Guideline for the laboratory diagnosis of functional iron deficiency

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Functional iron deficiency (FID) is a state in which there is insufficient iron incorporation into erythroid precursors in the face of apparently adequate body iron stores, as defined by the presence of stainable iron in the bone marrow together with a serum ferritin value within normal limits (Macdougall *et al*, 1989). In its broadest sense this definition encompasses the partial block in iron transport to the erythroid marrow seen in subjects with infectious, inflammatory and malignant diseases, and is a major component of the anaemia of chronic disease (ACD). One form of FID, found in some subjects treated with erythropoiesis-stimulating agents (ESAs), has been the subject of numerous studies following the widespread use of these agents, especially in subjects with chronic kidney disease (CKD).

The clinical assessment of iron status has largely been focussed on the level of iron stores, as reflected in the serum ferritin concentration. However iron in stores is metabolically inactive and is not only unavailable for immediate use but may be

difficult to bring into use at all. The real clinical issue lies in active iron metabolism, the movement of iron from effete red cells and into further generations of developing red cells. It is nevertheless true that replenishment of iron lost from the red cell pool will be compromised and iron supply to the erythroid marrow will be suboptimal as iron stores become depleted. Irrespective of cause, inadequate iron supply leads to impaired haemoglobin production and a reduction in the mean cell haemoglobin (MCH) value that becomes apparent after several weeks of impairment. In contrast, it has long been evident that the adequacy of iron supply might be estimated from the haemoglobin content of the reticulocyte within a time span of a few days. With the widespread introduction of automated cell counters capable of measuring the numbers, volume and haemoglobin content of reticulocytes, many laboratories are now in a position to detect the early indications of a failure of iron supply in this way.

In 2006 the National Institute for Health and Clinical Excellence (NICE) published a guideline entitled, 'Anaemia management in people with chronic kidney disease (CKD).' (NICE, 2006). Among tests recommended for the assessment of iron status was the percentage of hypochromic red cells (%HRC). This variable, which continues to be recommended in the updated guideline (guideline 114, NICE, 2011), has limited availability, whilst the reticulocyte measures mentioned above have become more widely available. It is thus timely to review the use of these newer variables, together with more established measures of iron status, in the management of patients with FID.

Guideline writing methodology

The guideline group was selected to be representative of UK-based experts in the clinical and laboratory fields of iron metabolism, CKD, quality control and method evaluation. MEDLINE was searched systematically for publications in English from 1966-2011 using key words: functional iron deficiency and each of the parameters discussed. The writing group produced the draft guideline, which was subsequently revised by consensus by members of the Task Force of the British Committee for Standards in Haematology (BCSH). The guideline was then reviewed by a sounding board of UK haematologists and members of both the BCSH and the British Society for Haematology. Comments were incorporated where appropriate. The 'GRADE' system was used to quote levels and grades of evidence, details of which can be found in Appendix 1. The object of this guideline is to provide healthcare professionals with clear guidance on the management of FID, in particular with respect to patients with CKD but also to other disease states in which ESAs have been used. The guidance may not be appropriate to patients with inflammatory diseases and in all cases individual

patient circumstances may dictate an alternative approach. This guideline is only applicable to adults, not children.

Summary of recommendations

- **Mean cell volume (MCV) and mean cell haemoglobin (MCH) values are useful at diagnosis and in assessing trends over periods of weeks or months. They have no use in assessing acute changes in iron availability secondary to therapy with erythropoiesis-stimulating agents (ESAs). (1B)**
- **The percentage of hypochromic red cells (%HRC) is the best-established variable for the identification of functional iron deficiency (FID) and thus has the greatest level of evidence. Reticulocyte haemoglobin content (CHr) is the next most established option. Both tests have limitations in terms of sample stability or equipment availability. Other parameters may be as good but there is no evidence that they are any better, and generally there is less evidence for newer red cell and reticulocyte parameters. (1B)**
- **A CHr value <29 pg predicts FID in patients receiving ESA therapy. A reticulocyte haemoglobin equivalent (Ret-He) value <25 pg is suggestive of classical iron deficiency and also predicts FID in those receiving ESA therapy. (1B) A Ret-He value <30·6 pg appears to have the best predictive value for likelihood of response to intravenous iron therapy in chronic kidney disease (CKD) patients on haemodialysis. (1B)**
- **The measurement of red cell zinc protoporphyrin concentration provides a sensitive index of FID and may be used as an alternative to indices of RBC hypochromia or reticulocyte haemoglobin content, although it is less sensitive than these to acute changes in iron availability. If used in the assessment of FID in CKD patients it is essential that measurements be made on washed cells, with the use of appropriate reference limits. (1B)**
- **Bone marrow examination for the sole purpose of assessing iron stores is rarely justifiable. It may be helpful if there are concerns that a high serum ferritin value (>1200 μg/l) is not a true reflection of the bone marrow iron storage pool. (1B)**
- **The serum ferritin assay is essential in the assessment and management of patients with all forms of iron-restricted erythropoiesis (IRE) including FID. Values <12 μg/l indicate absent iron stores. (1A) Values as high as 1200 μg/l in CKD patients do not exclude the possibility of FID and some such patients may respond to intravenous iron therapy. No recommendation as to**

the highest serum ferritin concentration beyond which it is unsafe to give a trial of intravenous iron therapy can be given. A serum ferritin concentration <100 μg/l in non-dialysed patients or <200 μg/l in chronic haemodialysis patients is associated with a high likelihood of iron deficiency and a potentially good response to intravenous iron therapy. (1A) Values above the suggested cut-offs given above should therefore not be used to guide iron therapy. Serum ferritin values >1200 μg/l should be used to ascertain whether investigation of potential iron overload should be undertaken. (1B)

- The serum ferritin concentration is not useful in predicting ESA responsiveness in cancer-related anaemia (1A)

- The soluble transferrin receptor (sTfR) assay is relatively expensive, not widely available, and is not currently subject to external quality assessment (EQA) in the UK. An International Standard may improve assay standardization. The treatment of renal anaemia with ESAs, which increase sTfR, is a complicating factor. The assay may have a role, either alone or in combination with the ferritin assay, if automated measures such as %HRC, CHr or Ret-He are unavailable. (1B)

- In isolation the percentage saturation of transferrin is not recommended as a predictor of responsiveness to intravenous iron therapy in patients with CKD. It may be used to monitor response to ESA and/or iron therapy in CKD. When used with either the serum ferritin concentration or measurements such as %HRC and CHr it may be useful in the diagnosis of FID. (1A)

- The measurement of serum erythropoietin concentration in the setting of anaemia has limited value in the diagnosis of FID. (1A)

- The utility of hepcidin measurement as a diagnostic tool is currently uncertain and for the time being this technique remains a research investigation.

Functional iron deficiency

The laboratory classification of iron status breaks down in the presence of inflammatory disease, as most of the variables used to define iron deficiency become abnormal despite the presence of adequate body iron reserves. ACD typically develops, a consistent feature of which is the retention of iron within body stores. As a result the supply of iron to the erythroid marrow becomes inadequate. This is the major form of FID; a second type often occurs when the erythroid marrow is stimulated by ESAs. Since the

discovery of the iron regulatory peptide, hepcidin, a 25-amino acid peptide synthesized in the liver, our understanding of the biology of ACD has greatly improved (Goodnough *et al*, 2011). Hepcidin is upregulated in the setting of chronic inflammation and cancer, resulting in its increased synthesis in the liver stimulated by cytokines of which interleukin (IL) 6 is the most important. By degrading ferroportin, hepcidin decreases iron absorption from the gastrointestinal tract and decreases the accessibility of stored iron from macrophages. Where FID and inflammatory illness coexist it is likely that increased hepcidin synthesis will restrict the absorption of oral iron. Intravenous iron preparations might overcome this block.

In patients treated with ESAs for the anaemia associated with CKD, the response rate improves when intravenous rather than oral iron supplements are given, and often allows a reduction in the ESA dose (Nemeth *et al*, 2004; van Wyck *et al*, 2004; Henry, 2010; Qunibi *et al*, 2010). Intravenous iron is routinely used in ESA-treated patients with CKD on dialysis and this practice is endorsed in national and international guidelines (NICE, 2011; National Kidney Foundation, 2006; Kidney Disease: Improving Global Outcomes (KDIGO) Anemia Work Group 2012).

Variables for the assessment of functional iron deficiency or iron-restricted erythropoiesis

Red cell variables

There are now a considerable number of red cell indices available for the assessment of iron status. Used in isolation as a diagnostic test, none is capable of differentiating between iron deficiency and FID. All are confounded by α°- and β-thalassaemia heterozygosity and in homozygotes and some heterozygotes for α+ thalassaemia. Patients with combined iron and vitamin B12/folate deficiencies or sideroblastic anaemia may also prove problematic. Once a diagnosis has been made, however, some of these variables may be used to monitor the response to ESA therapy and the requirement for iron.

These indices can be divided into five groups; the traditional measures of MCV, MCH and mean cell haemoglobin concentration (MCHC): measures based on increased

hypochromia: indices of reticulocyte volume and Hb content: red cell zinc protoporphyrin (ZPP) concentration: and recently introduced indices, such as red cell size factor (Rsf).

MCH, MCV and MCHC

The MCH is derived from the red blood cell (RBC) count and haemoglobin concentration (Hb), both of which are measured with considerable accuracy and precision by modern analysers. Values obtained from differing types of analyser are therefore largely interchangeable whereas MCV values, which are derived using differing analytical principles, are much less so. Unlike MCV, MCH is unaffected by several days of storage.

As both MCV and MCH are derived from the entire circulating red cell mass, they are slow to change and have no value in detecting either the acute development of iron lack or the early response to iron therapy in patients treated with ESAs. The MCHC is of limited or no use in assessing changes in iron availability associated with ESA therapy.

Recommendation

- MCV and MCH should be determined at diagnosis and are useful in assessing trends over periods of weeks or months. They have no use in assessing acute changes in iron availability secondary to ESA therapy.

Hypochromic red cells: %HRC, %Hypo-He, Low Haemoglobin Density

As originally identified using Siemens technology, hypochromic red cells are those with Hb <280 g/l. The clinical utility of this variable in detecting FID in patients with chronic renal failure treated with ESAs has long been recognized (Macdougall *et al*, 1992). A value of ≥6% was found to be superior to measurements of soluble transferrin receptor (sTfR), ZPP, ferritin and total iron-binding capacity (TIBC) in differentiating between iron-deficient and iron-sufficient patients with chronic renal failure receiving maintenance doses of ESAs (Tessitore *et al*, 2001). This value was incorporated into UK guidelines on anaemia management in this group (NICE, 2006). The measure is effective in the detection and monitoring of FID secondary to ESA therapy in anaemic subjects with advanced acquired immunodeficiency syndrome (Matzkies *et al*, 1999) and rheumatoid arthritis (Arndt *et al*, 2005). An increase in %HRC in subjects with low-risk myelodysplastic syndromes treated with ESAs may however reflect improved

survival of a pre-existing population of abnormal hypochromic red cells, rather than ESA-induced FID (Ljung *et al*, 2004).

Two other variables are now available for the assessment of hypochromia. One, %Hypo-He, is produced by some Sysmex blood counter analysers (XE 5000 and XN), and defines those red cells having MCH <17 pg. The measure has clinical utility in the differential diagnosis of anaemia (Urrechaga *et al*, 2009). The second, Low Haemoglobin Density (LHD%), is a variable based on a mathematical transformation of the MCHC value and is available on some Beckman Coulter instruments. Values correlate highly with those of %HRC (Urrechaga, 2010).

Reticulocyte count, immature reticulocyte fraction, CHr and Ret-He

The great increase in precision of the automated reticulocyte count and the provision of measures of immature reticulocyte fraction and the reticulocyte-specific indices of volume and haemoglobin content provide an opportunity to assess the effects of changing iron status on this transient population.

The reticulocyte count itself cannot provide information about a patient's iron status. However a reticulocyte increase of $\geq 40 \times 10^9/l$ from baseline by week four of ESA therapy in cancer patients has been shown to predict an adequate response, defined by a $\geq 6\%$ rise in haematocrit above baseline, and it implies adequate iron stores (Henry *et al*, 1995). Automated blood counters capable of producing reticulocyte data generally group cells depending on RNA content. The immature reticulocyte fraction (IRF) is the component with highest RNA content. Immature reticulocytes are released during periods of intense erythropoietic stimulation, such as following haemorrhage or haemolysis, or in response to therapy with iron or ESAs. The IRF increases several days before the reticulocyte count (Davies, 1996) and is thus an early indicator of response to therapy. Nevertheless, the test is little used, probably because of a lack of standardization of methods and instrument- or method-specific reference ranges.

CHr, the term used to describe the reticulocyte MCH as derived by Siemens analysers, was the first automated reticulocyte measure available for routine use. Among patients undergoing bone marrow examination for diagnostic purposes CHr had a better predictive value for iron depletion than MCV, serum ferritin or transferrin saturation values (Mast *et al*, 2002). CHr has been used as the standard against which other emerging variables have been assessed (Brugnara *et al*, 2006). Among subjects with

presumed FID, CHr compared favourably with other measures of iron status in predicting a response to intravenous iron (Mittman *et al*, 1997; Chuang *et al*, 2003). The variable received US Federal Drug Administration approval in 1997 and was incorporated into the revised European Best Practice Guidelines for the management of patients with chronic renal failure (Locatelli *et al*, 2004). A target CHr of 29 pg was recommended (evidence level B). This value is indicative of the adequacy of iron incorporation into the developing erythron, although some patients with CHr values >29 pg responded to intravenous iron therapy, leading to a suggested cut-off value of 32 pg (Fishbane *et al*, 2001). In a study of sample stability, a small but statistically insignificant fall in CHr values over 24 h was demonstrated (Lippi *et al*, 2005).

An alternative measure of reticulocyte haemoglobin content, Ret-He, is available on some analysers manufactured by the Sysmex Corporation. Although expressed in the standard unit of cellular haemoglobin content, (pg), Ret-He is a natural log transformation of Ret-Y, a measure of volume obtained from measurement of forward light scatter of reticulocytes and itself expressed in arbitrary units. A number of studies (Canals *et al*, 2005; Thomas *et al*, 2005; Brugnara *et al*, 2006; Garzia *et al*, 2007; Maconi *et al*, 2009; Miwa *et al*, 2010) have found excellent concordance between Ret-He and CHr in subjects with both iron deficiency and chronic renal failure. However Brugnara (2003) has reported that it is less clear that either low Ret-He or CHr values are predictive of response to intravenous iron or whether ESA usage can thus be reduced to the minimum required (Mast *et al*, 2008). Canals *et al* (2005) studied 504 patients with ACD or other iron-restricted states. Ret-He alone was able to distinguish iron-deficient and iron-sufficient subjects using a cut-off of 25 pg with reasonable sensitivity (0·76). However the groups that included ACD, mild iron deficiency anaemia and reduced iron stores showed significant overlap. The interquartile range for the ACD group in this study was below that of the reference range and the values of the ACD group were significantly different from those of the iron deficiency group. Although less sensitive (80% agreement with sTfR and sTfR/log ferritin values), a Ret-He cut-off of 25 pg may also help to distinguish iron deficiency (values <25 pg) from ACD (values >25 pg). A Ret-He cut-off value of 30·6 pg is a better predictor of response to intravenous iron than baseline serum ferritin or transferrin saturation values in CKD patients undergoing thrice-weekly haemodialysis (Buttarello *et al*, 2010).

Red blood cell size factor (Rsf)

This variable is derived from the square root of the product of the MCVs of mature RBC and reticulocytes. It shows good correlation with CHr, with slightly better

sensitivity and identical specificity for the detection of iron-restricted erythropoiesis (IRE). Patients with values >87·7 fl were more likely to have ACD, those with lower values to have iron deficiency (Urrechaga, 2009).

Recommendation

- The %HRC is the best-established variable for the identification of functional iron deficiency (FID) and thus has the greatest level of evidence (Tessitore *et al*, 2001). CHr is the next most established option. Both tests have limitations in terms of sample stability or equipment availability. Other parameters may be as good but there is no evidence that they are any better, and generally there is less evidence for newer red cell and reticulocyte parameters.
- A CHr value <29 pg predicts IRE in patients with iron deficiency anaemia, FID and those receiving ESA therapy. A Ret-He value <25 pg predicts FID in those receiving ESA therapy. Among reticulocyte variables, a Ret-He value <30·6 pg appears to be the best predictive value for response to intravenous iron in CKD patients on haemodialysis.

Zinc Protoporphyrin

Zinc protoporphyrin (ZPP) is a trace by-product of haem synthesis and any condition that limits iron supply to the erythroid marrow or stimulates porphyrin synthesis leads to an increased concentration of ZPP in circulating red cells. The incorporation of iron into protoporphyrin IX is the final stage of haem synthesis, and an increase in ZPP is an indicator of defect(s) at any point along this pathway. The measure is therefore non-specific and raised values occur in iron deficiency, FID, lead poisoning and in many iron-sufficient subjects with $\alpha°$- and β-thalassaemia traits (Graham *et al*, 1996).

There are two important limitations of the measurement of ZPP by the commonly used method of haematofluorometry. First, plasma constituents contribute to the magnitude of the ZPP value, with potentially misleading elevations being found in the presence of hyperbilirubinaemia (Buhrmann *et al*, 1978) and in chronic renal failure (Garrett & Worwood, 1994). Second, a spurious and progressive increase in ZPP values is seen as Hb falls below about 100 g/l and many subjects with moderate or severe anaemia have raised ZPP values irrespective of iron status. These shortcomings can be overcome by

washing RBC prior to testing or adjusting the Hb concentration to a standard value, but sample manipulation is time-consuming. Due to lack of specificity, ZPP should not be used in isolation as a diagnostic test, but once a diagnosis is made it may be used to monitor response to therapy. The ZPP concentration reflects the entire circulating red cell population and is thus less sensitive than %HRC or CHr to acute changes in iron availability (Fishbane & Maessaka, 1997).

Recommendation

- The measurement of ZPP concentration provides a reliable index of FID and may be used as an alternative to indices of red cell hypochromia or reticulocyte haemoglobin content, although it is less sensitive to acute changes in iron availability. If used in the assessment of FID in CKD patients, it is essential that measurements be made on washed RBC, with the use of appropriate reference limits.

Assessment of iron stores

Assessment of body iron stores is essential both as a diagnostic tool and to monitor the effects of therapy with iron and/or ESAs. This may be achieved by cytological evaluation of the iron content in aspirated bone marrow, or by use of the serum ferritin assay. Ferrokinetic studies using radiolabelled iron have been excluded from discussion as they are rarely employed these days and can be cumbersome.

Cytological assessment of iron stores

An assessment of iron stores in the bone marrow can be made by use of Perls' Prussian blue reaction. Although considered a 'gold standard' test, assessment can be misleading if insufficient material is available: seven or more particles should be available for review (Hughes et al, 2004), and few haematologists can honestly say they invariably manage this number on their aspirate films. Inadequate material was a major factor in a study that concluded that over 30% of reports of absence of stainable iron were inaccurate (Barron et al, 2001). Also, the presence of stainable iron does not define how much can be re-solubilized and incorporated into the developing erythron. Furthermore, bone marrow examination is uncomfortable and not without complications, such as post-biopsy pain and bleeding.

Recommendation

- Bone marrow examination for the sole purpose of assessing iron stores is rarely justifiable in CKD patients. It may be helpful if there are concerns that a high ferritin value (>1200 μg/l) is not a true reflection of the bone marrow iron storage pool.

Serum ferritin

The serum ferritin assay has become the standard test for the assessment of iron stores (Cavill, 1999). Ferritin in serum results from leakage from tissue or intracellular fluids and in health there is a relationship between the two, such that each μg/l of ferritin in serum is equivalent to ~8–10 mg of iron in stores (Cook & Skikne, 1982; Worwood, 1997). Confounding variables may alter this leakage and mask the level of stored iron or, in some cases, the organ(s) it is derived from. Most clinicians are aware of the 'acute phase' nature of serum ferritin but the degree by which this variable deflects from the true measure of the iron stores is less often considered. What the ferritin value cannot do, particularly in CKD, is to indicate when sufficient stores exist to supply erythropoiesis. Here the term 'sufficient' implies the patient will not respond to additional iron supplementation, usually intravenously. Some authors have argued it may be counterproductive to set an upper limit of serum ferritin concentration to provide a 'sufficiency level' (Kalantar-Zadeh et al, 2005), such that it may still be safe to give intravenous iron, and some CKD patients may respond to this therapy despite raised ferritin values. There is also evidence to suggest that CKD patients with low serum ferritin concentrations have a poorer outcome compared to patients with values in the 200–1200 μg/l range (Kalantar-Zadeh et al, 2005). Additionally, some patients with CKD may have excess iron in the liver and spleen, yet paucity within the bone marrow available for erythropoiesis (Ali et al, 1980, 1982).

Therefore the setting of an upper limit of serum ferritin concentration above which intravenous iron supplementation is not advised is not evidence-based (Dukkipati & Kalantar-Zadeh, 2007). Most guidelines use a value of >500 μg/l (NICE, 2006) or >800 μg/l (target range 200–500 μg/l; National Kidney Foundation, 2002) for CKD patients on ESA therapy, citing that above these values there is a greater risk of exacerbated iron overload with further therapy. However anaemic CKD patients (Hb <110 g/l) with ferritin concentrations of 500-1200 μg/l showed an increase in Hb when treated with intravenous iron (Coyne et al, 2007). These patients all had transferrin

saturation (TSat) levels <25%, taken to indicate the presence of FID. The best indicator of underlying IRE in this group was the response to intravenous iron. The serum ferritin concentration was unhelpful in predicting response to ESA therapy in cancer-related anaemia (Littlewood *et al*, 2003).

Recommendation

- The serum ferritin assay is essential in the initial assessment and ongoing management of patients with FID. Values <12 μg/l are indicative of absent iron stores. Values as high as 1200 μg/l in CKD patients do not exclude IRE, as patients with such levels may have FID and respond to intravenous iron. No recommendation as to the highest ferritin concentration beyond which it is unsafe to give a trial of intravenous iron can be given. A ferritin concentration <100 μg/l in non-haemodialysis patients or <200 μg/l in chronic haemodialysis patients is associated with a high likelihood of iron deficiency and a potentially good response to intravenous iron. Values above the suggested cut-offs given above should therefore not be used to guide iron therapy. Values >1200 μg/l should be used to ascertain whether investigation of potential iron overload should be undertaken.
- The serum ferritin concentration is not useful in predicting ESA responsiveness in cancer-related anaemia.

Soluble transferrin receptor

The transferrin receptor is highly expressed on erythroid precursors. Increased levels are found in disorders associated with an expanded erythroid marrow (Kohgo *et al*, 1987; Huebers *et al*, 1990) and also in iron deficiency.

The clinical utility of sTfR measurement has been hampered by the lack of agreement concerning the source both of standards and of antigens used to raise antibodies. Use of the recently developed first World Health Organization Reference Reagent for sTfR should improve this situation (Thorpe *et al*, 2010).

The major clinical role of the assay is in differentiating the anaemia of iron lack from that caused by inflammation, which has little or no effect on sTfR values, and in detecting the presence of iron lack when the two coexist (Ferguson *et al*, 1992; Punnonen *et al* (1997). Although in some studies estimation of sTfR did not prove superior to the serum ferritin assay in the detection of iron deficiency in patient groups

typical of those seen in clinical practice (Mast *et al*, 1998; Means *et al*, 1999; Lee *et al*, 2002), a recent systematic review concluded that its use improves the diagnosis of iron deficiency, especially in the presence of chronic disease or gastrointestinal malignancy (Koulaouzidis *et al*, 2009). In patients with stable chronic renal failure and stable kidney disease not receiving iron or ESAs, the sTfR concentration alone proved inferior to that of serum ferritin in detecting those with coexisting iron deficiency (Fernandez-Rodriguez *et al*, 1999). However in a group of patients receiving maintenance doses of ESAs, and with stable erythropoiesis, the measure proved superior to ZPP, transferrin saturation and serum ferritin, but inferior to %HRC and CHr, in differentiating between iron-deficient and -sufficient subjects (Tessitore *et al*, 2001).

The TfR index, a ratio of the ferritin concentration to that of sTfR (Punnonen *et al*, 1997), was found to be superior to ferritin alone in predicting response to intravenous iron in renal patients on long-term ESA therapy (Chen *et al*, 2006). This approach helps to overcome one drawback to the use of sTfR as a single test in monitoring iron status during ESA therapy, that of the therapy itself leading to increased concentrations via expansion of the erythroid marrow (Chiang *et al*, 2002).

The TfR index has been used, along with a measure of iron availability to the erythroid marrow, such as %HRC or CHr, in the production of a so-called diagnostic plot (Thomas & Thomas, 2002). Sequential testing can be used to monitor the effects of, or need for, supplementation with iron or ESAs (Thomas *et al*, 2006).

Recommendation

- The sTfR assay is relatively expensive, not widely available, and is not currently subject to external quality assessment (EQA) in the UK. An International Standard may improve assay standardization. The treatment of renal anaemia with ESAs, which increase sTfR, is a complicating factor. The assay may have a role, either alone or in combination with the ferritin assay, if automated measures such as %HRC, CHr or Ret-He are unavailable.

Serum iron, TIBC and %Saturation (%Sat)

The %Sat value is derived from serum iron and TIBC values and is the most widely used of the three. The serum iron concentration falls markedly within hours of the onset of inflammatory illness and TIBC also falls but to a lesser degree. This results in reduced %Sat values that persist for the duration of the illness. Although a measure of

iron in transport and not of iron in stores, %Sat values of <20 indicate the need for parenteral iron in the setting of anaemia treated with ESAs (Macdougall *et al*, 1990). Used in isolation, %Sat has poor sensitivity and specificity in detecting those who respond to intravenous iron (Low *et al*, 1997; Tessitore *et al*, 2001), although combination with another variable (e.g sTfR) produces improved accuracy and may be a useful alternative where RBC and reticulocyte variables are not available.

Recommendation

- In isolation, %Sat is not recommended as a predictor of responsiveness to parenteral iron therapy in patients with CKD. It may be used to monitor response to ESAs and/or iron in CKD (Macdougall *et al*, 1990). When used with either the ferritin concentration or RBC/reticulocyte variables it may be useful in the diagnosis of FID.

Erythropoietin

Serum erythropoietin (Epo) levels are not routinely measured, particularly in the setting of CKD and are not recommended in patients with anaemia and CKD (National Kidney Foundation, 2006). What is clear is that for patients with the various stages of CKD with anaemia there is a relative lack of serum Epo. However low Epo levels have limited clinical value given that some cancers and arthritides are associated with suppression of Epo levels (Hochberg *et al*, 1988; Miller *et al*, 1990). In a study by Rose *et al* (1995), patients with myelodysplasia and baseline Epo levels <100 μ/ml were most likely to respond to ESA therapy. It remains to be seen whether such patients' refractoriness to ESA therapy relates to FID in cancer patients, but supplementation with iron appears to improve response rates (Henry, 2010).

Recommendation

- The measurement of serum Epo in the setting of anaemia has limited clinical value in the laboratory diagnosis of FID.

Hepcidin

Recently, hepcidin has emerged as the master regulator of iron availability to the bone marrow (Ganz, 2007). Assays for its measurement in serum or plasma have improved

considerably and this has generated optimism that hepcidin quantitation might be a superior alternative to traditional markers of iron status.

Broadly speaking, the assays that have been developed include radioimmunoassay, enzyme-linked immunosorbent assays and mass spectrometry techniques (Macdougall *et al*, 2010). The main advantage of immunoassays is that they are technically easier to perform, readily accessible (several are commercially available) and cheaper to implement. Their main disadvantage is that the antibodies used cross-react with both the biologically inactive hepcidin-22 and -20 fragments, thus overestimating the true bioactive hepcidin-25 level (Macdougall *et al*, 2010). This is acceptable when the same assay is used to measure changes over time, with or without intervention, but is less satisfactory if absolute hepcidin values are required. Mass spectrometry techniques are more accurate, detecting only hepcidin-25, but they are costly, labour-intensive and time-consuming.

Serum levels of hepcidin are elevated in acute and chronic inflammatory states, such as infection, rheumatoid arthritis, inflammatory bowel disease and CKD, and are usually undetectable in conditions causing iron overload, such as hereditary haemochromatosis (Ganz, 2007). Other factors, however, may affect hepcidin levels, and it is not yet clear whether this novel biomarker has any advantages in determining iron status or in ascertaining the need for supplemental iron. Indeed, in a study of haemodialysis patients, hepcidin measurement was inferior to %HRC in predicting the response to intravenous iron (Tessitore *et al*, 2010). The hepcidin level in CKD patients may not be of greater diagnostic value than the ferritin level, but further studies are needed (Coyne, 2011; Peters *et al*, 2012).

At present there are no UK EQA schemes for hepcidin assays. There is also a lack of harmonization of reference values across the different assay platforms.

Recommendation

- The utility of hepcidin measurement as a diagnostic tool is currently uncertain and for the time being this technique remains a research investigation.

Functional iron deficiency and the anaemia of chronic disease

The failure of adequate iron incorporation into the developing erythron is just one component of ACD and cancer-related anaemia. With these disorders the actions of γ-interferon, transforming growth factor-β and tumour necrosis factor produce a down-regulation of the erythron, early erythroid precursor cell death and reduced Epo sensitivity. Increased levels of both hepcidin and IL6 have the additional effect of reducing iron transfer to developing erythroblasts. It is therefore difficult to predict the response to intravenous iron therapy in these patients. Despite this however there is evidence to suggest that ACD patients with biochemical markers of FID do benefit from iron supplementation (Thomas *et al*, 2005). The development of FID in anaemic cancer patients blunts the response to ESA therapy unless supplementary iron is provided (Cazzola *et al*, 1992).

Oral iron therapy has been the mainstay of treatment for patients with iron deficiency. Intravenous iron is safe and effective and in patients with ACD is superior to oral iron when used in conjunction with ESAs.

Physicians treating patients with chronic inflammatory diseases or cancer with ESAs should be aware of the potential development of FID. Using the variables discussed above to help guide therapy seems entirely appropriate even though evidence is less clear than for CKD. Fig 1 provides an example of an algorithm for use in CKD patients.
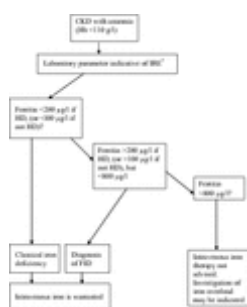
Figure 1. Management of iron-restricted erythropoiesis in patients with CKD on ESA. *Where IRE (iron-restricted erythropoiesis) is defined by: Percentage of hypochromic red cells (%HRC) > 6%. Reticulocyte haemoglobin content (CHr) < 29 pg. Reticulocyte haemoglobin equivalent (Ret-He) < 30·6 pg Or indicative values from other red cell or reticulocyte parameter. CKD, chronic kidney disease; ESA, erythropoiesis stimulating agent; FID, functional iron deficiency; HD: haemodialysis.

Quality control

Internal quality control (IQC) and EQA for all reported variables should be available. This is the case for traditional variables such as Hb, MCV, MCH and reticulocyte count but, for newer variables used to detect or monitor FID and IRE, EQA is not available and in some instances neither is IQC.

Recommendation

- Ideally results on patient samples should not be reported unless it can be demonstrated through the use of IQC that the analytical procedure is valid and free of problems. Some instruments have reportable variables available in the absence of stated ranges for the manufacturer's IQC material: these should be reported with caution. EQA allows comparison of results between laboratories and methodologies, making it possible to identify the best method(s) for performing a test and identifying unreliable ones. The newer variables of use in the assessment of iron status are mostly instrument-specific, limiting the cost-effectiveness of providing a national EQA scheme. However it may be possible to devise some form of inter-laboratory comparison, e.g. by using the manufacturer's own control material or by local laboratories with the same instrumentation sharing blood samples. Whilst this is not ideal, it would provide laboratories with some reassurance that the results they are reporting are within consensus with those of others using the same technology.

Disclaimer

While the advice and information in these guidelines is believed to be true and accurate at the time of going to press, neither the authors, the British Society for Haematology nor the publishers accept legal responsibility for the content of these guidelines.

Appendix 1

Strength of recommendation

Strong (grade 1): Strong recommendations (grade 1) are made when there is confidence do or do not outweigh harm and burden. Grade 1 recommendations can be applied uniformly to most patients. Regard as 'recommend.'

Weak (grade 2): Where the magnitude of benefit or not is less certain a weaker grade 2 recommendation is made. Grade 2 recommendations require judicious application to individual patients. Regard as 'suggest.'

Quality of evidence and definitions

The quality of evidence is graded as high (A), moderate (B) or low (C). To put this in context, it is useful to consider the uncertainty of knowledge and whether further research could change what we know or our certainty.

A. High: Further research is very unlikely to change confidence in the estimate of effect. Current evidence derived from randomized clinical trials without important limitations.

B. Moderate: Further research may well have an important impact on confidence in the estimate of effect and may change the estimate. Current evidence derived from randomized clinical trials with important limitations (e.g. inconsistent results, imprecision – wide confidence intervals or methodological flaws – e.g. lack of blinding, large losses to follow-up, failure to adhere to intention to treat analysis), or very strong evidence from observational studies or case series (e.g. large or very large and consistent estimates of the magnitude of a treatment effect or demonstration of a dose-response gradient).

C. Low: Further research is likely to have an important impact on confidence in the estimate of effect and is likely to change the estimate. Current evidence from observational studies, case series or just opinion.

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