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Original Article

Antibodies to Erythropoietin Are Associated with Erythropoietin Resistance in Hemodialysis Patients in KwaZulu-Natal (South Africa)

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ABSTRACT. Recombinant human erythropoietin (rHuEPO) is a glycoprotein and biological equivalent to the endogenous compound administered to treat anemia of end-stage renal disease patients. Resistance to rHuEPO has been reported, whereby patients require higher and higher doses of rHuEPO to maintain an adequate hemoglobin level. In this study, assessment of native and administered erythropoietin (EPO), antibody and hemoglobin levels was carried out on a sample of patients with renal failure on hemodialysis (HD). This is a randomized controlled trial where consecutive subjects attending HD units at Addington Hospital and King Edward Hospital, Durban (South Africa) were included until the target number was reached. Forty patients with renal failure on HD and receiving recombinant EPO Beta (Recormon) for treatment of anemia via the subcutaneous route in weekly doses of 2000 IU, 4000 IU, 6000 IU, 8000 IU, 12,000 IU, or 18,000 IU according to the severity of the anemia were included after obtaining informed consent. Also included in the study were 10 HD patients not on rHuEPO therapy and 10 healthy individuals from the Durban University of Technology, recruited as described above to form the control group. ELISA was used to measure serum levels of EPO as well as antibodies to EPO. Results were analyzed by descriptive, inferential methods and by logistic regression analysis using IBM SPSS Statistics for Windows version 22.0. Antibodies to EPO were found in almost all patients who were receiving EPO. The highest levels of antibody to EPO were found to be associated with patients receiving the highest weekly dose of EPO (18,000 IU). Logistic regression analysis also revealed that serum levels of EPO, gender or age were not associated with any significant variation of serum antibody level. High levels of serum antibodies to EPO are a risk factor for EPO resistance.

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Introduction

Recently, many reports have highlighted the benefits of erythropoietin (EPO) not only in end-stage renal disease (ESRD) but also in many other clinical situations such as cancer-related anemia, nonuremic conditions including

hematological and oncological disorders, prematurity, HIV infection and preoperative therapies. Further, recombinant human EPO (rHuEPO) reduces the need for blood transfusion and hence exposure to donor blood products. In addition, it improves the quality of life.[1,2] In ESRD the use of EPO therapy is indicated since these patients cannot synthesize enough of it to correct severe anemia.[3]

EPO deficiency causing anemia occurs commonly in chronic kidney disease (CKD), and there is clinical evidence which shows that rectifying anemia can slow the advancement of CKD.[4] Measuring EPO concentrations improve the diagnostic value of recognizing relationship of EPO deficiency and renal anemia.[5] The decrease in production of EPO correlates well with the declining glomerular filtration rate.[6]

Resistance to erythropoiesis-stimulating agents (ESAs) has been observed in patients with CKD and it is associated with poor clinical outcomes. The presence of ESA resistance cannot always be explained by the known risk factors of the condition, suggesting that additional factors may be involved.[7]

The administration of higher doses of EPO can possibly override the inhibitory effect of circulating cytokines on EPO hence overcoming EPO resistance to a degree.[8] In other studies also, patients who were treated with rHuEPO to correct anemia developed anti-EPO antibodies. These antibodies did not completely neutralize EPO possibly because they are low-affinity antibodies.[9]

Although treatment with rHuEPO has been generally successful, a small number of patients produce antibodies that can neutralize both endogenous EPO and recombinant proteins.[10] Most cases of antibody production have been associated with the formulation of epoetin alfa when administered subcutaneously.[11]

In this study, EPO concentrations were measured together with anti-EPO antibodies, hemoglobin (Hb) levels, and various clinical, nutritional, and inflammation parameters with the aim to evaluate the association of the levels of anti-EPO antibodies with the above-listed parameters.

Materials and Methods

This is a prospective, randomized controlled study. Hemodialysis (HD) patients were recruited from the HD units at Addington Hospital and King Edward Hospital (South African State Hospitals), after obtaining permission from the Health Department of KwaZulu-Natal and ethical approval from the research ethics committee of Durban University of Technology, Durban. Forty consecutive HD patients who agreed to participate in the study were included. They were divided into three groups. CKD patients on HD receiving recombinant EPO (Group 1); one control group consisting of CKD patients on HD not receiving recombinant EPO (Group 2); (these patients were recently started on dialysis and had Hb of at least 10 g/dL while receiving IV iron treatment without recombinant EPO). The other control group consisted of healthy volunteers (Group 3) from a student group who agreed to participate following an interview. The inclusion criteria for the patient group were: CKD patients on HD, age between 18 and 60 years old, the demographics were recorded (Table 1). Patients' pre- and post-blood pressures were measured using the sphygmomanometer Kenz 700 (Table 1). The exclusion criteria included, workup for live related transplant within the year, pregnancy, and acute kidney injury. Those with pure red cell aplasia were also excluded from the study.

Blood sampling and processing

Blood sample collection was done on a monthly basis for a total of six months. Venous whole blood samples (15 mL) were obtained from patients using a 20 mL sterile syringe from the arterial line of the HD blood circuit on commencement of the HD procedure and transferred into three purple top tubes. All blood samples were labeled accordingly. They were kept in a fridge at 5°C, before being transported. All blood samples tubes were couriered in a cooler bag within 2 h of collection to the immunology research laboratory of

Table 1. Patient demographics and clinical parameters.

Parameters	**Mean**	**SEM**
Age	48.71 years	1.22
Gender		
Male	20	
Female	16	
Hemoglobin	9.64 g/dL	±0.33
%Saturation	34	±4
Transferrin	1.73 g	±0.61
Ferritin	932.50	± 110
Erythropoietin dose	10.200 IU	1.00
Erythropoietin levels		
Male	119.49	±7.54
Female	139.42	±15.24
Antibodies to erythropoietin		
Male	0.44	±0.01
Female	0.43	±0.02
Systolic blood pressure (Pre)	152 mm Hg	±4.0
Diastolic blood pressure (Pre)	88 mm Hg	±2

the Nelson Mandela Medical School at Doris Duke Medical Research Institute. The blood samples were centrifuged at 3000 RPM at 5°C. Plasma was collected and stored at −20°C and then −80°C in the freezer until being used.

The Hb, percentage saturation of transferrin and ferritin blood levels were obtained from patient's routine monthly blood tests performed at the Hospital Laboratory.

Enzyme linked immunosorbent assay for the detection of erythropoietin Levels

All reagents were prepared as described by the manufacturer (Quantikine^{R} *in vitro* diagnostic kit, R&D Systems, Oxon, UK). The sensitivity of the quantikine assay was reported to be 95% to detect EPO at 0.6 mIU/mL and the specificity was 97% (Quantikine).[12] One hundred µL of EPO assay diluent were added to each well, followed by the addition of 100 µL of either standard, control, or specimen in each well. Plates were sealed and incubated at room temperature (20°C–25°C) for 1 h on the orbital microplate shaker at 500 ± 50 RPMI. Solution was aspirated then 200 uL of conjugate was added to each well. Plate was sealed and incubated at room temperature (20°C–25°C) using the orbital shaker. Wells were then aspirated and washed four times with 1X Wash solution. Two hundred microliter of substrate solution were added to each well, and then the plate was incubated at room temperature for 20–25 min. One hundred microliter stop solution was then added to each well and within 15 min, the reading of plate was done at 450 nm filter using a microplate ELISA reader (EL X 800, Universal Microplate Reader, BioTek Instruments).

Enzyme linked immunosorbent assay for the detection of recombinant erythropoietin antibodies

Ninety-six wells of polystyrene microfiber plates were coated with rHuEPO-beta (Roche, Sandton SA) at 10 mg/L in phosphate buffer solution (PBS) pH 7.4 and then incubated overnight at 4°C. The plates were emptied and washed five times with PBS using an electronic washer (EL X 50, Autostrip Washer, and Biotek Instruments). The plates were then postcoated until the top of each well with PBS containing 30 g/L bovine serum albumin and incubated for 4 h at room temperature.

The contents of the wells were flicked out and 200 µL of serum dilutions of 1:50 was added to the wells. Conjugate was added to the positive control wells and PBS was added to the negative control wells, the no EPO wells and in the nonspecific binding wells. The wells were incubated for 1 h at room tempe-

rature. Plates were then washed five times as described previously by an electronic washer). After washing, 100 µL of freshly prepared substrate solution was added to each well. The plates were then washed five times.

Subsequently, 100 µL of horseradish peroxidase conjugate goat anti-human (IgG), (Sigma, Missouri USA) were added to the wells, including the wells that were labeled as non-specific binding wells. Phosphate buffer solution was added in the positive control wells, negative control wells, no EPO wells, and nonspecific binding wells. The plate was then incubated for 1 h at room temperature. The reactions were then stopped by adding 100 µL stop solution (sulphuric acid). The absorbance was measured with a microplate reader (EL X 800, Universal Microplate Reader, BioTek Instruments) at 450 nm.

Statistical Analysis

Statistical analysis was carried out by using the IBM SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics methods were used to characterize the data (means, standard error of the mean) and linear regression analysis was used to investigate the association between the antibodies and various clinical and laboratory parameters.

Results

Patient demographics and clinical parameters

The demographics and clinical characteristics of subjects are summarized in Table 1.

Antibody levels of hemodialysis patients according to weekly recombinant human erythropoietin doses and gender

The antibody levels presented in optical density (OD) were consistent in their levels in both the male and female groups (Figure 1). The levels of antibodies were similar in the male and female groups of those on zero doses of rHuEPO as compared to the other weekly doses of rHuEPO. All patients represented here had antibodies.

Antibody levels according to hemoglobin levels

The antibody levels versus the haemoglobin levels were scattered from the months of August 2006 to January 2007 of the trial. There was no significant correlation between the antibody levels and the hemoglobin levels (Figure 2).

Erythropoietin concentration according to antibody levels

No correlation was found between antibody levels and EPO levels in the HD patients receiving rHuEPO (Figure 3).

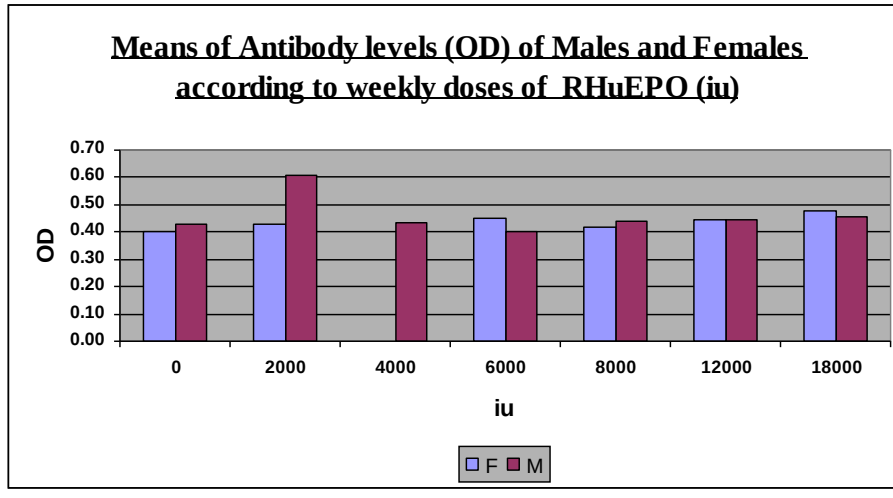

Figure 1. Antibody levels (OD, Y axis) of male and female hemodialysis patients according to weekly recombinant human erythropoietin doses (antibody according to weekly doses), (iu X axis).

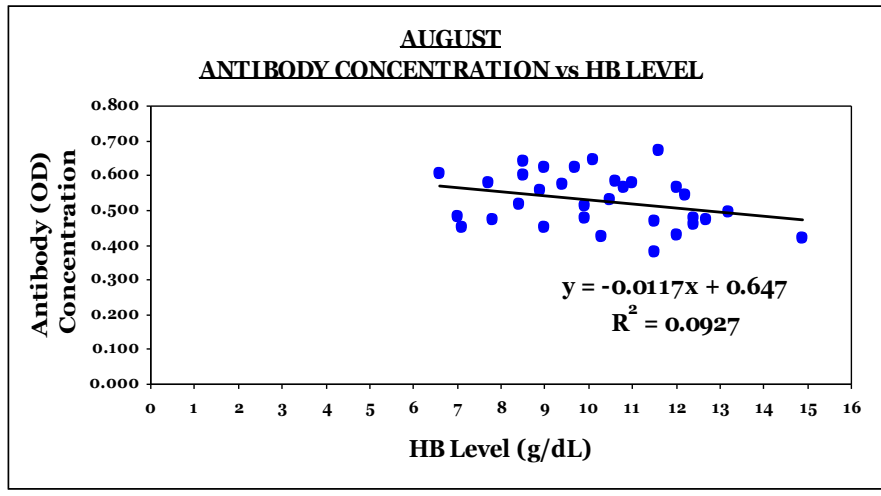

Figure 2. Antibody levels versus hemoglobin levels in the month of August.

Erythropoietin antibodies levels and weekly of erythropoietin doses

In order to correct or to improve the levels of hemoglobin, the weekly doses of Recormon were increased from 0 IU to 18,000 IU. When the total weekly doses of EPO received by patients was analyzed, antibodies to EPO were found in all patients groups (Figure 4).

Patients were divided into two groups: patients requiring the highest doses of Recormon (EPO resistant group) and the rest (EPO responsive group). Logistic regression analysis performed on the two groups showed that no significant association was found in serum levels of EPO, gender or age with EPO weekly doses (Table 2).

However, a high anti-EPO antibody level has a significant association with and highest weekly EPO doses, odds ratio (OR) = 3.975 (E +6) (8.233–1.920 E +12) (P = 0.026). Low hemoglobin level was also found to be associated with highest weekly EPO doses [OR = 0.523 confidence interval (CI) = (0.309–0.885], P = 0.026) (Table 2).

Discussion

Our results showed that antibodies to EPO

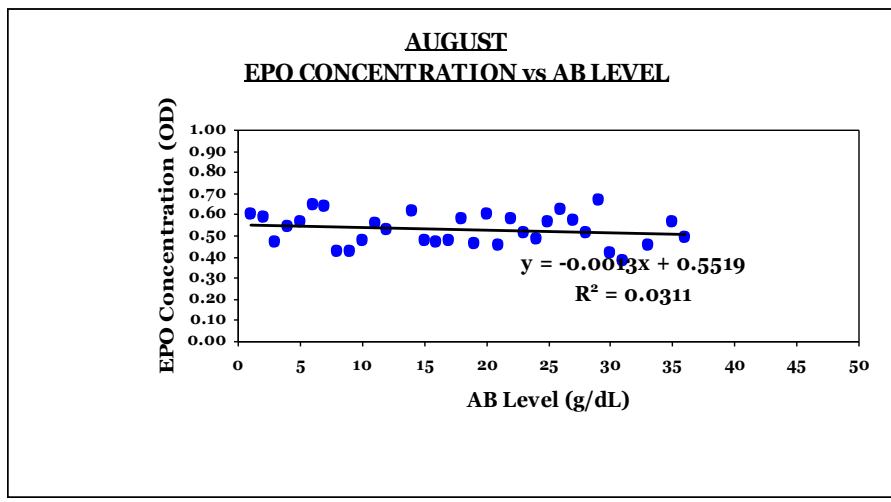

Figure 3. Erythropoietin concentration according to antibody levels.

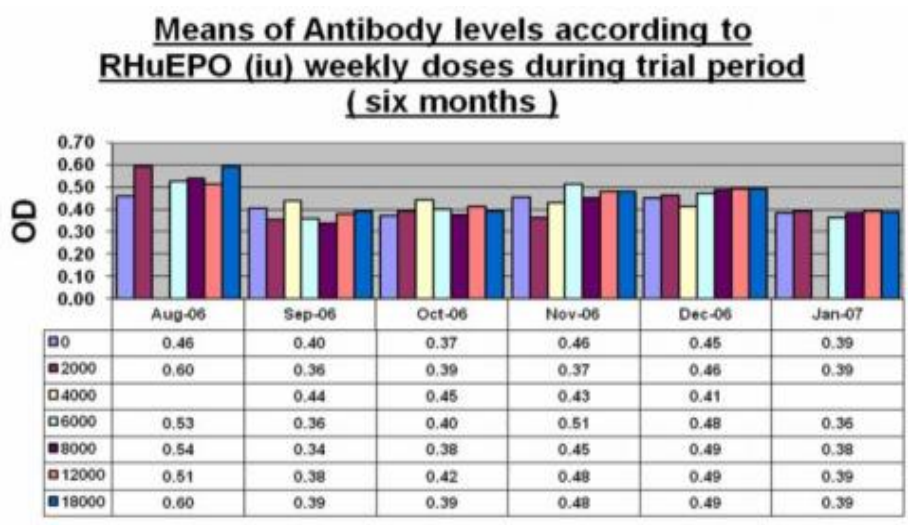

Figure 4. Erythropoietin concentration of hemodialysis patients according to weekly recombinant human erythropoietin doses.

were present in CKD HD patients receiving rHuEPO for the treatment of anemia. This presence of antibody to EPO in patients receiving recombinant EPO therapy (rHuEPO) corresponded with previous studies.[9] It was also found that the patients treated with rHuEPO to correct anemia developed anti-EPO antibodies, but they did not completely neutralize EPO, probably because they may have been low-affinity antibodies.[9] This was also observed in another study in a rat model with CKD, where long-term treatment with a high rHuEPO doses was associated with anti-EPO antibodies formation, resistance to rHuEPO therapy, and development of a hypoxic, inflammatory, and fibrotic milieu in the kidney tissue.[13]

Antibodies to rHuEPO and pure red cell aplasia (PRCA) were also reported in a liver transplant patient on immunosuppressive therapy, antiviral therapy for hepatitis C with ribavirin and interferon following one week of EPO treatment.[14] Another patient with chronic liver disease developed anti-EPO associated pure red cell aplasia, but recovered after liver transplantation and immunosuppression.[15] Recombinant EPO has been used to improve anemia associated with antiviral therapy and to minimize dose reductions. There are several cases in the literature of patients with chronic hepatitis C who developed PRCA while not on recombinant EPO.[16]

However, in our patients, the presence of antibodies was not associated with progression to PRCA. The characteristics of antibodies present should be further investigated for the possibility that these antibodies are neutralizing or not. Neutralizing antibodies could bind to the portions of the drug molecules involved in receptor binding or cell activation, thereby blocking the therapeutic effect of the drug.[17] In addition, neutralizing Abs formed

Table 2. Logistic regression analysis of erythropoietin weekly doses and various variability.

Variables	Odd ratio	Confidence interval	*P*
Erythropoietin level	1.005	(0.997–1.013)	0.200
Hemoglobin	0.523	(0.309–0.885)	0.016
Transferrin	0.782	(0.075–8.136)	0.837
Gender	0.701	(0.173–2.849)	0.620
Age	0.201	(−0.001–0.004])	0.25
Erythropoietin antibody	3.975 (E+6)	(8.233–1.920 E+12)	0.023

against a recombinant protein may also inhibit the activity of the corresponding endogenous factor, resulting in a failure to respond to that factor. We had monitored these patients only over a six-month period; therefore, further studies with a larger group of patients for a longer trial period is needed to evaluate these findings further.

The fact that patients with the highest EPO antibody have a degree of resistance to EPO, may have a significant clinical implication. Although the degree of resistance is not as extreme as with PRCA cases, it may represent a form of pure red cell hypoplasia. As supramaximal doses of EPO may have undesirable side effects such as hypertension, promotion of malignancy, strategy to reduce the need of supramaximal doses of EPO is warranted. Further research could evaluate the role of short-term immunosuppressive therapy in patients requiring the highest EPO doses.

A recent study showed that three patients had PRCA and antibodies against EPO in serum. There was no correlation between age, gender, cause of renal failure, HD duration, Hb level, (rHuEPO) dose and the serum levels of anti-rHuEPO antibody.[18]

Using the ELISA was useful in detecting antibodies, similar to other studies where the ELISA was used. The amounts of antibodies were measured accordingly and were used to compare the immune responses. Those with high antibodies had a greater immune responses and vice versa.[19]

In previous studies, antibodies were associated with PRCA, which was highlighted as a serious complication in EPO therapy and screening for antibodies were considered.[20] Global outcome guidelines have suggested that the testing of antibodies should be done if patients Hb decreases to less than five in a period of eight weeks of therapy.[20]

It has been discussed in previous studies that the polysorbate 80 formulation was less stable, causing aggregation of epoetin alfa molecules, especially at increased temperatures and immunogenicity. Other literature explained that the leachates from uncoated rubber syringe stoppers in prefilled syringes could have been significant in the increased immunogenicity and increasing the presence of antibodies.[21] Due to the outcomes for increasing immunogenicity in patients, the formulations of Eprex replaced uncoated rubber stoppers with fluoroscein-coated stoppers, and the polysorbate was removed.[11,21]

Studies showed that biopharmaceuticals are based on naturally occurring proteins such as antibodies, receptors, cytokines, enzymes, and toxins, nucleic acids (DNA, RNA) or attenuated microorganisms. Immunogenicity has been described as a major factor impairing the efficacy of biopharmaceuticals due to biopharmaceutical neutralization. EPO beta was used in our patients, as in previous studies.[22] Recent findings on different recombinant EPOs done simultaneously depicted differences in content, isoform profiles, and potency was observed not only in products from different manufacturers but also in different batches of the same product.[23] Although the debate on the plausible PRCA factors is still ongoing, the increasing use of polysorbate in biologicals should be monitored for any possible chemical degradation leading to the formation of acids and peroxides.[24]

Caution against undiscriminated adoption of biosimilars was advised to decrease costs and increase access to therapy because the patients could develop anti-rHuEPO antibodies after subcutaneous injection of biosimilar rHuEPO.[25] Despite having similar therapeutic effects of different recombinant EPO, these follow on biologics may have slightly different biochemical qualities eliciting varying degrees of immunogenicity.[26]

The increased doses and frequency of EPO presented in our study have a parallel association in the analysis of the results. Antibodies showed an association with the increased use of rHuEPO. Our results showed a presence of higher levels of antibodies with the increase of weekly EPO doses. This was also previously reported by Rahbar et al.[18]

As discussed in an article that set Hb levels are used and may be misleading because many patients do not have stable Hb levels over time. Hence, in some observational studies of

CKD patients, time-averaged assessments rather than single Hb values were used when analyzing their impact on patients' survival.[27,28]

Conventionally, rHuEPO was administered three times weekly. Studies evaluating less frequent administration regimens have demonstrated that once-weekly subcutaneous administration of epoetin beta during the maintenance phase of therapy has the same efficacy in maintaining Hb levels as the three-times-weekly regimen. In particular, two large-scale, randomized, controlled studies[29,30] showed that stable Hb levels could be maintained with once-weekly epoetin beta treatment without an increase in dose compared with administration two or three times weekly.

This could be due to the varying degrees of resistance in patients receiving rHuEPO. Hemoglobin targets may vary in patients. Although our study has concentrated on the association of anti-EPO antibodies, other factors may play a significant role in EPO resistance. It was observed that only when the weekly doses of rHuEPO increased the desirable or target Hb were achieved. This could be due to the higher doses of rHuEPO being able to reduce the inhibitory effect of circulating cytokines on EPO, thereby overcoming EPO resistance to a degree.[8] In their study, Horl et al[8] were able to achieve similar hemoglobin levels in patients with neoplasia or hepatitis as in his entire population sample of HD patients. It was postulated that excessive cytokine production reduces the epoetin response.[31] Patients with chronic inflammatory conditions will respond sluggishly to rHuEPO. The resistance to high doses of EPO was seen in 35%–65% of HD patients whom showed signs of inflammation that could be a cause of anemia through the suppression of bone marrow erythropoiesis by a number of cytokines.[32] Additional factors could be looked for in future studies as suggested by a recent article based on the hypothesis that anemia, partially attributable to a reduced response to ESA, could be the link between malnutrition, inflammation, and the poor outcome of CKD patients.[32]

Furthermore, iron deficiency has been described as being one of the main causes of inadequate response to treatment with rHuEPO.[33] Another study showed that in functional iron deficiency, despite having enough stores of functional iron, the mobilization of iron to the bloodstream was insufficient to meet the requirements of the erythroid marrow. This is probably due to the fact that in inflammatory states, Cytokines may block the release of iron from deposits.[34]

Conclusion

In conclusion, a significant number of anemic patients with ESRD, treated with rHuEPO, raises an easily detected low-affinity immune response directed to the recombinant protein. The antibodies were present in all groups of the sample population selected. Higher levels of rHuEPO antibodies were associated with patients receiving higher EPO weekly doses. This finding indicates that EPO resistance could possibly be associated with rHuEPO antibodies. These findings warrant further studies with a larger population and evaluate the possible potential role for immunosuppressive therapy, which may act against the antibodies to rHuEPO.

Conflict of interest: None declared.

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