Impact of C-reactive protein on absolute reticulocyte count in haemodialysis patients: the role of iron status

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Abstract

\textbf{Background.} The exact mechanisms by which the effects of inflammation on erythropoesis occur are still to be determined. We aimed to examine the relation between C-reactive protein (CRP) and erythropoesis as quantified by the absolute reticulocyte count (RTC) and the possible effect of iron status on this relationship.

\textbf{Methods.} As part of a study that follows the changes of haematologic parameters after the intravenous (IV) administration of iron in 93 stable haemodialysis (HD) patients, we made a cross-sectional analysis of baseline measurements and an analysis of changes in RTC 1 week after baseline measurements and iron administration.

\textbf{Results.} Multiple linear regression analysis revealed that RTC had a positive correlation with CRP; RTC had a negative correlation with reticulocyte haemoglobin content (CHr). An interaction was also found between CRP and CHr in that CRP had a significant relation to RTC only in those patients whose CHr was more than 31.2 pg. At lower values of CHr, the correlation between CRP and RTC was not significant. Five days after the IV administration of 200 mg iron sucrose, a significant increase of


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RTC was observed, only in those patients with elevated baseline CRP levels who also showed an increase in CHr levels from \( \leq 31.2 \) pg at baseline to \( \geq 31.2 \) pg post-administration, supporting the presence of an independent positive correlation between CRP and RTC when iron is adequate.

**Conclusions.** It is indicated that, in HD patients, elevated CRP values are associated with increased erythropoietic production only when CHr is quite satisfactory.

**Keywords:** C-reactive protein; functional iron deficiency; haemodialysis; intravenous iron therapy; reticulocyte haemoglobin content

## Introduction

Anaemia is nearly universal in patients on haemodialysis (HD). It is mainly due to the abnormally low erythropoietin (EPO) levels, but other factors may also be involved, including inflammation [1–4].

Anaemia of inflammation is thought to primarily reflect a reduction in red blood cells (RBC) production by the bone marrow. A number of factors are thought to contribute to this hypoproliferative state among which are the abnormal iron metabolism with trapping of iron in macrophages [5], the inability to increase erythropoiesis due to increased apoptotic death of RBC precursors within the bone marrow [6] and the relative decrease in EPO production [5]. Furthermore, an independent association between inflammation and circulating EPO has recently been reported. Ferrucci et al. [7] suggested that elevated inflammatory markers were related to a significant increase in EPO levels, resulting in the maintenance of erythrocyte count and haemoglobin (Hb) within normal levels in persons without anaemia, while in subjects with anaemia, the higher inflammation markers were associated with a blunted EPO response. Thus, despite the improved understanding of erythropoiesis, the exact mechanisms by which the effects of inflammation on erythropoiesis occur are still to be determined.

In the present study, we aimed to check the relation between C-reactive protein (CRP), which is a commonly used marker of inflammation, and erythropoiesis as quantified by absolute reticulocyte count (RTC) in a population of HD patients. We also wanted to further examine this relation not only with respect to Hb levels and conventional iron status markers, but also in relation to reticulocyte haemoglobin content (CHr) levels. CHr provides an indirect measure of the functional iron available for new RBC production over the preceding 3–4 days [8–12]. Reticulocytes are the first form of RBC that enters circulation. RTC more directly assesses erythropoiesis, as opposed to Hb levels, which take a relatively long time to change and, therefore, might not track short-term variations in inflammation markers.

Until recently, measurement of RTC has seldom been used in clinical practice, despite the fact that reticulocytes constitute an important parameter in the pathophysiology of anaemia. The main reason for this is the lack of precision of the common microscopic counting technique. However, the introduction of automated flow cytometric methods has greatly improved the precision and accuracy of reticulocyte counting [13–16].

Therefore, the aim of the present study was to examine in chronic HD patients the relationship between CRP levels and erythropoiesis, as quantified by RTC, and the possible effect of iron status on this relationship.

## Materials and methods

### Study population

The present study is part of a study that follows the changes of haematologic parameters after the intravenous (IV) administration of iron in stable HD patients.

Study subjects were recruited among the patients of a single chronic HD centre. Inclusion criteria included: (i) age \( > 18 \) years, (ii) HD duration \( > 3 \) months, (iii) HD performed three times per week, for 3.5–4 h/session, (iv) stable clinical condition without blood loss of any reason for at least 2 months prior to recruitment, (v) administration of a stable dose of erythropoiesis-stimulating agents (ESAs), without blood transfusions or IV iron therapy, for the same period, (vi) vascular access obtained through arteriovenous fistulae, arteriovenous graft or cuffed-tunnelled dialysis catheters, (vii) absence of any haematologic disorder other than anaemia and (viii) normal thyroid function and serum aluminium \( < 60 \) μg/L.

The study protocol was approved by the Institutional Review Board of the Papageorgiou General Hospital. Written informed consent was obtained from each subject prior to enrolment in the study.

### Study design

The study comprised two stages. The first stage was a cross-sectional assessment of the patient population. With the enrolment of a patient, information on demographics, HD care, medical history and laboratory parameters were registered. Demographic characteristics included age, gender and diabetes status. HD care information included patient’s weight, duration of HD, type of vascular access, HCV and HBV seropositivity, ESAs dose, angiotensin-converting enzyme inhibitors (ACEi) therapy and adequacy of HD (quantified using the Kt/V index).

Laboratory assays performed on arterial line blood samples collected prior to the midweek HD session included haematocrit, Hb, RTC, CHr, low-, middle- and high-fluorescence reticulocytes, serum ferritin, transferrin saturation (TSAT), soluble transferrin receptor (sTfR), serum folate levels, vitamin B12 concentration, CRP levels, albumin, aluminium concentration and intact parathyroid hormone (iPTH) levels. Immature reticulocyte fraction (IRF) was calculated as the sum of middle- and high-fluorescence reticulocytes [16].

In the second stage of the study, all enrolled patients received intravenously a total dose of 200 mg iron sucrose on two consecutive HD sessions. The first 100 mg was given 2 days after the baseline measurements and the second 100 mg was given during the next HD session. Seven days after the initial measurements, RTC and CHr were measured again (first week RTC and CHr, respectively).

Reticulocyte indices and standard haematologic parameters were determined using the ADVIA®120 Haematology System (Bayer Corporation, Tarrytown, NY, USA). Briefly, ADVIA 120 determined the mean cellular RBC and reticulocyte volume (MCV, MCVr) and Hb concentration (CHCM, CHCMr). In analogy to the calculation of Hb content of mature RBC, CHr was calculated as the product of MCVr and CHCMr. Absorption data were used for sorting reticulocytes as low-, middle- and high-fluorescence reticulocytes.

Serum level of CRP was determined quantitatively by rate nephelometry in the Immage Beckman Coulter analyte with normal range \( < 0.8 \) mg/dL.

### Statistical analysis

Most variables followed a Gaussian distribution and their results are expressed as mean \( \pm \) SD. For the non-normally distributed variables (CRP, ferritin, iPTH, folate and HD duration), the results were presented as median (range) and were log\(_{10}\)-transformed in order to normalize their dis-
distribution prior to subsequent statistical analysis. As an initial assessment of the unadjusted effect of the age, gender, diabetes mellitus, log-HD duration, HD access, ACEi therapy, EPO dose, CHr, IRF, log-ferritin, TSAT, sTfR, log-CRP, log-iPTH, Kt/V, log-folate and albumin levels on RTC, univariate linear regression analysis was performed. Variables that had a relationship to the RTC with an associated significance value of <0.10 and variables that were considered clinically relevant according to the current literature were used as candidates for building a multiple regression model. A manual stepwise approach was used to build the model with probability value <0.10 for variable entry and removal. Comparisons of data for RTC before and after iron administration were performed by using paired Student’s t-test. Statistical analysis was performed using SPSS 11 for Mac OS X. Statistical significance was defined as a P < 0.05.

Results

Study population

Ninety-three subjects were enrolled in the study, out of a total of 187 patients treated in an HD unit. The most common reasons for exclusion from the study were prior administration of ESAs at a non-stable dose, blood transfusion or iron administration during the preceding 2-month period or a change in clinical condition. All patients were of Caucasian origin. Demographic, clinical and laboratory data of the enrolled patients are given in Table 1.

Forty-one patients (44%) had Hb levels between 11 and 12 g/dL, 14 patients (15%) had Hb levels above 12 g/dL and 38 patients (41%) had Hb levels below 11 g/dL. Thirty patients (32%) had ferritin levels below 200 ng/mL and 20 patients (22%) had TSAT below 20%. Thirty-two patients (34%) had CRP levels above the upper normal limit of 0.8 mg/dL, 10 patients (11%) had albumin levels below 3.6 g/dL and 31 patients (33%) had Kt/V <1.2. All patients had normal vitamin B₁₂ levels, whereas 61 patients (66%) had serum folate levels below the lower normal limit.

Univariate correlations and multiple linear regression analysis

The unadjusted associations between possible confounding variables and RTC revealed that IRF (r = 0.35, P = 0.001), log-CRP (r = 0.23, P = 0.03) and serum sTfR (r = 0.23, P = 0.03) were all positively correlated with RTC, whereas age (r = −0.34, P = 0.001) and CHr (r = −0.32, P = 0.002) were negatively correlated with RTC. No other variable correlated significantly with RTC.

A manual stepwise approach was used to build the model of multiple linear regression analysis. Age, IRF, log-CRP, CHr and sTfR significantly predicted RTC in a

Table 1. Demographic, clinical and laboratory characteristics of the 93 HD patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65.1 ± 12.8</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>50 (54)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.8 ± 14.6</td>
</tr>
<tr>
<td>Diabetics, n (%)</td>
<td>14 (15)</td>
</tr>
<tr>
<td>HD duration* (months)</td>
<td>44 (3–345)</td>
</tr>
<tr>
<td>HD access, n (%)</td>
<td></td>
</tr>
<tr>
<td>AV fistula</td>
<td>69 (74)</td>
</tr>
<tr>
<td>AV graft</td>
<td>13 (14)</td>
</tr>
<tr>
<td>Permanent catheter</td>
<td>11 (12)</td>
</tr>
<tr>
<td>EPO dose (IU/kg/week)</td>
<td>127.0 ± 81.5</td>
</tr>
<tr>
<td>iPTH* (pg/mL)</td>
<td>165 (3–1249)</td>
</tr>
<tr>
<td>Folate* (ng/mL)</td>
<td>4.6 (1.8–19.2)</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>RTC (×10⁹ cells/L)</td>
<td>49.3 ± 23.8</td>
</tr>
<tr>
<td>CHr (pg)</td>
<td>32.5 ± 3.0</td>
</tr>
<tr>
<td>IRF (%)</td>
<td>16.9 ± 9.3</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>241 (14–1437)</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>27.1 ± 10.0</td>
</tr>
<tr>
<td>sTfR (mg/L)</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>CRPa (mg/dL)</td>
<td>0.48 (0.09–8.46)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>9.7 ± 2.3</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>HCV and/or HBV seropositivity, n (%)</td>
<td>10 (11)</td>
</tr>
<tr>
<td>Most recent Kt/V</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>ACEi administration, n (%)</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or number (percent) unless otherwise indicated.

*Data are shown as median (range).
univariate analysis and, therefore, they were included in the multiple linear regression analysis. EPO dose, administration of ACEi, folate levels, Hb and iPTH along with patient’s weight and creatinine levels were all considered clinically relevant variables and were included in the model, although they did not significantly predict RTC in the univariate analysis. During the process, the presence of a significant interaction between log-CRP and CHR, in terms of their effect on RTC, arose and the interaction term log-CRP \( \times \) CHR was included in the model. This interaction meant that the correlation between RTC and log-CRP changes depending on the levels of CHR. We wanted to determine the cut-off CHR value at which this change in RTC–log-CRP correlation occurs. For this, the CHR levels range was first divided into halves, then into tertiles and finally into quartiles, and three separate sensitivity analyses were performed. In patients with CHR values in the upper half and in the two upper tertiles, the correlation between RTC and log-CRP was significant; in contrast, it was not significant in patients with CHR values in the lower half and in the lower tertile. To test whether the optimal cut-off was at even lower CHR levels, the correlation between RTC and log-CRP was assessed at CHR quartiles. However, patients of the two intermediate quartiles did not show significant correlation between RTC and log-CRP; therefore, the threshold between the first (lowest) and second quartile was not optimal, while the threshold between the lowest and intermediate tertile was considered optimal. The univariate correlation between log-CRP and RTC according to CHR is shown in Figure 1.

The results of the multiple regression analysis are shown in Table 2. Patient age, IRF and the interaction between log-CRP and CHR all independently predicted RTC (adjusted \( R^2 = 0.32, P < 0.001 \)).

### Effect of IV iron on RTC

In the second stage of the study, IV iron administration resulted in a significant increase in mean CHR levels from 32.5 pg at baseline to 33.2 pg at first week (\( P < 0.001 \)). Patients with baseline CHR at the two higher tertiles did not show a significant change in RTC with iron administration. This was true both for patients with normal baseline CRP levels (\( N = 46 \), mean baseline RTC = \( 43.3 \times 10^9 \) cells/L and mean first week RTC = \( 43.7 \times 10^9 \) cells/L, \( P = 0.8 \)) and for patients with baseline CRP \( \geq 0.8 \) mg/dL (\( N = 16 \), mean baseline RTC = \( 51.1 \times 10^9 \) cells/L and mean first week RTC = \( 43.2 \times 10^9 \) cells/L, \( P = 0.2 \)).

Furthermore, patients with baseline CHR at the lower tertile had a more complex response to iron administration. They were stratified into three groups, according to their baseline CRP and their first week CHR levels (Table 3). There was no significant difference of mean baseline RTC among the three groups. In the first group (\( N = 9 \)) comprising of patients with baseline CRP \( \geq 0.8 \) mg/dL whose first week CHR increased to \( \geq 31.2 \) pg, RTC after iron administration increased by 37% (\( P = 0.03 \)). In the second group (\( N = 7 \)) comprising of patients with baseline CRP \( \geq 0.8 \) mg/dL and first week CHR \( < 31.2 \) pg and in the third group (\( N = 15 \)) comprising of patients with normal baseline CRP values, RTC did not change significantly (Table 3).

### Discussion

In this study, elevated CRP was associated with increased RTC only in the presence of a quite satisfactory iron status. There was a significant interaction between CRP and CHR, such that CRP had a significant relation to RTC only when CHR levels were more than 31.2 pg. At lower CHR values, the correlation between CRP and RTC was not significant.

The increase in RTC and erythropoiesis when CRP levels were elevated is mentioned for the first time and could be supported by the increased EPO levels in patients with elevated CRP, reported by Ferrucci et al. [7]. It is known that EPO promotes erythropoiesis and, therefore, increases RTC. In that study [7], it was shown that, in the presence of proinflammatory state, Hb levels may be initially main-

### Table 2. Multiple regression analysis of variables associated with RTC in the 93 HD patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstandardized β coefficient</th>
<th>SE</th>
<th>Standardized β coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.72</td>
<td>0.22</td>
<td>-0.39</td>
<td>0.001</td>
</tr>
<tr>
<td>IRF</td>
<td>1.06</td>
<td>0.27</td>
<td>0.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CHR</td>
<td>-0.91</td>
<td>1.05</td>
<td>-0.11</td>
<td>0.4</td>
</tr>
<tr>
<td>Log-CRP</td>
<td>-154.40</td>
<td>54.67</td>
<td>-2.99</td>
<td>0.006</td>
</tr>
<tr>
<td>CHR ( \times ) log-CRP*</td>
<td>5.10</td>
<td>1.70</td>
<td>3.21</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Model adjusted for EPO dose, Hb, TSAT, sTfR, log-iPTH, log-folate, sex, weight, creatinine, log-ferritin and ACEi therapy (adjusted \( R^2 = 0.32; P < 0.001 \)).

*CHR \( \times \) log-CRP indicates the interaction between log-CRP and CHR.

### Table 3. Changes in RTC in 31 patients with baseline CHR \( \leq 31.2 \) pg after IV administration of 200 mg iron sucrose

<table>
<thead>
<tr>
<th></th>
<th>Group 1 CRP ( \geq 0.8 ) mg/dL and first week CHR ( \geq 31.2 ) pg (( N = 9 ))</th>
<th>Group 2 CRP ( \geq 0.8 ) mg/dL and first week CHR ( &lt; 31.2 ) pg (( N = 7 ))</th>
<th>Group 3 CRP ( &lt; 0.8 ) mg/dL (( N = 15 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean baseline RTC (( \times 10^9 ) cells/L)</td>
<td>46.8</td>
<td>70.0</td>
<td>57.7</td>
</tr>
<tr>
<td>Mean 1st wk RTC (( \times 10^9 ) cells/L)</td>
<td>60.1</td>
<td>65.6</td>
<td>51.6</td>
</tr>
<tr>
<td>Percent change</td>
<td>+37.2</td>
<td>-2.7</td>
<td>+3.2</td>
</tr>
<tr>
<td>P-value</td>
<td>0.03</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

These patients were stratified into three groups according to baseline CRP levels and their first week CHR levels.
tained by a compensatory rise in EPO, but, at a more advanced stage of inflammation, the production of EPO fails to respond adequately and circulating levels of Hb progressively decline. In our study, elevated CRP was associated with increased RTC in patients with quite satisfactory CHR. When CHR levels fell, the positive correlation between CRP and RTC ceased to exist. It is known that erythropoiesis involves the close interaction of iron and EPO. When these two components are coupled, RBC production moves briskly and efficiently. If one component is absent (e.g. iron deficiency), anaemia results. This could explain why in patients with lower CHR levels, that is, patients with less available iron for erythropoiesis, there was no significant positive correlation between CRP and RTC.

The positive correlation between CRP and RTC only when CHR levels were >31.2 pg does not conflict with the knowledge that chronic inflammation is associated with hypoproliferative anaemia. It simply reflects the fact that, in early stages of inflammatory states, before iron available for erythropoiesis is reduced, inflammation is associated with increased erythrocyte production. However, inflammation is also accompanied by increased hepcidin levels, resulting in a decrease in iron absorption and in iron release from macrophages [17–19]. This results in a gradual decline in iron available for erythropoiesis, and thus, the positive association between CRP and RTC disappears.

The presence of a positive correlation between CRP and RTC only when CHR levels were >31.2 pg is further supported by the findings in the second stage of our study. In this hypothesis-generating sub-groups analysis, administration of 200 mg iron sucrose resulted in a prompt increase in RTC only in those patients who had increased CRP levels at baseline and an increase of CHR to ≥31.2 pg post-administration. Patients in all other groups did not show any prompt significant change in RTC with iron administration. It is possible that increasing CHR levels ≥31.2 pg with iron administration allowed elevated CRP to exert its effect on RTC. The small number of patients in the three groups is certainly a limitation of our study. However, of the nine patients of Group 1, seven showed an increase of RTC at the end of the first week after the iron administration (range of increase, 15–110%), one patient showed a decrease (by 28%) and in another one there was no change. The mean increase in RTC in Group 1 was 37%; changes in the other groups were statistically and clinically insignificant (~3% in Group 2, 3% in Group 3; Table 3). These findings are also consistent with studies showing that patients with chronic inflammation present substantial improvement of their anaemia with IV iron alone [20–23]. However, iron administration improved anaemia in individuals with and without inflammation in our study. This is suggested by the fact that all patients with low CHR showed an increase in RTC 2 weeks after the administration of iron, independently of their CRP levels (data not shown). The fact that, of those patients, only those with increased CRP showed a more immediate increase in RTC within 5 days after iron administration is suggestive of an independent positive correlation between CRP and RTC when there is iron adequacy.

This increase of RTC with increased CRP levels could be attributed to stress erythropoiesis. Stress erythropoiesis results in an early release of reticulocytes, which remain in the circulation for longer, before they convert to mature RBC, thereby increasing total RTC [16,24]. However, that increase reflects a premature release of reticulocytes in the circulation, rather than increased erythroid production. Stress reticulocytes are large cells and they contain more residual RNA than normal reticulocytes, staining more intensely. High- and middle-fluorescence reticulocytes with more RNA, corresponding to IRF, are regarded as indicators of increased stress erythropoiesis [8,16]. In our statistical model, in order to correct for the possible presence of stress erythropoiesis, we used the IRF parameter as a predictor for RTC. We found that the positive correlation between RTC and CRP was independent of IRF, and therefore, independent of premature release of RTC in the circulation.

In principle, a change in CRP because of iron administration could contribute to the increased RTC in the first group. However, the same should have happened in the patients of the second group who had similarly increased CRP levels and received the same dose of iron. Regarding the influence of IV iron on CRP, data are limited and somewhat conflicting. There are studies where IV administration of iron resulted in a decrease in CRP levels [25], while in other studies this had no effect [26,27]. CRP was not measured after administration of iron, and this is a limitation of the study.

It is worth keeping in mind that increased use of parental iron might be an important factor contributing to the occurrence of bacterial infections [28]. Therefore, in the face of an acute episode of bacteraemia, IV iron therapy should be temporarily discontinued until bacteraemia has resolved. On the other hand, recent studies have found that IV iron administration is not associated with increased risk of bacterial infection or mortality in HD patients [29,30]. However, despite conflicting data, there is compelling mechanistic evidence for the role of iron in endothelial cell injury and atherosclerosis, including the ability of iron to cause endothelial cell damage [31]. Therefore, until more definite findings on the relationship between iron treatment, infection risk and cardiovascular disease are available, specific risks and benefits should be considered on an individual patient basis.

Serum folate levels of our patients were frequently low; however, studies have shown that serum folate is underestimated in chronic kidney disease due to retention of folate binding proteins and also varies widely with dietary intake [32,33]. Erythrocyte folate concentration is in general a more reliable indicator of folate status than serum folate [34]. The multiple linear regression analysis in our study was adjusted for folate levels to exclude any impact of folate levels on the final model.

The sample size for this study was 93 HD patients; as a result, the number of patients in some of the sub-groups in the second stage of the study was small, and the results reported herein must be interpreted with caution. The study of factors involved in haemopoiesis in HD patients is particularly challenging because of the complex interactions during HD treatment (ESAs or iron dose, loss of blood during sessions). Although fairly rigid criteria were used to select subjects in this study, our results must be regarded as hypothesis-generating only, especially due to the fact that the study design was open-label and non-
randomized. An additional limitation of this study was the lack of EPO measurements, although the exogenous administration of EPO would have complicated the interpretation of this measurement in our patients. Randomized controlled intervention trials with IV iron are needed to confirm the present hypothesis.

**Conclusion**

In conclusion, the results of this study provide novel evidence that, in HD patients, elevated CRP is associated with increased RTC only in the presence of a quite satisfactory iron status, whereas in its absence, this association is not evident. This finding, after its verification by randomized controlled studies, could serve as the theoretical basis for intensive iron treatment of anaemia in chronic HD patients and other chronic diseases. The exact mechanisms by which inflammation interferes with erythropoiesis and the role of iron status should be delineated in further experimental studies.

**Conflict of interest statement.** None declared.

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