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Hepcidin: a real-time biomarker of iron need

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There are numerous blood-based biomarkers for assessing iron stores, but all come with certain limitations. Hepcidin is a hormone primarily produced in the liver that has been proposed as the 'master regulator' of dietary uptake and iron metabolism, and has enormous potential to provide a 'real time' indicator of body iron levels. In this Minireview, the biochemical function of hepcidin in regulating iron levels will be discussed, with a specific focus on how hepcidin can aid in the assessment of iron stores and clinical diagnosis of iron deficiency, iron deficiency anaemia and other iron-related disorders. The role hepcidin itself plays in diseases of iron metabolism will be examined, and current efforts to translate hepcidin assays into the clinic will be critically appraised. Potential limitations of hepcidin as a marker of iron need will also be addressed, as well as the development of new therapies that directly target the hormone that sits atop the hierarchy of systemic iron metabolism.

Introduction

Human hepcidin is a 25-amino acid hormone that is believed to be the body's primary regulator of iron metabolism.[1] The peptide was independently discovered by two groups in 2000[2] and 2001,[3] both of whom were studying antimicrobial peptides in human body fluids. As its name suggests, hepcidin is primarily synthesised in hepatocytes, though other tissue types have been shown to produce the peptide in smaller amounts.[4]

The direct influence of hepcidin on circulating iron levels has led to significant interest in developing clinical assays for the hormone as a biomarker of iron stores. In this Minireview, the current status of hepcidin as a marker of body iron need will be critically appraised with respect to our understanding of hepcidin and its interactions with other iron regulatory proteins, diseases involving impaired hepcidin function, the opportunities and limitations of analytical methods for hepcidin detection, and potential confounding factors that could hamper interpretation of hepcidin levels and its effect on iron metabolism. With this in mind, hepcidin still has enormous potential as an indicator of iron need in 'real time', and will certainly complement the common panel of iron-associated factors used in routine pathology.

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Hepcidin and iron regulation

Initial efforts to identify the gene encoding hepcidin culminated in animal studies that revealed mice lacking upstream stimulatory factor 2 (*USF2*) exhibited tissue iron overload.[5] This lead to the discovery that the downstream *HAMP* gene, which was silenced by *USF2* knockout, was responsible for encoding hepcidin.[6] Mice possess two *HAMP* orthologs: *HAMP1* and *HAMP2*, although the role of *HAMP2* in regulating iron levels is less well defined.[7] Upregulation of human *HAMP* in the liver increases hepcidin expression, which acts to inhibit iron efflux from cells *via* the transmembrane export protein ferroportin (Fpn). Hepcidin binds to Fpn on the outer cell membrane, internalising the protein and promoting its degradation by lysosomal-associated membrane protein 1.[8] Endocytosis of the hepcidin–Fpn couple is dependent on ubiquitination of Fpn on the cytoplasmic side of the cell membrane,[9] which recently supplanted the previous suggested mechanism of internalisation that was reliant on phosphorylation of Y302/Y303 residues by Janus kinase 2 (Jak2).[10] This can have the

effect of limiting dietary iron intake by blocking export of iron from enterocytes; preventing release of iron liberated from erythrocytes in macrophages; or restricting the release of iron from the significant reservoir stored in the liver.[11] Thus, expression of hepcidin can have profound and varied effects on systemic iron metabolism, promoting iron retention within cells whilst simultaneously decreasing circulating iron levels.

HAMP transcription is primarily influenced by two pathways: the bone morphogenetic protein (BMP)–SMAD pathway that directly regulates iron metabolism; and a separate pathway regulated by inflammation (see below). Additionally, intracellular BMP–SMAD signalling may also be influenced by iron sensing by transferrin receptors 1 and 2 (TfR1; TfR2). Working backwards and focusing on BMP–SMAD (which involves the SMAD1, 5 and 8 proteins), this pathway relies on the ability for intra- and extracellular iron levels to be monitored and relayed to hepatocytes, inducing expression of hepcidin when the feedback loop is reporting iron-replete conditions. SMAD signalling is regulated by SMAD anchor molecules, which interact with SMAD proteins to induce phosphorylation and increased *HAMP* transcription, such as the recently identified SMAD anchor endofin.[12] These anchor molecules on the inner membrane of hepatocytes stabilise SMAD, which is phosphorylated by a complex of BMP receptors 1 and 2, both trans-membrane proteins with extracellular domains that can be both activated or inhibited.[13] Genetic deletion of BMP receptor 1 effectively blocks hepcidin expression, leading to systemic iron overload.[14]

In the case of BMP receptor complexes, the repulsive guidance molecule hemojuvelin (also known as RGMC or HFE2) forms a structural bridge between BMPs and neogenin-1,[15] to produce an active signalling complex,[16] which promotes hepcidin expression *via* the SMAD pathway. Neogenin-1 itself can inhibit hemojuvelin expression,[17] thereby having its own unique impact on iron metabolism. Neogenins are not exclusive iron-regulatory molecules; their interactions with BMPs are a key component of cell differentiation[18] and their overexpression can inhibit tumour growth, making them a potential diagnostic and therapeutic target for some cancers.[19] Tissue iron load can also influence hepcidin levels *via* the BMP–SMAD signalling pathway, with endothelial cells secreting BMP6 that acts on hepatocyte BMP receptors to regulate hepcidin expression.[20] There is also evidence of a sexual dimorphism related to hepcidin expression, as testosterone indirectly decreases *HAMP* transcription *via* promotion of epidermal growth factor receptor (EGFR).[21]

Hepcidin is also mediated to some degree by proprotein convertases, most notably furin, which cleaves membrane-bound hemojuvelin and releases it as a soluble molecule able to form a ligand with BMP receptors, though this likely is of relevance to hepcidin expression only in the liver (see below), where neogenin is essential for furin-mediated cleavage of hemojuvelin and its interaction with BMP receptors on the cell surface.[22] Furin itself is upregulated in iron-deficient and hypoxic conditions,[23] highlighting how multiple pathways are intertwined with regard to hepcidin expression.

Recent evidence has suggested that hemojuvelin expression is not essential for iron sensing, supporting a system with multiple redundancy mechanisms in place to regulate systemic iron levels separate to BMP receptor signalling. Mice with the *HJV* gene ablated demonstrated systemic iron overload with high levels of transferrin (Tf) saturation, regardless of dietary iron intake, although deposition of iron within the liver was proportional to dietary exposure, and hepcidin mRNA expression responded to changing dietary iron levels, and the BMP–SMAD signalling pathway was preserved, albeit at reduced levels.[24] Outside of the liver, hemojuvelin is also expressed at high levels in skeletal muscle, although conditional knockout of *HJV* in mouse muscle tissue showed no measurable effect on either hepcidin expression nor systemic iron levels.[25]

Thus, activation of the BMP–SMAD pathway can also be induced by sensing of systemic iron levels *via* interactions between holo-Tf and TfR1 and 2, independent of the BMP receptor complex, although the precise mechanism by which this occurs remains unclear. It is suggested that the hereditary haemochromatosis protein (HFE), which interacts with TfR1 during periods of low circulating iron levels, is displaced as holo-Tf levels rise.[26] The HFE protein then interacts with TfR2,[27] activating the ERK/MAPK pathway that is proposed to promote hepcidin expression *via* SMAD signalling or an independent, as-yet undiscovered pathway.[28] Whether extracellular binding of Tf to TfR1 and TfR2 is relayed to BMP receptor ligands, or if iron sensing by TfR1 and 2 is even related to these cell surface receptors remains unclear,[29] as is whether the ERK/MAPK pathway participates in SMAD signalling.[30]

Separate to systemic iron levels, hepcidin expression can also be driven by erythropoietic signals.[28] Growth differentiation factor 15 (GDF15)[31] and twisted gastrulation 1 (TWSG1)[32] are both erythroblast-expressed proteins that suppress hepcidin expression by acting on the BMP/SMAD pathway. GDF15 is of particular interest, as expression is increased in β-thalassemia patients and correlates with serum ferritin levels.[33] Erythropoietin (EPO) stimulates the release of the hormone erythroferrone (EFRE) from bone marrow and the spleen, which reduces hepcidin synthesis.[34] β-Thalassemia patients have abnormal erythropoietic activity, requiring regular transfusions that can lead to iron overload. In transfusion-dependent thalassemia patients, both erythropoiesis and GDF15 expression are decreased,[35] highlighting the importance of this protein in hepcidin, and thereby iron, regulation. Mutation to transmembrane serine protease 6 (TMPRSS6; also known as matripase-2) can cause pathological activation of the BMP/SMAD signalling pathway, resulting in iron retention and increased EPO levels, even in cases of severe iron deficiency.[36] The precise mechanism by which matripase-2 influences BMP/SMAD signalling is still the subject of some contention; a recent report linked matripase-2 activity to EPO-induced expression of EFRE,[37] while another suggested that matripase-2 and EFRE affect hepcidin expression independent of one another.[38]

An emerging model involving TfR1 is also suggested to play a role in erythropoietic regulation of hepcidin expression, as TfR1 levels are high on the surface of erythroblasts to meet the demand for iron. Mice haploinsufficient for TfR1 compensate by increasing erythroblast numbers, resulting in equivalent

soluble TfR1 levels in serum compared to controls. When *TFR1*$^{+/-}$ bone marrow cells were implanted in wild-type animals to examine the specific effects of erythroid signalling on hepcidin levels, iron-mediated erythropoiesis was restricted and hepcidin levels increased, suggesting that TfR1 regulated hepcidin expression in a manner independent of EFRE and GDF15.[39]

Inflammation also induces hepcidin expression *via* interleukin inflammatory cytokines, leading to internalisation and degradation of Fpn and inhibition of iron export necessary for erythropoiesis, which can lead to anaemia of chronic disease (also known as anaemia of inflammation). Interleukin-6 (IL-6) activates the IL-6/Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway[40] that directly regulates the inflammation response of the hormone and indirectly promotes BMP-mediated transcription of hepcidin.[8,28] Since IL-6 was identified as playing a role in hepcidin expression, other inflammatory cytokines have been studied, with IL-1α,[41] IL-1β[42] and IL-22[43] identified as promotors of hepcidin expression, and conflicting evidence regarding IL-10,[41] which appears to have a cell-specific response within primary macrophages.[44]

In addition to iron sensing, erythropoiesis and inflammation, all of which are intrinsically linked to iron metabolism, hepcidin expression is also regulated by hypoxia. There is some contention as to whether hepcidin expression is directly mediated by the well-characterised hypoxia-inducible factor (HIF) 1α and 2α pathways, or *via* a more indirect route regulated by erythropoiesis.[45] Hypoxic patients have decreased circulating hepcidin levels.[46] *In vitro* studies in hepatoma cells showed neither HIF1α or HIF2α influenced hepcidin expression, nor did it influence TfR1 expression.[47] Human subjects native to high altitude environments (>4000 m above sea level) suffering chronic mountain sickness assessed for markers of hepcidin regulation (*e.g.* EPO, soluble TfR1, GDF-15 *etc.*) showed that only EPO was associated with hepcidin expression in cases of hypoxia,[48] supporting a erythropoiesis-driven mechanism of iron regulation as opposed to direct effects of oxygen depletion. This paradigm is further reinforced by the identification that platelet derived growth factor (PDGF) BB inhibits hepcidin expression in extreme hypoxic conditions *via* downregulating cAMP response element-binding protein (CREB) transcription.[49] The identification of CREB-mediated hepcidin expression presents yet another independent pathway by which iron levels are regulated in response to a physiological stress.

The major downstream effect of increased hepcidin expression is reduced Tf saturation levels, as a reduction in transit of iron from within cells through Fpn limits the amount of iron available for loading onto the two binding sites of Tf. Consequently, delivery of iron to tissue is reduced, with less iron-laden Tf available for import into the cell *via* TfR1-mediated endocytosis. It is somewhat ironic that hepcidin does indeed exhibit antimicrobial properties, as its original discoverers were searching for, though its mechanism of action is more likely an indirect result of lower circulating iron suppressing iron-dependent bacterial proliferation, as opposed to direct cysteine-mediated permeabilisation of microbial cytoplasmic membranes[50] (hepcidin's amino acid sequence contains eight cysteine residues[51]). Hepcidin-null mice exposed to high iron levels are extremely susceptible to bacterial infection, showing a 100% mortality rate when inoculated with siderophilic *Yersinia enterocolitica* and *Vibrio vulnificus*, which can be prevented or limited by exogenous delivery of a hepcidin analogue.[52]

The rate of Tf turnover in plasma is shorter than that of erythrocytes (Tf half-life is approximately 9 days[53]), though the protein undergoes cycling between holo- and apo-states more than 10 times per day, thus cycling around 30 mg of iron.[54] After delivering iron to a cell *via* pH-induced release and reduction of bound ferric (Fe^{3+}) iron to the ferrous (Fe^{2+}) state within the endosome by six transmembrane epithelial antigen of the prostate-3 (STEAP3),[55] which then passes through divalent metal transporter-1 (DMT1), Tf is expelled from the cell by undocking from TfR1 and returning to the circulation. Now, Tf is free to accept non-Tf bound iron (NTBI), loaded onto the glycoprotein by the circulating a multi-copper ferroxidase ceruloplasmin (Cp),[56] thereby completing the feedback loop when iron bound to Tf again interacts with TfR1/2 on the surface of hepatocytes, initiating the cellular events that lead to hepcidin expression by *HAMP*.

One important aspect of hepcidin activity when describing it as a 'master regulator' of iron metabolism that should be considered is how the hormone also regulates dietary intake. As mentioned above, Fpn expressed in duodenal enterocytes facilitates the export of inorganic dietary iron into the circulatory system, which is oxidised by the membrane-bound ferroxidase hephaestin; and hepcidin-induced inhibition has the expected effect of lowering absorption by internalising Fpn. Approximately 40% of absorbed dietary iron is haem-based,[57] which crosses the brush-border *via* the haem carrier protein-1 (HCP1).[58] HCP1 was subsequently shown to also act as a folate transporter,[59] though subsequent studies have confirmed its central role in haem transport.[60] Within enterocytes, haem it is metabolised by haem oxygenase 1 (HO1)[61] and thus enters circulation in the same manner as inorganic iron species: regulated by hepcidin (Fig. 1).

Hepcidin and diseases of iron metabolism

Dysfunction of hepcidin can cause serious health issues resulting from iron overload. When hepcidin is unable to adequately inhibit iron export from Fpn, circulating iron levels rapidly increase. In addition to its role in systemic iron regulation, hepcidin is also an inflammatory response factor, and can contribute to anaemia of inflammation by preventing the release of iron stores needed for erythropoiesis.[62] Diseases that promote inflammatory induction of hepcidin expression and associated anaemia include renal failure,[63,64] cardiovascular disease,[65] myelofibrosis[66] and *Helicobacter pylori* infection,[67] among others.[68] Liver dysfunction itself can also result in hepcidin-induced anaemia; hepatic adenomas result in abnormally high levels of hepcidin expression,[69] again restricting the iron available to bone marrow.

Hepcidin is also affected in several hereditary disorders of iron metabolism. Mutations to the *HFE*, *TFR2* and *HJV* all result in decreased expression of hepcidin,[62] and direct mutation to *HAMP*, known as juvenile haemachromatosis, leads to severe

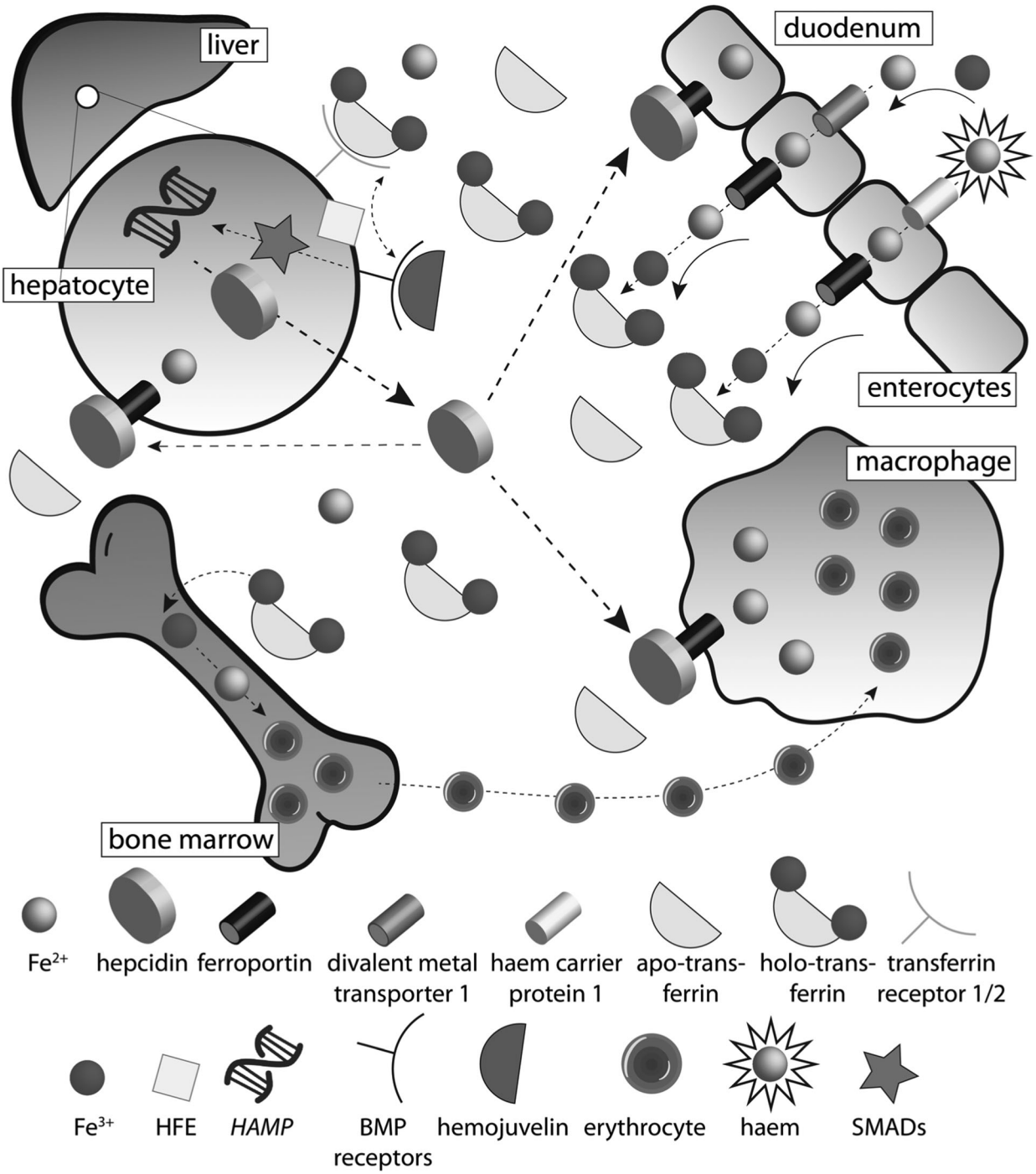

Fig. 1 Hepcidin-mediated mechanism of iron regulation. Hepcidin expression in hepatocytes is increased in response to iron sensing by TfR1, HFE protein, BMP receptors and SMAD signalling. Release of hepcidin into the circulatory system promotes the internalisation and degradation of the iron exporter Fpn, essentially blocking iron efflux from cells. This includes enterocytes at the brush border of the duodenum, where dietary iron is taken up by either DMT1 or haem carrier protein 1, and macrophages recycling erythrocytes. Reduced iron availability decreases Tf saturation, which in turn reduces the rate of erythropoiesis. As iron levels in the circulatory system return to normal levels, iron sensing in the liver reduces hepcidin expression.

tissue iron overload and associated damage from iron-mediated oxidative stress.[70] The two most common mutations to *HFE* (C282Y and H63D) both interfere with BMP–SMAD signalling by inhibiting translocation of the HFE protein to the cell surface, preventing hepcidin expression in response to iron sensing, resulting in increased dietary iron uptake and generalised iron overload.[71] It should be noted, however, that homozygous C282Y carriers (approximately one in 200 in European populations) do not necessarily develop the disorder or exhibit symptoms later in life, and that numerous lifestyle and environmental factors appear to dictate clinical manifestation of iron overload.[72] Mutations to the target protein of hepcidin can also cause severe iron overload;

the C326S point mutation to the *FPN* gene encoding Fpn prevents hepcidin from internalising the exporter, leading to increased circulating iron levels and specific accumulation of iron and associated degeneration of pancreatic cells.[73] Point mutations to *FPN* cause a systemic response *via* hepcidin expression; both the C326S[74] and G80S[75] mutations induce increased levels of circulating hepcidin (most likely due to an intact TfR–HFE sensing pathway), and a recent case study described a rare A69T mutation that resulted in increased hepicidin and the removal of 21 grams of iron (*via* phlebotomy) over a 16 month period.[76]

Hepcidin may also have play an important role in neurological disorders that feature brain iron accumulation. Neurodegenerative disorders like Alzheimer's and Parkinson's disease both feature elevated levels of brain iron and marked inflammation,[77] both of which should induce a hepcidin-mediated response. C6 glioma cells have been shown to express hepcidin *in vitro*,[78] and histological examination of normal brains has identified hepcidin expression in both neurons and astrocytes, indicating that iron metabolism in the relatively isolated central nervous system produces hepcidin independent of the liver.[79] In the same study, hepcidin was shown to be significantly decreased in Alzheimer's disease brain tissue lysates, in addition to colocalising with haem-containing deposits in damaged brain vasculature. This has given rise to the 'hepcidin–Fpn' hypothesis of iron accumulation in Alzheimer's disease,[80] which also encompasses the amyloid precursor protein (APP; which is cleaved in the neuronal membrane to produce the β-amyloid 'hallmark' protein of Alzheimer's disease). APP is thought to play a role in iron export from neurons[81] by stabilising Fpn on the cell surface.[82] This hypothesis is not without limitations, as it is difficult to isolate iron accumulation in the brain as being a potential causative factor or simply a result of disease-related inflammation.[83]

Studies of hepcidin mRNA in the murine brain identifies widespread expression of the peptide, with an age-dependent increase,[68] which would be consistent with a homeostatic response to natural brain iron accumulation with age. In Parkinson's disease, where a pathogenic role of iron is better defined,[84] relatively little work has investigated the possible role of hepcidin in neuronal iron accumulation. Cell culture models of parkinsonian neurodegeneration using 6-hydroxydopamine, which is an iron-mediated neurotoxic metabolite of dopamine,[85] showed that knock-down of hepcidin facilitated neuronal iron export and reduced the severity of oxidative damage stemming from the neurotoxin insult.[86] Iron-induced oxidative stress in rats *via* acute iron exposure could be partially attenuated by pre-treatment with recombinant hepcidin adenovirus,[87] which may represent an interesting avenue of therapeutic development to address brain iron overload, particularly in light of recent successes with iron chelation therapy in human Parkinson's disease patients.[88]

Acute inflammation, such as that observed in subarachnoid haemorrhage, elicits the same increase in hepcidin expression observed in the rest of the body, with downstream effects including decreased Fpn and Cp levels. Long-term retention of iron in the brain following a traumatic injury can induce iron-mediated oxidative stress;[89] an effect accentuated with age.[90]

Hepcidin assays: clinical translation and potential limitations

As noted by Arezes and Nemeth,[91] despite hepcidin having enormous potential as a reliable biomarker of iron stores, particularly for assessing iron deficiency and iron deficiency anaemia in otherwise healthy individuals (*i.e.* no history of hereditary iron metabolic disorders or acute inflammation), current available assays are for 'research-only' purposes. In their review of current approaches to hepcidin analysis, Arezes and Nemeth outline the reasons why clinical translation of these assays has been delayed: mass spectrometry methods are comparatively low-throughput compared to ELISAs, which themselves are often limited by the inability to distinguish the active full length (25 amino acids) hormone from truncated variants that may result from biological degradation or break-down between sample collection and assessment. Initial attempts to establish a reliable quantitative assay in a global round-robin study that employed eight separate methods (all ELISA or mass spectrometry-based) in independent laboratories found significant variation in measured hepcidin levels between techniques used, though the precision of each method was considered analytically acceptable.[92] This study encouraged further efforts to establish a standardised method for measuring hepcidin, and a major step forward took place in 2016 when methods recommended by the International Consortium for Harmonization of Clinical Laboratory Results were employed to further investigate inter-laboratory variation. While equivalence between laboratories was still lacking the necessary accuracy and precision (inter-measurement procedure CV was 28.6%), it was identified that a common calibrator could be used to improve analytical validity, and a native lyophilised plasma with cryolyoprotectant was developed. Simulated harmonisation using this material predicted an achievable equivalence of 7.7%.[93]

Although progress has been made to unify global efforts for a validated, clinically-applicable analytical assay for hepcidin, there is still significant work to be undertaken to meet the exacting standards of medical regulatory bodies. It is possible to speculate on why a clinically approved assay has yet to be developed, even with the efforts of International Consortium for Harmonization of Clinical Laboratory Results, an initiative of the American Association of Clinical Chemistry which itself is still establishing the necessary network of international laboratories for robust method validation.[94] There are a range of biochemical assays that have been developed for hepcidin, including competitive and sandwich-based ELISA approaches, mass spectrometry-based detection, and techniques employing advances in nanotechnology. Here, only an update on new methods to detect hepcidin-25 (the biologically active 25 amino acid form of the hormone) are summarised in Table 1; for examples prior to 2014 see the comprehensive review by Konz *et al.*[95] However, many of the issues raised by Malyszko in a 2009 editorial[96] are still faced today. In this author's opinion, liquid chromatography and tandem mass spectrometry is the preferred candidate for a reliable clinical assay. Advances in mass spectrometry have ensured sensitivity is no longer a

significant issue for detecting hepcidin in biological fluids, though the lack of certified reference materials and the comparative expense of both infrastructure and sufficiently qualified operators undoubtedly limits uptake within clinical chemistry laboratories. Outside of the laboratory, mass spectrometry is also impractical, and the need for rapid, field-portable detection likely drives the continued development of ELISA-based assays, which are cheaper and can be performed in a range of settings.

Translation of research-based assays of hepcidin into the clinic for assessing iron stores has been slow not only due to the aforementioned issues surrounding analytical validity and cost; there are also concerns regarding the potential confounding effects introduced due to hepcidin's role in innate immunity. Iron is crucial in the body's self-defence mechanisms against pathogens, thus it is not surprising that hepcidin is an acute phase II response factor that can contribute to the manifestation of anaemia of chronic disease.[109] It is entirely possible that hepcidin will face the same biological hurdles as other biomarkers of iron deficiency due to its multifaceted role. At best, hepcidin will be a complementary assay for differentiating between anaemia of iron deficiency and that of chronic disease. Thomas *et al.*[110] reported that hepcidin-25 determination alone could distinguish iron deficiency anaemia from combined iron deficiency anaemia and anaemia of chronic disease, but not the reverse state. However, inclusion of reticulocyte haemoglobin levels in the model was able to discriminate all three conditions. Conversely, hepcidin and ferritin was less accurate at determining iron deficiency, (which is an asymptomatic condition that can be considered 'prodromal' iron deficiency anaemia if iron store continue to decline) than the soluble TfR1/log ferritin ratio.[111]

Hepcidin, iron regulatory proteins and their combined use in diagnostics

New functional relationships between numerous proteins and hepcidin expression are being reported each year, primarily due to our better understanding of systems biology and the synergistic relationship between multiple regulatory systems in the human body. As mentioned previously, the major downstream effect of low hepcidin expression is Tf saturation. This 80 kDa glycoprotein has two iron-binding sites, which are rapidly occupied by iron as it transits Fpn. Like hepcidin, this protein is primarily synthesised in the liver, though other tissue types (including the brain[112]) are able to produce their own endogenous supply. Iron released by Fpn is loaded onto Tf by ferroxidases hephaestin (membrane-bound) and Cp (circulating),[113] and is released by the ferric reductase STEAP3.[114]

Recent evidence has suggested that, in addition to inducing the internalisation and degradation of Fpn, hepcidin has direct effects on other iron regulatory proteins. *Ex vivo* experiments have shown that hepcidin down-regulates mRNA levels of several genes involved in iron regulation in the intestine, including those encoding hephaestin, DMT1, duodenal cytochrome *b* (Dcytb) and HCP1.[115]

The current panel of biomarkers recommended by the American Academy of Pediatrics (iron deficiency is a significant health concern for children) for assessing iron deficiency and iron deficiency anaemia include serum ferritin, Tf saturation, soluble TfR1, haemoglobin, reticulocyte haemoglobin, and mean corpuscular volume.[116] However, in isolation, each of these markers has limitations. Ferritin is an acute phase inflammatory response protein, and should be viewed in conjunction with other markers of inflammation, such as c-reactive protein. Additionally, even though ferritin has a high capacity for iron (a single molecule can bind over 4500 iron atoms[117]), its comparatively low concentration in serum contributes only a small proportion of circulating iron levels. Further, recent developments for assessing ferritin iron saturation have shown that its iron load has little correlation with protein levels.[118] There is also debate as to whether circulating ferritin levels are at all related to iron storage capacity; its role in inflammation may dwarf antecedent iron storage roles. Ferritin is not synthesised in blood, and Kell and Pretorius[119] recently suggested that its presence in the circulatory system is a marker of 'leakage' from damaged cells.

Transferrin saturation is a good indicator of iron availability, though routine clinical assays are insensitive to small perturbations in iron load and are calculated using the assumption that iron from other sources (*e.g.* NTBI and ferritin) are negligible.[120] TfR1, being so tightly connected to hepcidin expression is a useful marker, though solubilised protein (released by proprotein convertase 7, which is in the same family as furin[121]) is only present at low levels. TfR1 is also expressed on the surface of erythroblasts and reticulocytes and this likely contributes to the detectable pool of soluble TfR1.[122] Soluble levels of TfR1 decrease when the concentration of holo-Tf is elevated.[123]

Haemoglobin (Hb) has been the mainstay for assessing iron stores for many years, and remains the gold standard according to World Health Organization guidelines,[124] though it has poor sensitivity and specificity for detecting iron deficiency[125] and should be used in conjunction with other markers. Erythrocytes are 35% Hb[126] with a lifespan of 110–120 days, and thus are not an ideal measure of immediate iron stores. Reticulocyte haemoglobin (CHr) is a more reliable measure, as it represents the iron content of immature erythrocytes within a 1–2 day period.[127] However, consensus has yet to be reached on appropriate CHr cut-off levels for diagnosis of iron deficiency and related anaemias. Mean corpuscular volume is a measure of erythrocyte volume, though its interpretation as a measure of direct iron stores is confounded by its role in multiple non-iron related anaemias. Although these limitations can be significant, combined they can provide a good overall picture of current iron stores and risk of iron deficiency anaemia (Fig. 2). Considering that hepcidin expression has direct influence over all of these markers, from Tf saturation to regulating iron available to bone marrow, it stands to reason that this hormone has significant utility as a marker of iron stores, particularly in assessing immediate iron needs.

Hepcidin is also has great potential for nutritional studies as a readout of immediate response to iron supplementation. Prentice *et al.*[128] used a univariate statistical model to compare hepcidin, ferritin, c-reactive protein, soluble TfR1 and soluble TfR1/log ferritin as predictors of incorporation of iron into

Table 1 Selected examples of hepcidin-25 assays from 2014 to present. ELISA = enzyme-linked immunosorbent assay; LC-MS/MS = liquid chromatography tandem mass spectrometry; MALDI = matrix assisted laser desorption/ionisation; SELDI = surface enhanced desorption/ionisation; TOF-MS = time-of-flight mass spectrometry

Study	Method	Standard type and sample matrix	Limit of detection	Limit of quantification	Upper limit	Precision (% CV)	Comments
Cenci *et al.*[97]	Molecularly imprinted polymer nanoparticles/ surface plasmon resonance	Human serum	13.5 pg mL^{-1}	Not reported	2.1 ng mL^{-1}	18% (intra-assay)	Biotinylated nanoparticles immobilised to a surface plasmon resonance chip.
Dahlfors *et al.*[98]	Competitive ELISA	Human serum, human liver biopsies	60 pg mL^{-1}	Not reported	50 ng mL^{-1}	18–13% (intra-assay); 24–22% (inter-assay)[a]	Validated against LC-MS/ MS (R^2 = 0.805); assessed cross-reactivity for hepcidin-20 and -22.
Delaby *et al.*[99]	Nano LC-MS/MS	Human serum	2 ng mL^{-1}	6 ng mL^{-1}	100 ng mL^{-1}	7–28% (intra-assay)[a] 9–10% (inter-assay)	Validated against competitive ELISA method approved for clinical use in France[100] (Pearson's R^2 = 0.960).
Delaby *et al.*[101]	LC-MS/MS	Human CSF	0.14 ng mL^{-1}	0.16 ng mL^{-1}	25 ng mL^{-1}	9.5–2.5% (intra-assay)[a]	—
Grebenchtchikov *et al.*[102]	Antibody mimetic	Human serum and plasma	43 pg mL^{-1}	Not reported	8.37 ng mL^{-1}	6.4% (intra-assay); 8.7% (inter-assay)[b]	ANTICALIN® human lipocalin binding assay. Validated against weak cation exchange TOF-MS (Spearman R^2 = 0.951) and competitive ELISA (Spearman R^2 = 0.980).
Gutschow *et al.*[103]	Competitive ELISA	Murine serum and urine	0.18 ng mL^{-1} (synthetic hepcidin); 1.30 ng mL^{-1} (5% serum dilution); 1.80 ng mL^{-1} (10% serum dilution)	10.3 ng mL^{-1} (5% serum dilution); 5.10 ng mL^{-1} (10% serum dilution)	333 ng mL^{-1} (synthetic hepcidin); 6666 ng mL^{-1} (5% serum dilution); 3333 ng mL^{-1} (10% serum dilution)	3.1% (intra-assay); 5.9% (inter-assay)	Applied to murine models of β-thalassemia intermedia, hereditary hemochromatosis, hypotransferrinemia, heterozygous TfR1 deficiency and iron refractory iron deficiency anaemia.
Gutschow *et al.*[104]	Competitive ELISA	Human serum and plasma	Not reported	Not reported	200 ng mL^{-1}	7–4% (intra- and inter-assay)[a]	In-house produced monoclonal antibody to hepcidin.
Lefebvre *et al.*[105]	LC-MS/MS	Synthetic hepcidin (murine, human and isotope-labelled); murine serum	0.75 ng mL^{-1}	1.5 ng mL^{-1}	200 ng mL^{-1}	2.6–6.5% (inter-day)	—
Scarano *et al.*[106]	Affinity sensing/surface plasmon resonance	Synthetic human hepcidin	1.8 ng mL^{-1}	Not reported	100 ng mL^{-1}	12% (type not reported)	Low antibody stability (<3 days) reported.
Uelker *et al.*[107]	Manual and automated competitive ELISA	Human serum and plasma	0.15 ng mL^{-1} (manual)	Not reported	81 ng mL^{-1} (manual and automated)	6.79% (intra-assay, manual); 12.7% (inter-assay, manual); 2.60% (intra-assay, automated); 13.7% (inter-assay, automated)	Test of two commercially available ELISA kits.
Wang *et al.*[108]	Nanopore film enrichment for MALDI-TOF-MS	Human serum	Not reported	54 ng mL^{-1}	270 ng mL^{-1}	Not reported	Study of correlation with prolactin levels

[a] Low and high concentration hepcidin samples. [b] Means for low, mid and high hepcidin samples.

erythrocytes in an elegantly-designed stable-isotope iron supplementation trial in an anaemic population with high rates of inflammation. Concluding that, over the course of the 30 day trial, hepcidin was the most accurate determinant of iron incorporation, the authors noted that the development of cost-effective assays for hepcidin would be of great value for intervention studies in low-income countries. This need was further emphasised in the recent perspective by Ganz on hepcidin and the burden of disease resulting from iron deficiency, particularly in the developing world.[129]

Beyond measures of body iron stores, hepcidin also has potential utility in other clinical settings (several of which are reviewed by Arezes and Nemeth[91]). Novel applications of hepcidin determination have recently been proposed for assessing if iron supplementation is necessary for pregnant women;[130] as a biomarker of blood doping in sports;[131] (particularly pertinent considering that testosterone increases iron absorption in erythrocytes[132]) and as a predictive indicator of breast tumour growth.[133] In the chirrhotic liver, where generalised hepatic function is impaired, the ratio of serum hepcidin to ferritin is decreased in line with increasing fibrosis.[134] Chronic kidney disease can cause anaemic of chronic disease with corresponding increased hepcidin expression, due to both extended periods of inflammation and impaired ability of the kidneys to excrete excessive hepcidin buildup.[135,136] In recent years, hepcidin has been emerging as a potential marker of inflammatory status in type 2 diabetes mellitus and comorbid obesity, where it is elevated in line with several other cytokines,[137] including IL-6, which (as discussed above) can directly induce hepatic hepcidin expression. However, attention is likely better directed to regulators of hepcidin expression as opposed to the hormone alone for early disease diagnosis; for instance, increased GDF15 expression is associated with glucose intolerance (*i.e.* a pre-diabetic state) in the absence of anaemia, and prior to corresponding increased hepcidin levels.[138]

Therapeutic opportunities involving hepcidin

If hepcidin is involved in a range of pathological processes, it is not surprising that attention is being directed to targeting hepcidin function as novel therapies.[139] As of early 2017, six separate therapies that directly target hepcidin are in varying stages of development (Table 2).[11] All are biomolecules, including peptides, an ANTICALIN®, a L-RNA spiegelmer and an antibody; and focus on both ends of the hepcidin function spectrum. Hepcidin-mimetics are being developed to address iron overload and β-thalassemia, while other therapies are being designed to capture hepcidin prior to complexing with Fpn, thereby increasing available iron for the anaemic. Whether these approaches will be safer and more efficacious than conservative iron chelation therapies[140] for iron overload disorders, or provide better outcomes than simple iron supplementation for the deficient remains to be seen; the L-RNA spiegelmer NOX-H94 for treating iron deficiency anaemia is the most advanced of the six therapies being developed and is currently in four phase II trials, while the other five are either in preclinical development or are still being assessed for safety and tolerability.[11]

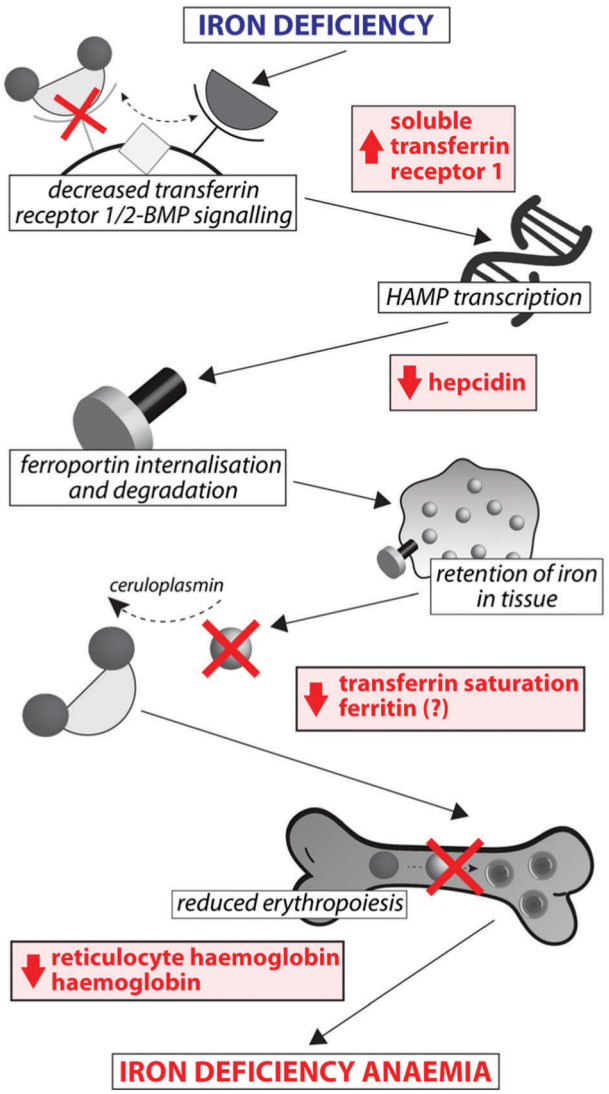

Fig. 2 Biomarkers of iron stores and response during transition from iron deficiency to iron deficiency anaemia. Soluble TfR1 is released when levels of holo-Tf are decreased, which in turn signals decreased expression of hepcidin by inhibiting the formation of Tf/TfR1/HFE/hemojuvelin/BMP receptor complexes. If retention of iron within cells and tissues increases or iron stores are depleted, circulating iron levels are decreased further, lowering transferrin saturation levels (and possibly ferritin). Decreased accessibility to iron reduces rates of erythropoiesis, decreasing reticulocyte haemoglobin levels in the short term, and total haemoglobin levels during chronic iron deficiency. Continued disruption of systemic iron levels eventually leads to iron deficiency anaemia.

Circumventing the effects of inflammatory cytokines on hepcidin expression has potential for treating anaemic of chronic disease that results from extended periods of systemic inflammation. The identification and characterisation of enodgenous BMP receptor antagonist complexes has led to the development of synthetic BMP receptor inhibitors,[141] which could in future be used to treat anaemia of chronic disease by preventing hepcidin expression *via* the BMP–SMAD pathway, enabling release of iron to bone marrow.

Table 2 Current therapies under development targeting hepcidin function. Adapted from Crielaard *et al.*[11]

Compound name	Developing company	Molecule type	Intended function	Targeted disease	Status
M012	Merganser Biotech	Peptide	Hepcidin mimetic	β-Thalassaemia; low-risk myelodysplastic syndrome; polycythaemia	Phase I
LJPC-401	La Jolla Pharmaceutical Company	Peptide	Hepcidin mimetic; hepcidin agonist	Iron overload	Phase I
PTG-300	Protagonist Therapeutics	Peptide	Hepcidin mimetic; hepcidin agonist	Iron overload	Preclinical
PRS-080	Pieris Pharmaceuticals	ANTICALIN®	Hepcidin capture	Iron deficiency anaemia; functional iron deficiency	Phase Ib
NOX-H94	NOXXON Pharma	L-RNA spiegelmer	Hepcidin capture	Iron deficiency anaemia	Phase II
12B9m	Amgen	Human IgG2-specific antibody	Hepcidin capture	Iron deficiency anaemia	Preclinical

Patients with chronic kidney disease also have decreased vitamin D levels; thus, there has been recent interest in a possible link between vitamin D and iron metabolism and whether this essential micronutrient could be used in a therapeutic setting to limit anaemia from kidney dysfunction. Treatment of cultured hepatocytes showed increased interaction between vitamin D and its receptor, with resultant decreased hepcidin expression. In a pilot clinical trial of healthy volunteers, a single oral dose of vitamin D decreased circulating hepcidin levels by 34% within 24 hours.[142] Subsequent randomised, double-blind, placebo-controlled trials of high dose vitamin D_3 in healthy adults have confirmed this potentially therapeutic effect one week post-supplementation,[143] paving the way for longer-term trials for treating inflammatory anaemia. Intervention with vitamin D supplementation has also been proposed as a means to prevent anaemia of chronic disease in patient during early-stage renal failure.[144]

Iron in real time: why does hepcidin matter?

As the 'master regulator' of iron homeostasis, hepcidin levels provide the most immediate indicator of iron status and need. Sudden changes in circulating iron levels result in a rapid response; haemodialysis patients given an intravenous injection of iron sucrose showed a 25–200% increase in hepcidin levels 15 minutes after administration, with Tf saturation levels increasing by approximately one-third during the same time.[145] This emphasises the importance of Tf in dictating hepcidin expression *via* the BMP–SMAD pathway; an increase in NTBI elicited increased uptake of iron onto Tf, which was then appropriately sensed in the liver through interactions between holo-Tf and TfR1 and TfR2, leading to increased hepcidin release to prevent further supplementation of the elevated NTBI pool by inhibiting Fpn. Thus, in cases of acute iron exposure, Tf can be considered to be equivalent in importance to regulating iron levels as hepcidin, if not more, as uptake of excess iron by the iron transporter dictates hepcidin transcription. This is supported by studies in a murine model deficient in haemoglobin, which exhibits severe anaemia yet shows increased hepcidin expression in line with increased Tf saturation and concentration.[146]

Why is it so important to have a real-time indicator of iron need, and how does it differ from the information provided currently? As discussed previously, the current panel of biomarkers for iron deficiency and iron deficiency anaemia have several limitations, which are shared to some degree with hepcidin. However, the major advantage of hepcidin is that, in the absence of any potential confounding factors, it provides a snapshot of immediate iron requirements, as opposed to markers like haemoglobin that do not respond in an acute manner to changes in iron levels. Thus, longitudinally assessing hepcidin levels can provide a better indication of how iron requirements change over time: static hepcidin levels with the absence of any clinical symptoms of iron deficiency anaemia likely indicates that a suitable equilibrium state has been reached, while steadily decreasing levels over several time points suggest a systemic deficit in iron metabolism that may require therapeutic or nutritional intervention. While it is unlikely that hepcidin levels alone will be labelled as the 'gold standard' for assessing circulating iron levels, adding it to the current biomarker panel will undeniably improve future diagnostic accuracy. Although results from initial attempts to integrate hepcidin with other circulating iron biomarkers have been mixed, it is likely that global harmonisation of analytical methods for detecting hepcidin, as well as studies that encompass the two main respondents to hepcidin expression (Tf saturation and TfR1 levels), will see that this unique hormone becomes an integral player in both research and clinical disciplines related to iron in biology.

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References

1 T. Ganz, *Blood*, 2003, **102**, 783–788.
2 A. Krause, S. Neitz, H.-J. Mägert, A. Schulz, W.-G. Forssmann, P. Schulz-Knappe and K. Adermann, *FEBS Lett.*, 2000, **480**, 147–150.

3 C. H. Park, E. V. Valore, A. J. Waring and T. Ganz, *J. Biol. Chem.*, 2001, **276**, 7806–7810.
4 J. F. Collins, M. Wessling-Resnick and M. D. Knutson, *J. Nutr.*, 2008, **138**, 2284–2288.
5 G. Nicolas, M. Bennoun, I. Devaux, C. Beaumont, B. Grandchamp, A. Kahn and S. Vaulont, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8780–8785.
6 N. C. Andrews, *Semin. Hematol.*, 2002, **39**, 227–234.
7 J. Truksa, P. Lee and E. Beutler, *Blood Cells, Mol., Dis.*, 2007, **39**, 255–262.
8 E. Nemeth, M. S. Tuttle, J. Powelson, M. B. Vaughn, A. Donovan, D. M. Ward, T. Ganz and J. Kaplan, *Science*, 2004, **306**, 2090–2093.
9 B. Qiao, P. Sugianto, E. Fung, A. del-Castillo-Rueda, M.-J. Moran-Jimenez, T. Ganz and E. Nemeth, *Cell Metab.*, 2012, **15**, 918–924.
10 I. De Domenico, E. Lo, D. M. Ward and J. Kaplan, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 3800–3805.
11 B. J. Crielaard, T. Lammers and S. Rivella, *Nat. Rev. Drug Discovery*, 2017, DOI: 10.1038/nrd.2016.248.
12 J. B. Goh, D. F. Wallace, W. Hong and V. N. Subramaniam, *Sci. Rep.*, 2015, **5**, 13986.
13 D. Yadin, P. Knaus and T. D. Mueller, *Cytokine Growth Factor Rev.*, 2016, **27**, 13–34.
14 A. U. Steinbicker, T. B. Bartnikas, L. K. Lohmeyer, P. Leyton, C. Mayeur, S. M. Kao, A. E. Pappas, R. T. Peterson, D. B. Bloch, P. B. Yu, M. D. Fleming and K. D. Bloch, *Blood*, 2011, **118**, 4224–4230.
15 E. G. Healey, B. Bishop, J. Elegheert, C. H. Bell, S. Padilla-Parra and C. Siebold, *Nat. Struct. Mol. Biol.*, 2015, **22**, 458–465.
16 J. L. Babitt, F. W. Huang, D. M. Wrighting, Y. Xia, Y. Sidis, T. A. Samad, J. A. Campagna, R. T. Chung, A. L. Schneyer, C. J. Woolf, N. C. Andrews and H. Y. Lin, *Nat. Genet.*, 2006, **38**, 531–539.
17 D.-H. Lee, L.-J. Zhou, Z. Zhou, J.-X. Xie, J.-U. Jung, Y. Liu, C.-X. Xi, L. Mei and W.-C. Xiong, *Blood*, 2010, **115**, 3136–3145.
18 Z. Huang, D. Sun, J.-X. Hu, F.-L. Tang, D.-H. Lee, Y. Wang, G. Hu, X.-J. Zhu, J. Zhou, L. Mei and W.-C. Xiong, *J. Neurosci.*, 2016, **36**, 5833–5849.
19 Q. Zhang, F. Liang, Y. Ke, Y. Huo, M. Li, Y. Li and J. Yue, *Oncol. Rep.*, 2015, **34**, 258–264.
20 S. Canali, K. B. Zumbrennen-Bullough, A. B. Core, C.-Y. Wang, M. Nairz, R. Bouley, F. K. Swirski and J. L. Babitt, *Blood*, 2017, **129**, 405–414.
21 C. Latour, L. Kautz, C. Besson-Fournier, M. L. Island, F. Canonne Hergaux, O. Loréal, T. Ganz, H. Coppin and M.-P. Roth, *Hepatology*, 2014, **59**, 683–694.
22 N. Zhao, J. E. Maxson, R. H. Zhang, M. Wahedi, C. A. Enns and A.-S. Zhang, *J. Biol. Chem.*, 2016, **291**, 12322–12335.
23 L. Silvestri, A. Pagani and C. Camaschella, *Blood*, 2008, **111**, 924–931.
24 K. Gkouvatsos, C. Fillebeen, A. Daba, J. Wagner, G. Sebastiani and K. Pantopoulos, *PLoS One*, 2014, **9**, e85530.
25 W. Chen, F. W. Huang, T. B. de Renshaw and N. C. Andrews, *Blood*, 2011, **117**, 6319–6325.
26 P. J. Schmidt, P. T. Toran, A. M. Giannetti, P. J. Bjorkman and N. C. Andrews, *Cell Metab.*, 2008, **7**, 205–214.
27 E. Tolosano, *Haematologica*, 2015, **100**, 565–566.
28 M. W. Hentze, M. U. Muckenthaler, B. Galy and C. Camaschella, *Cell*, 2010, **142**, 24–38.
29 D. Meynard, J. L. Babitt and H. Y. Lin, *Blood*, 2014, **123**, 168–176.
30 A. C. G. Chua, D. Trinder and J. K. Olynyk, *Hepatology*, 2011, **54**, 16–19.
31 T. Tanno, N. V. Bhanu, P. A. Oneal, S.-H. Goh, P. Staker, Y. T. Lee, J. W. Moroney, C. H. Reed, N. L. Luban, R.-H. Wang, T. E. Eling, R. Childs, T. Ganz, S. F. Leitman, S. Fucharoen and J. L. Miller, *Nat. Med.*, 2007, **13**, 1096–1101.
32 T. Tanno, P. Porayette, O. Sripichai, S.-J. Noh, C. Byrnes, A. Bhupatiraju, Y. T. Lee, J. B. Goodnough, O. Harandi, T. Ganz, R. F. Paulson and J. L. Miller, *Blood*, 2009, **114**, 181–186.
33 R. Athiyarath, B. George, V. Mathews, A. Srivastava and E. S. Edison, *Ann. Hematol.*, 2014, **93**, 2093–2095.
34 L. Kautz, G. Jung, E. V. Valore, S. Rivella, E. Nemeth and T. Ganz, *Nat. Genet.*, 2014, **46**, 678–684.
35 S.-R. Pasricha, D. M. Frazer, D. K. Bowden and G. J. Anderson, *Blood*, 2013, **122**, 124–133.
36 A. J. Ramsay, J. D. Hooper, A. R. Folgueras, G. Velasco and C. López-Otín, *Haematologica*, 2009, **94**, 840–849.
37 A. Nai, A. Rubio, A. Campanella, O. Gourbeyre, I. Artuso, J. Bordini, A. Gineste, C. Latour, C. Besson-Fournier, H. Y. Lin, H. Coppin, M.-P. Roth, C. Camaschella, L. Silvestri and D. Meynard, *Blood*, 2016, **127**, 2327–2336.
38 S. Aschemeyer, V. Gabayan, T. Ganz, E. Nemeth and L. Kautz, *Am. J. Hematol.*, 2017, DOI: 10.1002/ajh.24672.
39 S. B. Keel, R. Doty, L. Liu, E. Nemeth, S. Cherian, T. Ganz and J. L. Abkowitz, *Exp. Hematol.*, 2015, **43**, 469–478.
40 D. M. Wrighting and N. C. Andrews, *Blood*, 2006, **108**, 3204–3209.
41 P. Lee, H. Peng, T. Gelbart, L. Wang and E. Beutler, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 1906–1910.
42 N. K. N. Shanmugam, K. Chen and B. J. Cherayil, *J. Biol. Chem.*, 2015, **290**, 30637–30647.
43 D. F. Wallace and V. N. Subramaniam, *Int. Immunol.*, 2015, **27**, 281–287.
44 H. Huang, A. A. Lamikanra, M. S. Alkaitis, M. L. Thézénas, A. Ramaprasad, E. Moussa, D. J. Roberts and C. Casals-Pascual, *PLoS One*, 2014, **9**, e88408.
45 N. Zhao, A.-S. Zhang and C. A. Enns, *J. Clin. Invest.*, 2013, **123**, 2337–2343.
46 G. Ravasi, S. Pelucchi, F. Greni, R. Mariani, A. Giuliano, G. Parati, L. Silvestri and A. Piperno, *Blood Cells, Mol., Dis.*, 2014, **53**, 204–210.
47 M. Volke, D. P. Gale, U. Maegdefrau, G. Schley, B. Klanke, A.-K. Bosserhoff, P. H. Maxwell, K.-U. Eckardt and C. Warnecke, *PLoS One*, 2009, **4**, e7875.
48 N. P. Talbot, T. G. Smith, S. Lakhal-Littleton, C. Gülsever, M. Rivera-Ch, K. L. Dorrington, D. R. Mole and P. A. Robbins, *Blood*, 2016, **127**, 1206–1207.
49 T. Sonnweber, D. Nachbaur, A. Schroll, M. Nairz, M. Seifert, E. Demetz, D. Haschka, A.-M. Mitterstiller,

A. Kleinsasser, M. Burtscher, S. Trübsbach, A. T. Murphy, V. Wroblewski, D. R. Witcher, K. Mleczko-Sanecka, C. Vecchi, M. U. Muckenthaler, A. Pietrangelo, I. Theurl and G. Weiss, *Gut*, 2014, **63**, 1951–1959.
50 J.-L. Dimarcq, P. Bulet, C. Hetru and J. Hoffmann, *Biopolymers*, 1999, **47**, 465–477.
51 H. N. Hunter, D. B. Fulton, T. Ganz and H. J. Vogel, *J. Biol. Chem.*, 2002, **277**, 37597–37603.
52 J. Arezes, G. Jung, V. Gabayan, E. Valore, P. Ruchala, P. A. Gulig, T. Ganz, E. Nemeth and Y. Bulut, *Cell Host Microbe*, 2015, **17**, 47–57.
53 M. Awai and E. B. Brown, *J. Lab. Clin. Med.*, 1963, **61**, 363–396.
54 K. Gkouvatsos, G. Papanikolaou and K. Pantopoulos, *Biochim. Biophys. Acta*, 2012, **1820**, 188–202.
55 A. K. Sendamarai, R. S. Ohgami, M. D. Fleming and C. M. Lawrence, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 7410–7415.
56 L. L. Dunn, Y. S. Rahmanto and D. R. Richardson, *Trends Cell Biol.*, 2007, **17**, 93–100.
57 G. J. Anderson, D. M. Frazer, A. T. McKie, C. D. Vulpe and A. Smith, *BioMetals*, 2005, **18**, 339–348.
58 M. Shayeghi, G. O. Latunde-Dada, J. S. Oakhill, A. H. Laftah, K. Takeuchi, N. Halliday, Y. Khan, A. Warley, F. E. McCann, R. C. Hider, D. M. Frazer, G. J. Anderson, C. D. Vulpe, R. J. Simpson and A. T. McKie, *Cell*, 2005, **122**, 789–801.
59 A. Qiu, M. Jansen, A. Sakaris, S. H. Min, S. Chattopadhyay, E. Tsai, C. Sandoval, R. Zhao, M. H. Akabas and I. D. Goldman, *Cell*, 2006, **127**, 917–928.
60 S. Le Blanc, M. D. Garrick and M. Arredondo, *Am. J. Physiol.: Cell Physiol.*, 2012, **302**, C1780–C1785.
61 K. A. Duck and J. R. Connor, *BioMetals*, 2016, **29**, 573–591.
62 P. L. Lee and E. Beutler, *Annu. Rev. Pathol.: Mech. Dis.*, 2009, **4**, 489–515.
63 T. Ganz and E. Nemeth, *Semin. Nephrol.*, 2016, **36**, 87–93.
64 M. R. Hanudel, M. Rappaport, V. Gabayan, G. Jung, I. B. Salusky, E. Nemeth, T. Ganz and J. Zaritsky, *Haematologica*, 2016, **102**, e85–e88.
65 R. Pechlaner, S. Kiechl, M. Mayr, P. Santer, S. Weger, D. Haschka, S. S. Bansal, J. Willeit and G. Weiss, *Clin. Chem. Lab. Med.*, 2016, **54**, 151–161.
66 M. Asshoff, V. Petzer, M. R. Warr, D. Haschka, P. Tymoszuk, E. Demetz, M. Seifert, W. Posch, M. Nairz, P. Maciejewski, P. Fowles, C. J. Burns, G. Smith, K.-U. Wagner, G. Weiss, J. A. Whitney and I. Theurl, *Blood*, 2007, **129**, 1823–1830.
67 L. Hudak, A. Jaraisy, S. Haj and K. Muhsen, *Helicobacter*, 2017, **22**, e12330.
68 C.-Y. Wang and J. L. Babitt, *Curr. Opin. Hematol.*, 2016, **23**, 189–197.
69 D. A. Weinstein, C. N. Roy, M. D. Fleming, M. F. Loda, J. I. Wolfsdorf and N. C. Andrews, *Blood*, 2002, **100**, 3776–3781.
70 A. Roetto, G. Papanikolaou, M. Politou, F. Alberti, D. Girelli, J. Christakis, D. Loukopoulos and C. Camaschella, *Nat. Genet.*, 2003, **33**, 21–22.
71 A. Pietrangelo, *Gastroenterology*, 2015, **149**, 1240–1251.
72 L. W. Powell, R. C. Seckington and Y. Deugnier, *Lancet*, 2016, **388**, 706–716.
73 S. Altamura, R. Kessler, H.-J. Gröne, N. Gretz, M. W. Hentze, B. Galy and M. U. Muckenthaler, *Cell Metab.*, 2014, **20**, 359–367.
74 R. L. Sham, P. D. Phatak, E. Nemeth and T. Ganz, *Blood*, 2009, **114**, 493–494.
75 F. Wolff, B. Bailly, B. Gulbis and F. Cotton, *Clin. Chim. Acta*, 2014, **430**, 20–21.
76 L. Ferbo, P. M. Manzini, S. Badar, N. Campostrini, A. Ferrarini, M. Delledonne, T. Francisci, V. Tassi, A. Valfrè, A. M. Dall'Omo, S. D'Antico, D. Girelli, A. Roetto and M. De Gobbi, *Blood Transfus.*, 2016, **14**, 531–534.
77 O. Myhre, H. Utkilen, N. Duale, G. Brunborg and T. Hofer, *Oxid. Med. Cell. Longevity*, 2013, **2013**, 1–19.
78 R. C. McCarthy and D. J. Kosman, *PLoS One*, 2014, **9**, e89003.
79 A. A. Raha, R. A. Vaishnav, R. P. Friedland, A. Bomford and R. Raha-Chowdhury, *Acta Neuropathol. Commun.*, 2013, **1**, 55.
80 T. Hofer and G. Perry, *J. Trace Elem. Med. Biol.*, 2016, **38**, 1–9.
81 J. A. Duce, A. Tsatsanis, M. A. Cater, S. A. James, E. Robb, K. Wikhe, S. L. Leong, K. Perez, T. Johanssen, M. A. Greenough, H.-H. Cho, D. Galatis, R. D. Moir, C. L. Masters, C. McLean, R. E. Tanzi, R. Cappai, K. J. Barnham, G. D. Ciccotosto, J. T. Rogers and A. I. Bush, *Cell*, 2010, **142**, 857–867.
82 B. X. Wong, A. Tsatsanis, L. Q. Lim, P. A. Adlard, A. I. Bush and J. A. Duce, *PLoS One*, 2014, **9**, e114174.
83 D. J. Hare, E. P. Raven, B. R. Roberts, M. Bogeski, S. D. Portbury, C. A. McLean, C. L. Masters, J. R. Connor, A. I. Bush, P. J. Crouch and P. A. Doble, *Neuroimage*, 2016, **137**, 124–131.
84 D. J. Hare, S. Ayton, A. Bush and P. Lei, *Front. Aging Neurosci.*, 2013, **5**, a54.
85 D. J. Hare and K. L. Double, *Brain*, 2016, **139**, 1026–1035.
86 Q. Xu, A. G. Kanthasamy, H. Jin and M. B. Reddy, *J. Parkinson's Dis.*, 2016, **2016**, 1–7.
87 J. Gong, F. Du, Z. M. Qian, Q. Q. Luo, Y. Sheng, W. H. Yung, Y. X. Xu and Y. Ke, *Free Radical Biol. Med.*, 2016, **90**, 126–132.
88 D. Devos, C. Moreau, J. C. Devedjian, J. Kluza, M. Petrault, C. Laloux, A. Jonneaux, G. Ryckewaert, G. Garçon, N. Rouaix, A. Duhamel, P. Jissendi, K. Dujardin, F. Auger, L. Ravasi, L. Hopes, G. Grolez, W. Firdaus, B. Sablonnière, I. Strubi-Vuillaume, N. Zahr, A. Destée, J.-C. Corvol, D. Pöltl, M. Leist, C. Rose, L. Defebvre, P. Marchetti, Z. I. Cabantchik and R. Bordet, *Antioxid. Redox Signaling*, 2014, **21**, 195–210.
89 S. D. Portbury, D. J. Hare, C. Sgambelloni, D. I. Finkelstein and P. A. Adlard, *Metallomics*, 2016, **8**, 193–200.
90 S. D. Portbury, D. J. Hare, C. Sgambelloni, D. P. Bishop, D. I. Finkelstein, P. Doble and P. A. Adlard, *Metallomics*, 2017, **9**, 402–410.
91 J. Arezes and E. Nemeth, *Int. J. Lab. Hematol.*, 2015, **37**, 92–98.
92 J. J. C. Kroot, E. H. J. M. Kemna, S. S. Bansal, M. Busbridge, N. Campostrini, D. Girelli, R. C. Hider, V. Koliaraki, A. Mamalaki, G. Olbina, N. Tomosugi, C. Tselepis, D. G. Ward, T. Ganz, J. C. M. Hendriks and D. W. Swinkels, *Haematologica*, 2009, **94**, 1748–1752.
93 L. N. van der Vorm, J. C. M. Hendriks, C. M. Laarakkers, S. Klaver, A. E. Armitage, A. Bamberg, A. J. Geurts-Moespot, D. Girelli, M. Herkert, O. Itkonen, R. J. Konrad, N. Tomosugi,

M. Westerman, S. S. Bansal, N. Campostrini, H. Drakesmith, M. Fillet, G. Olbina, S.-R. Pasricha, K. R. Pitts, J. H. Sloan, F. Tagliaro, C. W. Weykamp and D. W. Swinkels, *Clin. Chem.*, 2016, **62**, 993–1001.
94 G. L. Myers and W. G. Miller, *eJIFCC*, 2016, **27**, 30–36.
95 T. Konz, M. Montes-Bayon and S. Vaulont, *Metallomics*, 2014, **6**, 1583–1590.
96 J. Malyszko, *Clin. J. Am. Soc. Nephrol.*, 2009, **4**, 1015–1016.
97 L. Cenci, E. Andreetto, A. Vestri, M. Bovi, M. Barozzi, E. Iacob, M. Busato, A. Castagna, D. Girelli and A. M. Bossi, *J. Nanobiotechnol.*, 2015, **13**, 51.
98 G. Dahlfors, P. Stål, E. C. Hansson, P. Bàràny, C. Sisowath, L. Onelöv, D. Nelson, G. Eggertsen, J. Marmur and S. Beshara, *Scand. J. Clin. Lab. Invest.*, 2015, **75**, 652–658.
99 C. Delaby, J. Vialaret, P. Bros, A. Gabelle, T. Lefebvre, H. Puy, C. Hirtz and S. Lehmann, *EuPa Open Proteomics*, 2014, **3**, 60–67.
100 T. Ganz, G. Olbina, D. Girelli, E. Nemeth and M. Westerman, *Blood*, 2008, **112**, 4292–4297.
101 C. Delaby, P. Bros, J. Vialaret, A. Moulinier, V. Delatour, A. Gabelle, S. Lehmann and C. Hirtz, *Bioanalysis*, 2017, **9**, 337–347.
102 N. Grebenchtchikov, A. J. Geurts-Moespot, S. Trentmann, N. Andersen, R. S. B. Aiba, A. Allersdorfer, C. M. Laarakkers, F. C. Sweep, H. Tjalsma, A. M. Hohlbaum and D. W. Swinkels, *Clin. Chem.*, 2014, **60**, 897–899.
103 P. Gutschow, P. J. Schmidt, H. Han, V. Ostland, T. B. Bartnikas, M. A. Pettiglio, C. Herrera, J. S. Butler, E. Nemeth, T. Ganz, M. D. Fleming and M. Westerman, *Haematologica*, 2015, **100**, 167–177.
104 P. Gutschow, K. Westerman, H. Han, V. Ostland, G. Olbina, W. Paxton and M. Westerman, *Blood*, 2016, **128**, 2456.
105 T. Lefebvre, N. Dessendier, D. Houamel, N. Ialy-Radio, C. Kannengiesser, H. Manceau, C. Beaumont, G. Nicolas, L. Gouya, H. Puy and Z. Karim, *Clin. Chem. Lab. Med.*, 2015, **53**, 1557–1567.
106 S. Scarano, A. Vestri, M. L. Ermini and M. Minunni, in *Lecture Notes in Electrical Engineering – Sensors: Proceedings of the First National Conference on Sensors, Rome 15–17 February, 2012*, ed. F. Baldini, A. D'Amico, C. Di Natale, P. Siciliano, R. Seeber, L. De Stefano, R. Bizzarri and B. Andò, Springer, New York, 2014, vol. 162, pp. 95–98.
107 B. Uelker, I. Cushman, T. Dudek, D. M. Herkert and D. C. E. Geacintov, *Blood*, 2016, **128**, 4820.
108 J. Wang, G. Liu, Z. Xu, J. Dai, P. Song, J. Shi, Y. Hu, Z. Hu, G. Nie, Y. Z. Chang and Y. Zhao, *Nanomedicine*, 2015, **11**, 871–878.
109 R. T. Means Jr, *Eur. J. Haematol.*, 2015, **94**, 2–3.
110 C. Thomas, U. Kobold, S. Balan, R. Roeddiger and L. Thomas, *Int. J. Lab. Hematol.*, 2010, **33**, 187–193.
111 D. Enko, H. Wagner, G. Kriegshäuser, C. Kimbacher, R. Stolba, E. Worf and G. Halwachs Baumann, *Eur. J. Haematol.*, 2015, **95**, 507–513.
112 T. Moos, T. R. Nielsen, T. Skjørringe and E. H. Morgan, *J. Neurochem.*, 2007, **103**, 1730–1740.
113 G. O. Latunde-Dada and R. J. Simpson, in *Iron Deficiency and Overload*, ed. S. Yehuda and D. I. Mostofsky, Humana Press, Totowa, 2009, pp. 31–49.
114 R. S. Ohgami, D. R. Campagna, E. L. Greer, B. Antiochos, A. McDonald, J. Chen, J. J. Sharp, Y. Fujiwara, J. E. Barker and M. D. Fleming, *Nat. Genet.*, 2005, **37**, 1264–1269.
115 G. Bergamaschi, A. Di Sabatino, A. Pasini, C. Ubezio, F. Costanzo, D. Grataroli, M. Masotti, C. Alvisi and G. R. Corazza, *Clin. Nutr.*, 2016, DOI: 10.1016/j.clnu.2016.09.021.
116 R. D. Baker, F. R. Greer and The Committee on Nutrition, *Pediatrics*, 2010, **126**, 1040–1050.
117 K. Honarmand Ebrahimi, P.-L. Hagedoorn and W. R. Hagen, *Chem. Rev.*, 2015, **115**, 295–326.
118 T. Konz, E. Añón-Alvarez, M. Montes-Bayón and A. Sanz-Medel, *Anal. Chem.*, 2013, **85**, 8334–8340.
119 D. B. Kell and E. Pretorius, *Metallomics*, 2014, **6**, 748–773.
120 D. J. Hare, J. D. Doecke, N. G. Faux, A. Rembach, I. Volitakis, C. J. Fowler, R. Grimm, P. A. Doble, R. A. Cherny, C. L. Masters, A. I. Bush and B. R. Roberts, *ACS Chem. Neurosci.*, 2015, **6**, 398–402.
121 J. Guillemot, M. Canuel, R. Essalmani, A. Prat and N. G. Seidah, *Hepatology*, 2013, **57**, 2514–2524.
122 K. Harms and T. Kaiser, *Best Pract. Res., Clin. Endocrinol. Metab.*, 2015, **29**, 799–810.
123 K. Dassler, M. Zydek, K. Wandzik, M. Kaup and H. Fuchs, *J. Biol. Chem.*, 2006, **281**, 3297–3304.
124 *Iron deficiency anaemia: assessment, prevention and control. A guide for programme managers*, World Health Organization, United Nations Children's Fund, United Nations University, Geneva, 2001.
125 M. B. Zimmermann and R. F. Hurrell, *Lancet*, 2007, **370**, 511–520.
126 R. I. Weed, C. F. Reed and G. Berg, *J. Clin. Invest.*, 1963, **42**, 581–588.
127 A. E. Mast, M. A. Blinder, Q. Lu, S. Flax and D. J. Dietzen, *Blood*, 2002, **99**, 1489–1491.
128 A. M. Prentice, C. P. Doherty, S. A. Abrams, S. E. Cox, S. H. Atkinson, H. Verhoef, A. E. Armitage and H. Drakesmith, *Blood*, 2012, **119**, 1922–1928.
129 T. Ganz, *Clin. Chem.*, 2015, **61**, 577–578.
130 A. Bah, R. Wegmuller, C. Cerami, L. Kendall, S.-R. Pasricha, S. E. Moore and A. M. Prentice, *BMC Pregnancy Childbirth*, 2016, **16**, 157.
131 N. Leuenberger, E. Bulla, O. Salamin, R. Nicoli, N. Robinson, N. Baume and M. Saugy, *Drug Test. Anal.*, 2016, DOI: 10.1002/dta.2122.
132 W. Guo, E. Bachman, M. Li, C. N. Roy, J. Blusztajn, S. Wong, S. Y. Chan, C. Serra, R. Jasuja, T. G. Travison, M. U. Muckenthaler, E. Nemeth and S. Bhasin, *Aging Cell*, 2013, **12**, 280–291.
133 R. Orlandi, M. De Bortoli, C. M. Ciniselli, E. Vaghi, D. Caccia, V. Garrisi, S. Pizzamiglio, S. Veneroni, C. Bonini, R. Agresti, M. G. Daidone, D. Morelli, C. Camaschella, P. Verderio and I. Bongarzone, *Ann. Oncol.*, 2014, **25**, 352–357.
134 T. C. H. Tan, D. H. G. Crawford, M. E. Franklin, L. A. Jaskowski, G. A. Macdonald, J. R. Jonsson, M. J. Watson, P. J. Taylor and L. M. Fletcher, *Liver Int.*, 2012, **32**, 1391–1399.
135 J. Zaritsky, B. Young, H.-J. Wang, M. Westerman, G. Olbina, E. Nemeth, T. Ganz, S. Rivera, A. R. Nissenson and I. B. Salusky, *Clin. J. Am. Soc. Nephrol.*, 2009, **4**, 1051–1056.

136 L. Mercadel, M. Metzger, J. P. Haymann, E. Thervet, J.-J. Boffa, M. Flamant, F. Vrtovsnik, P. Houillier, M. Froissart and B. Stengel, *PLoS One*, 2014, **9**, e99781.
137 M. Andrews, N. Soto and M. Arredondo-Olguín, *Nutrition*, 2015, **31**, 51–57.
138 M. M. Yalcin, A. E. Altinova, M. Akturk, O. Gulbahar, E. Arslan, D. Ors Sendogan, I. Yetkin and F. B. Toruner, *J. Diabetes Res.*, 2016, **2016**, 1240843.
139 E. Fung and E. Nemeth, *Haematologica*, 2013, **98**, 1667–1676.
140 Z. I. Cabantchik, A. Munnich, M. B. Youdim and D. Devos, *Front. Pharmacol.*, 2015, **4**, a167.
141 P. B. Yu, C. C. Hong, C. Sachidanandan, J. L. Babitt, D. Y. Deng, S. A. Hoyng, H. Y. Lin, K. D. Bloch and R. T. Peterson, *Nat. Chem. Biol.*, 2007, **4**, 33–41.
142 J. Bacchetta, J. J. Zaritsky, J. L. Sea, R. F. Chun, T. S. Lisse, K. Zavala, A. Nayak, K. Wesseling-Perry, M. Westerman, B. W. Hollis, I. B. Salusky and M. Hewison, *J. Am. Soc. Nephrol.*, 2014, **25**, 564–572.
143 E. M. Smith, J. A. Alvarez, M. D. Kearns, L. Hao, J. H. Sloan, R. J. Konrad, T. R. Ziegler, S. M. Zughaier and V. Tangpricha, *Clin. Nutr.*, 2016, DOI: 10.1016/j.clnu.2016.06.015.
144 S. M. Zughaier, J. A. Alvarez, J. H. Sloan, R. J. Konrad and V. Tangpricha, *J. Clin. Transl. Endocrinol.*, 2014, **1**, 19–25.
145 N. Kitsati, D. Liakos, E. Ermeidi, M. D. Mantzaris, S. Vasakos, E. Kyratzopoulou, P. Eliadis, E. Andrikos, E. Kokkolou, G. Sferopoulos, A. Mamalaki, K. Siamopoulos and D. Galaris, *Haematologica*, 2015, **100**, e80–e83.
146 S. J. Wilkins, D. M. Frazer, K. N. Millard, G. D. McLaren and G. J. Anderson, *Blood*, 2006, **107**, 1659–1664.

