

## Detection of Anti-Erythropoietin Antibodies in Pediatric Hemodialysis Patients

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### ABSTRACT

**Background:** Anemia develops early in the course of renal failure, becomes prominent as the disease progresses, and contributes substantially to disability. The availability of recombinant human erythropoietin (rHuEPO) has led to the almost complete disappearance of the severe anemia of end stage renal disease.

**Objectives:** The aim of this work is detection of anti-EPO antibodies in pediatric hemodialysis (HD) patients under EPO therapy and its relation to type, dose and duration of therapy.

**Methods:** Our subjects were divided into two groups: Group (1) included randomly selected HD patients from the Pediatric Dialysis Unit, Children's Hospital, Ain Shams University. Their ages ranged from 8-22 years (mean  $14.83 \pm 3.4$  years). Twenty-five were females and thirty-four were males. Their duration of illness ranged from 1 month to 12 years (mean  $3.9 \pm 3$  years). Twelve patients were receiving regular injections of EPO  $\beta$  and 47 were receiving EPO  $\alpha$ . Group (2) included healthy subjects age and sex matched with group 1 as control group. In addition to clinical evaluation, venous samples were collected from the patients before dialysis session and examined for the presence of anti-EPO antibodies using ELISA method. Complete blood count on T-540 cell counter was also done for all patients.

**Results:** In our results we found positive anti-EPO antibodies in 44.1% of our subjects. In patients with positive antibodies the blood indices for anemia were significantly lower than in patients with negative antibodies. Also anti-EPO antibodies were positive in 48.9% and 25% of patients receiving EPO  $\alpha$  and EPO  $\beta$  respectively. No relationship could be detected between the presence of anti-EPO antibodies and factors affecting the efficacy of dialysis (duration of dialysis, length of dialysis session, dialyzer surface area). Not a single patient with positive anti-EPO antibodies had a Hb concentration equal to or more than the target value in renal failure patients (11 g/dL) while in patients with negative anti-EPO antibodies only one patient (3%) did not reach the target Hb level.

**Conclusions:** Anti-EPO antibodies were found in 44.1% of our sample of HD patients. The prevalence of antibodies was more in patients receiving EPO  $\alpha$  than EPO  $\beta$ . No relationship could be found between factors affecting the efficacy of dialysis and the presence of anti-EPO antibodies. We could not find a relation between dose and duration of EPO therapy and the development of such antibodies. Our patients did not show any of the features suspicious of pure red cell aplasia.

### INTRODUCTION

Patients with chronic renal failure have subnormal endogenous erythropoietin (EPO) production. Clinical studies have shown that recombinant human erythropoietin (rHuEPO) therapy corrects the anemia of chronic renal failure, avoids blood transfusions and improves quality of

life. Furthermore, it optimizes a patient's hemodynamic status thus minimizing the risk of progression to left ventricular hypertrophy and its associated mortality<sup>(1)</sup>.

The peak concentration of EPO in serum after a subcutaneous dose is approximately one-tenth of the immediate peak increase produced by the same dose

administered intravenously. However, subcutaneous rHuEPO (epoetin) maintains the concentration of EPO in serum above that derived from endogenous production for a longer period, usually for at least 48 hours, (though dependent on dose) and so may prolong the stimulus for red cell production<sup>(2)</sup>.

The first dose of epoetin should be calculated on a per kilogram basis and adjusted thereafter according to the response. A starting dose of 100 IU/kg, administered weekly by subcutaneous injection, lies well within the range of 80-100 IU/kg recommended by the National Kidney Foundation - Dialysis Outcomes Quality Initiative (NKF-DOQI) clinical practice guidelines for the treatment of anemia of chronic renal failure and that of 50-150 IU/kg recommended by the European best practice guidelines for the management of anemia in patients with chronic renal failure<sup>(3)</sup>.

Erythropoietin resistance may be due to vitamin deficiencies especially vitamin B12 and folic acid and supplementation should be provided if a deficiency is present<sup>(4)</sup>. Iron deficiency may occur due to accelerated erythropoiesis coupled with ongoing dialysis-related blood losses or occult bleeding sources<sup>(5)</sup>.

The hypothesis of a potential role of hyperparathyroidism in rHuEPO resistance was initially based on observations that primary hyperparathyroidism is sometimes associated with anemia and that surgical ablation of a parathyroid adenoma has been shown to correct such anemia<sup>(4)</sup>.

Adequate dialysis may contribute to correction of anemia by removing inhibitors

of erythropoiesis. This effect appears to be much more prominent in patients receiving a very low dialysis dose and experiencing an increase of dialysis adequacy. Dialysis time per se, independent from dialysis adequacy, may have a role in the correction of anemia, but this has never been tested properly<sup>(6)</sup>.

During the first 10 years of therapy, three cases of epoetin-associated pure red cell aplasia (PRCA) were published. Since 1998, a significant increase has been noted in the number of patients developing severe anemia during the course of epoetin treatment due to neutralizing antibodies<sup>(7)</sup>.

Several studies have reported the presence of IgG antibodies against erythroblast or EPO responsive cells. These antibodies bound to both native and deglycosylated EPO, inhibited the binding of EPO to the EPO receptor, and blocked the ability of EPO to induce the growth of an EPO-responsive cell line<sup>(8)</sup>.

Patients must not be switched to another form of erythropoietic therapy as the antibodies cross-react with all erythropoietic therapies available. In around 70% of cases, immunosuppressive regimens are effective in eliminating the antibodies; cessation of epoetin therapy without concomitant immunosuppression is rarely effective. Kidney transplantation seems to provide an immediate and effective cure<sup>(9)</sup>. PRCA responds poorly to immunosuppressive therapy and results in prolonged transfusion dependence<sup>(10)</sup>.

## AIM OF THE WORK

The aim of this work was the detection of anti-EPO antibodies in pediatric hemodialysis (HD) patients under EPO therapy

and its relation to type, dose and duration of therapy.

## SUBJECTS AND METHODS

The present study was carried out at the Pediatric Dialysis Unit, Children's Hospital, Ain Shams University during the period from January 2003 to June 2004.

Subjects were classified into two groups: **Group (1):** Comprised fifty-nine children under regular HD and receiving EPO therapy. Their ages ranged from 8-22 years (mean  $14.83 \pm 3.4$  years). Twenty-five were females and thirty-four were males. Their duration of illness ranged from 1 month to 12 years (mean  $3.9 \pm 3$  years). The length of dialysis session ranged from 2-3 hours and the frequency of dialysis ranged from 2-3 times/week. In our study 12 patients were receiving regular injections of epoetin  $\beta$  and 47 were receiving epoetin  $\alpha$ . The doses of epoetin ranged from 1000-7000 units/week (mean  $3936.51 \pm 1389.74$  units) and the duration of therapy ranged from 1 month to 8 years (mean  $3.7 \pm 2.37$  years). The dialyzer surface area used in HD for these patients ranged from 0.3-1 m<sup>2</sup> (mean  $0.858 \pm 0.11$  m<sup>2</sup>).

**Group (2):** Included fifty-nine subjects age and sex matched with group 1, who presented to the out patient clinic, Children's Hospital, Ain Shams University with minor illnesses. They never received EPO therapy, and their ages ranged from 3-17 years (mean  $8.88 \pm 4.27$  years).

All patients were subjected to:

### 1- Clinical evaluation for

- History of renal failure: onset, course, duration and cause of the renal failure.

- History of dialysis: duration of dialysis, frequency, duration of session and dialyzer surface area used in HD.
- Type of EPO, its dose and duration of therapy.
- Symptoms and signs of anemia and pure red cell aplasia.

### 2- Laboratory investigation:

- Complete blood count (CBC) on Coulter T-540 cell counter.
- Estimation of anti-EPO antibodies in patients' serum by a developed enzyme immunoassay method (ELISA).

### Specimen collection

Venous samples were collected from all patients at room temperature (15-28°C) before beginning the dialysis session and the separated serum was not hemolysed and stored at -20°C.

### Reagents used

1. Epoetin beta 2000 IU/ml (Recormone, Roche, USA).
2. Epoetin alfa 4000 IU/ml (Eprex, Janssen-Cilag, Greece).
3. Phosphate buffered saline (PBS).
4. Bovine serum albumin (BSA) (Sigma, USA).
5. P-nitrophenyl phosphate (PNPP) (Sigma, USA).
6. Anti-human IgG conjugated with alkaline phosphatase (Sigma, USA).
7. Tween 20%.
8. Polystyrene plates of 96 wells (Nunc, Roskilde, Denmark).

### Reagents preparation

1. rHuEPO-alfa or beta was diluted at 10 mg/l in PBS pH 7.4.
2. Dilution of samples 1:25 in 2% PBS-BSA-0.2% Tween 20%.

3. Dilution of anti-human IgG (1:2000) with PBS.

#### *Steps*

1. 100  $\mu$ l of rHuEPO  $\alpha$  or  $\beta$  was put into all strips.
2. 100  $\mu$ l of 5% BSA was added for blocking of non specific binding.
3. Then the plates were washed three times with PBS.
4. 100  $\mu$ l of the diluted sample was added to the wells for one hour at room temperature.
5. Then the plates were washed five times.
6. Then 100  $\mu$ l of anti-human IgG conjugated with alkaline phosphatase was added for one hour at room temperature.
7. Then 100  $\mu$ l of PNPP (2 mg/ml) was added to the wells for  $\frac{1}{2}$  hour at room temperature.
8. The final reaction was read at 405 nm using an ELISA reader.
9. The cut-off point of positivity was calculated as 3SD above the mean optical density of the control samples.

#### **Statistical Analysis**

Data were analyzed using statistical software SPSS for windows, quantitative variables were presented as mean  $\pm$  standard deviation (mean  $\pm$  SD), Spearman's correlation test was used for correlation within the studied groups, comparison of mean values of various variables between studied groups was done using student t-test for normally distributed values and Mann-Whitney U-test for non-parametric data, for all tests a probability (p value)  $< 0.05$  was considered significant. Graphic presentation of the obtained results was also done.

#### **RESULTS**

In the present study, comparison of patients receiving EPO  $\alpha$  and EPO  $\beta$  revealed no significant difference regarding the duration of illness, duration of dialysis, details of dialysis sessions and duration or dose of EPO therapy ( $p > 0.05$ ). Furthermore, no difference was found concerning hemoglobin (Hb) concentrations, RBC counts and hematocrit (Hct) values ( $p > 0.05$ ).

Anti-EPO antibodies were detected in 26 out of the 59 studied patients (44.1%). These were 23 out of 47 patients receiving EPO  $\alpha$  (48.9%) and 3 out of 12 patients receiving EPO  $\beta$  (25%) a proportion which was statistically not significant ( $p > 0.05$ ) (Fig. 1).

Comparison of patients with positive anti-EPO antibodies to those with negative antibodies revealed no significant difference regarding the duration of illness, duration of dialysis, details of dialysis sessions and duration or dose of EPO therapy ( $p > 0.05$ ). The patients with positive antibodies had significantly lower hemoglobin (Hb) concentrations, RBCs counts and hematocrit (Hct) values ( $p < 0.05$ ) (Table 1).

Not a single patient with positive anti-EPO antibodies had a Hb concentration more than the target value in renal failure patients (11 g/dL) while in patients with negative anti-EPO antibodies only one patient (3%) did not reach the target Hb level (Fig. 2).

Studying patients receiving EPO  $\alpha$  and those receiving EPO  $\beta$  as separate groups revealed no significant difference regarding the duration of illness, duration of dialysis, details of dialysis sessions and duration or

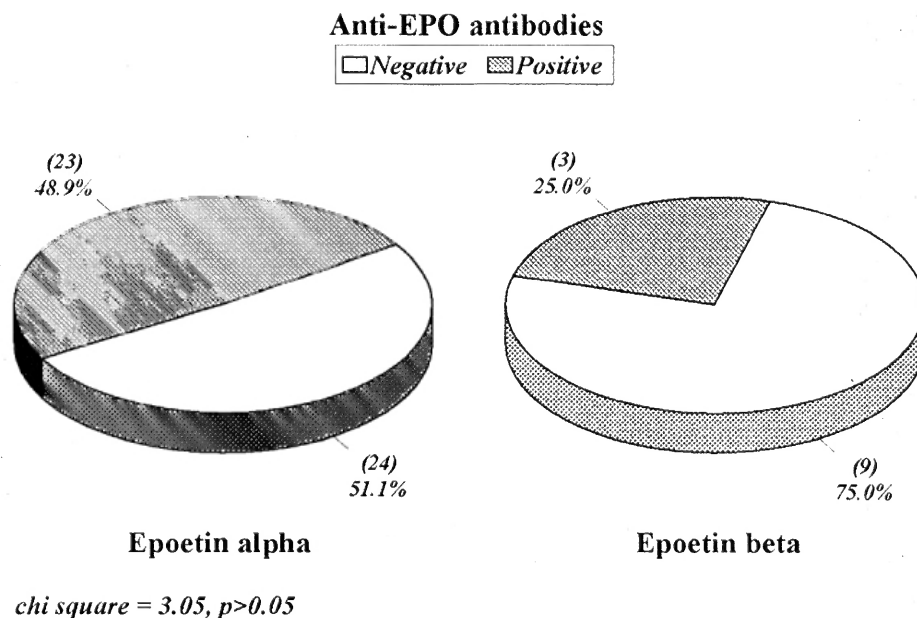
dose of EPO therapy ( $p > 0.05$ ) when comparing patients with positive anti-EPO antibodies to those with negative antibodies. While the patients with positive antibodies had significantly lower hemoglobin (Hb) concentrations, RBCs counts and hematocrit (Hct) values ( $p < 0.05$ ) in each group separately.

Correlations between duration of illness,

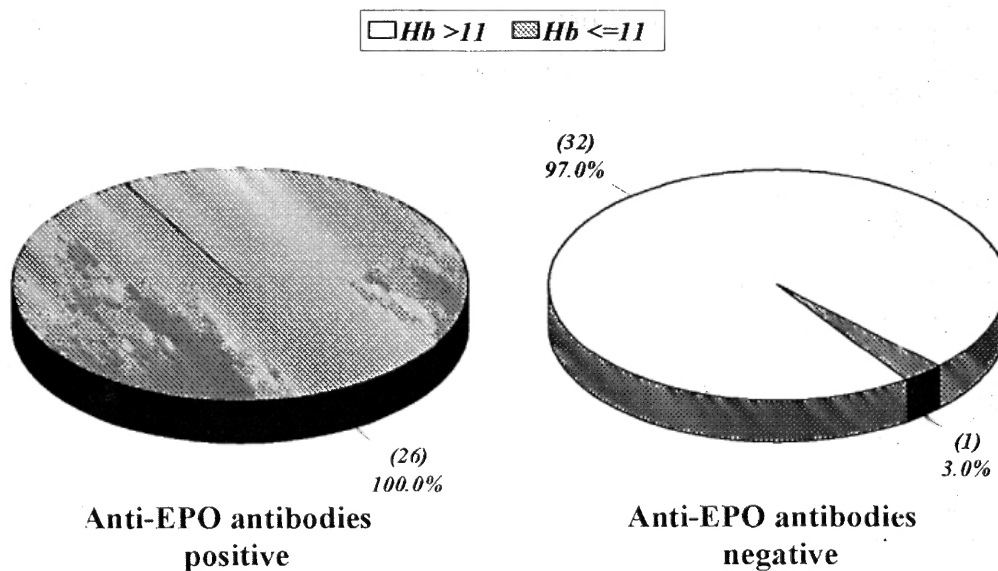
duration of dialysis, length of dialysis session, dialyzer surface area, duration and dose of EPO therapy on the one hand and blood indices (RBCs counts, Hb concentrations, Hct values) on the other hand did not show any significant result when studied in all patients and in those receiving EPO  $\alpha$  or EPO  $\beta$  as separate groups ( $p > 0.05$ ).

**Table 1: Comparison of patients with positive and negative anti-EPO antibodies regarding their RBC counts, Hb level and Hct values.**

Variable	Positive anti-EPO Antibodies (n = 26) Mean $\pm$ SD	Negative anti-EPO Antibodies (n = 33) Mean $\pm$ SD	t	p
RBC counts ( $\times 10^6/\text{mm}^3$ )	3.06 $\pm$ 0.63	4.04 $\pm$ 0.51	6.59	< 0.05 (S)
Hb level (g/dL)	8.47 $\pm$ 1.51	12.04 $\pm$ 1.12	10.37	< 0.05 (S)
Hct values (%)	25.34 $\pm$ 5.67	33.72 $\pm$ 3.44	6.4	< 0.05 (S)



**Fig. 1: Comparison of proportions of positive and negative anti-EPO antibodies among patients receiving the two types of epoetin.**



*chi square = 55.13, p < 0.05*

**Fig. 2: Comparison of proportions of patients with hemoglobin less than 11 g/dL to those equal to or more than 11 g/dL among patients with positive and negative anti-EPO antibodies.**

## DISCUSSION

Resistance to rHuEPO in patients with chronic renal failure can be due to iron or folate deficiency, aluminum toxicity, or hyperparathyroidism with bone marrow fibrosis. Inadequate HD can also contribute to resistance to EPO. The presence of antibodies against EPO was reported in a patient with transient pure red cell aplasia. This report provides clear evidence that the patient's IgG antibody was directed against the EPO. Thus, antibodies against EPO may induce high-grade resistance to the hormone<sup>(11)</sup>.

Since the introduction of rHuEPO therapy, patients have been screened for the antibodies against EPO. Until 1998, there have been only three fully documented reports of anti-EPO antibodies following administration of rHuEPO. These reports came from Germany<sup>(12)</sup>, Spain<sup>(13)</sup>, and USA<sup>(14)</sup>.

Peces et al.<sup>(13)</sup> described the development of antibodies against EPO in a patient treated by HD who was resistant to rHuEPO. An initial adequate response to rHuEPO (20 U per kilogram of body weight subcutaneously three times a week) was followed eight months later by a lack of response, despite the administration of higher doses of the drug (35 U per kilogram three times a week). The absence of any other cause of anemia suggested the possibility of anti rHuEPO antibodies as a cause. An ELISA test revealed IgG anti-rHuEPO antibodies in the patient's serum that bound to both the alpha and beta forms of the molecule. The binding of these antibodies was inhibited by prior incubation with pure rHuEPO.

Guest and Levitt<sup>(15)</sup> described the development of EPO resistance in HD patients who received EPO for one year, and after exclusion of other causes of EPO

resistance, testing for neutralizing antibody was performed with the use of an immunoassay and a bioassay involving a murine cell line requiring erythropoietic agents for growth. Anti EPO antibodies were detected with IgG1, IgG2, and IgG4 isotypes.

In our study, 44.1% of pediatric HD patients were positive to anti-EPO antibodies detected by the ELISA method. Castelli et al.<sup>(16)</sup> found positive anti-EPO antibodies in 67% of their patients. In an attempt to correlate the presence of anti-EPO antibodies with dose and duration of treatment with rHuEPO, they did not find any relationship between anti-EPO antibodies and these pharmacological parameters.

Valtuille et al.<sup>(17)</sup> studied the prevalence of anti-EPO antibodies in 217 HD patients. They did not observe any case of EPO resistance or PRCA. They found anti-EPO antibodies in 5 patients only (2.3%) and most of them had low titres: 1/1 in two patients, 1/10 in two patients and 1/100 in one patient. The discrepancy between their results and ours is most probably due to different methodology as they used radio-immunoprecipitation reaction for detection of the antibodies.

The most likely explanation for anti-EPO antibodies formation is a subtle change in the EPO molecule that may occur during the manufacturing and the formulation processes, or in the handling and distribution processes. Apparently a change in the product leads to the induction of antibodies neutralizing the endogenous EPO in these patients causing a complete block in the differentiation of red blood cells<sup>(18)</sup>.

In the present study we found a higher

incidence of anti-EPO antibodies in patients receiving EPO  $\alpha$ . The antibodies were found in 53% and 25% of patients receiving EPO  $\alpha$  and EPO  $\beta$  respectively. We could not find any relationship between the dose or the duration of EPO therapy and development of anti-EPO antibodies.

No previous study has compared the prevalence of anti-EPO antibodies among patients receiving EPO  $\alpha$  and EPO  $\beta$ . However Chng et al.<sup>(19)</sup> reported a series of Chinese renal patients who developed PRCA after treatment with EPO-alpha, suggesting that this is a problem worldwide. They were treated successfully with cyclosporine and became transfusion independent.

An increase in the number of PRCA cases has been reported from four studies in the period from 1988, when rHuEPO was first introduced to the market, through 1997 and eighty two cases between July 1997 and December 2001 (seventy eight of these cases were EPO alfa). This coincides with the removal of human serum albumin from the previous USA formulation of epoetin alfa, in order to comply with new regulations from the European regulatory authorities. It has been proposed that the new formulation is less stable, allowing aggregates of EPO molecules to form, which increases the possibility of antibody formation<sup>(20)</sup>.

In our study, both EPO  $\alpha$  and  $\beta$  were equally effective in controlling anemia of CRF. The same results were reported by Jeren-Strujic et al.<sup>(21)</sup>

Studying the efficacy of dialysis as a contributing factor for the occurrence of resistance to EPO, we could not find any relationship between duration of dialysis,

length of dialysis session and dialyzer surface area when compared to mean RBCs counts, mean Hb concentrations and mean Hct values.

Localleti et al.<sup>(22)</sup> in their randomized study for the effect of high flux dialysis on anemia of HD patients found no significant difference in Hb level increase between patients treated for 3 months with a high-flux biocompatible membrane in comparison with those treated with a standard membrane, while Villaverde et al.<sup>(23)</sup> found that a switch from low-flux to high-flux membrane, without changing the dialysis dose, improved response to epoetin by about 14% in 31 HD patients with target Hct 35% however, the study was not randomized, making it difficult to draw definite conclusions from the data.

Massoud and Aboul Fottouh<sup>(24)</sup> tested the effect of parental iron in the treatment of anemia of HD patients without the use of EPO and found that the anemia of chronic renal failure can be adequately treated in a significant number of patients without the use of rHuEPO. This can be achieved by the parental iron therapy, adequate dialysis and correction of other contributing factors to the development of anemia.

The present study showed that the presence of anti-EPO antibodies significantly affected the achievement of target Hb (11 g/dL) as proven by the fact the target Hb was achieved in 97% of patients with negative EPO antibodies as compared with 0% of patients with positive EPO antibodies. This target Hb concentration was chosen according to European best practice guidelines recommendations to maintain a target hemoglobin concentration

of > 11 g/dL (Hct > 33%) or reach this target Hb within 4 months of starting treatment in HD patients treated with rHuEPO regardless of age, gender or ethnicity<sup>(25)</sup>. In the present study, failure of achievement of target Hb in anti EPO antibodies positive patients was most probably not due to PRCA as its features (Drop of Hb of > 0.1 g/dL per day without transfusions or transfusion need of > 1 U per week to keep Hb level stable with no major drop of leucocytes and platelets)<sup>(7)</sup> were not present among studied patients.

The presence of strong neutralizing anti-EPO antibodies is considered the most important cause of PRCA as proven by Casadevall<sup>(9)</sup> who described twenty one cases of PRCA occurring in patients receiving rHuEPO to correct anemia of CRF. After an initial normal response to rHuEPO, the patients became severely anemic and needed repeated RBCs transfusions. An ELISA test revealed IgG anti rHuEPO antibodies in the patient's serum.

In our patients there were three patients with renal failure secondary to SLE, two of them had positive anti-EPO antibodies and they were on regular HD for three and four years respectively. Tzioufas et al.<sup>(26)</sup> found autoantibodies against endogenous EPO in SLE patients not receiving EPO therapy. This raises the question whether the antibodies detected in our SLE patients were autoantibodies or not. The occurrence of autoantibodies is further supported by Sipsas et al.<sup>(27)</sup> who found circulating EPO antibodies in HIV patients. They observed a significant association between the occurrence of EPO autoantibodies and anemia. Circulating anti-EPO auto antibodies were



an independent predictor of anemia. They were seen as strong as other known causes of anemia and associated with lower Hb and higher EPO levels. The association of anti-EPO autoantibodies with anemia became stronger when the analysis was limited to the group of patients without any other medical condition explaining their anemia.

The importance of detection of EPO antibodies is further supported by the fact that all antibodies detected so far are cross-reactive with the endogenous and all recombinant EPO molecules and hence, when PRCA is suspected, treatment with EPO needs to be stopped, as patients do not respond to an increase in dose. It is

important that the patient is not switched to another recombinant erythropoietic protein (epoetin alfa, beta or darbepoetin alfa)<sup>(7)</sup>.

In conclusion, anti-EPO antibodies were found in 44.1% of our pediatric HD patients. The prevalence of antibodies was more in patients receiving EPO alfa than EPO beta. We could not find a relation between dose and duration of EPO therapy and the development of such antibodies. Our patients did not show any of the features suspicious of PRCA. No relationship could be found between factors affecting the efficacy of dialysis and the development of anti-EPO antibodies.

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