Ferritin and Iron Studies in Anaemia and Chronic Disease

ABSTRACT

Anaemia is a condition in which the number of red cells necessary to meet the body's physiological requirements is insufficient. Iron deficiency anaemia (IDA) and the anaemia of chronic disease (ACD) are the two most common causes of anaemia worldwide[1]; iron homeostasis plays a pivotal role in the pathogenesis of both diseases. An understanding of how iron studies can be used to distinguish between these diseases is therefore essential, not only for diagnosis but also in guiding management. This review will primarily focus on IDA and ACD; however iron overload in anaemia will also be briefly discussed.

IRON HOMEOSTASIS

The average human adult contains approximately 3 to 4g iron[2]. There is no excretory system for iron (aside from blood loss and mucosal shedding - which is not regulated by homeostasis) so absorption of iron from the gastrointestinal system is tightly controlled.

Iron absorption into the enterocyte occurs primarily in the terminal duodenum through the divalent metal transporter (DMT1)[3]. Export of iron from the enterocyte is through the basolateral membrane by ferroportin-1[4]. Ferroportin-1 is also highly expressed at sites involved in iron transfer including macrophage membranes and the sinusoidal surfaces of hepatocytes[5].

Once iron is released from the enterocyte, it is transported to sites of usage and storage by transferrin[2]. Transferrin is a 75-80 kDA glycosylated protein that can carry up to two ferric ions; under physiological conditions around 30-40% of the iron binding capacity of transferrin is used in[6]. Transferrin then delivers the bound iron through transferrin receptor-1 (TfR1) to the sites of usage and storage; TfR1 mediated iron import is the main pathway used by erythrocytes and hepatocytes[7]. Iron transport into the macrophages of the reticuloendothelial system (RES) is primarily through erythrophagocytosis of senescent red blood cells[6].

Free iron is cytotoxic; if it is not immediately utilised after internalisation it will associate with ferritin, the main iron storage protein in the body[8]. The main site for iron storage is within the macrophages of the reticuloendothelial system (particularly of the liver, spleen and bone marrow) and hepatocytes[6]. Export of iron from sites of storage is through ferroportin-1.

Regulation of iron homeostasis is mainly via iron regulatory proteins (IRP)/iron responsive elements (IRE) and hepcidin. The former control uptake and storage of iron whilst the latter regulates iron export. Hepcidin plays a central role in iron homeostasis through its effect on ferroportin-1; after hepcidin binds to ferroportin-1, ferroportin-1 is internalised and degraded by lysosomes; the overall effect is to decrease ferroportin-1 expression and block iron export[9].

It is beyond the remit of this review to fully cover iron homeostasis, a recent review by Tomas Ganz[2] provides a comprehensive overview of this topic.

IRON STUDIES

Iron studies are a panel of tests used to assess the amount of circulating iron and storage iron. These tests should be interpreted together. Below is a summary of the routine iron studies performed in most laboratories.

Ferritin

As the main iron storage protein in the body, the majority of ferritin is intracellular. However, a soluble form is found in the blood and can be assayed[10].

Ferritin concentrations vary by age and gender. From adolescence, males have higher values than females, a trend that persists into late adulthood. In women, ferritin concentrations remain relatively low until menopause and then rise[11]. In both sexes, ferritin increases from around 70 years of age[12].

A ferritin concentration <15µg/L in adults[13] is almost always diagnostic of iron deficiency. An elevated ferritin may reflect iron overload; however ferritin is an acute phase protein, so may also be increased in liver disease, malignancy, infection and inflammation[14]. Therefore, a normal ferritin concentration alone does not necessarily exclude iron deficiency.

Serum iron

Serum iron is a measure of the amount of iron bound to transferrin in the plasma. Only a small proportion of the body's iron is bound to transferrin at any one time[15]. There is a rapid turnover of transferrin-bound iron and circulating iron concentration can be affected by dietary intake; as a result there is significant variation in iron concentration within each day and between days[16]. For this reason, assessment of serum iron alone provides little helpful clinical information.

Total Iron Binding Capacity (TIBC) / Transferrin

TIBC is an assay which determines the amount of iron that can be bound to unsaturated transferrin i.e. the total number of transferrin binding sites per unit volume of plasma or serum. Historically, it was assessed by adding an excess of iron to plasma and measuring the amount of iron retained[17]. Therefore TIBC is a proxy measure of transferrin.

Unlike serum iron, TIBC does not have rapidly changing concentrations in the plasma. However it is not a useful marker of early iron deficiency as values do not change until stores are depleted[18].

Transferrin is the transporter protein for iron and its concentration can be determined by immunological methods[18]. Both TIBC and transferrin rise in iron deplete states and fall in inflammatory and iron overload disorders.

Transferrin saturation

This is derived by dividing serum iron by TIBC. As the name suggests, it is the percentage of transferrin bound to iron. In iron deplete states the amount of iron is reduced and therefore the transferrin saturation will be reduced (and vice versa). A transferrin saturation of <15% in association with an elevated TIBC is indicative of iron deficiency anaemia. A transferrin saturation of

>45% is suggestive of iron overload and will usually require further investigation[19]. As previously mentioned, the variation in plasma concentration of iron is considerable, and therefore there will be daily variation in the transferrin saturation; as a result transferrin saturation must be interpreted alongside other iron studies.

IRON DEFICIENCY ANAEMIA

Iron deficiency anaemia is due to the lack of sufficient iron to form normal red blood cells; it is the most common cause of anaemia worldwide[1]. Iron deficiency may be the result of blood loss, inadequate dietary intake or malabsorption. The gold standard for diagnosing iron deficiency is the absence of stainable iron on bone marrow biopsy; however this is impractical and iron deficiency is usually assessed by laboratory parameters on a peripheral blood sample.

Laboratory diagnosis of iron deficiency anaemia

Full blood count (FBC) and blood film

By WHO criteria, anaemia is defined as a haemoglobin concentration (Hb) of <120g/L in a female or <130g/L in a male[13]. In the early stages of iron deficiency, haematopoiesis is not affected; as stores diminish further, the red cells become microcytic first and then hypochromic before the Hb falls. As well as microcytosis and hypochromia, the blood film may feature poikilocytosis (variation in shape, including pencil cells) and anisocytosis (variation in size)[20]. Microcytosis is reflected in the FBC as a reduction in the mean cell volume (MCV) and hypochromia as a reduction in the mean corpuscular haemoglobin concentration (MCHC).

Iron Studies

Hepcidin feedback is regulated by concentrations of iron; in iron deplete states, circulating concentrations of this hormone fall[21]. As hepcidin falls, ferroportin expression increases, leading to increased absorption of iron from enterocytes and increased iron export from storage cells. The IRP/IRE system also works to reduce the conversion of cytosolic iron into ferritin. Lastly, in order to optimise delivery of exported iron to areas of high demand, the production of transferrin is upregulated in the liver.

Iron studies can reflect this physiological response. Circulating transferrin and TIBC are elevated. Serum iron falls; the relative decrease in supply compared to demand reduces the circulating pool. Transferrin saturation is reduced (typically <15%) due to increased TIBC and reduced serum iron. The increased export of iron from stores and decreased ferritin production lead to a fall in circulating ferritin; a concentration of <15µg/L is diagnostic of iron deficiency[13].

Although a low serum ferritin is both a highly specific and sensitive marker of iron deficiency, a normal ferritin can be falsely reassuring. As previously discussed, ferritin may rise with advancing age and inflammation, therefore diagnosing iron deficiency in these states can be challenging; however a ferritin concentration above 100µg/L is unlikely to be associated with iron deficiency[22]. The British Society of Gastroenterology suggests that the threshold for diagnosing iron deficiency

should be raised to a serum ferritin concentration of 50µg/L in people who have comorbidities[23]. Table 1 summarises these changes.

There are assays which can be helpful in diagnosing iron deficiency in cases when it is not clear from conventional iron studies; these are discussed below.

Soluble transferrin receptor (sTfR)

sTfR results from the proteolysis of TfR and occurs following the binding of transferrin to Tf R, this produces monomers that are measurable in plasma or serum. The concentration of sTfR is therefore an indirect measure of total TfR[24]. TfR mediated iron import is the main pathway used by erythrocytes and hepatocytes; most TfRs are located on erythroid progenitors[25]. As a result sTfR concentration is believed to reflect erythroid turnover and is determined by erythroid proliferation rate and iron demand; sTfR concentrations will increase in iron deficiency[26]. Concentrations can also be increased in other high erythroid turnover states such as haemolytic anaemia and thalassaemia[27].

Unlike ferritin, sTfR is not an acute phase reactant, so serum concentrations do not rise in inflammatory states; therefore sTfR can be useful in diagnosing iron deficiency in such cases. In addition, sTfR/log ferritin index can be useful in diagnosing early iron deficiency and may have a higher sensitivity and specificity than sTfR alone[28].

sTfR is not a widely available assay. There is no uniform standard for measuring serum concentration or a universally established reference range. Therefore, whilst this may eventually be useful in determining iron status, validation is still necessary in population studies[27].

Zincprotoporphyrin (ZPP)

In the last step of haemoglobin production, ferrous protoporphyrin is combined with globin to make haemoglobin. When there is a lack of iron, zinc replaces iron to produce zinc protoporphyrin. The normal ratio of iron to zinc in protoporphyrin is approximately around 30000:1, but ZPP will increase to measurable concentrations with progressive iron deficiency[18]. Currently this assay is not widely available but could be considered when conventional iron studies are not diagnostic.

ANAEMIA OF CHRONIC DISEASE

Anaemia of chronic disease (ACD) is the second most common cause of anaemia worldwide[1]; it was first identified in 1962 after studies on anaemia associated with infection[29]. ACD is expected to become more prevalent in the future as the number of elderly patients with chronic inflammatory conditions rises.

A variety of clinical conditions can lead to ACD such as infection, inflammatory disorders (including inflammatory bowel disease and rheumatological conditions) and malignancy; these three causes account for 75% of cases[30]. ACD is immune driven. Cytokines induced by activated leucocytes exert multiple effects that contribute to the fall in haemoglobin; these include changes in iron homeostasis, erythropoietic activity, erythropoietin production and the life span of erythrocytes[1].

A particular case of ACD is the anaemia of chronic renal failure. This is mediated by a decrease in circulating erythropoietin, which leads to a reduction in erythropoietic activity; this anti-proliferative effect is enhanced by accumulating uraemic toxins[31]. In patients with end stage disease, chronic inflammation has also been shown to correlate with the degree of anaemia[32]. The activation of immune cells may stem from repeated infection and/or contact activation from dialysis membranes. In these patients, the changes of iron homeostasis mirror those found in ACD[1].

The diagnosis of ACD can be challenging and is perhaps best explained in conjunction with the pathophysiological mechanisms underlying this disease.

<u>Dysregulation of iron homeostasis and its effect on laboratory markers</u>

Disturbance of iron homeostasis is a hallmark of ACD and is driven by inflammatory cytokines.

There is an increase in interferon-ϒ (IFN-ϒ), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10 (IL-10)[1]. IL-6 and lipopolysaccharide (endotoxin found on the outer membrane of gram negative bacteria) are strong inducers of hepatic hepcidin production[33], this results in reduced ferroportin-1 expression and sequestration of iron within the enterocytes, hepatocytes and macrophages[9]. Iron import is unregulated in the macrophages by increased DMT-1 expression (mediated by IFN-ϒ and lipopolysacharide), upregulation of TfR expression (mediated by IL-10) and lastly phagocytosis of senescent erythrocytes, a process which is enhanced by TNF-α mediated damage of erythrocyte membranes[1]. Lastly, TNF-α, IL-1, IL-6 and IL-10 all induce ferritin expression and stimulate the storage of iron[6].

The overall effect is increased iron storage, particularly in the macrophages, and decreased availability of iron which ultimately leads to iron restricted erythropoiesis[34]. This can be assessed by laboratory markers.

Full blood count (FBC) and blood film

ACD varies in severity but patients typically present with mild (Hb >100g/L) or moderate (Hb 85-100g/L) reductions in haemoglobin concentrations[35]. Microscopically, the erythrocytes are usually normocytic and normochromic. Concurrent haematinic deficiencies, haemoglobinopathies and the underlying disease can all affect the red cell indices and blood film features, therefore the FBC alone is not sufficient in the diagnosis of ACD.

Iron Studies

Serum iron is reduced in ACD, reflecting the decreased availability of iron. Serum transferrin is typically normal or low, and its fall in acute inflammation is thought to be due to increased degradation[36]. Depending on transferrin concentration, TIBC can be low or normal. Transferrin saturation is typically low and is a reflection of the decreased serum iron. Serum ferritin is either normal or elevated, in part due to ferritin's role as an acute phase protein but also the net effect of diversion of the body's iron into this storage protein within the reticuloendothelial system in ACD. Table 1 summarises these changes.

Soluble transferrin receptor (sTfR)

As previously discussed, sTfR is not affected by inflammatory cytokines and therefore can be useful in differentiating between isolated ACD (in which the concentration would be normal) and ACD associated with true iron deficiency when sTfR would be elevated.

Zincprotoporphyrin (ZPP)

In patients with impaired iron supply for erythropoeisis, regardless of the cause, ZPP concentrations will rise. Therefore, ZPP concentrations rise in ACD and cannot be used to assess whether there is superimposed iron deficiency[37].

Hepcidin

Hepcidin plays a central role in the dysregulation of iron homeostasis seen in ACD. Hepcidin is usually be elevated in ACD, however the increase in production may be opposed by the effects of iron deficiency[38]. Therefore, concentration may be useful in distinguishing patients with pure ACD from those with superimposed iron deficiency. However, the long-term effects of hepcidin may be to induce iron deficiency and therefore its use in diagnosing ACD needs to be more carefully evaluated and standardised[39].

IRON OVERLOAD

Iron overload in the setting of anaemia is commonly iatrogenic (repeated red cell transfusion in patients with thalassaemia major for example). However, it is also a well-documented phenomenon in certain diseases such as non transfusion dependent thalassaemias and sideroblastic anaemia.

In iron overload, the capacity for transferrin to transport iron is exceeded; this results in an increase in non-transferrin-bound iron within the plasma, leading to direct oxidative damage to tissues and organs[40]. Iron accumulation in the parenchyma can lead to significant organ damage including liver cirrhosis, diabetes and myocardial damage; early diagnosis and treatment is particularly important for patients in whom iron overload is the main factor in limiting survival.

While it is beyond the remit of this review to describe the pathogenesis of iron overload in these conditions, the effect of iron overload on iron studies will be discussed.

Diagnosing Iron overload

Typically in iron overload, iron studies show elevated ferritin, serum iron and transferrin saturation; there is a decrease in both TIBC and transferrin. A raised transferrin saturation is often an early marker of iron overload; a saturation of >45% is highly suggestive of iron overload[19]. Table 1 summarises these changes.

While there is evidence that serum ferritin concentration correlates with the degree of parenchymal loading in organs such as the liver, its accuracy can be compounded by factors such as inflammation and the underlying disease process[41]. Determination of liver iron concentration through biopsy is a reliable indicator of total body iron stores in patients with thalassaemia major; however this procedure is invasive[42]. Non invasive techniques such as MRI T2* have been shown to quantify iron

in both the liver and myocardium; MRI can be useful in diagnosing iron overload and guiding response to treatment[43].

SUMMARY

Iron is an essential element required for growth and survival. Deficiency and dysregulation of iron homeostasis forms the basis of the two commonest causes of anaemia worldwide: iron deficiency anaemia and anaemia of chronic disease. Iron studies can be useful in the differentiation between the two disease processes and be used to guide diagnosis and treatment.

Table 1

	Iron Deficiency Anaemia	Iron deficiency and inflammation	Anaemia of chronic disease	Iron overload
Serum iron	Decreased	Decreased	Decreased	Increased
TIBC, Transferrin	Increased	Decreased/Normal	Decreased/Normal	Decreased
Transferrin saturation	Decreased	Decreased/Normal	Decreased	Increased
Serum ferritin	Decreased (Diagnostic if <15µg/L)	Normal (Usually <100µg/L)	Normal/Increased	Increased
sTfR	Increased	Increased	Normal	Decreased
ZPP	Increased	Increased	Increased	Decreased

REFERENCES

1. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med 2005*; **352**:1011-23
2. Ganz T. Systemic Iron Homeostasis. *Physiol Rev 2013*; **93**:1721-41
3. Illing AC, Shawki A, Cunningham CL, Mackenzie B. Substrate profile and metal-ion selectivity of human divalent metal-ion transporter-1. *J BiolChem 2012*; **287:**30485–96
4. McKie AT, Marciani P, Rolfs A, et al. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell 2000*; **5:**299–309
5. Gulec S, Anderson GJ, Collins JF. Mechanistic and regulatory aspects of intestinal iron absorption. *Am J Physiol Gastrointest Liver Physiol 2014*; **307**:G397-409
6. Muñoz M, García-Erce JA, Remacha AF. Disorders of iron metabolism. Part 1: molecular basis of iron homeostasis. *J Clin Pathol 2011*; **64**:281-6
7. Merle U, Theilig F, Fein E, et al. Localization of the iron-regulatory proteins hemojuvelin and transferrin receptor 2 to the basolateral membrane domain of hepatocytes. *Histochem Cell Biol 2007*; **127**:221-6
8. Waldvogel-Abramowskia S, Waeber G, Gassner C, et al. Physiology of Iron Metabolism. *Transfus Med Hemother 2014*; **41**:213-21
9. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science 2004*; **306**:2090–3

10. Cohen LA, Gutierrez L, Weiss A, et al. Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. *Blood 2010*; **116:**1574–84
11. Gibson R. *Principles of nutritional assessment*. 2nd ed. Oxford: Oxford University Press; 2005
12. Loría A, Hershko C, Konijn AM. Serum Ferritin in an Elderly Population. *J Gerontol 1979*; **34**:521-524
13. WHO. *Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity.* Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization, 2011 (WHO/NMH/NHD/MNM/11.1) (http://www.who.int/vmnis/indicators/haemoglobin.pdf, accessed [27th May 2016])
14. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med 1999*; **340**:448-54
15. Takami T, Sakaida I. Iron regulation by hepatocytes and free radicals. *J Clin Biochem Nutr 2011*; **48**:103–6
16. Dale JC, Burritt MF, Zinsmeister AR. Diurnal Variation of Serum Iron, Iron-Binding Capacity, Transferrin Saturation, and Ferritin Levels. *Am J Clin Pathol 2002*; **117**:802-9
17. Ramsay WN. The measurement of serum transferrin by iron-binding capacity. *J Clin Pathol 1973*; **26**:691–6
18. World Health Organization, Centers for Disease Control and Prevention. *Assessing the iron status of populations*. 2nd ed. 2007. ISBN: 978 92 4 1596107 (electronic version) (http://apps.who.int/iris/bitstream/10665/75368/1/9789241596107_eng.pdf?ua=1, accessed [27th May 2016])
19. van Bokhoven MA, van Deursen CT, Swinkels DW. Diagnosis and management of hereditary haemochromatosis. *BMJ 2011*; **342**:c7251
20. Bain, B. *Blood Cells: A Practical Guide*. 4th edn. Blackwell Oxford; 2006
21. Ganz T, Nemeth E. Hepcidin and Iron Homeostasis. *Biochim Biophys Acta 2012*; **1823**:1434–43
22. Guyatt GH, Oxman AD, Ali M, Willan A, McIlroy W, Patterson C. Laboratory diagnosis of iron deficiency anaemia: an overview. *J Gen Intern Med 1992*; **7**:145-53
23. Goddard AF, James MW, McIntyre AS, Scott BB. Guidelines for the Management of Iron Deficiency Anaemia. *Gut 2011*; **60**:1309-16
24. Cook JD, Skikne BS, Baynes RD. Serum transferrin receptor. *Annu Rev Med 1993*; **44**:63–74.
25. Feelders R. Structure, function and clinical significance of transferrin receptors. *Clin Chem Lab Med 1999*; **37**:1–10
26. Baillie FJ, Morrison AE, Fergus I. Soluble transferrin receptor: a discriminating assay for iron deficiency. *Clin Lab Haematol 2003*; **25**:353-7
27. WHO. *Serum transferrin receptor levels for the assessment of iron status and iron deficiency in populations.* Vitamin and Mineral Nutrition Information System. Geneva: World Health Organization; 2014 (WHO/NMH/NHD/MNM/14.6; http://apps.who.int/iris/bitstream/10665/133707/1/WHO_NMH_NHD_EPG_14.6_eng.pdf?ua=1, accessed [27th May 2016])
28. Infusino I, Braga F, Dolci A, Panteghini M. Soluble transferrin receptor (sTfR) and sTfR/log ferritin index for the diagnosis of iron-deficiency anemia. A meta-analysis. *Am J Clin Pathol 2012*; **138**:642-9
29. Cartwright GE, Wintrobe MM. Anemia of infection. *Adv Intern Med 1962*; **5**:165-226
30. Fitzsimons EJ. The anaemia of chronic disease. *BMJ 2001*; **322**:811–2

31. Eschbach JW. Anemia management in chronic kidney disease: role of factors affecting epoetin responsiveness. *J Am Soc Nephrol 2002*; **13**:1412-4
32. de Francisco AL, Stenvinkel P, Vaulont S. Inflammation and its impact on anaemia in chronic kidney disease: from haemoglobin variability to hyporesponsiveness. *NDT Plus 2009*; **2(Suppl_1)**: i18–i26
33. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood 2003*; **101**:2461-63
34. Gangat N, Wolanskyj AP. Erratum: "Anemia of Chronic Disease". *Semin Hematol 2013*; **50**:232-238
35. Zarychanski R, Houston DS. Anemia of chronic disease: A harmful disorder or an adaptive, beneficial response? *CMAJ 2008*; **179**:333-7
36. O'Shea MJ, Kershenobich D, Tavil lAS. Effects of Inflammation on Iron and Transferrin Metabolism. *Br J Haematol 1973*; **25**:707-14
37. Hastka J, Lasserre JJ, Schwarzbeck A, Strauch M, Hehlmann R. Zinc protoporphyrin in anemia of chronic disorders. *Blood 1993*; **81**:1200–4
38. Cullis JO. Diagnosis and management of anaemia of chronic disease : current status. Br J Haematol 2011; **154**:289-300
39. Goodnough LT. The new age of iron: evaluation and management of iron-restricted erythropoiesis. *Sem Hematol 2009*; **46**:325-7
40. Walter PB, Fung EB, Killilea DW, et al. Oxidative stress and inflammation in iron-overloaded patients with beta-thalassaemia or sickle cell disease. *Br J Haematol 2006*; **135**:254–63
41. Nielsen P, Günther U, Dürken M, Fischer R, Düllmann J. Serum ferritin iron in iron overload and liver damage: correlation to body iron stores and diagnostic relevance. *J Lab Clin Med 2000*; **135**:413-8
42. St Pierre TG, Clark PR, Chua-anusorn W, et al. Noninvasive measurement and imaging of liver iron concentrations using proton magnetic resonance. *Blood 2005*; **105**:855-61
43. Cazzola M, Della Porta MG, Malcovati L. Clinical Relevance of Anemia and Transfusion Iron Overload in Myelodysplastic Syndromes. *Hematology Am Soc Hematol Educ Program 2008*; **2008**:166-75