*	0.35 ± 0.17*
Neutrophils (x 10^9/L)	3.03 ± 1.02	4.17 ± 1.87*	4.11 ± 1.73*
Albumin (g/dL)	ND	4.0 ± 0.4	3.7 ± 0.4§
CRP (mg/dL)	1.75 (0.76-4.70)	3.20 (1.73-7.23)*	10.14 (3.82-38.99)*§
Elastase (µg/L)	28.29 (26.03-34.74)	34.13 (28.76-39.16)*	39.75 (31.15-64.84)*§
Elastase/Neutrophil ratio	10.86 (7.44-12.12)	8.70 (7.32-11.42)	10.25 (7.56-17.41)

Table 2. Haematological data and neutrophil activation markers, for controls and for responders and non-responders HD patients.* $p<0.05$, *vs* controls; § $p<0.05$, *vs* responders. NM: not done. Results are presented as mean ± standard deviation and as median (interquartile ranges). Hb: Haemoglobin; CRP: C-reactive protein. Adapted from Costa, 2008c.

CXCR1 is a receptor that recognizes CXC chemokines, particularly the pro-inflammatory IL-8 (Pay, 2006; Sherry, 2008). The decreased expression of this receptor in neutrophil surface is associated to the release of components of neutrophil granules and reflects the need for inotropic support. Recently, it was shown that the levels of the neutrophil chemoattractant receptor, CXCR1, are mildly diminished in pediatric patients, as a consequence of end stage renal disease itself, and that recurrent serial bacterial infection markedly exacerbated the loss of CXCR1 by neutrophils (Sherry, 2008). This loss of CXCR1 on neutrophils can be due to the uremic state, to changes in leukocyte adhesion molecule expression or membrane microvilli and/or to cross-desensitization of this receptor, due to prior exposure to several unrelated chemoattractants, including N-formylated peptides and the complement cleavage product C5a. Chronic exposure of circulating inflammatory cells to these mediators may lead also to loss of this chemokine receptor expression and/or function via cross-desensitization.

The HD procedure, itself, seems to lead to neutrophil activation found in HD patients (Costa, 2008c). However, the rise in neutrophil activation products observed after the HD procedure does not explain the higher neutrophil activation found in non-responders patients. Actually, a significant positive correlation between elastase levels and CRP, suggests that the rise in neutrophil activation is part of the inflammatory process found in HD patients, which is particularly enhanced in non-responders. The statistically significant correlation that we found between elastase levels and the weekly rhEPO doses also strengthens this hypothesis; in fact, non-responders to rhEPO therapy patients, requiring higher weekly rhEPO doses to achieve target hemoglobin levels, present an increased inflammatory process (Costa, 2008c).

4. Iron status

Iron is an essential trace element that is required for growth and development of living organisms, but excess of free iron is toxic for the cell (Arth, 1999; Atanasio, 2006). Mammals lack a regulatory pathway for iron excretion, and iron balance is maintained by the tight regulation of iron absorption from the intestine (Park, 2001; Atanasio, 2006). The intestinal

iron absorption is regulated by the level of body iron stores and by the amount of iron needed for erythropoiesis (Arth, 1999; Park, 2001; Atanasio, 2006; Nemeth, 2003).

In HD patients, iron absorption is similar to that found in healthy individuals; however, when under rhEPO therapy, the absorption of iron increases as much as 5 times (Skikne, 1992). This increased iron absorption is not sufficient to compensate for the iron lost during the HD procedure, and with the frequent blood draws performed on these patients. For this reason, and because the association of rhEPO with iron therapy achieves a better erythropoietic response, intravenous iron administration has become a standard therapy for most patients receiving rhEPO. To avoid iron overload, with potentially harmful consequences, there is a need to monitor iron therapy by performing regular blood tests reflecting body's iron stores. However, analytical and intra-individual variability of classical iron markers, limits its value. For instance, in case of inflammation, several parameters (transferrin and ferritin) used to study iron status, are misleading. This, triggered the search for more useful new markers to monitor patients with disturbances in iron status. One of these new markers is the soluble transferrin receptor (sTfR), which reflects the iron needs of the erythroid cells and is independent of an on-going inflammatory process. More recently, a complex regulatory network that governs iron traffic emerged, and points to hepcidin as a major evolutionary conserved regulator of iron distribution (Nicolas, 2002; Kemma, 2005; Nemeth, 2006). This small hormone produced by the mammalian liver has been proposed as a central mediator of dietary iron absorption, due to its inhibitory effect in iron uptake from the small intestine, and in iron release from macrophages and hepatocytes, leading to decreased iron availability for erythropoiesis; a decreased placental iron transport was also observed (Kulaksiz, 2004). The synthesis of hepcidin is regulated by anemia/hypoxia, inflammation and iron overload.

The *in vitro* stimulation of fresh human hepatocytes by pro-inflammatory IL-6 showed a strong induction of hepcidin mRNA, indicating that this cytokine is an important mediator of hepcidin induction, in inflammation (Fleming, 2001; Dallilio, 2003; Hsu, 2006; Domenico, 2007). Moreover, it was shown that hepcidin expression is also regulated by other hepatic proteins, including the hereditary hemacromatosis protein (HFE), transferrin receptor 2, hemojuvelin, bone morphogenic proteins, transferrin and EPO (Fig. 3).

Hepcidin is synthesized as preprohepcidin, a protein with 84 amino acids. This peptide is cleaved, leading to prohepcidin with 60 aminoacids, which is further processed, giving rise to the 25 aminoacids protein, hepcidin (Dallilio, 2003; Hsu, 2006). Hepcidin was reported to bind to the transmembrane iron exporter ferroportin, which is present on macrophages, on the basolateral site of enterocytes, and also on hepatocytes. *In vitro* studies showed that hepcidin induces the internalization and degradation of ferroportin, crucial for cellular iron export (Domenico, 2007). By diminishing the effective number of iron exporters on the membrane of the enterocytes and of the macrophages, hepcidin inhibits iron uptake and release, respectively. This is the phenotype of ferroportin disease, in which the deficiency in ferroportin leads to iron accumulation, mainly in macrophages, and, usually, to anaemia (Njajou, 2002).

Increased hepcidin expression along an inflammatory process, 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 iron-refractory (Nicolas, 2002; Kulaksiz, 2004; Hsu, 2006). This decreased availability in iron may be a host defence mechanism against invading microorganisms.

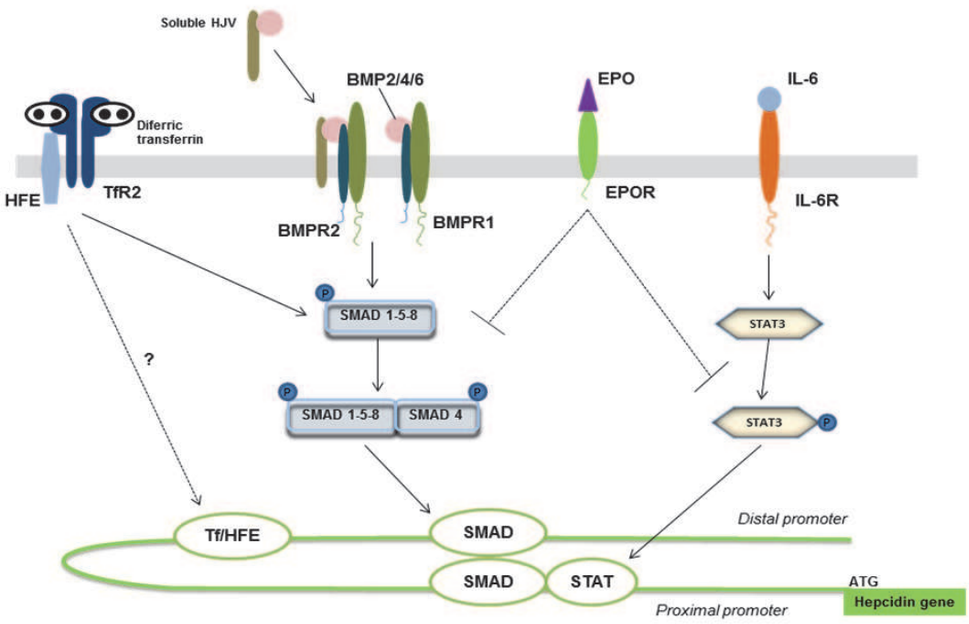

Fig. 3. Schematic pathways involved in hepcidin gene expression. Interleukin (IL)- 6 stimulates hepcidin synthesis via STAT activation; bone morphogenic proteins (BMP) stimulate hepcidin gene expression in a pathway dependent on hemojuvelin (HJV), BMP receptors (BMPR1 and BMPR2), and SMAD activation; transferrin also stimulates hepcidin gene expression in a pathway dependent on transferrin receptor 2 (TfR2), HFE protein, and SMAD activation. Erythropoietin (EPO) has an inhibitory effect in hepcidin expression in a pathway dependent on EPO receptor (EPOR), and STAT and SMAD inhibition. For simplicity, only some factors associated with hepcidin expression are shown.

The resistance to rhEPO therapy has been associated with disturbances in iron metabolism. Actually, the main cause for rhEPO resistance described in literature in HD patients, is iron deficiency, which persists in some patients, even after iron supplementation (Drueke, 2001). This iron deficiency can be absolute, with serum ferritin concentration less than 100 mg/dL, or functional.

We recently reported that HD patient's non-responders to rhEPO therapy present a mild to moderate anaemia, even with the administration of higher rhEPO doses (Costa, 2008d). This anaemia is hypochromic (decreased mean cell haemoglobin and mean cell haemoglobin concentration), and presents with a more accentuated anisocytosis than in HD patients that are good responders to rhEPO therapy. The haematological changes in non-responders seem to reflect a "functional" iron deficiency, as they presented adequate iron stores, as defined by conventional criteria, and an apparent inability to mobilize the iron needed to adequately support erythropoiesis. Actually, no statistically significant differences were found in serum iron status markers between responders and non-responders HD patients, except for the soluble transferrin receptor (s-TfR), which was significantly higher in non-responders (Table 3). The levels of this soluble receptor may be increased in two clinical settings, in case of increased erythropoietic activity and of iron deficiency (Atanasio, 2006; Deicher, 2006). We observed in our HD patients a positive and significant correlation between s-TfR and the weekly rhEPO/Kg doses, suggesting that s-TfR was an indicator of the erythropoietic stimuli of the administrated rhEPO, and not an indicator of iron body deficiency. Moreover,

no differences were found for transferrin saturation, between responders and non-responders HD patients, excluding, therefore, iron deficiency as the principal cause of the elevated s-TfR found in HD patients non-responders to rhEPO therapy.

	Controls (n=25)	Responders (n=25)	Non-responders (n=25)
Iron (µg/dL)	73.42 ± 25.24	60.24 ± 22.97	50.40 ± 29.27*
Ferritin (ng/mL)	85.10 (37.88-123.95)	380.30 (252.30-543.75)*	452.00 (163.00-674.50)*
Transferrin (mg/dL)	231.50 (205.00-268.00)	173.00 (152.50-186.00)*	161.00 (139.00-211.00)*
TS (%)	21.83 ± 7.97	25.05 ± 9.69	20.73 ± 12.09
s-TfR (nmol/L)	20.85 ± 8.56	19.56 ± 6.83	34.13 ± 11.4§
Prohepcidin (ng/mL)	92.11 ± 18.28	165.72 ± 36.69*	137.77 ± 46.03*§
CRP (mg/dL)	1.75 (0.76-4.70)	3.20 (1.73-7.23)*	10.14 (3.82-38.99)*§
s-IL2R (nmol/L)	758.83 ± 234.95	4005.71 ± 1835.70*	4394.17 ± 1701.80*
IL-6 (pg/mL)	1.90 (0-3.75)	5.75 (3.83-13.95)*	8.80 (4.55 – 21.30)*

Table 3. Serum markers of iron status and of inflammation, for controls, responders and non-responders HD patients.* $p<0.05$, *vs* controls; § $p<0.05$, *vs* responders. Results are presented as mean ± standard deviation and as median (interquartile ranges). TS: Transferrin saturation; CRP: C-reactive protein; s-IL2R: Soluble interleukin-2 receptor; IL-6: Interleukin-6. Adapted from Costa, 2008d.

Inverse correlations between CRP and mean cell volume, mean cell haemoglobin, serum iron and transferrin saturation were also found in non-responders patients, suggesting that the "functional" iron deficiency may be related with the enhanced chronic inflammation found in these patients. Actually, as previously referred hepcidin may have an import key role in "anaemia of inflammation" by limiting iron availability for erythropoiesis and, in that way provides a direct link between inflammation and iron metabolism.

In literature there is evidence that HD patients present increased serum levels of prohepcidin and hepcidin (Costa, 2008d; Costa, 2009). As non-responders patients present high inflammatory markers, it would be expected that prohepcidin and hepcidin serum levels were increased in non-responders patients. However, in our studies, we found that non-responders patients present lower prohepcidin, and a trend to lower hepcidin serum levels, when compared with responder's patients (Fig. 4). These findings might result from the downregulation of liver hepcidin expression induced by high doses of rhEPO, acting, therefore, as a hepcidin inhibitory hormone. Since non-responders were treated with much higher doses of rhEPO, as compared with responders, the lower prohepcidin and hepcidin levels among non-responders could be explained by this inhibitory effect of rhEPO.

Our data suggest that hepcidin serum levels are dependent on the degree of the inflammatory stimuli and of the therapeutic doses of rhEPO. In addition, the use of high doses of rhEPO, may induce increased iron utilization by the bone marrow, that may lead to depletion of iron stores and to a decrease in iron availability for erythroid cells, which will trigger a decrease in prohepcidin and hepcidin levels, in order to favour iron absorption (Costa, 2008d; Costa, 2009).

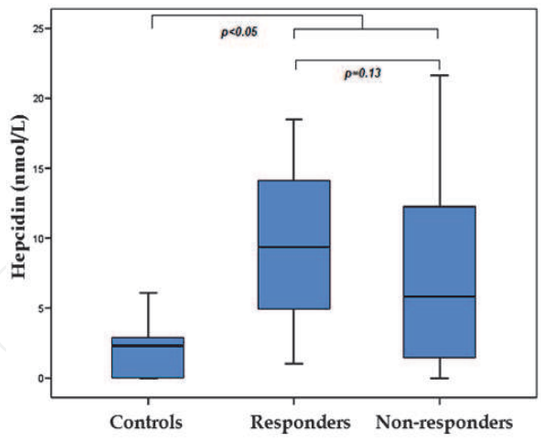

Fig. 4. Serum hepcidin levels for controls and for HD patients, responders and non-responders to rhEPO therapy. Boxplot shows median value (horizontal line in box) and first and third quartiles (inferior and superior line of the box, respectively). Adapted from Costa, 2009.

There is evidence in literature of a close interaction between inflammation, iron status and hepcidin serum levels, which, ultimately, regulates intracellular iron absorption and availability. It is also accepted that hepcidin plays a significant role in anaemia of HD patients; however, we wonder it is useful as a marker of resistance to rhEPO therapy, considering the overlap of the hepcidin levels between responders and non-responders HD patients, and the several influences and interrelations with other substances.
Clearly, more work is required for a better understanding about the role of iron metabolism in the development of resistance to rhEPO therapy and to provide useful therapeutic biomarkers of resistance.

5. Erythrocyte damage

The erythrocyte membrane is a complex structure comprising a lipidic bilayer, integral proteins and the skeleton. Spectrin is the major protein of the cytoskeleton, and, therefore, the major responsible for erythrocyte shape, integrity and deformability. It links the cytoskeleton to the lipid bilayer, by vertical protein interactions with the transmembrane proteins, band 3 and glicophorin A (Lucchi, 2000). In the vertical protein interaction of spectrin with band 3 are also involved ankyrin (known as band 2.1) and protein 4.2. A normal linkage of spectrin with the other proteins of the cytoskeleton assures normal horizontal protein interactions.
In HD patients, the erythrocytes are physically stressed during the HD procedure, metabolically stressed by the unfavourable plasmatic environment, due to metabolite accumulation, and by the high rate of haemoglobin autoxidation, due to the increase in haemoglobin turnover, a physiologic compensation mechanism triggered in case of anaemia (Lucchi, 2000; Stoya, 2002). The erythrocytes are, therefore, continuously challenged to sustain haemoglobin in its reduced functional form, as well as to maintain the integrity and deformability of the membrane.
When haemoglobin is denatured, it links to the cytoplasmic pole of band 3, triggering its aggregation and leading to the formation of strictly lipidic portions of the membrane, poorly

linked to the cytoskeleton. These cells are, probably, more prone to undergo vesiculation (loss of poorly linked membrane portions) whenever they have to circulate through the HD membranes or the microvasculature. Vesiculation may, therefore, lead to modifications in the erythrocyte membrane of HD patients (Reliene, 2002; Rocha, 2005).

Erythrocytes that develop intracellular defects earlier during their life span are removed prematurely from circulation (Santos-Silva, 1998; Rocha-Pereira, 2004). The removal of senescent or damaged erythrocytes seems to involve the development of a senescent neoantigen on the membrane surface, marking the cell for death. This neoantigen is immunologically related to band 3 (Kay, 1994). The deterioration of the erythrocyte metabolism and/or of its antioxidant defences may lead to the development of oxidative stress within the cell, allowing oxidation and linkage of denatured haemoglobin to the cytoplasmatic domain of band 3, promoting its aggregation, the binding of natural antiband 3 autoantibodies and complement activation, marking the erythrocyte for death. The band 3 profile [high molecular weight aggregates (HMWAg), band 3 monomer and proteolytic fragments (Pfrag)], differs between younger, damaged and/or senescent erythrocytes. Older and damaged erythrocytes present with higher HMWAg and lower Pfrag. Younger erythrocytes show reduced HMWAg and higher Pfrag (Santos-Silva, 1998). Several diseases, known as inflammatory conditions, present an abnormal band 3 profile, suggestive of oxidative stress development (Santos-Silva, 1998; Belo, 2002; Rocha-Pereira, 2004).

Leukocyte activation is part of an inflammatory response, and is an important source of ROS and proteases, both of which may impose oxidative and proteolytic damages to erythrocyte and plasma constituents. Actually, oxidative stress has been reported to occur in HD patients and has been proposed as a significant factor in HD-related shortened erythrocyte survival.

Erythrocyte membrane protein studies performed in HD patients, using cuprophane and polyacrylonitrile dialysis membranes, showed a reduction in spectrin and band 3, and an isolated reduction in band 3, respectively (Delmas-Beauvieux, 1995).

As referred, we hypothesized that non-responders patients to rhEPO therapy could have an enhanced erythrocyte damage and/or senescence; we, actually, found an altered erythrocyte membrane band 3 profile in HD patients, with a decrease in HMWAg, Pfrag and in Pfrag/band 3 monomer and HMWAg/band 3 monomer ratios, as compared to control. This profile presents changes reflecting the co-existance of an increased number of younger and damaged erythrocytes. Non-responders patients also showed a decrease in Pfrag and in Pfrag/band 3 monomer ratio (Fig. 5), suggesting that they present a higher number of damaged erythrocytes that may result from an even more adverse plasmatic microenvironment (Costa, 2008e).

We also found some changes in erythrocyte membrane protein composition of HD patients using high-flux polysulfone FX-class dialysers of Fresenius, being the decrease in spectrin the most significant change. This reduction in spectrin may account for a poor linkage of the cytoskeleton to the membrane, favoring membrane vesiculation, and, probably, a reduction in the erythrocyte lifespan of these patients (Reliene, 2002). Significant increases in protein bands 6 and 7 were also observed, which may further reflect an altered membrane protein interaction and destabilization of membrane structure. This membrane destabilization was further strengthened by the significant changes observed for spectrin/band 3 ratio (table 4). In non-responders HD patients these changes were more accentuated than in responders, presenting a trend to lower values for spectrin (table 4) and significantly lower value for ankyrin/band 3 and spectrin/ankyrin ratios (Costa, 2008f; Costa, 2008g). These enhanced alterations may be due to a higher erythrocyte metabolic stress and/or to changes resulting from the HD procedure *per se*.

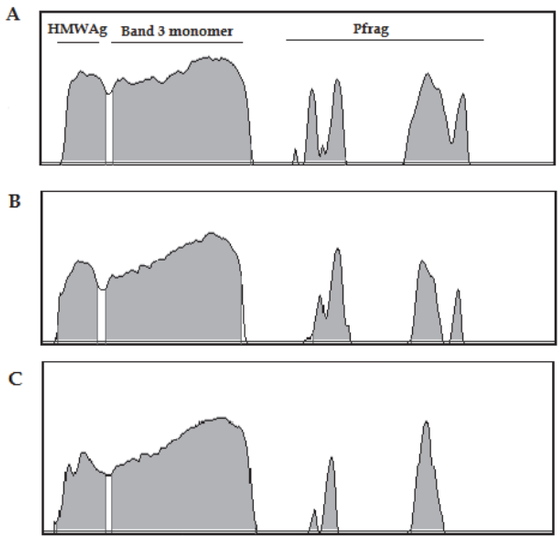

Fig. 5. Examples of densitometer tracing of immunoblots for band 3 profile. A- Control; B- Responder HD patient; C- Non-responder HD patient.

	Controls (n=26)	Responders (n=32)	Non-responders (n=31)
Spectrin (%)	27.63 (26.41-28.79)	24.75 (22.38-26.63)*	22.35 (18.95-25.92)*
Ankyrin (%)	6.97±1.62	6.09±2.07	6.97±1.60§)
Band 3 (%)	38.57 ± 3.99	39.92±4.03	38.65±3.70
Protein 4.1 (%)	7.56±1.45	7.18±1.33	7.31 ±1.63
Protein 4.2 (%)	5.51±0.72	5.54±1.57	5.35±1.29
Band 5 (%)	6.82±0.86	6.70±1.02	7.04±1.00
Band 6 (%)	5.19±1.04	6.61±1.30*	7.37±1.32*
Band 7 (%)	2.20±0.65	3.16±0.98*	3.49±1.43*
Protein 4.1/Spectrin	0.276 ± 0.624	0.310 ± 0.105	0.340 ± 0.130*
Protein 4.1/Band 3	0.192 (0.154–0.227)	0.183 (0.154-0.208)	0.183 (0.159-0.205)
Protein 4.2/Band 3	0.149 (0.125-0.162)	0.135 (0.110-0.169)	0.142 (0.110-0.161)
Spectrin/Band 3	0.707 (0.649-0.822)	0.572 (0.541-0.685)*	0.544 (0.486 -0.687)*
Ankyrin/Band 3	0.185 ± 0.585	0.155 ± 0.060	0.183 ± 0.052§
Spectrin/Ankirin	4.18 ± 1.07	4.44 ± 2.25	3.10 ± 0.94*§

Table 4. Erythrocyte membrane protein profile for controls, responders and non-responders HD patients.* $p<0.05$, *vs* controls; § $p<0.05$, *vs* responders. Results are presented as mean ± standard deviation and as median (interquartile ranges). Adapted from Costa, 2008f.

Although HD procedure seems to have an important role in these alterations in erythrocyte membrane protein composition, their exact origin(s) are not fully understood. We hypothesized that the increased plasma levels of elastase found in HD patients could induce alterations in erythrocyte membrane proteins, leading to a decrease in erythrocyte lifespan in HD patients, particularly enhanced in non-responders, and, consequently, to an increase in the degree of the anaemia, in these patients (Fig. 6).

To establish the value of elastase in the erythrocyte membrane changes observed in HD patients, we performed in a more recent study (unpublished data), some *in vitro* assays using erythrocytes from 18 HD patients (10 responders and 8 non-responders) and from 8 healthy controls; erythrocyte suspensions in phosphate buffered saline, pH 7.4, were incubated at 37º C, under gentle rotation, in the presence of 0.03, 0.1 and 0.5 µg/mL of neutrophil elastase. These assays used erythrocytes collected before and immediately after HD procedure. Before the HD procedure, the erythrocytes from responders and non-responders HD patients are more susceptible to the proteolytic action of elastase than the erythrocytes from the controls, and this susceptibility is more pronounced for the erythrocytes from non-responders. As after the HD procedure the composition of the erythrocyte membrane from both responders and non-responders did not change, it seems that the more susceptible erythrocytes are removed during the HD procedure.

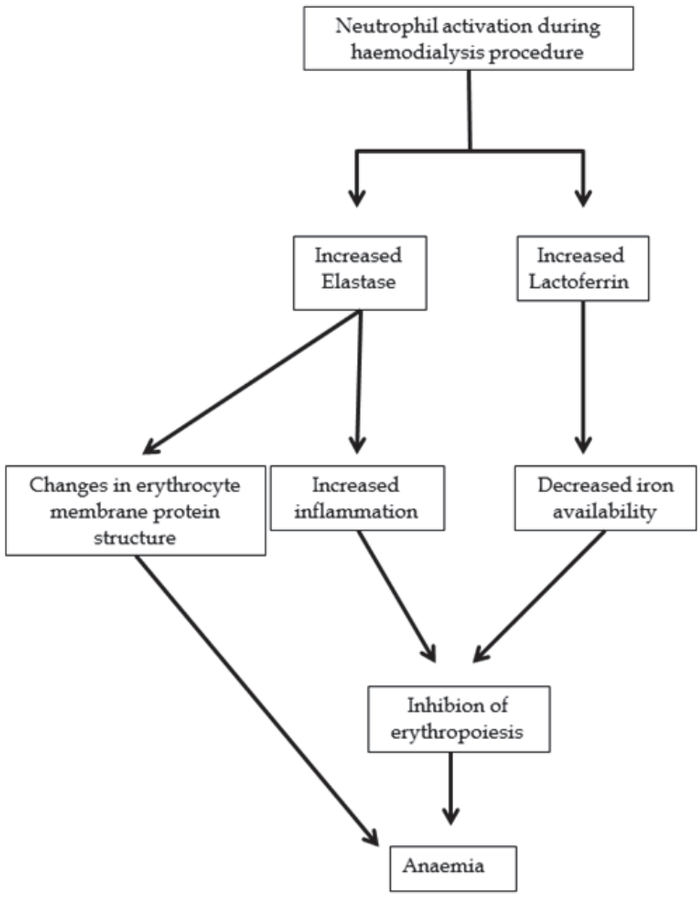

Fig. 6. In HD patients, the increased plasma levels of elastase can induce changes in erythrocyte membrane proteins, leading to a decrease in the erythrocyte lifespan and, consequently, to increase the degree of anaemia in these patients. Moreover, the increased levels of elastase might exacerbate the inflammatory process that has an inhibitory effect on erythropoiesis. The release of lactoferrin during the HD procedure may contribute to decrease iron availability for erythropoisis. These changes are enhanced in non-responders HD patients.

5. Conclusions

Although the etiology of resistance to rhEPO therapy is still unknown, inflammation seems to have an important role in its pathophysiology. Resistance to rhEPO therapy is also associated with "functional" iron deficiency, neutrophil activation, and with changes in erythrocyte membrane protein structure.

The exact origins of the inflammatory process remain unclear. We wonder if the release of elastase during the HD procedure could amplify the inflammatory process in HD patients, particularly in non-responders, and if this elastase release has a role in the alterations observed in the erythrocyte membrane protein structure, further contributing to worsening of anaemia (Fig 6). The inflammatory process, the rhEPO doses administrated, and the lactoferrin release during the haemodialysis procedure, seem to play 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.

Further studies are needed to better understand the rise in inflammation and the associated need for higher doses of rhEPO and reduced iron availability. It is also important to clarify the effect of higher levels of elastase in the inflammatory process, and in the alterations in the erythrocyte membrane protein composition and in the band 3 profile.

New therapeutic options, in order to decrease rhEPO doses, are currently under investigation, namely the protein product of the growth arrest-specific gene 6, the mixture of herbal extracts - jusen-taiho-to, the growth hormone, the insulin-like growth factors-1, and the development of an inhibitor of hepcidin.

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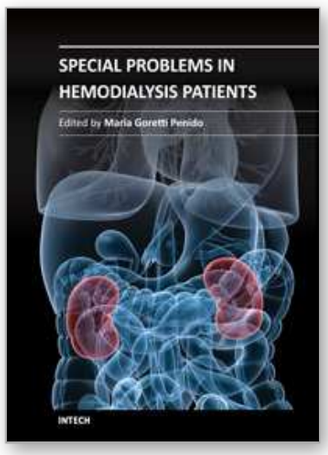

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This book provides an overview of special cases in hemodialysis patients. Authors have contributed their most interesting findings in dealing with patients suffering of other diseases simultaneously, such as diabetes, cardiovascular disease and other health problems. Each chapter has been thoroughly revised and updated so the readers are acquainted with the latest data and observations in these complex cases, where several aspects are to be considered. The book is comprehensive and not limited to a partial discussion of hemodialysis. To accomplish this we are pleased to have been able to summarize state of the art knowledge in each chapter of the book.

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