# EXHIBIT 4

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# Pure red cell aplasia secondary to treatment with erythropoietin

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**ABSTRACT: Pure red cell aplasia (PRCA) is a rare condition defined as severe anemia secondary to the virtual absence of red blood cell precursors in the bone marrow. In the setting of patients treated with rHuEPO, the disease is generated by epoetin-induced antibodies that neutralise all the exogenous rHuEPO and cross-react with endogenous erythropoietin. As a result, serum erythropoietin levels are undetectable and erythropoiesis becomes ineffective. Only 4 cases of PRCA associated with rh-EPO have been reported before 1998. Thereafter, a sharp increase in the incidence of this rare condition has been reported, mainly associated with epoetin alpha use outside the United States. A number of possible mechanisms leading to PRCA development have been identified. Among these, modification of drug formulation and down stream processing probably has had a major role. Indeed, in 1998 the formulation of epoetin alpha in Europe was modified because of the fear of the "mad cow" syndrome. However, differences in molecule structure and glycosylation among different epoetins can not be excluded. It should also be underlined that the rise in the incidence of PRCA cases has been coincident with a major shift from intravenous to subcutaneous administration of rHuEPO.**

**The abrupt rise in the incidence of PRCA cases observed in the last few years, deserves particular attention; however, we have to balance its severity, but extreme rarity, with the high number of chronic kidney disease patients who die each year because of cardiovascular disease that could partially be reduced by anemia treatment.**

**Key words:** *Pure red cell aplasia, Anemia, Chronic kidney disease, Erythropoietin, Anti-erythropoietin antibodies, Molecular structure*

## INTRODUCTION

Therapy with recombinant human erythropoietin (rHuEPO) is a well established treatment for renal anemia. This product of molecular genetic technology has been used for more than 15 years and has an excellent therapeutic index (a selective and potent effect on erythropoiesis against only mild to moderate adverse events such as aggravation of hypertension or thrombotic complications). However, the increasing incidence of acquired pure red cell aplasia (PRCA) in patients with chronic kidney disease (CKD) treated with rHuEPO in the last few years has given rise to a number of concerns about this pharmacological agent.

PRCA is a rare condition defined as severe anemia secondary to the virtual absence of red blood cell precursors in the bone marrow. In the setting of patients treated with rHuEPO, the disease is generated by epoetin-induced antibodies that neutralize all the exogenous rHuEPO and cross-react with endogenous erythropoietin. As a result, serum erythropoietin levels are undetectable and erythropoiesis become ineffective.

In this review we will try to summarize the main features of the disease, its incidence and prevalence and the possible causes of increased immunogenicity which have been identified so far.

### *Pure red cell aplasia: Main features and differential diagnosis*

Acquired PRCA is an immune-mediated disorder of erythropoiesis that can result in severe anemia, low reticulocyte counts (< 10,000/mm$^3$), normal platelet and granulocyte counts, and bone marrow smears exhibit an almost complete absence of red cell precursors without any other remarkable signs. Antibodies can recognise either erythroid precursor cells or erythropoietin: in the first case serum erythropoietin is high, in the second case it is low (1). As a result of almost complete interruption of erythropoiesis, the

hemoglobin concentration declines very fast, at a rate corresponding to a red cell life span (nearly 0.1 g/dL/day, slightly less than 1g/dL/week) (2); mean corpuscular volume is usually normal or slightly increased.

PRCA has been associated with a number of different conditions that are to be kept in mind in the evaluation of a suspected PRCA case associated with rHuEPO therapy. This rare disease has often been reported in association with thymoma, myelodysplasia, B- and T-cell chronic lymphatic leukemia and chronic myeloid leukemia, autoimmune hemolytic anemia, lupus erythematosus systemicus, rheumatoid arthritis, and viral infections (in particular parvovirus B19 and hepatitis B virus). In addition, several drugs can cause PRCA. The commonest drugs described in the literature were anticonvulsants, antibiotics and anti-thyroid agents (3). Of interest, a number of drugs commonly used in nephrological clinical practice have been reported to rarely cause PRCA. Among these, allopurinol (3), azathioprine (4), and mycophenolate mofetil (5).

It is also worth noting that PRCA has been reported in patients with no underlying disease (6, 7).

PRCA associated with treatment with rHuEPO has been described as the abrupt appearance of severe anemia refractory to treatment. The median age of the patients was 61 years, with a slight predominance of males (8). The median duration of treatment with rHuEPO before diagnosis was seven months, ranging from one month to five years (8). No correlation was found with cause of kidney failure, CKD treatment, age or gender as such, but there was a disproportionally higher incidence in males over 70 years. However, it is of note that elderly male patients are very common in the ESRD population and the Authors did not clarify whether they corrected the analysis taking into account this increased prevalence.

The majority of the patients had relatively stable clinical conditions, hemoglobin levels and rHuEPO doses before the onset of the disease (9-12). Many of them were switched to treatment with another rHuEPO after the worsening of anemia, hindering the recognition of the real cause-effect relationship between a given type of erythropoietin and PRCA. For this reason, and also because anti-erythropoietin antibodies cross react with other erythropoietins, treatment with rHuEPO must be stopped immediately in suspected cases. Indeed, dose increases are ineffective and expose patients to the risk of systemic reactions (11).

All PRCA cases associated with rHuEPO have been solely reported in CKD patients, in spite of the large use of this drug also in the setting of oncology. Patients with cancer are probably less likely to develop the disease because of a decrease in immune-competency, concomitant treatment and reduced time of exposure to the drug.

### *Incidence of pure red cell aplasia associated with erythropoietin treatment*

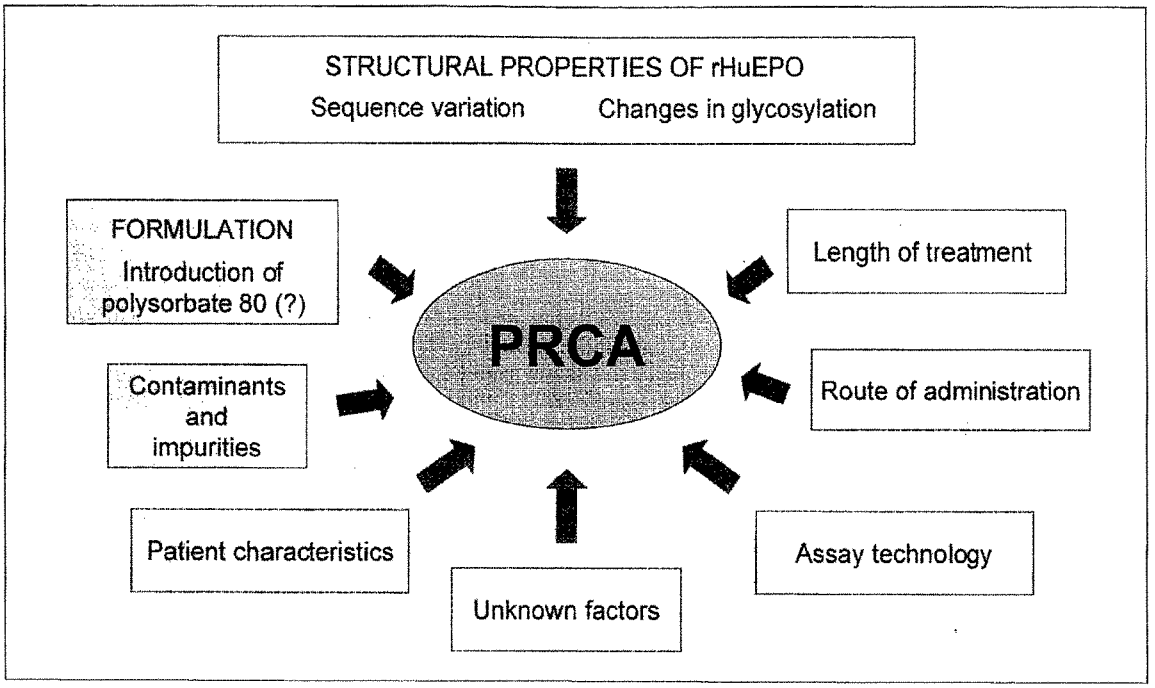
Only 4 cases of PRCA associated with rHu-EPO have been reported before 1998 (9,13-15). Between 1998 and 2000, French investigators identified new 13 PRCA patients after taking rHuEPO, 12 receiving epoetin alpha and 1 receiving epoetin beta (10). Shortly thereafter, the Food and Drug Administration reported 82 cases of PRCA from July 1997 to December 2001 (8). The majority of cases were observed in patients treated with Eprex®, (epoetin alpha), which is a product of Johnson & Johnson outside the United States (8). The observed increase in the distribution of Eprex®, in this time period can not account for differences among the brands in the number of reported cases (8). Up to March 24, 2003 Johnson & Johnson confirmed 163 cases of antibody positive PRCA; 142 in patients exposed only to Eprex® and 21 in patients exposed to another erythropoietic protein in addition to Eprex®. Further 61 cases are under investigation (Johnson & Johnson press release). A stabilization in the worldwide exposure-adjusted rate of antibodypositive PRCA case reports was observed in 2002 versus 2001 (Johnson & Johnson press release). A very recent official editorial by the European Renal Association-European Dialysis Transplant Association (ERA-EDTA) (16) reported 5 PRCA cases in patients solely treated with epoetin beta and 4 cases treated with Epogen® (epoetin alpha produced by Amgen in the United States). In addition, three more cases treated with epoetin beta and three cases treated with darbepoetin alpha were considered unlikely to be associated with the disease (16). The incidence per 10,000 treated patient years was confirmed to be much higher for Eprex® (1.11) than for epoetin beta (0.12), Epogen® (0.02) and darbepoetin alpha (0.5) (16). Possible additional cases, i.e. one patient treated with epoetin beta (17) and three Chinese patients treated with epoetin alpha, have recently been published (12).

All these data clearly suggest that the rise in the incidence of PRCA cases in recent years is mainly associated with epoetin alpha use outside the United States.

### *Characteristics of anti-erythropoietin antibodies*

Casadevall et al (10) extensively studied the characteristics of anti-erythropoietin antibodies identified in a number of PRCA cases. These antibodies were polyclonal, homogeneous in the single patient and all able to neutralize very high concentrations of the native protein. After deglycosylation, the affinity of the antibodies increased in all the patients, excepting in the

Fig. 1




only one who was treated exclusively with epoetin beta. In this patient, the antibodies were probably directed against linear and conformational epitopes (10), suggesting that mechanisms of immunization can differ among rHuEPO types.

Figure 1 summarizes possible mechanisms leading to PRCA development.

*Molecular structure of different erythropoietins*

Human erythropoietin (EPO) is a hydrophobic protein of 165 aminoacids stabilized by three N-glycans (18). One O-linked sugar chain is also invariably present (19). Deglycosylation of the N-linked oligosaccharides made rHuEPO remarkably susceptible to aggregation on heat-treatment, indicating that the carbohydrate chains are essential to stability (20). The carbohydrate content also plays some important roles in the activity and biosynthesis of the molecule. Given this high glycosylation content (almost 40% of total mass) and the requirement for sialic acid for optimal in vivo activity, mammalian cells are essential to produce rHuEPO (Chinese hamster ovary cells).

At present, three different types of rHuEPO are available on the market: epoetin alpha, epoetin beta and epoetin omega (the last one only in Eastern Europe). All these three molecules have the same aminoacid composition, which is identical to that of EPO, but differ in their carbohydrate content. Epoetin alpha has a slightly lower sialylation than epoetin beta; this accounts for the small differences observed in the pharmacokinetics and pharmacodynamics of the two molecules (21), but it does not explain the different immunogenicity. Skibeli et al (22) reported a more reduced sialylation of glycans in EPO than in rHu-EPO. Even if these differences might be involved in the etiology of PRCA, artefacts due to extraction procedures of human erythropoietin explaining these differences can not be completely ruled out (23).

The molecule of epoetin-omega is a sialoglycoprotein with smaller amounts of O-bound sugars, it is less acidic and has different hydrophylity than the other 2 epoetins (24). At present, no cases of PRCA have been reported in patients treated with epoetin omega, but only a small number of patients have received the drug.

Darbepoetin alfa has recently been introduced onto the market. Like rHuEPO, it is produced by recombinant DNA technology in Chinese hamster ovarian cells and it contains five N-linked carbohydrate chains(two more than rHuEPO). To introduce new carbohydrate attachment sites into the polypeptide backbone, the DNA sequence of the erythropoietin gene was modified, obtaining an aminoacid sequence that differs from EPO at five positions (25). As a result, darbepoetin alpha has a greater molecular weight, sialic acid content and negative charge than rHu-EPO (9). Since the aminoacid sequence and the carbohydrate content of darbepoetin alpha differ from EPO, it is theoretically possible that this new molecule could be immunogenic. However, the location of the aminoacid substitutions proximal to the carbohydrate addition site probably hides them from immune surveillance (9). Furthermore, the fact that these addi-

tion sites are distal to the receptor binding sites reduces the likelihood of developing neutralizing antibodies (9). At present, no PRCA cases have been reported in patients solely treated with this agent. Even if the number of patients who have been treated so far with darbepoetin alpha is much lower than that of patients treated with rHuEPO, thus significantly reducing the number of patients at risk of developing PRCA, the majority of the patients enrolled in clinical studies have been tested for the development of antibodies against darbepoetin alpha, but these have never been detected.

Two other new erythropoietic agents are under evaluation. A pegylated version (i.e. the covalent addition of the water soluble polyethylene glycol moiety) of epoetin beta, Ro 50-3821, has recently been synthesized and is under evaluation in a phase II clinical trial (26). Its aminoacid sequence is identical to EPO. No antibodies against this new drug have been reported, but clinical experience is still very limited.

When considering the risk of developing PRCA, the total chemical synthesis of new erythropoietic agents can add further concerns compared with EPO analogues obtained with the DNA recombinant technique. The "synthetic erythropoiesis protein" (SEP), a polymer consisting of a 166-amino-acid polypeptide chain and two covalently attached, branched, polymer moieties that are negatively charged has recently been synthesized (27). In cell and animal assays for erythropoiesis, SEP displayed potent biological activity and had significantly prolonged duration of action *in vivo*, but there is still no information about its possible immunogenicity.

### *Protein glycosylation and immunogenicity*

Protein glycosylation is an ubiquitous post-translational modification. Oligosaccharide units of glycoproteins serve a variety of functions (28), including modulation of the biologic activity and the clearance of proteins from the plasma. Structures of many oligosaccharide chains linked to proteins are identical or closely similar amongst species and are not or are only weakly immunogenic. However, interest in glycobiology has increased dramatically amongst immunologists during the last few years due to the fact that oligosaccharides also play a central role in adhesion and homing events during inflammatory processes, comprise powerful xenotransplantation antigens, and may provide targets for tumor immunotherapy (29). In addition, alterations in glycosylation are now known to occur in a number of autoimmune diseases (29).

Antibodies raised against glycoprotein antigens may be specific for their carbohydrate units which are recognized irrespective of the protein carrier (carbohydrate epitopes), or in the context of the adjacent amino acid residues (glycopeptidic epitopes) (30). Conformation or proper exposure of peptidic epitopes of glycoproteins is also frequently modulated by glycosylation due to intramolecular carbohydrate-protein interactions. In highly glycosylated proteins, peptidic epitopes are either apparently masked by glycans, or conversely glicosylation may enhance the reaction (30). The enhancement of antibody binding to partially or totally deglycosilated antigen suggest recognition of a peptidic epitope (31). This appears to be the case for the majority of antibodies against rHuEPO studied so far (10). Also considering that the glycate content of epoetin alpha and beta has not been modified in recent years, it seems unlikely that altered glycosylation or differences in the carbohydrate content among erythropoietin types can explain the rise in the incidence of PRCA cases observed in the last three years.

### *Drug formulation and down stream processing*

rHu-EPO is a poorly water-soluble drug. For this reason, excipients are required to enhance its dispersion or to inhibit precipitation once the solution is mixed with water.

Excipients differ among rHuEPO types, possibly explaining various degree of immunogenicity of these molecules and in particular the recent rise in the incidence of PRCA cases occurring mainly in patients treated with Eprex®. In 1998 the formulation of this drug was modified in Europe because of the fear of the "mad cow" syndrome. Following regulatory guidance, human serum albumin (HAS) was removed from the initial formulation and substituted by polysorbate 80, which is a surfactant, and glycine. Since its registration in 1990, epoetin beta has contained polysorbate 20, which is another surfactant. Darbepoetin alpha contains polysorbate 80, but at a lower concentration than Eprex®. The HAS-free formulation epoetin alpha may be more susceptible to formation of aggregates under stress conditions or may be more susceptible to denaturation, leading to increased immunogenicity. This formulation may also be more susceptible to formation of aggregates in the presence of silicon oil, which is used to lubricate syringes. Even if no aggregate formation was seen at storage conditions of 2-8°C, higher amounts of silicon oil, with higher temperature over time, increases aggregate formation (32). For this reason, tighter controls on product to minimize exposure to higher temperatures during manufacturing, shipping, and prior to patient use have been developed to increase awareness about the importance of appropriate handling of the drug (32). The presence of contaminants or impurities seem not to be a main cause, since no changes in product before and after the process of bulk manufacturing were detected (32).

***Route of administration***

The rise in the incidence of PRCA cases has been coincident with a major shift from intravenous to subcutaneous administration of rHuEPO, given the possibility of reducing costs with the latter. This occurred especially outside the United States and could have influenced the rise in the number of PRCA cases by two mechanisms. First of all, it is well known that, the subcutaneous route is more likely to give immunogenicity than the intravenous one (33). This because skin has a highly developed immune system with different cell types contributing to antigen presentation and immune responses. It is possible that prolonged exposure of these cells to epoetin after subcutaneous administration can increase immunogenicity (11).
Secondly, the subcutaneous route increases the possibility of self-administration, making home storage more common and increasing the risk of inappropriate handling or storage. It is a matter of fact that the majority of the patients developing PRCA received rHuEPO subcutaneously. Furthermore, different reporting rates across various countries, all utilizing identical Eprex®, could support a role of increased immunogenicity for the subcutaneous route. For instance, in Germany the number of PRCA cases is low and only about 30% of the patients receive Eprex® subcutaneously (Johnson & Johnson press release). However, it is also true that in Italy the majority of the patients receive Eprex®, subcutaneously, but PRCA is rare. In December 2002, regulatory authorities contraindicated the subcutaneous use of Eprex®, in CKD patients. The impact of this measure on the incidence of new PRCA cases is still unknown.

***What to do?***

Even if PRCA secondary to rHuEPO treatment is still a very rare condition, physicians must constantly be aware of the possibility this disease can occur and consider it in the differential diagnosis of a patient who has rapid worsening of anemia or does not respond to the drug. After a complete workup for anemia has been done (including assessment of the reticulocyte count) and other known causes of anemia have been excluded, if PRCA is still suspected, bone marrow evaluation and testing for erythropoietin antibodies are indicated. In the meantime, rHuEPO therapy must be discontinued, without switching treatment to another type of erythropoietin, in order to avoid misinterpretation of the causative agent and occurrence of crossreactivity with the new compound. After stopping treatment, a slow decrease in the autoantibody titre is usually observed (10), but serum erythropoietin is detectable only when autoantibodies completely disappear. Preliminary observations suggest that nearly 40% of the patients remain transfusion-dependent. The treatment of PRCA is still an open question, due to the little experience accumulated so far. More than half of the patients appear to respond to immunotherapy. Corticosteroids alone, steroids associated with cyclosporine or cyclophosphamide, immunoglobulin, plasmapheresis have been used. Steroid plus cyclophosphamide probably give the best results (34). Cyclosporine also seems to be effective (34). Patients who received kidney transplantation have had the best outcome. This is probably because immunosuppressive regimens instituted after transplantation are effective on PRCA as well.

## Conclusions

In CKD patients, rHuEPO is not only highly effective at correcting the underlying anemia, restoring energy levels, and increasing patient well-being and quality of life, but it also has considerable secondary benefits in terms of morbidity and mortality reduction, particularly in relation to improved cardiac function. The abrupt rise in the incidence of PRCA cases observed in the last few years deserves particular attention from the nephrological community. However, putative causative mechanisms are very complex and perhaps nobody will be able to completely clarify them. For this reason, in the near future we will probably have to live with PRCA, balancing its severity but extreme rarity with the high number of CKD patients who die each year because of cardiovascular disease that could partially be reduced by anemia treatment. Also we must not forget the impressive benefits on quality of life and patient wellbeing obtained by anemia correction.

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