Review

Erin S. Grant*, Danielle B. Clucas, Gawain McColl, Liam T. Hall and David A. Simpson*

Re-examining ferritin-bound iron: current and developing clinical tools

https://doi.org/10.1515/cclm-2020-1095
Received July 16, 2020; accepted October 1, 2020; published online October 22, 2020

Abstract: Iron is a highly important metal ion cofactor within the human body, necessary for haemoglobin synthesis, and required by a wide range of enzymes for essential metabolic processes. Iron deficiency and overload both pose significant health concerns and are relatively common worldwide health hazards. Effective measurement of total iron stores is a primary tool for both identifying abnormal iron levels and tracking changes in clinical settings. Population based data is also essential for tracking nutritional trends. This review article provides an overview of the strengths and limitations associated with current techniques for diagnosing iron status, which sets a basis to discuss the potential of a new serum marker – ferritin-bound iron – and the improvement it could offer to iron assessment.

Keywords: diagnostics; ferritin; ferritin-bound iron; iron; pathology.

Introduction

Iron is a critical metal within the body, used for oxygen transport by haemoglobin and myoglobin, as well as acting as a cofactor for numerous essential enzymes, including those involved in mitochondrial energy production [1–4]. The average person houses between 3 and 5 g of iron, most of which (65–75%) is found in the haemoglobin of erythrocytes [3]. The rest resides primarily in the tissues – mostly the liver, spleen and muscles – but approximately 0.1% can be found in blood serum [5]. Regulation of iron levels is notable in that it lacks an active excretion mechanism. Rather, in a healthy individual, losses are incidental and occur due to processes such as enteric desquamation, menstruation, or through the skin [1]. They can also be pathological, for example, in the case of gastrointestinal bleeding. In healthy adult males and post-menopausal females typically only 1–2 mg of iron is lost daily, and this is replenished by dietary absorption. Therefore, most iron used in biological processes comes from recycling of iron from senescent red blood cells [3].

Interruption of the fine balance between iron absorption, usage and loss results in the pathologic states of either iron deficiency (ID) or overload. These are both relatively common conditions which may be diagnosed incidentally or present symptomatically, and treatment of both is important for maintaining health and quality of life.

The gold standards for identifying abnormal iron levels are bone marrow aspirate in cases of ID, and tissue biopsy of the liver or heart in cases of overload. However, these techniques are not desirable in either clinical or research settings because of their invasive nature and the difficulty in obtaining an ideal sample [6–10]. Therefore, they have primarily been used as reference techniques to establish the validity and specificity of more appropriate methods and may be used in complex diagnostic cases where more conventional measures do not return a clear result.

The biomarkers and methods used to assess abnormal iron status depend on whether the investigation is initiated for clinical diagnosis of an individual, or for research purposes such as in a nutritional survey, as well as whether deficiency or overload is under investigation. However, in all cases, assessment will involve measurement of biomarkers present in peripheral blood, as presented in Figure 1. In clinical settings, these biomarkers are commonly serum iron, serum ferritin, serum transferrin and transferrin saturation [11], and red cell indices [12]. Additional
measurements including soluble transferrin receptor, inflammatory markers such as α-1-acid glycoprotein (AGP) and C-reactive protein (CRP), and hepcidin, may also be done, to clarify the aetiology of an abnormal value of one of the main biomarkers [13]. Clinical evaluation of iron overload also often involves magnetic resonance imaging to determine the burden on overloaded tissues, including the liver, heart and brain, and to track treatment progress. Therefore, determination of both deficiency and overload, whether in a clinical or research setting, is conducted using analysis of multiple indicators as highlighted by Pfeiffer and Looker [14]. In this review, we provide a brief overview of the biomarkers used in this multivariate approach and look to the potential improvements offered by additional markers. This provides a basis to discuss another novel biomarker – ferritin-bound iron – as a further enhancement to the multivariate approach to tracking iron status.

**Current and prospective biomarkers – role in iron homoeostasis**

Without an active excretion mechanism, introduction of iron into the body and movement between the three major compartments (active use, transit and storage) is tightly controlled. Absorption, transport, recycling and storage are mediated by a complex combination of receptors, hormones, transport proteins and storage proteins which function together to ensure that the availability of iron for active use remains sufficient for requirements. The biomarkers used in both clinical and research settings are chosen for their essential roles in this homoeostatic iron cycle and have been explored in detail in many reviews [1, 5, 15, 16]. For our purposes, only aspects relevant to the assessment of iron status will be discussed here.

Iron absorption from the gut, and mobilisation out of the storage compartment is initiated and suppressed by the peptide hormone hepcidin [3]. Hepcidin expression, which is regulated by hepatocellular iron stores, erythropoietic signals, hypoxia, and inflammation, results in less iron absorption from the gut and reduced release of stored iron [5, 17].

Iron transport includes delivery to facilitate erythropoiesis and other iron-related processes, as well as conveyance to and from storage in tissues [1]. Plasma iron transport is performed by the protein transferrin (Tf) which is capable of binding two iron ions. However, this capacity is only filled under severe overload or iron toxicity, with usual saturation below 50% [18]. The iron that is bound to Tf is known as serum iron, and when combined with a measurement of the Tf concentration (or total iron-binding capacity (TIBC)), the Tf saturation (TSAT) can be determined.

Iron in the serum may also be present in severe cases of toxicity or overload in a “free” form bound only to small ligands [19]. Collectively this iron pool is termed non-transferrin bound iron (NTBI) and it may include iron bound to citrate, acetate and albumin, however, the exact makeup of its forms likely differs depending on the aetiology of its existence [20]. This is because NTBI is not usually present in serum and only forms when iron is in excess, as in iron overload pathologies. A subset of NTBI, termed “labile iron” interacts in redox cycling, making it potentially the most detrimental form, however, labile NTBI does not appear to be present in all overload pathologies [21].

Internalisation of the iron delivered to cells is mediated by binding of Tf to cellular transferrin receptor (TfR) [3]. After generation of the TfR-Tf complex, transit across the cell membrane occurs via endocytosis. Iron is then released under low pH and the Tf is returned to the extracellular environment. Expression of the cellular receptor increases when iron stores are low and erythropoiesis is iron-limited [18]. A cleaved and truncated version of cellular TfR, which is soluble (sTfR), is present in the serum and reflects cellular levels during ID [22]. It is also dependent on erythropoietic activity, rising with increased erythropoiesis, such as during thalassaemia major or intermedia, and falling with lower rates, for example with hypertransfusion and chronic renal failure [23].

**Figure 1: An overview of the clinical analyses used to evaluate iron status and the compartments from which these samples are taken. They are divided into direct measures of iron in the tissues, and biomarkers taken from a blood test. Both current and new/potential biomarkers are included and are discussed in further detail in the text.**
A normal iron status requires a reserve of storage iron which can be drawn on when iron demands increase, such as after blood loss. This means that the storage compartment is preferentially affected by deficiency or overload in an attempt to limit consequences on iron in active use. Iron is stored in tissues inside the cage-like protein ferritin, which protects the intracellular environment from oxidative stress posed by “free” iron [24]. Although located primarily within tissues, there is also a small amount of ferritin present in serum which reflects tissue iron stores during otherwise normal functioning.

**Multivariate approach to determining iron status**

Current analysis of abnormal iron levels involves consideration of multiple biomarkers depending on whether a deficiency or overload is being evaluated [14].

**Deficiency**

Investigation for iron deficiency is often initiated by the presentation of symptoms such as fatigue but may also be performed after identification of another abnormality such as a low haemoglobin, or if red cell indices suggest iron deficiency. A low mean cell volume (MCV) and mean cell haemoglobin (MCH) and an elevated red cell distribution width (RDW) are the typical findings in iron deficiency [25]. Abnormal red cell morphology, such as microcytic hypochromic red cells or elongated cells, on a blood film may also point to a diagnosis of iron deficiency [26]. In clinical diagnostic settings iron deficiency is typically confirmed using a panel of markers usually comprising biochemical measures including serum ferritin (SF), serum iron, transferrin concentration and TIBC or TSAT [11].

The biochemical measures are more sensitive and specific for ID, with the majority of analyses containing a measure of SF concentration. SF has been shown to correlate well with iron stores in cases of simple ID, as confirmed by bone marrow aspirate measurements [27–31]. However, despite this, the commonly used cutoff value of 15 μg/L (in adults) has been noted to be specific but non-sensitive for ID [32, 33]. Additionally, the literature contains a variety of other cutoffs, provided by different expert groups and individual laboratories [33], with one review finding cutoff values between 12 and 200 μg/L for ID [34]. These difficulties have gained much interest in recent years, resulting in re-evaluation of the evidence to support current cutoffs provided by the World Health Organisation (WHO) [35]. This review found that WHO cutoffs appeared valid for healthy populations, but more data is needed for specific age groups and individuals with particular concurrent pathologies. This is highlighted in the recent update to WHO cutoff values, provided in Table 1, which identified the strength of evidence for these values to be of “low certainty” [36]. Nevertheless, SF remains the most important biomarker of ID, and in combination with a low Hb value is indicative of ID anaemia (IDA) [32, 37]. Measures related to transferrin, such as TIBC and TSAT, are also often included in the evaluation of iron status and may offer further insight into the stage of a deficiency. For example, a TSAT below 25% can be indicative of low iron stores [11]. However, TIBC and TSAT require measurement of serum iron for their calculation. Serum iron levels are subject to diurnal variation and fluctuations with oral intake [38], and themselves have no role in the diagnosis of iron deficiency. As a consequence, values derived from serum iron measurements (TIBC, TSAT) are also subject to these limitations, are poorly specific, and should not be interpreted alone [11].

**Difficulties in assessing ID**

Assessment of ID has reached a fairly advanced stage by combining multiple biomarkers to account for the fact that none can provide a full picture of the homoeostatic iron

<table>
<thead>
<tr>
<th>Serum ferritin, μg/L</th>
<th>Iron deficiency</th>
<th>Risk of iron overload</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent healthy</td>
<td>Concurrent infection/inflammation</td>
<td>Apparent healthy</td>
</tr>
<tr>
<td>Infants and young children (0–5 years)</td>
<td>&lt;12</td>
<td>&lt;30</td>
</tr>
<tr>
<td>&gt;5 years</td>
<td>&lt;15</td>
<td>&lt;70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant women</td>
<td>&lt;15</td>
<td>–</td>
</tr>
</tbody>
</table>
cycle if measured in isolation. Despite this, there are still situations in which it is hard to reach a precise conclusion.

The most pervasive confounder of accurate identification of ID is the presence of an acute or chronic infection or inflammatory state. During the body’s response to infection, inflammation, or malignancy, iron availability is limited to deprive pathogens [15, 39]. Reduction in available iron is initiated by upregulation of hepcidin [15], which has a flow-on effect that results in changes to many of the biomarkers used to assess iron status, including low serum iron, and TIBC. Additionally, SF is an acute phase protein, increased in the acute phase response to infection or inflammation as well as in liver disease, without representing an increase in iron stores or liver iron respectively [40]. Of these, the change to SF is the most significant for assessment purposes, providing a common source of difficulty for measuring iron status in this setting.

These difficulties are exemplified in a systematic review by Garcia-Casal and colleagues [35] where the correlation between SF and stainable bone marrow was compared between healthy individuals or those who had an inflammatory condition. In patients, SF levels varied widely, however, the mean ferritin concentration of patients with inflammatory conditions who were iron-deficient based on bone marrow assessment was 82.43 μg/L, well within the usual “normal” reference range, compared to ID in otherwise healthy individuals which presented with a mean serum ferritin of 15.1 μg/L.

To account for these challenges, serum ferritin values must be interpreted in the context of patients’ comorbidities and clinical states. Guidelines have been published by a number of medical associations regarding the interpretation of ferritin values in certain disease groups. For example, in chronic heart failure a trial of intravenous iron supplementation is recommended in symptomatic patients with SF<100 μg/L or between 100 and 300 μg/L with a TSAT<20% [41, 42], while in adult patients with chronic kidney disease and anaemia, a trial of iron supplementation is suggested for patients SF≤500 μg/L and TSAT≤30% [43]. In patients with malignancy, The European Society for Medical Oncology defines absolute iron deficiency as SF<100 μg/L and functional iron deficiency as SF>100 μg/L and TSAT<20% [44]. Finally, in the elderly, concomitant disease is highly prevalent and SF tends to increase with age, regardless of inflammatory comorbidity. These factors make diagnosis of iron deficiency in the elderly especially difficult. SF cut-offs of less than 45–100 μg/L have been recommended [45].

In recent years both the clinical and research spaces have sort to meet these challenges, improving assessment of iron status during inflammation and infection by use of additional biomarkers such as stTR, as well as biomarkers of inflammation. The two most common inflammatory markers are CRP and AGP, however others include α1-antichymotrypsin (ACT), haptoglobin, and fibrinogen [46–49].

Biomarkers of inflammation are extremely important for identifying the potential aetiology of elevated SF. This was evidenced in a study comprising infants, children and adults which measured SF during the incubation, early convalescence and late convalescence periods of inflammation, as determined by measures of CRP and AGP [50]. This study found increases in SF of approximately 30, 90 and 36% in each stage respectively, providing a means for linking high SF to an inflammatory state.

The use of stTR as an additional biomarker is supported by research showing that when combined with SF it provides a better measure of iron status [51]. This is because stTR is not affected by inflammation in the way ferritin is; stTR is increased in iron deficiency with or without inflammation, but is normal in anaemia of chronic disease (ACD) [52]. This makes it useful, especially when combined with SF, for diagnosing ID in the setting of inflammation, and for distinguishing IDA from ACD [53–55]. However, the fact that stTR is also influenced by erythropoietic activity makes it, like the other biomarkers, unsuitable in isolation, and care must be taken to account for concurrent conditions which result in erythroid hypo- or hyperplasia [23, 56].

Additionally, combining stTR with SF in the body iron model, as implemented in the National Health and Nutrition Evaluation Survey (NHANES) of the United States [13], provides a quantitative measure of the storage iron within the body in units of mg of iron/kg of body weight [57]. Unlike SF, stTR continues to respond in accordance with the extent of a deficiency [52]. A negative value using the body iron index indicates the deficit to be recovered before iron accumulates in the body’s stores, thereby demonstrating the degree of ID.

In clinical settings, an individual’s concurrent pathologies can be assessed in concert with their iron status. Therefore, inflammatory markers such as CRP can be considered within the nuanced context of the individual and consideration of these values is now included in WHO cutoff ranges [36]. Additionally, the availability and usage of stTR is growing in the clinical space, allowing the effects of patient pathology to be assessed in more detail. For example, stTR in combination with hepcidin (discussed below) has been used to identify iron deficiency in patients after acute heart failure [58].

In research settings interpretation of SF, particularly in settings where inflammatory conditions are highly
prevalent, is complex and nuanced approaches used in the clinical care of individual patients are not feasible at the population level. Use of additional biomarkers and/or adjustment of SF for the presence of inflammation is required to provide a more accurate account of ID prevalence estimates. Approaches based on incorporation of both inflammatory markers and sTfR have been studied in detail, notably by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia (BRINDA) project [59, 60]. One study from this project investigated the best practice from four possible approaches [60]: increasing the SF cutoffs in populations known to be high in inflammatory conditions, excluding individuals based on CRP and AGP measures, applying arithmetic correction factors, and use of regression correction. This study determined that regression correction was the most reliable method of adjustment, although more research is needed to corroborate the finding. Another study conducted as part of the BRINDA project looked at similar adjustment methods, this time applied to the body iron model [59]. Again, the regression correction method was found to be the most useful.

Although sTfR and inflammatory markers have been the focus of much research, there is still no consensus on exactly how to implement them. For example, there are at least three derived indicators based on sTfR and SF. These are a simple ratio (sTfR/SF) [61], the ratio of sTfR to the log of SF [62], or the body iron index [57]. Additionally, as sTfR concentration is influenced by erythropoietic rate as well as iron status [22, 63], these ratios/indices are subject to the same difficulty from confounding pathologies as sTfR alone.

Although there have been significant advances towards the development of a precise identification process for iron deficiency, unconfounded by concurrent inflammation, there undoubtedly remains room for improvement. One avenue is the identification of new biomarkers which could clarify the presence of iron deficiency in hard to diagnose cases [13].

**Overload**

The same panel of tests may be performed in cases of iron overload to initiate further investigations or track the progress of treatment. Assessment may be triggered by symptoms such as fatigue or weakness but iron overload is often asymptomatic during the early stage [11]. More commonly, knowledge of familial predisposition in the case of genetic haemochromatosis [64], or sustained therapeutic transfusions to treat haemoglobinopathies [65], will lead to testing. Iron overload is indicated by a high SF, combined with a high TSAT (above 50%) [11]. However, in some rare iron-overload disorders, such as ferroportin disease or aceruloplasminemia, TSAT may be normal or low [66]. Once iron overload has been identified by further investigations, the progress of treatment, either by phlebotomy or iron chelation, can be tracked by measuring SF regularly [64, 65]. Researchers looking at overload trends at the population level also use SF cut-offs as the biomarker of choice [13].

While SF and the other biomarkers are used for identifying individuals at risk of overload and tracking treatment, they are indirect measures and are often regarded as secondary compared to magnetic resonance imaging (MRI) which can directly quantify and map organ-specific iron accumulation in vivo. MRI protocols have been developed for hepatic [67], myocardial [68, 69], and neurological iron [70], which are also commonly used to identify structural changes to the imaged tissue during disease. This allows simultaneous identification of iron accumulation and functional deterioration, especially in the myocardium. The advantages of MRI have seen it widely implemented in clinical settings to both diagnose and track the progress of iron overload disorders.

**Difficulties in assessing iron overload**

As in ID diagnosis, elevation of SF which is unrepresentative of an increase in iron stores, such as is seen with infection, inflammation, liver disease and obesity, complicate the diagnosis of iron overload. While this may cause cases of iron deficiency to be missed, it can also lead to over investigation of iron overload if used as a sole marker. This was demonstrated by McKinnon et al. [71], who found that mean SF values increased over a 10 year span between 1995 and 2005 in a population of Australian adults. This occurred in correlation with pathologies such as fatty liver disease, metabolic syndrome and simple obesity and translated to 31% of men having a SF above the standard upper cut-off for iron overload, compared to 23% in 1995. Correspondingly, more patients are being referred for investigation of potential iron overload.

MRI protocols for assessing iron accumulation are also continually being improved to address limitations. For example, a full theoretical description of how iron impacts the MRI signal has not yet been developed [67], which necessitates experimental calibration, requiring large sample sizes. Additionally, the calibration method varies with the type of protocol used and the physical specifications of the MRI machine.
Further to these technical challenges, practical limitations of MRI machines include their expense, large size, and technological sophistication, making them unsuited to resource-poor settings. They may also be poorly tolerated by some patients, with acquisition times requiring individuals to be immobile from as little as a single breath-hold up to 20 min [67].

Finally, an important area for iron accumulation appears to be the brain, where it may play a role in some neurodegenerative diseases as well as being extremely important for proper neural development [72, 73]. Currently, the only way to detect brain iron is through MRI, which has been used to study iron accumulation in Parkinson's disease and other patients for research purposes [74–77]. However, MRI does not have sufficient specificity or resolution for application to brain iron deficiency. In the case of neural development the current approach to identifying brain iron deficiency is to infer it through the presence of both behavioural changes and evidence of IDA [13].

The limitations associated with MRI and biomarkers used for clinical evaluation of iron overload mean that identification of additional biomarkers is also important. One potential biomarker for iron overload is NTBI, with the most obvious application being to track the progress of chelation therapy [21, 78, 79]. However, MRI does not have sufficient specificity or resolution for application to brain iron deficiency. In the case of neural development the current approach to identifying brain iron deficiency is to infer it through the presence of both behavioural changes and evidence of IDA [13].

The limitations associated with MRI and biomarkers used for clinical evaluation of iron overload mean that identification of additional biomarkers is also important. One potential biomarker for iron overload is NTBI, with the most obvious application being to track the progress of chelation therapy [21, 78, 79]. However, significant differences between assay protocols and the need for further standardisation and validation of these assays means that at present the utility of NTBI is still unclear [13, 80, 81]. More work must be done for it to become a clinical biomarker.

Therefore, similarly to ID, there is a need for the identification if new biomarkers which would improve the accuracy and specificity of diagnosis and monitoring of iron overload.

Hepcidin

Hepcidin is currently being studied to determine its use as a new biomarker of iron status, which is motivated by its importance in iron homoeostasis. Abnormal levels of hepcidin have been observed in iron-related pathologies, for example, low expression is seen in some overload disorders [82] and IDA, while increased expression occurs with inflammation and the rare genetic condition iron-refractory iron deficiency anaemia (IRIDA) [83]. These changes may mean that hepcidin measurement can improve clinical differentiation of IDA from ACD, diagnosis of IRIDA, and evaluation of some iron overload pathologies [84, 85].

However, creation of a simple test for hepcidin has been mitigated somewhat by consideration of its number of isoforms, only one of which, hepcidin-25, appears to be biologically active [5]. It can also be bound to α-2-macroglobulin and albumin, but exactly how much hepcidin is attached to these carrier molecules remains unclear, as well as whether they affect hepcidin function [84, 85]. Both details must be accounted for in the development of a clinical test.

Initially, hepcidin detection was conducted using mass spectrometry, however, immunoassays have become available in recent years [86–89]. Isoform specificity [87], adhesion to laboratory plastics [88], and difficulties in antibody generation [87, 90], have been barriers for hepcidin immunoassay development.

Current laboratory performance of both mass spectrometry and immunoassay tests for hepcidin is quite varied, as assessed in two international round-robins [91, 92]. Additionally, there have been two attempts at development of a standard reference material. Although the first attempt, a synthetic hepcidin [92], was found to be non-commutable with the native form, a more recent reference material generated and tested by Diepeveen and colleagues [93], increased equivalence between methods. However, between-laboratory variation was still less than ideal. Finally, while studies to develop reference ranges based on healthy populations have been conducted [94, 95], further work is needed to clarify the impact of factors other than iron status on plasma hepcidin levels. These challenges mean that hepcidin is currently not considered a standard biomarker of iron status and further work must be conducted to see it more widely used.

Ferritin-bound iron: a prospective marker

Future improvements iron status assessment will likely turn to the identification of new biomarkers. Here, we consider one such potential biomarker: ferritin-bound iron (FBI). Despite the difficulties with SF, it remains the fundamental biomarker in diagnostic iron assessment. Therefore, it is worth considering whether current evidence suggests that further focus be given to FBI as a way to enhance the information that can be collected from SF, improving its diagnostic utility.

Ferritin-bound iron: current knowledge

Structurally, ferritin is roughly spherical and cage-like, with a generally accepted maximum iron storage capacity of ~4000/4500 iron atoms [16, 96], as represented in
Figure 2. A representation of the continuum of iron loading in ferritin.

The cage-like structure of ferritin gives it the ability to store between 0 and 4000/4500 iron atoms in a crystalline core. The core has a magnetisation (M) which flips direction due to thermal energy. The magnetic signature is proportional to the number of iron atoms present, providing an avenue for direct sensing of ferritin-bound iron, non-destructively, in situ.

Figure 3: Plot of the ferritin-bound iron (FBI) content of samples representing the entire range of possible iron statuses. A difference can be seen between the overloaded, inflammation, and positive balance cases, as compared to normal or iron depleted/deficient cases. FBI was measured by a destructive method wherein ferritin was digested in 0.2 mL of HNO₃ at 75 °C, followed by quantitative iron analysis via atomic absorption spectroscopy. Reproduced from [100].

FBI across patients who were iron deficient, iron replete and iron overloaded as shown in Figure 3 [100]. Interestingly they found that a FBI saturation >15% excluded inflammation as the sole reason for elevated SF.

Additionally, studies which looked at healthy individuals did not find a consistent FBI saturation. Work by ten Kate et al. found SF from healthy individuals was 24.3% filled, compared to 19.3% in patients with an acute phase response [104], while Herbert et al. found the opposite relationship between FBI in healthy individuals and those with an inflammatory response [100]. Further work by ten Kate et al. found healthy controls to have an average FBI saturation of 17.8% compared to 9.1% in patients with adult onset Still’s disease [102].

When combined, the large variation between FBI measurements across all these studies does not provide a conclusive picture of its clinical utility. Further work needs to be done with larger cohorts and additional comparison to healthy controls is necessary to establish normal levels. Cross-referencing with bone marrow iron, liver iron and other markers of iron status in serum, is also important. At present, the true utility of FBI for providing clinical, or research-specific information is unclear and more work, which can offer clarity, is needed.

Ferritin-bound iron measurement techniques

The relatively limited data surrounding FBI is linked to technical inadequacies; currently, there is no robust,
simple method which can measure FBI in situ, (current and prospective methodologies are summarised in Table 2). Instead, the majority of extant measurements come from a two-step process, first requiring purification and measurement of ferritin concentration, followed by liberation of the iron core which can be assessed by a variety of methods, with the most common being atomic absorption spectroscopy (AAS) [100–102, 104, 105]. The need for a two-step measurement protocol introduces an opportunity for loss of the Fe atoms, as well as contamination from other sources, such as iron from haem [106]. Additionally, AAS has a detection limit above much of the physiological range, with one study reporting the requirement of an initial sample with a ferritin concentration of >200 μg/L to adequately measure its corresponding iron content [101]. These difficulties have restricted AAS analysis of FBI and prevent it from being a practical protocol in clinical settings.

True assessment of the utility of FBI requires development of more reliable, sensitive and practical technologies, which remove the difficulty of contamination and loss that may be introduced during a separation step. One recent publication attempts to mitigate this, using species-specific isotope dilution mass spectrometry (SS-IDMS) [110]. In SS-IDMS a sample of apoferritin is spiked with a known isotopic spike of ferritin in the same ratio, reducing overall error. Mass spectrometry of the iron after the purification process then provides a ratio of isotopic Fe to sample Fe, which can be used to determine the ferritin saturation [114]. The necessity of the isotopic spike in this technique limits its clinical practicality, however, it benefits from a low limit of detection (18 ng of Fe in total) making it a good option for standardisation of clinically viable techniques.

Future methods which can successfully analyse FBI under clinically relevant conditions, should ideally be direct measurements of the iron core in situ. To achieve such a measurement with the necessary sensitivity, a magnetic-based detection scheme seems likely. Aggregation of Fe3+ ions in the ferritin core is, in simplest terms, superparamagnetic. This means that unless completely devoid of iron, each ferritin molecule generates a fluctuating magnetic field, whose frequency spectrum is correlated with its thermal energy [115]. At a specific temperature, known as the blocking temperature, dependent on the number of iron atoms in the core, the magnetisation becomes static. Ferritin has a blocking temperature of ~40 K when ~50% loaded [116]. The magnetic nature of the core provides a means of measuring the iron content of the protein without the need for an initial separation step.

Knowledge of the magnetic behaviour and morphology of the iron core of ferritin has been investigated using highly sensitive methods such as superconducting quantum

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity</th>
<th>Sample size</th>
<th>Clinical applicability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destructive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colourimetry</td>
<td>15 μg Fe/L [98]</td>
<td>2 mL serum [98]</td>
<td>Limited</td>
</tr>
<tr>
<td>AAS</td>
<td>15 μg Fe/L requires</td>
<td>300 μL serum [101]</td>
<td>Limited</td>
</tr>
<tr>
<td></td>
<td>200 μg SF/L [101]</td>
<td>20 μL [104]</td>
<td>Challenges – loss and contamination</td>
</tr>
<tr>
<td></td>
<td>100 μg SF/L with 20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-destructive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>5–15 g/L ferritin [107]</td>
<td>Not specified</td>
<td>Limited</td>
</tr>
<tr>
<td>Mössbauer spectroscopy</td>
<td>100 mg/mL [108]</td>
<td>Not specified</td>
<td>High protein concentration</td>
</tr>
<tr>
<td>SQUID</td>
<td>100 mg/mL ferritin</td>
<td>Not specified</td>
<td>Limited</td>
</tr>
<tr>
<td></td>
<td>[109]</td>
<td></td>
<td>High protein concentration</td>
</tr>
<tr>
<td>SS IDMS</td>
<td>18 ng Fe in total [110]</td>
<td>12 mL serum [110]</td>
<td>Limited</td>
</tr>
<tr>
<td>AFM</td>
<td>Individual proteins</td>
<td>1 ng/L ferritin [111]</td>
<td>Low throughput and isotopic spike required</td>
</tr>
<tr>
<td>Diamond quantum sensing</td>
<td>A single protein [112]</td>
<td>~1 mg/L ferritin [113]</td>
<td>Low throughput and scalability</td>
</tr>
</tbody>
</table>

AFM, atomic force microscopy; AAS, atomic absorption spectroscopy; NMR, nuclear magnetic relaxometry; SQUID, superconducting quantum interference device.
interference device (SQUID) magnetometers [109, 117], Mössbauer spectroscopy [108], and nuclear magnetic relaxometry (NMR) [107]. While these techniques could be used to determine the iron content of ferritin, they are specialised and have relatively poor sensitivity, making them unsuitable for translation to a clinical test. For example, SQUID magnetometers require cryogenic operating temperatures and high serum ferritin concentrations (e.g., 100 mg/mL [109]). Likewise, NMR requires protein concentrations of 5–15 mg/mL, while Mössbauer spectroscopy also needs a frozen sample and requires ferritin of approximately 100 mg/mL [108]. These protein concentrations are all several orders of magnitude larger than the physiological range of SF.

There are currently two simpler techniques under development, which also detect the iron core via its magnetic signature. The first of these is atomic force microscopy (AFM).

Conventional AFM utilises Van Der Waals forces between a scanning probe or cantilever and a surface of interest, to chart surface topography. Two modifications to the basic function of AFM allow it to distinguish between apo and holoferritin, these are bimodal phase imaging and magnetic force microscopy (MFM) [111, 118]. To date, decent headway has been made into the application of both AFM and MFM to ferritin detection. Beginning with initial proof-of-concept measurements using AFM, to demonstrate the detection of individual ferritin molecules adsorbed to a surface [119], MFM and bimodal phase imaging have since been used to distinguish between individual apo- and holoferritin, shown in Figure 4 [118]. Detection has also been demonstrated in both air and liquid [118].

One of the main strengths of AFM, is that it requires small sample volumes, of less than 50 µL, enabling the precise evaluation of a small number of particles with up to 5 nm resolution [111, 118]. However, this is also a limitation in terms of diagnostic applicability, as it cannot be used to generate statistically significant averaging of particles in a serum sample. Therefore, it seems likely that AFM-based techniques are more applicable to research settings, with the potential to provide highly specific spatial, and magnetic information.

The second potential measurement technique arises out of the rapid development of ultrasensitive magnetometers based on the nitrogen-vacancy (NV) defect in diamond. The defect – a disruption to the regular crystal lattice of diamond – occurs naturally but can be engineered for sensing purposes. It is useful because it can be optically excited, with the resulting fluorescence intensity correlated with local magnetic noise. A theoretical foundation for this method was first proposed some ten years ago [120], and early experimental work has shown that ferritin can be detected by individual nanodiamonds [112], as well as a bulk diamond chip [121]. It has also been combined with AFM to detect ferritin aggregates inside a cell [122].

The NV defect is an attractive system to provide sensing of FBI because unlike AFM, it has the potential to sense diagnostically relevant numbers of ferritin, generating statistically significant averages. It could also be implemented in a clinical setting with throughput times, pricing, and interfacing, equivalent to that of conventional immunoassays of SF.

Continued work to provide a clinically applicable measurement protocol for FBI in situ could remove the
ambiguity inherent in current SF analysis and provide a path for robust assessment of the true utility of FBI in the diagnosis of iron status.

Conclusions

Current assessment of iron status for clinical diagnosis and population surveys depends on a multivariate approach which combines measurement of multiple biomarkers involved in the regulation and mobilisation of iron within the body. These biomarkers, which are essentially indirect correlates of storage iron, may be supplemented with direct techniques, most notably MRI. Although adequate in conventional ID and overload, diagnostic cases involving complicated anaemia and concurrent pathologies, or population surveys taking place in settings with high rates of infectious disease or obesity, are instances in which the accurate assessment of iron status is still difficult. Among approaches to improve assessment in these circumstances, such as using regression modelling and correction factors, the identification of new biomarkers which can offer further insight is an important goal. Ferritin-bound iron may prove to be one such biomarker. However, a focused effort must be made to produce an adequate technique for measuring ferritin-bound iron before its true utility can be properly assessed.

Research funding: None declared.

Author contributions: E.G and D.C prepared the manuscript with input from G.M, L.H, and D.S. All authors participated in revising and approving the final manuscript.

Competing interests: Authors state no conflict of interest.

References


61. Grant et al.: Re-examining ferritin-bound iron


