New Red Cell Parameters on the Sysmex XE-2100 as Potential Markers of Functional Iron Deficiency

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The ideal approach to monitor the haematological response of patients with the anaemia associated with chronic renal failure during erythropoietin therapy is controversial. The reticulocyte haemoglobin content (CHr) and percentage hypochromic red cells (% Hypo) may be used as sensitive and specific indicators of functional iron deficiency in clinical situations with otherwise normal red cell indices. In the reticulocyte channel of a new automated haematology analyser using fluorescence flow cytometry, the Sysmex XE-2100, two novel parameters are determined (RBC-Y and RET-Y) by measuring the mean value of the forward scatter histograms of red cells and reticulocytes. These values seem to equate with red cell/reticulocyte haemoglobin content.

40 normal healthy males, 70 patients with iron deficiency (selected by indices), 90 patients with chronic renal failure before the start of dialysis or receiving continuous ambulatory peritoneal dialysis and 115 patients on long-term haemodialysis were studied on two different automated haematology analysers. A soluble transferrin receptor assay and measurements of ferritin were used to assess iron status.

A normal and iron-deficient range for RBC-Y and RET-Y has been defined and compared to the values for CHr and % Hypo. Excellent correlation between CHr and RET-Y (r = 0.94) is obtained in all groups and reasonable correlation with % Hypo and RBC-Y (r = 0.84). The soluble transferrin receptor had similar correlation with RET-Y and RBC-Y (r = 0.68) and % Hypo (r = 0.66) but less good with CHr (r = 0.55).

Ferritin was an unreliable indicator of functional iron deficiency.

The two new parameters on the XE-2100 seem to indicate functional iron deficiency, correlate with soluble transferrin receptor levels and are acceptable alternatives to CHr and % Hypo on a routine haematology analyser.

Key Words: Functional Iron Deficiency, Automated Hematology Analyzer, Hypochromic Red Cells, Reticulocytes, Transferrin Receptor

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INTRODUCTION

Patients with chronic renal failure classically develop a normochromic normocytic anaemia, often with a reduced reticulocyte response and a bone marrow erythroid hyperplasia compatible with the degree of anaemia. Recombinant erythropoietin (rHuEpo) therapy can potentially fully correct the anaemia of renal failure but this may be limited by sub-clinical iron deficiency leading to an impaired response. Early detection of iron deficiency in these circumstances is essential to ensure optimal utilisation of rHuEpo dosage and concomitant iron therapy. Failure to supply iron at a sufficient rate adversely affects patient outcome.

Simple iron deficiency is detected in the clinical laboratory by a series of sequential changes, with the body entering a state of progressive negative iron balance and manifesting certain biochemical markers of iron depletion. As frank iron deficiency anaemia develops, the red cell mean cell volume (MCV) and mean cell haemoglobin (MCH) become progressively reduced, the serum ferritin, serum transferrin saturation and serum iron levels fall and there is a rise in transferrin level. Accompanying an impaired iron supply, there is an increased cellular expression of transferrin receptors on erythroid cells leading to increased serum concentrations of the soluble receptor. As iron supply to the marrow erythroblasts declines the new red cells become increasingly hypochromic.

In the anaemia of chronic renal failure the routine biochemical markers of functional iron deficiency are completely unreliable. Serum ferritin is an indirect indicator of storage iron but not iron supply. As serum ferritin is also an acute-phase reactant, it may therefore be normal or even high despite developing iron deficiency. As biochemical tests are unreliable, in these circumstances the clinician has to rely on non-selective adjuvant iron replacement therapy or surrogate markers of functional iron deficiency from the automated haematology analyser.

The traditional impedance haematology analyser (Beckman Coulter GEN S, Sysmex NE-8000) simply provides the traditional red cell parameters and indices that have little bearing on iron availability to bone marrow ery-
throid cells. The Technicon H1, H2 and H3 series of cell counters used automated flow cytometry to measure the volume and haemoglobin concentration of individual red cells and then defined the percentage number of cells with a haemoglobin concentration of less than 28 g/dL as being hypochromic. The usual normal range of hypochromic red cells was less than 2.5 - 5%, and an increased percentage of hypochromic red cells (% Hypo) of greater than 10% was shown to correlate with functional iron deficiency. A more appropriate acute evaluation of marrow erythroid activity would directly measure reticuloocyte indices. Thus with the development of automated flow-cytometric reticulocyte counting it has become possible in the 1990s to quantitate precisely the reticulocyte haemoglobin content (CHR). Assessment of iron status during rHuEpo therapy has shown that a CHR of below 26 pg indicates actual iron deficiency.

The Sysmex XE-2100 (Sysmex, Kobe, Japan) is a recently introduced fully automated haematology analyser. This analyser utilises new technology to improve the range of quantitative cell analysis to include a nucleated red cell count, an immature granulocyte count and an optical fluorescent platelet count in situations where an red cell count, an immature granulocyte count and an range of quantitative cell analysis to include a nucleated

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ly introduced fully automated haematology analyser. This analyser utilises new technology to improve the

range of quantitative cell analysis to include a nucleated red cell count, an immature granulocyte count and an optical fluorescent platelet count in situations where an impedance count may be unreliable. The analyser uses automated fluorescent flow cytometry which in the reticuloocyte channel, using a new polyme thine dye, also measures the mean value of the forward light scatter histogram of mature red blood cells (RBC-Y) and reticuloocytes (RET-Y). These new parameters could potentially provide additional information of iron status and erythroid response following rHuEpo therapy in chronic renal failure.

We have therefore studied a group of healthy adult male volunteers, a group of newly diagnosed iron-deficient patients by standard haematological indices and a group of relatively stable patients with chronic renal failure, including a haemodialysis subgroup on regular rHuEpo therapy. The aim of this pilot study was to compare biochemical markers of iron status (ferritin and soluble transferrin receptor levels) with % Hypo, CHR and the new parameters RBC-Y and RET-Y in the above clinical groups and assess their potential predictive value in diagnosing iron deficiency.

**MATERIALS AND METHODS**

A total of 245 fresh peripheral blood specimens collected into K3 EDTA (Beckton Dickinson, Franklin Lakes NJ, U.S.A.) were analysed at University College Hospital London using the XE-2100 analyser for the RET-Y and RBC-Y. These are the mean value of the forward light scatter histogram of mature red blood cells (RBC-Y) and reticuloocytes (RET-Y) measured in the reticuloocyte channel and expressed in arbitrary units. The same samples were analysed within 4 hours on an Advia 120 for the CHr and % Hypo.

These samples consisted of 40 samples from normal healthy males, 50 untreated iron-deficient samples (selected by indices), 90 samples from patients with chronic renal disease before dialysis or on continuous ambulatory peritoneal dialysis (CAPD) and 65 samples from patients receiving long-term haemodialysis. 128 of the renal disease patients were on rHuEpo therapy and 64 on regular iron therapy.

An additional 70 samples from 20 iron-deficient and 50 chronic renal disease patients, were collected at the Royal Berkshire Hospital, Reading and analysed on the Sysmex XE-2100 and the Bayer Technicon H2 (Bayer Diagnostics, Tarrytown, N.Y, U.S.A) for % Hypo. There is no reticulocyte analysis on the H2 so CHR is unavailable. In total 315 samples were analysed. No patients included in the study had been transfused with red cells in the 4 weeks prior to the study. After blood count analysis, all samples were spun, and the EDTA plasma was frozen in aliquots for the soluble transferrin assay (sTfR) and ferritin levels, which were performed as a batch at the end of the study.

The sTfR was performed using the Bio-Stat diagnostics systems kit (Bio-stat Ltd., Stockport, Cheshire, U.K.). This is a particle-enhanced immunoturbidimetric assay (IdeA sTfR-IT) based on the detection of an immunoreaction between sTfR and TfR-specific antibodies in liquid phase. The immunoreaction is enhanced by latex particles coated with TfR antibodies. In the presence of sTfR the latex particles are agglutinated in a dose-dependent manner, causing increased turbidity. Measurement was performed on a Cobas Mira (Roche Diagnostics, Sussex, U.K.) at a wavelength of 600 nm. The amount of immunoprecipitate is proportional to the sTfR concentration in the sample. Results are calculated with the aid of a reference curve, and low and high sTfR concentration controls (provided by Bio-stat diagnostics) were run with each batch of samples analysed.

Ferritin was measured by an immunossay technique using an Abbott Architect analyser and Abbott reagents (Abbott, Maidenhead, U.K.) in the routine chemical pathology service.

**RESULTS**

Table 1 shows the ranges from the normal and iron-deficient patients for CHr, % Hypo, RBC-Y, RET-Y sTfR and ferritin, calculated using the results from the normal healthy male and the clinical iron-deficient samples. For CHr, % Hypo, RBC-Y and RET-Y there is a clear difference between the normal and iron-deficient ranges. With the sTfR and ferritin there is some overlap between the normal and iron-deficient range. 10 out of 70 apparently iron-deficient patients had a normal sTfR level. 6 of these samples also had a normal ferritin level, however all the samples had microcytic and hypochromic indices, low CHr, RBC-Y and RET-Y values and high % Hypo. It was decided to exclude those patients with a normal sTfR level (less than 2.29 mg/L) from the iron-deficient group. Additional 22 samples from the iron-deficient group had a normal or, in some cases, high ferritin level, 6 of these samples had a normal sTfR. Again all these samples had microcytic and hypochromic indices, low CHr, RBC-Y and RET-Y values and high % Hypo but those with a normal sTfR level were excluded. One sample from the iron-deficient group had a ferritin of

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804 µg/L with an iron-deficient value of 4.83 mg/L for the sTfR. All Advia and XE-2100 red cell and reticulocyte parameters indicated iron deficiency, and this sample was included in the data range. For the iron-deficient range, the number of samples investigated was n = 61 for all parameters, except CHr where 42 samples were tested as this parameter is not available on the Technicon H2. For all samples CHr was closely correlated to RET-Y and RBC-Y with r = 0.94 and r = 0.91 respectively (Fig. 1).% Hypo correlated to a better extent with RBC-Y (r = 0.84) and RET-Y (r = 0.81) than CHr (r = 0.75) (Fig. 2).

When comparing % Hypo to RBC-Y, there is a significant number of samples with a normal RBC-Y and abnormal % Hypo; these are all from renal patients, either pre-dialysis, on CAPD or haemodialysis. There are also some normal % Hypo results with an abnormal RBC-Y value. When comparing CHr with RET-Y there are 3 samples that have a normal CHr and abnormal RET-Y (2 of these patients on haemodialysis also had other haematological disease, 1 myeloma and 1 acute myeloid leukaemia). One haemodialysis sample has an abnormal CHr with a normal RET-Y.

Fig. 3 demonstrates the correlation of % Hypo with RBC-Y and CHr with RET-Y with the normal range cut-off values for all parameters marked on the graphs. This enables truth tables of agreement between each method to be visualised.

There was very weak correlation between ferritin and CHr and RBC-Y (r = 0.38 for both) but even worse correlation with % Hypo and RET-Y (r = 0.21 and 0.31 respectively). No overall correlation was demonstrated between sTfR and ferritin levels. The mean ferritin level for the normal samples was 101 µg/L. However for patients with renal disease, whether pre-dialysis, CAPD or haemodialysis, the mean ferritin level was higher at 544 µg/L with a very overall wide range. This was not unexpected as ferritin is an acute-phase protein and rises considerably and unpredictably in chronic disease, unrelated to iron status.

When comparing sTfR levels with CHr, % Hypo, RBC-Y and RET-Y there were reasonable correlations. sTfR had similar correlation values with RET-Y and RBC-Y (both r = 0.68) and % Hypo (r = 0.66) but slightly less good with CHr (r = 0.55). The correlation graphs for these parameters with sTfR are demonstrated in Fig. 4.

A normal or abnormal sTfR level does not always give a corresponding normal or abnormal red cell or reticulocyte parameter by the Advia or XE-2100 analysers. If iron deficiency is defined as a sTfR level of greater than 2.29 mg/L (the upper limit of our normal range), the ability of the four different cell counter parameters to correctly identify iron-deficient samples can be calculated. All parameters gave true-positive or true-negative results

### Table 1: Normal reference ranges (n = 40) and iron-deficient ranges for ferritin, sTfR, RBC-Y, RET-Y (each n = 61) and CHr (n = 42)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Iron-deficient</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Minimum</td>
</tr>
<tr>
<td>Ferritin, µg/L</td>
<td>101.4</td>
<td>14</td>
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<tr>
<td>sTfR, mg/L</td>
<td>1.4</td>
<td>0.93</td>
</tr>
<tr>
<td>CHr, pg</td>
<td>31.7</td>
<td>28</td>
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<tr>
<td>% Hypo</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>RBC-Y</td>
<td>175.6</td>
<td>168.7</td>
</tr>
<tr>
<td>RET-Y</td>
<td>184.4</td>
<td>171</td>
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</tbody>
</table>

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**Fig. 1. a** Correlation of CHr and RET-Y. **b** Correlation of CHr and RBC-Y. All patient groups.
in approximately 70% of samples, % Hypo showed fewer false-negative results than CHr, RET-Y and RBC-Y but more false-positive. CHr, RET-Y and RBC-Y all showed approximately the same number of false-negative results, but RBC-Y shows more false-positive results than CHr and RET-Y.

The figures for CHr and RET-Y are remarkably similar, and in every case of false results the samples were from the same patients. These results are presented in Table 2.
DISCUSSION

We have clearly defined ranges for CHr, % Hypo, RET-Y and RBC-Y in health and untreated simple iron deficiency disease. Our study shows that the mean normal CHr value is 31.7 pg, RET-Y 184.4 and RBC-Y 175.6. CHr as well as RET-Y and RBC-Y values progressively fall in iron deficiency anaemia. Value of less than 28 pg for CHr, less than 171 for RET-Y and less than 168.7 for RBC-Y would indicate iron deficiency. This is the first report of RBC-Y, RET-Y data on the XE-2100 to detect iron deficiency anaemia. These CHr values are in agreement with other published data that quote a CHr level of less than 28 pg to predict functional iron deficiency\(^5\) . This was slightly higher than other data where a mean normal CHr of 28.6 pg was calculated\(^9\) and a CHr of less than 26 pg being accepted as an indicator of iron-deficient erythropoiesis\(^8\). The value for % Hypo increases in iron deficiency, our mean normal value being 1.0%, and values greater than 4.8% represent true iron-deficiency. This correlates with other published data which suggested true iron-deficiency to be defined as a % Hypo of > 10% and a cut-off for functional iron-deficiency of 5%.\(^11\)

The primary purpose of this study was to determine whether the new parameters on the XE-2100 correlated with CHr and % Hypo in detecting potential functional iron deficiency in chronic renal failure. Despite the fact that CHr is a measurement of mean reticulocyte haemoglobin content and RET-Y is a measurement of reticulocyte size, the correlation between CHr and RET-Y in all patient groups was excellent and effectively interchangeable for clinical diagnostic purposes. There was also reasonably good correlation between % Hypo and RBC-Y (\(r = 0.84\)) to a better extent than % Hypo correlates with CHr (\(r = 0.75\)) although we have found better correlation between % Hypo and CHr than other workers (\(r^2 = 0.33, r = 0.57\))\(^2\).

Do these parameters therefore assist clinicians in detecting functional iron deficiency? sTfR assays were performed on all samples. This research test has been shown to sensitively detect iron-deficient erythropoiesis early in developing iron deficiency and to retain its specificity to changes in iron status irrespective of any concurrent inflammatory process or acute phase reaction\(^2\). It has also been shown to be a more sensitive and less variable index of iron status than the more conventional routine biochemical measurement of ferritin, serum iron, transferrin and total iron binding capacity.

The inaccuracy of the serum ferritin is due to the indirect

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Ability of CHr, % Hypo, RET-Y and RBC-Y to identify iron deficient patients, defined as having a sTfR level greater than 2.29 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHr</td>
<td>% Hypo</td>
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<tr>
<td>True-positive or true-negative result, %</td>
<td>69.6</td>
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<tr>
<td>Incorrect result, %</td>
<td>30.4</td>
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<tr>
<td>False-negative</td>
<td>22.2</td>
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<tr>
<td>False-positive</td>
<td>8.2</td>
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</table>

Fig. 4. a Correlation of sTfR with RET-Y. b Correlation of sTfR with RBC-Y. c Correlation of sTfR with % Hypo. d Correlation of sTfR with CHr. All patient groups.
method by which it assesses iron status. Ferritin is a storage complex, a small amount of which escapes into the circulation devoid of iron. However the serum ferritin level is frequently increased independent of iron status by factors such as inflammation, infection, malignancy, liver disease and alcohol abuse. Because of a non-specific serum ferritin patients increase in patients on chronic haemodialysis, iron deficiency may be overlooked, leading to failure of erythropoietin treatment. This is confirmed in this study. Patients with renal disease either pre-dialysis on CAPD or haemodialysis had a high mean ferritin level of 544 µg/L, but with a mean sTfR level of 2.49 mg/L which in contrast to the ferritin level, would indicate developing iron deficiency. No correlation was found between sTfR and ferritin levels, and only weak correlations were obtained between ferritin and CHr and RBC-Y and even less between ferritin and RET-Y and % Hypo. Reasonable correlation were found between sTfR and RET-Y, RBC-Y and % Hypo. CHr and sTfR showed a slightly less good correlation. However there were a significant number of samples that had an abnormal sTfR level with normal red cell and reticulocyte indices by both analysers and there were some samples with a normal sTfR and abnormal red cell and reticulocyte indices. % Hypo gave the most false-positive results and the least false-negative results, CHr and RET-Y gave exactly the same number of false-positive and false-negative results, and significantly these were all the same samples. RBC-Y gave slightly more false-positive results than CHr and RET-Y.

There is ongoing controversy amongst some renal physicians of whether CHr or % Hypo is the more appropriate parameter for detecting functional iron deficiency. It has been found that in individual patients that the % Hypo parameter for detecting functional iron deficiency. It has been found that in individual patients that the % Hypo parameter for detecting functional iron deficiency; sensitivity of 100% and specificity of 80% have been reported8 although we and some other workers5 did not found such good results. % Hypo and CHr give different information, % Hypo being a time-averaged measurement and CHr being a more acute parameter2. In all patient groups studied CHr and RET-Y are highly correlated, and % Hypo corresponds reasonably to RBC-Y. We have defined the normal range for these two new automated parameters on the XE-2100 and we have shown good or acceptable correlations with sTfR in patients with simple iron deficiency and chronic renal disease.

Future longitudinal studies are needed whereby iron therapy, whether orally or intravenously, is regularly monitored by standard haematological parameters in comparison with the new parameters RBC-Y and RET-Y, to determine their true predictive value for clinical diagnostic purposes.

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References