A systematic approach to the assessment of erythropoiesis

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Summary
The pathogenesis of anaemia may be simple or complex and the differential diagnosis can be difficult. An appreciation of the erythropoietic processes is required, together with regular review of investigations, to ensure that appropriate protocols are adopted. The application of tests, which define different facets of erythropoiesis, should be appropriate to the clinical circumstances. In some situations, such as the anaemia of chronic disorders, pregnancy and chronic renal failure, a detailed analysis of erythropoiesis is often required. Guidelines for investigating anaemia due to megaloblastosis or haemoglobinopathy are well established, whereas disturbances of iron metabolism are often difficult to classify. These require a clear distinction between storage and functional iron to differentiate whether the defect is due to readily treatable simple iron deficiency or more complex mechanisms, which do not respond to iron supplementation. Determination of red cell haemoglobin content, reticulocyte analysis and the assay of serum transferrin receptors are new generation parameters developed to address this. Practice pressures and new treatment options have contributed to investigations becoming more complex, especially those of the secondary anaemias, as new tests have become more readily available and often automated. This has resulted in reduced turnaround times and clinical demand has driven request patterns. Initiatives to develop evidence-based anaemia management protocols are welcomed but, wherever possible, should be developed through collaboration between the haematology department and the user unit, and based on available guidelines.

Keywords Erythropoiesis, review, anaemia, chronic, parameters, secondary

Introduction
Anaemia can be a feature of the disease processes in nearly 180 disorders. Whilst, in some of these, the pathogenesis may be simple, in others it can be complex, which can make differential diagnosis more difficult. An appreciation of the cell biology, physiology and pathophysiology of erythropoiesis is essential to ensure that appropriate investigation protocols are adopted in individual cases. This approach cuts across traditional clinical and laboratory classifications. As improvements in the understanding of erythropoiesis continue, it is also important to re-evaluate approaches and protocols periodically to incorporate new ideas and evaluate any new or improved analytical methods for the investigation of anaemia.

Normal erythropoiesis is the result of a steady-state balance between red cell mass (RCM) and erythropoietin (EPO), with each influencing the other (Figure 1). In uncomplicated sudden blood loss, RCM falls, EPO rises and RCM is restored by a phase of hyperproliferative erythropoiesis. Three main variations from this EPO/RCM relationship have been identified in anemia.

1. Hypoproliferative erythropoiesis in which either the bone marrow is unable to respond to an enhanced EPO signal, as seen in iron deficiency – sideropenic, or where
the EPO control mechanism or response to it is diminished, as seen in chronic renal failure (CRF) and the anaemia of chronic disease (ACD) – non-sideropenic. The latter two are complex conditions and are expanded upon later.

2. Ineffective erythropoiesis in which an increased response is found but the output of cells is inadequate, as seen in megaloblastic anaemia, aplastic anaemia, thalassaemia, abnormal haemoglobins and iron deficiency.

3. Hyperproliferative erythropoiesis in which an increased response occurs but the demand for cell replacement exceeds the compensatory capacity of the bone marrow, as seen in haemolytic anaemias.

Some types of anaemia show more than one type of pathogenesis, for example, thalassaemia can have both hyperproliferative and ineffective erythropoiesis, whereas iron deficiency can have hypoproliferative and ineffective elements. Analyses are available which define different facets of erythropoiesis. Some are basic descriptors, for example, the red cell count or haemoglobin concentration, whereas others are functional response markers, for example, reticulocytes or soluble transferrin receptors. When investigating anaemia, these analyses must be used in an appropriate sequence or algorithm. Initially it is sufficient to establish whether anaemia is due to an inability to make red cells or an inability to make haemoglobin. In some instances this approach will be all that is required. However, in others, a full profile of erythropoiesis is essential and may require quite different thinking, including using different reference ranges for some investigations. The approach will also differ depending on whether the investigation is trying to establish a diagnosis or looking for a transition point during the course of a disorder and its treatment, for example, checking iron availability when patients with CRF are treated with EPO. Table 1 summarizes the application of parameters described below.

In erythrocytosis, although the outcome is different, the approach is similar. There may be an appropriate relationship between EPO and RCM but, because of failure of O2 delivery to the kidney, RCM increases as in the secondary polycythaemias. Alternatively there may be a hyperproliferative response because of loss of the physiological link between RCM and EPO, as seen in primary polycythaemia when there is an erythropoietic clone with a reduced or absent dependency on EPO.

### Traditional parameters

**Investigations: past**

Clinical skills and observation originally formed the basis of a patient profile. These, together with a limited, manual, blood count, assessment of the reticulocyte response and examination of blood film and bone marrow morphology all played major roles. Diagnosis was presumptive and often unreliable in differentiating anaemias with different pathophysiologies.

**Investigations: present**

The differential diagnosis now includes a much extended blood count profile which includes a readily available, accurate measurement of the mean cell volume (MCV). Assay of circulating levels of ferritin, vitamin B12 and folate are carried out by most laboratories. Differential studies into the patient’s vitamin B12 absorptive capacity are undertaken routinely in most large hospitals. Tests for serum autoantibodies are offered by fewer centres, but are also readily available. Some functional tests tend to be available only from a few specialized laboratories.

**Blood film**

Automated erythrocyte analysis has reached the stage where examination of the peripheral blood film is used mainly to verify indices. This is particularly important in the case of mixed cell populations and for red cell changes which do not affect blood count parameters.

**Bone marrow examination**

This provides a general evaluation of erythropoiesis and has been largely superseded by non-invasive methods. Sufficient information can usually be derived from peripheral erythrocyte analysis to indicate erythropoietic drive. The estimation of stainable iron in bone marrow biopsies is a time-honoured method for assessing iron stores, although the procedure is traumatic for the patient.
and the method only semiquantitative. Observing iron in developing cells provides direct information to differentiate between reduced stores in iron deficiency and impaired functional release from reticuloendothelial cells in the chronic anaemias (Baynes, 1996; Worwood, 1997). For this reason most investigations, which attempt to evaluate methods for assessing iron stores and functional iron, have used bone marrow iron observation as the reference method.

### Mean cell volume and mean cell haemoglobin

A low MCV indicates a general decrease in the size of circulating red cells and this effect may be seen in more than one condition. In contrast, although discovery of a high MCV can facilitate the early diagnosis of megaloblastic anaemia, since this will often precede clinical anaemia by months or even years, up to 4% of all patients may have abnormally high MCVs in the absence of any other abnormality (Lindenbaum, 1983). Natural and biological variations, sometimes compounded by coexisting pathological conditions often result in great variation in the MCV rendering it an unreliable, if not misleading, measurement on occasion. Thus, a normal, lowered or raised MCV cannot always be equated to normo-, micro- or macrocytosis, respectively, often making examination of the blood film essential. Cavill (1997) describes the MCV as an ‘elastic parameter’ and suggests that, since the haemoglobin content of red cells is fixed throughout their lifespan, the

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**Table 1. Summary of parameters useful in evaluating erythropoiesis**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sample</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood film</td>
<td>Peripheral blood</td>
<td>Important for mixed cell populations and for red cell changes which do not affect blood count parameters.</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Marrow aspirate</td>
<td>General evaluation of erythropoiesis. Gold standard for iron stores.</td>
</tr>
<tr>
<td>MCV</td>
<td>Peripheral blood</td>
<td>General indication of the size of circulating red cells. Non-specific. Cut-off values need to be reconsidered in some situations.</td>
</tr>
<tr>
<td>Hypochromia</td>
<td>Peripheral blood</td>
<td>Refined MCH measurement indicating haemoglobin content of individual erythrocytes only available with some analysers. Provides a direct indication of iron delivery to the bone marrow.</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>Peripheral blood</td>
<td>Manual methods of limited value. Automated analysis provides a range of parameters including reticulocyte haemoglobin content, a sensitive indicator of functional iron deficiency.</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Serum</td>
<td>Reliable estimate of iron stores but can be disproportionately increased due to acute phase response. Used in haematinic screen.</td>
</tr>
<tr>
<td>sTfR</td>
<td>Serum</td>
<td>Generally considered a sensitive indicator of iron deficient erythropoiesis unaffected by acute phase response. Results more meaningful when expressed as a ratio with serum ferritin.</td>
</tr>
<tr>
<td>Serum iron/TIBC</td>
<td>Serum</td>
<td>Only reliable application is in screening for idiopathic haemochromatosis.</td>
</tr>
<tr>
<td>ZPP</td>
<td>Peripheral blood</td>
<td>Rapid analysis indicative of both absolute and functional iron deficiency. Not always reliable in monitoring iron supplementation.</td>
</tr>
<tr>
<td>B₁₂ assay</td>
<td>Serum</td>
<td>Differential diagnosis of macrocytosis. Reference ranges derived from white populations may not be generally applicable. Used in haematinic screen.</td>
</tr>
<tr>
<td>Folate assay</td>
<td>Serum/red cell</td>
<td>Differential diagnosis of macrocytosis. Serum and red cell assays each have limitations therefore both should be assayed. Used in haematinic screen.</td>
</tr>
<tr>
<td>IFAB</td>
<td>Serum</td>
<td>Diagnostic test for pernicious anaemia but only 70% sensitivity.</td>
</tr>
<tr>
<td>B₁₂ absorption studies</td>
<td>In vivo</td>
<td>Differential diagnosis of B₁₂ malabsorption. Does not exclude malabsorption of food-bound vitamin.</td>
</tr>
<tr>
<td>dU suppression test</td>
<td>Marrow aspirate</td>
<td>May be useful to confirm mild B₁₂ or folate deficiency.</td>
</tr>
<tr>
<td>Methylmalonic acid</td>
<td>Serum</td>
<td>Increased in B₁₂ deficiency.</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>Serum</td>
<td>Increased in B₁₂ and folate deficiency.</td>
</tr>
</tbody>
</table>

mean cell haemoglobin (MCH) provides a more reliable, and standardizable, index of cell size.

Reticulocytes

Reticulocyte analysis is undergoing fundamental change. Subjective visual investigations have restricted its usefulness in the past to situations where marked changes in bone marrow response may be occurring such as haemorrhage, haemolysis or the response to treatment. High levels of imprecision, with a coefficient of variation (CV) typically in excess of 25%, have severely limited the applicability. In an attempt to reduce method imprecision, the International Council for Standardization in Haematology (ICSH) has proposed a manual reference method based on the determination of the reticulocyte to red cell ratio (International Council for Standardization in Haematology, 1998). It is suggested that this method, which standardizes specimen collection, staining and counting procedures, should be used to determine the accuracy of automated counting systems and to provide a means for instrument calibration. The advent of analysis by flow cytometry has encouraged a re-evaluation of the role of reticulocyte analysis. This is discussed further, in ‘New Parameters’, below.

Ferritin assay

Body iron in excess of requirements comprises ferritin and haemosiderin. Most is in the form of ferritin, which is water-soluble. Iron may be mobilized from both forms, but is more accessible from ferritin. Found chiefly in the cytoplasm of cells of the reticuloendothelial system, it was once thought that ferritin did not appear in plasma or extracellular fluid under normal conditions but development of a sensitive immunoradiometric technique by Addison et al. (1972) enabled the demonstration that ferritin is a constituent of all normal human serum. In contrast to tissue ferritin, ferritin present in serum is low in iron content and glycosylated. However, the concentration in serum may be directly related to body iron stores and the assay has become the most widely applied evaluation of iron status. The original radioassay techniques have been largely superseded by automated enzyme-labelled techniques.

In most normal adults, concentrations range from 15 to 300 μg/l, but these vary with age and sex (Worwood, 1982, 1997; Baynes, 1996). Serum ferritin concentrations are relatively high at birth. As fetal red cells are removed from the circulation during the first few weeks of life, the haemoglobin concentration falls to 10–11 g/dl. At this time the rate of erythropoiesis is relatively low and iron released from the destruction of fetal cells is stored in the tissues. This process is accompanied by an increase in serum ferritin concentration. Synthesis of adult haemoglobin commences during the following 2 months, causing a rapid fall in both iron stores and the serum ferritin concentration. Both remain relatively low throughout childhood and adolescence.

Serum ferritin levels are higher in men than in women of childbearing age, reflecting differences in storage iron. Levels in postmenopausal women are closer to those found in men. The concentration falls during pregnancy. The initial reduction is thought to reflect increased maternal erythropoiesis, whereas in the latter stages transfer of iron to the fetus is the main cause. From 35 weeks, serum ferritin concentrations are lower in women who have not received iron therapy than in those who have.

Addison et al. (1972) found that patients with iron deficiency anaemia have serum ferritin levels approximately one-tenth of normal subjects, while patients with iron overload (haemochromatosis, haemosiderosis) have serum ferritin levels much higher than normal. Serum ferritin levels may serve as a tool to monitor the effects of iron therapy. However, raised concentrations must be interpreted with caution. Ferritin behaves as an acute phase protein and in some circumstances the serum level may not illustrate the true state of iron stores since immunoassay techniques reflect ferritin protein rather than iron. In both adults and children, chronic inflammation results in a disproportionate increase in ferritin levels in relation to iron reserves. Elevated ferritin levels, not directly related to iron stores, are also observed in acute and chronic liver disease, chronic renal failure and in some types of neoplastic disease (Baynes, 1996; Worwood, 1997).

Serum iron and total iron binding capacity

Measurement of the serum iron and total iron binding capacity (TIBC) can provide information relating to transport iron. Recommendations on methodology have been made by the ICSH (International Committee for Standardization in Haematology, 1978, 1990). Iron deficiency results in low serum iron concentrations, whereas high levels are found in iron overload conditions. Unfortunately this is not always the case. In the presence of inflammation or infection the serum iron is low irrespective of the level of storage iron present. High concentrations may also be encountered in liver disease, hypoplastic anaemias and in conditions where erythropoiesis is ineffective (Worwood, 1997).
Isolated sampling from the transport iron compartment can provide unreliable, if not misleading, information. Circulating iron, bound to transferrin, comprises only a very small part (0.01%) of the total body iron and has a very high turnover rate of 10–20 times per day in normal subjects (Cavill et al., 1977). These factors contribute to the variability of measurement encountered and severely limit the diagnostic usefulness of an individual serum iron measurement. The TIBC is indicative of the circulating transferrin concentration and is a more stable measurement than the serum iron (Cavill, 1982). Measurement of both the serum iron and TIBC provides more information than individual measurements (Worwood, 1997). Transferrin normally circulates about one-third saturated. Calculation of the percentage transferrin saturation from the serum iron and TIBC (serum iron/TIBC × 100) reflects the transport compartment but, for reasons outlined above, is also too variable as a single measurement (Baynes, 1996).

In iron deficiency, a low serum iron is associated with a raised TIBC, resulting in a low percentage transferrin saturation. A saturation level of 16% or less reflects inadequate transport iron to sustain erythropoiesis. Pregnancy and anaemia secondary to chronic disease are also associated with low transferrin saturation despite the presence of adequate iron stores. Iron overload results in high transferrin saturation levels due to the combination of an elevated serum iron and a normal or depressed TIBC.

The only viable clinical role for serum iron and TIBC is in screening for idiopathic haemochromatosis. The diagnosis of this condition, one of the most common inherited conditions in populations of European origin, should include the demonstration of a raised transferrin saturation (Males > 60%, Females > 50%) on at least two blood samples (Worwood, 1997). In the early stages of the disease this abnormality will be encountered before excessive iron accumulation and the resulting raised ferritin.

**Zinc protoporphyrin**

Zinc protoporphyrin (ZPP) is synthesized by developing erythrocytes when there is insufficient iron available to ferrochelatase for haem production. It can be quantified by spectroscopy and instruments are available for rapid analysis. Values are standardized by expressing the result as a ratio to haem. In healthy blood donors, a good inverse correlation between serum ferritin and ZPP has been found. Studies in a range of disorders suggest that ZPP is indicative of both absolute and functional iron deficiency and may substitute for percentage hypochromia where the instrumentation required for this analysis is unavailable. However, ZPP does not predict a response to iron supplementation in some renal patients. There are some instances when fluorescent substances in samples interfere with the assay, giving falsely elevated results. Washing the erythrocytes before analysis can reduce this but some of the test’s facility is lost (Braun, 1999).

**Vitamin B₁₂ and folate assays**

Vitamin B₁₂ and folate act as coenzymes in two important metabolic functions vital to normal cell growth and DNA synthesis: (1) the synthesis of methionine, and (2) the conversion of methylmalonyl CoA to succinyl CoA (Weir & Scott, 1983; Chanarin et al., 1992; Hoffbrand & Jackson, 1993). In the first of these, vitamin B₁₂ deficiency interferes with the regulation of folate metabolism, resulting in impaired DNA synthesis and consequently megaloblastic anaemia. Because both vitamins are linked by the reaction pathway for methionine synthesis, a deficiency in either will disrupt this and produce the same symptoms. However, the causal mechanism remains a topic of some discussion (Chanarin et al., 1992; Hoffbrand & Jackson, 1993).

Much of the research on this pathway resulted from the discovery that nitrous oxide inhibited thymidylate synthesis through inactivation of vitamin B₁₂ (Amess et al., 1975, 1978). In the second reaction, vitamin B₁₂ deficiency results in impaired fatty acid metabolism which may in turn lead to abnormal myelin lipid formation, contributing to neurological lesions (Weir & Scott, 1983).

In general, the serum or plasma vitamin B₁₂ concentration is related to the degree of tissue depletion, lowest levels occurring in the most severe cases (Chanarin, 1990; British Committee for Standards in Haematology General Haematology Task Force, 1994a). Normal levels vary depending on the method used and range between 120 and 1100 ng/l. Results falling within plus or minus 20% of the lower end of the reference interval should be regarded as ‘indeterminate’, and the sample repeated (British Committee for Standards in Haematology General Haematology Task Force 1994a). Carmel (1999) suggests that ethnic and racial factors should be considered when investigating vitamin B₁₂ disorders and metabolism, and questions the adequacy of reference ranges derived largely from white populations. Elevated vitamin B₁₂ levels have been associated with the use of oral contraceptives and multivitamins, and in myeloproliferative diseases such as chronic granulocytic leukaemia and myelomonocytic leukaemia. An elevated vitamin B₁₂ level in itself has not been known to cause clinical problems (Chanarin, 1979, 1990).

Folate levels in both serum and red cells are used to assess folate status. Normal serum concentrations range...
between 3.0 and 20.0 μg/l. In contrast, red cells have much higher folate levels, i.e. between 150 and 600 μg/l. A low serum folate is indicative of deficiency in the absence of recent folate intake. Red cell folate is the best indicator of long-term folate stores (Chanarin, 1979, 1990). A low red cell folate value is suggestive of a prolonged folate deficiency. However, a consequence of the common metabolic pathway is that B12 deficiency disrupts the uptake of folate into red cells. This can lead to a low red cell folate value even with adequate folate intake. For this reason, it is often necessary to measure both vitamins in a clinical workup. A case can be made for either the serum or red cell folate to be assayed but, because of the limitations of each, it would seem reasonable to assay both or to assay the red cell concentration whenever the serum folate is low. The true incidence of folate deficiency is not well known and most figures are derived from the frequency of anaemia in pregnancy. Globally, malnutrition is the most common cause of both folate and vitamin B12 deficiency (Dawson & Waters, 1994).

Both vitamins can be measured in serum by competitive protein binding assay. Most vitamin B12 methods use intrinsic factor (IF) as the specific binding protein, although radioimmunoassays have been described (O'Sullivan et al., 1992). β-Lactoglobulin from cow’s milk is the binding agent commonly used in folate assays. During the past 10 years, fully automated non-radioisotopic systems have largely replaced radioisotopic manual methods. In these systems, monoclonal antibodies are often used to set up the conditions whereby the specific binder is attached to a solid phase.

Intrinsic factor antibodies and assay

The demonstration of circulating intrinsic factor antibodies (IFAB) is virtually diagnostic of pernicious anaemia (Lindenbaum, 1983). They are rarely found in other autoimmune diseases such as disorders of the thyroid, diabetes mellitus and myasthenia gravis. Two types of IFAB are known to exist. Type I blocks the IF binding site for B12, preventing uptake of the vitamin, whereas type II reacts with an alternative antigen site on the IF molecule and may prevent attachment of the IF–B12 complex to the ileal binding sites (Roitt et al., 1964; Chanarin, 1979). Traditionally, techniques of testing for IFABs have been functional methods for the detection of type I antibody (Chanarin, 1979). Immunoassay methods have also been described which are capable of detecting both type I and type II IFAB (Conn, 1986; Waters et al., 1989), or type II alone (Sourial, 1988). Subsequent studies using this methodology have indicated that type II IFAB occur, alone and in combination with type I, in a much higher proportion of pernicious anaemia samples than was previously recognized (Conn, 1986; Waters et al., 1993). Assay of gastric IF is a direct measurement of the defect in pernicious anaemia but this requires an invasive technique and is rarely used.

Absorption studies

The urinary excretion method of Schilling (1953) remains the most commonly used and best standardized test for absorption of free vitamin B12. The recommended procedure (International Committee for Standardization in Haematology, 1981) is based on the Schilling test and employs a 1.0-μg aqueous oral dose of cyanocobalamin radiolabelled with cobalt-57. In this form, the test is carried out as a two-part procedure, without IF (part I) and with IF (part II) added to the oral dose. Dual-labelled methods, based on the Schilling test, where the two stages are combined by simultaneously administering two radiolabelled versions of cyanocobalamin, one bound to IF, the other unbound, were developed in the 1960s (Katz, DiMase & Donaldson, 1963; Bell, Bridges & Nelson, 1965). Although the original adaptations held potential advantages over the two-stage approach, particularly in terms of patient attendance and sample collection, doubts have been expressed over a later commercial modification (Payne & Finney, 1972; McDonald, Barr & Barton, 1975; Fairbanks, Wahner & Phyllyk, 1983; Zuckier & Chervu, 1984; Atrah & Davidson, 1989). This test has recently been withdrawn from the market.

Demonstration of normal absorption of free vitamin B12 does not exclude malabsorption of the food-bound vitamin. In patients with a subnormal serum B12, a normal Schilling test and satisfactory dietary intake, a food-bound absorption test should be considered (British Committee for Standards in Haematology General Haematology Task Force, 1994a). Vitamin B12 deficiency due to food-bound malabsorption does not reach the same degree of severity as that which may occur in pernicious anaemia because the enterohepatic circulation of the vitamin remains intact whilst IF is available. The incidence of the condition is unknown but Carmel (1995, 1996) believes it to be at least as common as pernicious anaemia and a major cause of vitamin B12 deficiency.

Deoxyuridine suppression test

The deoxyuridine suppression test can be used to confirm mild vitamin B12 or folate deficiency, although in some deficient patients the test can be normal. Bone marrow is
the recommended sample as opposed to peripheral blood lymphocytes. The test is labour-intensive and expensive, and is only available in a few specialized centres (British Committee for Standards in Haematology General Haematology Task Force, 1994a).

**Methylmalonic acid and homocysteine**

Both methylmalonic acid (MMA) and homocysteine (Hcy) are increased in the serum of patients with vitamin B₁₂ deficiency, whereas only Hcy is increased in folate deficiency. Measurement of MMA in a random urine sample can be used as a screening test, although the 24-h excretion level is more sensitive. Serum is the sample of choice. These metabolite assays can be automated using capillary gas chromatography-mass spectrometry, high performance liquid chromatography-mass spectrometry or immunoassay, but the equipment required is expensive and currently only available in a few specialized centres. However, new generation, integrated, systems are likely to become more available to a larger number of laboratories. Thin-layer chromatography is recommended as an inexpensive but sensitive screen for urinary MMA (British Committee for Standards in Haematology General Haematology Task Force, 1994a).

**Guidelines**

A clinical history and blood count with appropriate follow-up tests usually leads to a rapid laboratory diagnosis and protocols for investigating anaemia due to megaloblastosis (British Committee for Standards in Haematology General Haematology Task Force, 1994a) or haemoglobinopathy (British Committee for Standards in Haematology General Haematology Task Force, 1994b, 1994c, 1998) are well established. The pathogenesis of disturbances of iron metabolism is more difficult to classify, and requires a clear distinction between storage and functional iron. Most of the newer generation tests have been developed to address this. Therefore the remainder of this article will concentrate on hypoproliferative variants around functional iron deficiency and anaemia of chronic disorders.

**Problem areas**

The mechanism underlying some anaemias is relatively simple and the diagnosis straightforward, e.g. simple iron deficiency. In some situations a more detailed analysis of erythropoiesis may be necessary to ensure that an adequate response to treatment may be achieved. In this type of disorder the pathogenesis is typically more complex. The main situations where this arises are in the ACD in rheumatoid patients, pregnancy and chronic renal failure patients being treated with recombinant human erythropoietin (r-HuEPO).

**Anaemia of chronic disorders**

Means (1999) has reviewed the pathophysiology of this syndrome. Often confused with and sometimes wrongly referred to as ‘iron deficiency anaemias’, ACD has a much more complex pathogenesis than simple iron-deficient erythropoiesis. ACD is associated with inflammatory states, including chronic infection, rheumatoid arthritis and neoplasia. It is one of the most common disorders found in medicine and accounts for a significant proportion of anaemia investigations. There is evidence that the common factor causing anaemia is the change in immune and inflammatory mediators common to all of these disorders. Increased concentrations of tumour necrosis factor, interleukin-1 and interferons have been implicated and the mechanisms by which they disrupt erythropoiesis explored. The effect on erythropoiesis is threefold. A shortened red cell survival producing a slight increase in red cell production, impaired erythropoiesis due to reduced erythropoietin production and reduced progenator sensitivity, and impaired release of reticuloendothelial iron. The resulting hypoproliferative erythropoiesis has in the past been characterized by a low serum iron and a normal or raised serum ferritin. This conflicting information has made it difficult to draw conclusions about true iron status in relation to the anaemia. Recombinant erythropoietin has already been used to correct the anaemia in some cases of ACD. Better measures of erythropoiesis will have to be used to ensure adequate iron is available to support the erythropoietic drive and for monitoring treatment response. A fundamentally different approach is therefore required to differentiate whether the defective red cell output is due to simple iron deficiency, which is readily treatable, or more complex mechanisms which do not respond to iron supplementation.

**Pregnancy**

A number of physiological changes occur in pregnancy, including expansion of plasma volume, an EPO-driven increase in erythropoiesis to provide an expansion in RCM and increasing demands of the fetus for haematinics. These adaptations complicate the assessment of haematinc deficiency; for example, a reduction in haemoglobin concentration cannot be relied upon as a first-line
screening test. Iron stores are challenged by normal pregnancy. Research, by tracking EPO concentration, has shown that some benefit is likely to be gained by iron supplementation (Milman et al., 1997). Better criteria are therefore needed to provide a reliable indication of iron stores and functional iron in this situation. Using a study population which included anaemia, chronic inflammation and some thalassaemias, van den Broek et al. (1998) explored measures of iron status referenced against bone marrow-stainable iron. They confirmed that the MCV is unreliable as a screen due to the erythropoietic drive causing shortened transit times. Using a typical cut-off value of 1.2 μmol/l, these authors showed ZPP to have very poor specificity and that this could lead to significant over-diagnosis of iron deficiency. When referenced against bone marrow iron they suggest that serum ferritin can be used as a reliable indicator of stores provided that the cut-off value is raised to 30 μg/l. They also suggest specificity can be further improved (to 89%) by using multiple parameters, e.g. by combining serum ferritin with serum transferrin receptor (sTfR) and C-reactive protein measurements.

Similar questions have been raised about the significance of estimates of B₁₂ and folate status in pregnancy (House et al., 2000). Reliability of single estimates is questionable. It may be necessary to follow serum vitamin assays with biochemical indices (serum Hcy and MMA) before advising supplementation. In another study, Pardo et al. (2000) confirmed that serum B₁₂ assay alone in non-anaemic patients may be unreliable. Hcy and MMA values indicated there was no difference between a group with reduced B₁₂ and one with normal levels. More extensive studies are needed to define variability in all of these parameters in pregnancy before a protocol can be recommended.

Renal failure

Decreased production of erythropoietin is the main cause of anaemia in chronic renal failure. Introduction of r-HuEPO provided a major advance in the treatment of this form of anaemia. To achieve the optimum response from r-HuEPO therapy, the supply of iron to sites of erythropoiesis must be adequate to maintain the resulting increase in red cell mass. Failure to provide sufficient iron is considered to be the single most important reason for r-HuEPO hyporesponsiveness, as iron supply limits the stimulation rate of erythropoiesis (Hörl et al., 1996; Cavill et al., 1997). A small proportion of patients may respond poorly to r-HuEPO for reasons other than lack of iron. These may be infection, inflammatory disease, malignancy, blood loss or hyperparathyroidism. Aluminium overload can produce a functional deficiency that does not respond to iron therapy (Cavill et al., 1997; Tarng et al., 1999). Best practice guidelines on the strategies for managing anaemia in renal failure are available (Cameron, 1999).

The iron status of patients on r-HuEPO therapy may have to be assessed by a variety of methods at different stages of the treatment. Haemoglobin and ferritin concentrations may be used to assess iron stores. Transport iron is reflected by transferrin saturation but is variable. An assessment of the erythron iron supply can be obtained from the measurement of hypochromic cells (Hörl et al., 1996; Cavill et al., 1997). The serum ferritin level should be measured prior to r-HuEPO treatment and, where necessary, iron supplementation should be provided until the ferritin concentration is raised to more than 200 μg/l (Cavill et al., 1997).

New parameters

Hypochromic cell analysis

Functional iron deficiency arises when the rate of erythropoiesis exceeds the supply of iron to the erythron, irrespective of the level of storage iron present (Cavill et al., 1997). With the appropriate analyser it is possible to determine the haemoglobin content of individual erythrocytes. This measurement reflects the iron content of circulating red cells and provides a direct indication of iron delivery to the bone marrow (Macdougall et al., 1992). The emergence of a subpopulation of hypochromic erythrocytes is an indicator of lack of functional iron availability to developing cells. Normally less than 2.5% of erythrocytes have a haemoglobin concentration of less than 28 g/dL. In studies on renal patients this cut-off value gave a sensitivity in detecting iron deficiency of 91% but a specificity of only 54%. The specificity can be increased by raising the cut-off level but then sensitivity falls (Cullen et al., 1999). Baumann Kurer et al. (1995) used a cut-off of 11% in a study on iron deficiency in chronic inflammatory disease, giving a specificity of 90% but sensitivity was less than 77%. More studies are required on this parameter but at present availability is a limiting factor.

Reticulocyte analysis

Flow cytometry, in addition to providing information on reticulocyte numbers, also indicates the amount of RNA present on a cell-to-cell basis and hence their maturity.
This shows potential as a measure of erythropoiesis. Due to the much larger number of cells analysed, flow cytometry has made this investigation viable when the proportion of reticulocytes is around 1% of the red cell population, often where the interest is greatest. Some standardization of methodology is still required and a standard material for method comparison is needed (Cavill & Rowan, 1996). From this new analysis, two parameters are available to investigate anaemia. The reticulocyte count, which indicates effective erythropoiesis, and the proportion of reticulocytes with a high RNA content, which indicates the intensity of reticulocyte stimulation (d’Onofrio et al., 1996).

The clinical value of these parameters has been studied in a wide range of haematological disorders. Whilst they are useful, there is some question whether, for example, they give any better indication than white cell changes in a bone marrow transplant setting. Once reticulocytes could be isolated in flow cytometry, further analysis could be made on individual cells and population characteristics such as reticulocyte volume, reticulocyte haemoglobin concentration and reticulocyte haemoglobin content could be measured. It would appear that reticulocyte haemoglobin content, in particular, shows promise as a sensitive indicator of functional iron deficiency (Brugnara, 1998).

Cullen et al. (1999) have shown that reticulocyte haemoglobin content may have better diagnostic merit than hypochromic cell analysis. These authors used a cut-off for reticulocyte haemoglobin content of >26 pg in their renal study. This produced a sensitivity and specificity for detecting iron deficiency of 100% and 73%, respectively. Brugnara et al. (1999) have also found reticulocyte haemoglobin content to be a strong predictor of iron deficiency in children. It may also be useful for treatment monitoring as early changes are seen in response to therapy. Where the appropriate instrumentation is available this could be a valuable parameter for assessing effective iron availability to developing erythrocytes.

**Serum transferrin receptor assay**

Recent study of the control of iron metabolism at the cellular level, particularly the relationship between apo-ferritin and transferrin receptor (TfR) synthesis, has significantly improved understanding of iron metabolism in health and disease. Transferrin receptor molecules are eventually cleaved from the cell surface and form a free pool in plasma TfR (measured as sTfR). As erythropoietic cells have the highest concentration of receptors, they are the main contributors to sTfR concentration. In negative iron balance, serum ferritin concentration falls with iron stores. No significant change occurs in sTfR concentration until iron stores are exhausted or availability is restricted. At this point the sTfR concentration rises in response to the lack of iron. Unlike serum ferritin, sTfR levels are not influenced by infection or inflammation and have therefore been monitored in a wide range of anaemias including those with more complex pathogenesis, e.g. anaemias of chronic disease. The assay is generally considered to be a sensitive indicator of iron-deficient erythropoiesis which is not affected by the acute phase response. Reference values and changes in sideropenia and anaemia have been explored but the parameter has not gained favour as a standalone measure of iron status. It has been suggested that combination with ferritin is more informative.

Punnonen, Irijala & Rajmaki (1997) have suggested calculation of the ratio sTfR/log ferritin (sTfR-F Index) as a way of combining sTfR and ferritin results. They found this ratio was higher in iron-depleted patients with or without an accompanying infectious or inflammatory condition compared with iron replete ACD patients and provided a useful parameter for the identification of patients with depleted iron stores. Means et al. (1999) studied sTfR in patients with a wide range of anaemia aetiologies. Their study was referenced against bone marrow aspirate iron and found sTfR assay significantly more sensitive but less specific than other iron status assays in identifying the absence of stainable iron. In comparison, serum ferritin alone was highly specific but insensitive. Serum ferritin predicted aspirate iron stores accurately when values were above or below the reference range. They suggested a decision algorithm combining serum ferritin and sTfR sequentially. sTfR should be assayed when serum ferritin falls in the range of 25–300 µg/l. This improved sensitivity and specificity. Flowers & Cook (1999) used the ratio of sTfR/ferritin obtained from dried plasma spots to identify iron deficieny. These authors suggest that dried plasma spots may be used to study the prevalence of anaemia in epidemiological studies. However, they acknowledge that ACD patients were not included in the study.

In a study using response to iron supplementation as the main indicator, Suominen et al. (2000) compared bone marrow iron level, sTfR concentration, serum ferritin and sTfR-F index as indicators of functional iron deficiency in ACD patients. The sTfR concentration and sTfR-F index were both more effective indicators of patients who would benefit from supplementation than either bone marrow iron or serum ferritin. STfR and sTfR-F index both returned to normal after supplementation. One patient with zero bone marrow iron levels had normal baseline values and did not respond to supplementation. These
authors concluded that sTfR and sTfR-F index are useful parameters in detecting functional iron deficiency irrespective of storage iron level. At present, the assay is not widely used and it remains to be seen whether the sTfR assay, alone or in combination with the serum ferritin, gains wide acceptance.

**Practice pressures**

Non-invasive analyses, which describe different facets of the erythropoietic process, should be applied in an appropriate, sequential, manner to obtain maximum information. This investigative approach can be modified depending on whether a diagnosis is to be established or the patient’s condition or treatment are being monitored. Periodic re-assessment of existing procedures and evaluation of new, or modified, methods is necessary to improve the investigation and understanding of erythropoiesis. Since so many disorders have anaemia as a complication, investigation of these anaemias exerts significant demands on pathology services. With new treatment options making it possible to improve the quality of life (Florence, 1981; Scholz et al., 1997; Silverberg et al., 2001), the investigation has become more complex, especially that of the secondary anaemias. However, current trends towards non-investigative profiling can work against this. As new tests for the investigation of anaemia were developed, the traditional approach was to use these as follow-up tests and to treat them as ‘specials’ with particular attention from the haematologist guiding the investigation to conclusion. This practice is changing and, as tests have become more readily available, clinical demand has driven requesting patterns. Now results are often reported direct to the requesting clinician who will then make their own interpretation. The use (and abuse) of any test is directly related to availability. Establishment of a method, with increased confidence in the relevance of the results, usually leads to more requests, with a corresponding rise in laboratory workload.

In some respects a non-investigative strategy may be viewed as a positive factor. From the clinicians viewpoint a screening approach where most laboratory testing is carried out at the first visit, whether known at the time to be relevant or not, is efficient. In primary care this results in fewer journeys to the general practice surgery for the patient. In the outpatient clinic, or on the ward, fewer episodes will be required, resulting in fewer appointments or faster bed turnover, respectively. However, the transition to such a service also has drawbacks. In an uncontrolled service, relevant abnormal results will constitute an even smaller minority of the total produced, most of the remainder being unnecessary to the well-being of the patient. Because time spent examining data is inversely related to its volume there is a danger that the relevant may be obscured by the irrelevant, making it even more difficult to define the pathophysiology of anaemia (or any other disorder) in individual cases. Production of redundant information also has cost implications.

Advances in instrumentation have resulted in automation of many routine assays. Available systems fall into two main categories: ‘closed’ which restrict the user to the instrument manufacturer’s reagents and preclude development or running of in-house assays; and ‘open’ which perform liquid handling robotic functions, allowing any reagents to be used. Non-isotopic assay systems are available for use with random access automated analysers, offering the main advantages of increased throughput of samples and reduced turnaround times of patient results. The choice of system will depend upon the investigations carried out together with the circumstances within the individual department. Adequate independent evaluations of available systems are required, particularly for the large volume measurements such as vitamin B12, folate and ferritin. Precision for within-batch assay should be < 5% CV and for between-batches < 10% CV. The best precision is usually required at the lower end of the reference interval. Accuracy should be controlled by the standardization of calibrators against international standards [National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK, http://www.nibsc.ac.uk]. Reagent stability over a reasonable period is important for the laboratory carrying out small numbers of tests. Systems that can accept a variety of anticoagulated specimens as well as serum are beneficial.

With fully automated systems most errors are likely to be limited to the pre- and postanalytical stages. These may be dilutional, for example in the preparation of red cell lysate, or transcription errors. Consideration therefore needs to be given to sample labelling and tracing features. The use of barcoded labels reduces the possibility of transcription faults in addition to speeding up patient data input. Adequate interfacing to a host computer should allow two-way transfer and matching of patient identification and results. Linkage to an electronic transfer system reduces the chance of error at the request receipt and result delivery stages. Significant laboratory investment, either in terms of equipment capital costs, reagent rental arrangements or electronic data processing and transfer of results is required to maximize efficiency. A multidisciplinary or multicentre approach may have to be adopted to guarantee a sufficiently high throughput of samples to ensure that associated costs are maintained at
an acceptable level and the full potential of the instrument- 
ation is realized.

In the competitive world of automated analysers, manufacturers must keep pace with service developments by offering, and evolving, relevant test repertoires. As new tests are automated, turnaround times for results generally decrease and this tends to be accompanied by a further rise in workload. For example, this may yet prove to be the case with sTfR estimations. Should more manufacturers of automated immunoassay systems offer this assay, the use will be facilitated and is likely to rise. Developments in instrument software systems also allow manufacturers to provide the option of linking results, for example the sTfR to the serum ferritin level. This should encourage the adoption of an algorithm approach such as for a hypoproliferative–renal profile.

**Clinical acceptability**

Clinical governance quality initiatives include the development of evidence-based clinical guidelines aimed at agreement on local clinical activity, resulting in improved patient outcomes (Department of Health, 1999). The non-investigative approach outlined above cannot guarantee this without the laboratory becoming more involved in the interpretation of results and provision of advice on any further testing required. Experience of ACD, particularly in rheumatoid patients, and CRF patients being treated with r-HuEPO, has led to a growth of literature on anaemia pathogenesis in rheumatoid and renal journals. It has been suggested that renal physicians are now challenging the haematologists’ understanding of iron metabolism (Cavill, 1998). To standardize and optimize treatment of renal disease in patients with anaemia, some renal centres have developed anaemia management protocols (Senger, Trenkle & St John, 1998). Specialist clinical or nursing staff are also being employed to coordinate the assessment and treatment of anaemia in their patients. These initiatives should be welcomed but, wherever possible, should be developed through collaboration between the haematology department and the user unit, and based on available guidelines.

**References**


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