Dynamics of Iron Homeostasis in Health and Disease: Molecular Mechanisms and Methods for Iron Determination

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Abstract Iron is a versatile trace metal, indispensable for the survival of all living organisms. Despite its crucial role in vital biological processes, exceeded iron levels can be harmful for cellular and organismal homeostasis, due to iron's involvement in the generation of toxic hydroxyl radicals. As such, maintaining balanced iron levels is highly required in order for the organisms to avoid iron toxicity and at the same time preserve iron-dependent processes. This is achieved by the tight coordination of intricate systemic, cellular and subcellular mechanisms for iron absorption, excretion, utilization and storage. Those mechanisms decline during ageing, as well as in multiple human pathologies, leading to iron overload or deprivation, and eventually to death. To gain insight into how perturbations in iron homeostasis lead to disease, it is of great importance to use efficient methods for iron detection in distinct biological samples. Towards this direction, several biochemical and biophysical methods have been developed for the determination of iron and iron-containing compounds.

1 Introduction

Iron is one of the most important trace metals indispensable for life. Primarily found in the form of heme or iron-sulfur clusters (ISCs), it is highly required for numerous cellular and systemic biological processes. DNA replication and repair [1, 2], oxidative phosphorylation [3], and oxygen transport [4], are only some of the iron-dependent processes that highlight its crucial role in maintaining cellular and organismal homeostasis.

Iron's wide versatility mainly lies in its ability to easily cycle between two oxidation states: ferrus (Fe^{2+}) and ferric (Fe^{3+}) forms. Owing to this redox-state ability, excess iron can lead to the formation of hydroxyl radicals ($^{\bullet}OH$), through hydro-

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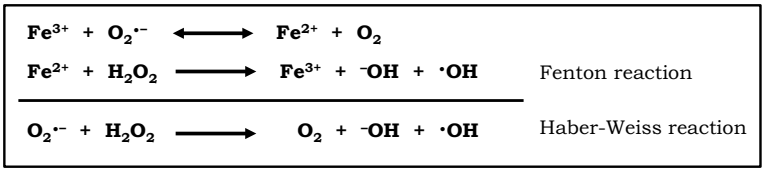

Fig. 1 Fenton and Haber-Weiss reactions that result in the generation of hydroxyl radicals in the presence of iron

gen peroxide (H_2O_2) reduction, a process widely known as Fenton reaction (Fig. 1) [5]. The resulted hydroxyl radicals are highly harmful, since they can cause severe oxidative damage and disruption of cellular constituents (including proteins, lipids and DNA), eventually leading to cell death. On the other hand, iron deprivation is also detrimental for life, since vital iron-dependent processes (i.e. oxygen distribution in tissues, cell proliferation and energy production) are perturbed. As such, maintaining balanced iron levels is of great importance, in order for the organisms to circumvent iron toxicity and at the same time ensure the function of significant iron-dependent processes. This is achieved by the existence of delicate mechanisms for the regulation of iron trafficking in tissues, cells and subcellular compartments. Particularly, there have been characterized mechanisms for iron sensing (Iron regulatory proteins—IRPs, hepcidin), acquisition (transferrins, divalent metal transporters, mitoferrins) excretion (ferroportin, multicopper oxidases) utilization (factors implicated in ISC and heme biogenesis), as well as storage (ferritins) [6, 7]. These molecular pathways have been found to be significantly dysregulated during ageing, and in various pathological conditions, including cancer, neurodegenerative and cardiovascular diseases [8]. In order to understand the exact molecular mechanisms through which iron dyshomeostasis leads to ageing and disease, it is critical to employ efficient techniques to determine iron quantification and distribution, as well as to assess its oxidative status in biological samples.

In this chapter we are going to analyze the mechanisms that govern iron homeostasis and discuss about how perturbations in these pathways lead to ageing and disease in humans. Finally, we are going to give an overview of the most common biochemical and biophysical methods, used to gain information concerning abundance, oxidation state and distribution of iron and iron-containing molecules.

2 Systemic Iron Homeostasis

The average amount of total iron in adults under physiological conditions is approximately 4 ± 1 g [9]. Iron absorption, storage, utilization and excretion need to be tightly regulated for the maintenance of optimal iron levels. So far, it is unclear whether specific mechanisms are required for iron excretion. Iron is mostly lost in uncontrolled ways and mainly through blood loss, menstruation, shedding from epithelial cells

and sweat. Thus, in order to avoid perturbation of systemic iron homeostasis, processes controlling intestinal iron uptake and recycling of existing iron pools need to be well orchestrated.

As expected, differences in total levels as well as in the homeostatic mechanisms, exist between infants, children, adult male, pregnant, pre- and post-menopausal women, aged individuals and centenarians [10–13]. Even under physiological conditions, it is imperative for the body to respond fast according to iron demands. In embryos, optimal iron concentration is required for normal development. Iron reaches the fetus through the placenta; however, how iron reaches the fetal blood remain largely unknown [14]. After birth, dietary iron absorption by the enterocyte is required to maintain physiological iron levels [15]. Infants are born with approximately 270 mg of iron. Initially and during development, the demand of iron is high and is subsequently stabilized at around 1 mg/day for men and 2 mg/day for women, reflecting the higher iron amounts required due to menstrual bleeding and pregnancy [9, 15].

The only way to compensate iron loss and high iron demands is through diet. Dietary iron is classified as heme (or haem) and non-heme iron. Heme is more bioavailable and highly abundant in meat and seafood. Free heme is not present in circulation since it is cytotoxic and its acquisition must be tightly regulated. Yet, the specific pathways involved for heme iron uptake remain enigmatic. Heme is released from the hemoproteins in the acidic environment of the stomach with the assistance of proteolytic enzymes and iron is released from heme in enterocytes, in a process that requires heme oxygenase, an enzyme that catalyzes heme degradation [15, 16]. However, how cells internalize heme iron is less well understood. Heme carrier protein 1 (HCP1) and heme responsive gene-1 (HRG-1) were initially thought to play important role in heme uptake, however HCP1 is believed to be primarily a folate transporter and only involved in low-affinity heme-Fe uptake [17]. HRG (heme responsive-gene) proteins have been proposed to participate in heme homeostasis [18–20]. *C. elegans* are natural heme auxotrophs and thus, they acquire heme from their environment. Taking advantage of this unique characteristic, heme trafficking can be studied without the mechanisms of heme biogenesis to interfere. In an evolutionary conserved mechanism, in *C. elegans*, HRG-4 mediates heme uptake at the plasma membrane, whereas HRG-1 has been proposed to transport heme into the cytosol of macrophages via the endocytosis pathway [18, 19]. In a more recent study, an inter-tissue mechanism of heme regulation was investigated [20]. Intestinal HRG-7 functions as a secreted signaling factor during heme starvation in extra-intestinal tissues. More interestingly, with the involvement of Smad transcription factor 9 (SMA-9), HRG-7 levels are repressed via a neuron-derived DBL-1-dependent signal (member of the transforming growth factor beta (TGF-beta) superfamily), suggesting that an analogous cell non-autonomous evolutionary conserved mechanism may exist for systemic heme iron homeostasis. Despite the recent progress in the field, further investigation is required to fully understand heme regulation and trafficking.

Non-heme iron refers to the rest inorganic iron, deriving mainly from plants. It is mainly found in its oxidized less soluble (ferric) form and its bioavailability

further declines in the presence of calcium and plant-derived compounds such as tannins, phenols and phytates. On the other side, ascorbic acid (vitamin C), vitamin A and citric acid may increase non-heme iron absorption [21]. Non-heme iron is also taken up on the apical brush border membrane of enterocytes in the small intestine, the duodenum and proximal jejunum via a distinct pathway. Dietary non-heme iron is initially reduced by reductases such as duodenal cytochrome B reductase to its ferrous form. Divalent metal transporter 1 (DMT1) at the apical membrane of intestinal epithelial cells is then responsible for ferrous iron acquisition, which is then stored within the iron-storage protein ferritin until needed, or exported through the basolateral epithelial membrane via ferroportin-1 (FPN1) into the blood stream [9, 15, 22, 23]. In cooperation with FPN1, hephaestin, which converts ferrous into ferric ions, enhances iron efflux. Then, plasma transferrin delivers iron to cells of all tissues for utilization [9]. Hepcidin a hepatocyte-secreted peptide hormone is considered the main regulator of systemic iron homeostasis. Hepcidin directly interacts with ferroportin, driving its internalization and degradation [24]. These events lead to decreased export of cellular iron and finally perturbed whole body iron homeostasis with implication in various iron-related diseases. Thus, current studies are focusing in the identification of novel hepcidin regulators as potential therapeutic targets of iron related diseases [25–28].

An underappreciated parameter that might play crucial role in iron uptake and whole body iron homeostasis is the existence of microbiota and most importantly of gut microflora, where iron acquisition happens. Iron is an essential mineral for pathogenic and non-pathogenic microbes. The role of iron in infection and immunity has been long appreciated and iron-withholding defense mechanisms exist to curtail iron as an innate immune mechanism against invading pathogens [29–32]. Microbes practically hijack iron from the host organism. Novel pharmacological approaches target iron acquisition from microbes to treat infectious diseases. On the other hand, the importance of symbiosis is increasingly appreciated. Interactions between host and microbiota may explain several phenomena that have so far eluded understanding. The importance of iron in bacterial growth and microflora composition under normal or pathological conditions is long acknowledged. However, how gut microflora may influence iron homeostasis is poorly studied. In a recent study, new aspects of the crosstalk between the microbiota and the intestinal epithelium in respect to iron sensing were revealed [33]. Gut microbiota altered the iron-related protein signature of mice, although they did not affect systemic iron load. Furthermore as discussed above, several proteins during various steps are required for efficient iron uptake. For instance, non-heme iron is first reduced to its ferrous form. Since, microbes contain evolutionary conserved mechanisms for iron homeostasis such as microbial ferric reductases, someone could assume that microbiota could provide important metabolically active material for more efficient iron uptake. Fungi, such as *Saccharomyces cerevisiae*, *Candida glabrata* and *Candida albicans* express cell surface ferric reductases and are capable of utilize iron from most ferric-containing molecules, including ferritin and transferrin [32]. Further investigation is required on how host-microflora interactions may influence iron homeostasis, with potential to provide answers to the so far unanswered questions.

3 Cellular Iron Homeostasis

In addition to the systemic control of iron abundance, cells have developed sophisticated mechanisms to maintain intracellular iron homeostasis (Fig. 2).

3.1 Cellular Iron Uptake and Utilization

The majority of mammalian cells incorporate blood-derived non-heme iron by using the Transferrin (Tf)-Transferrin Receptor (TfR) system. Each Tf molecule has the capacity to bind two ferric ions with high affinity. The binding of ferric ions to Tf is regulated by various factors, including temperature, pH and the presence of anionic species, like chloride [34–36]. Diferric Tf can be recognized by the specific receptor TfR1, which forms homodimers in the cell membrane of almost all mammalian cells. TfR2 is another TfR, which is predominantly expressed in hepatocytes and has a significantly lower affinity to diferric Tf [37, 38]. The entire Tf-TfR1 complex is subsequently internalized in cells, using the endocytotic machinery [39]. Importantly, the myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCKα) participates in the recruitment of the actin cytoskeleton, which supports endocytosis [40]. The endosomal acidic environment, generated by a proton pump, promotes the

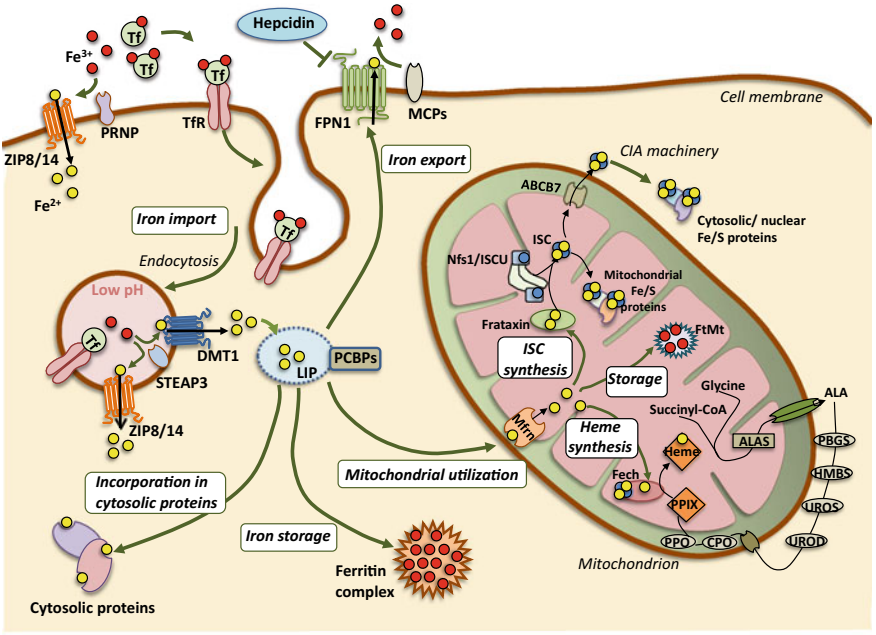

Fig. 2 Cellular mechanisms for iron homeostasis

dissociation of ferric ions from Tf, thus facilitating the recycling of apo-Tf back to the cell membrane. In addition to low pH, iron release from Tf is suggested to be enhanced by the reduction of ferric to ferrous ions. This process is mediated by six-transmembrane epithelial antigen of the prostate 3 (STEAP3), a ferrireductase resided at the endosomal membrane. Once reduced, ferrous ions are transported through DMT1 to the cytosol [41, 42].

Cells can also take up non-transferrin-bound iron (NTBI) from plasma. ZRT/IRT-like protein 8 (ZIP8) and ZIP14, members of the solute carrier 39 (SLC39) family can directly mediate the intracellular transport of plasma NTBI [43–45]. The prion protein (PRNP), localized in the cellular membrane is suggested to enhance the ZIP14-mediated NTBI transport due to its ferrireductase activity [46]. Apart from its role in NTBI transport, ZIP14 has also been implicated in transporting endosomal ferrous iron into the cytosol [47]. Although DMT1 and ZIP proteins seem to have similar roles in ferrous transport, their activity is highly pH-dependent. While ZIP8 and ZIP14 display maximal iron transport capacity at neutral pH [45, 48], DMT1 is more active in acidic environments (pH 5.5) [49]. This supports the hypothesis that ZIP8/14 may be the main NTBI transporters in the cell surface, as they directly communicate with the neutral pH-plasma, while DMT1 may be the main ferrous transporter in the acidic endosomes.

Cytosolic ferrous iron, released either by ZIP8/14 or DMT1 transporters, enters a low-molecular weight and chelatable labile iron pool (LIP). Due to the high redox activity of the ferrous state and the oxidative environment of the cytosol, LIP-derived iron is almost directly sequestered into cytosolic proteins, or delivered to mitochondria to support crucial iron-dependent processes. Cytosolic iron that is not utilized for iron-dependent processes can be stored into ferritin molecules. Their ferrireductase activity renders ferritins important factors of the cellular anti-oxidant response, as they transform the highly toxic ferrous ions into the less redox-active ferric form. Particularly, ferritins form shell-shaped 24-mers, comprised of ferritin light chain (FTL) and ferritin heavy chain (FTH) polypeptides. The composition of FTLs and FTHs in the 24mer may vary, and this variation modulates accordingly the iron-binding capacity of the entire complex. Intriguingly, each 24-mer can incorporate up to 4500 ferric ions [50]. Upon iron-depleted conditions, ferritin-incorporated iron is released to meet the cellular iron demands, through a process involving ferritinophagy, a selective type of autophagic degradation [51]. The remaining cytosolic ferrus iron that is neither stored nor utilized can be exported to the extracellular space through the hepcidin-regulated FPN1. Iron export is enhanced by the ferroxidase activity of multicopper-oxidases (MCPs). Although differentially expressed in human tissues, the MCPs ceruloplasmin, hephaestin and zyklopen are similarly involved in cytosolic iron export [52]. Members of the Poly-(rC)-binding protein (PCBP) seem to be important regulators for the fate of cytosolic ferrus iron, since they have been identified to act as iron chaperones [53]. Particularly, PCBP1 and PCBP2 have been involved in the metalation of iron-containing proteins [54, 55], but also in the delivery of ferrous ions to ferritins [56, 57] and FPN1 [58].

3.2 Regulation of Cellular Iron Homeostasis

The majority of the above-mentioned cellular mechanisms are under the tight control of the Iron Regulatory Protein (IRP) system. IRPs modulate the expression of iron-related genes to regulate iron import, export, storage and utilization. This is achieved by their ability to associate with specific stem-loop structures within the untranslated regions (UTRs) of the respective mRNAs, called iron-responsive elements (IREs). IRPs' binding to 5′-IREs blocks mRNA translation, whereas their binding to 3′-IREs induces it, through mRNA degradation blockage. As such, the expression of iron-related proteins is differentially regulated, depending on the IRE-type their mRNAs contain [59]. There have been identified two IRPs in mammals, IRP1 and IRP2, both of which have similar affinity to IRE-containing transcripts [60].

The RNA binding activity of IRPs is strongly dependent on the intracellular iron levels. Upon iron deprivation, the IRE-binding affinity of IRPs is highly induced leading to the translational suppression of the 5′IRE-containing mRNAs encoding for ferritins [61, 62] and FPN1 [22] and concurrently to the stabilization of the 3′IRE-containing mRNAs encoding for TfR1 [63], MRCKα [64] and DMT1 [65]. This homeostatic mechanism promotes iron uptake pathways and ensures that the remaining cytosolic iron will not be stored or exported, in order to be used for crucial iron-dependent processes. Under iron-replete conditions, IRPs' RNA-binding activity is strongly inhibited. When iron is intracellularly abundant, IRP1 is predominantly found assembled with a cubane type [4Fe–4S] of iron-sulfur cluster (ISC). The particular ISC assembly enables IRP1 to function as a cytosolic aconitase, and at the same time diminishes its affinity for IRE-containing transcripts [63]. A totally different inhibition mechanism has been characterized for IRP2, which lacks aconitase activity. Specifically, IRP2 is post-translationally regulated by an iron-dependent Ubiquitin proteasome mechanism. F-Box And Leucine Rich Repeat Protein 5 (FBXL5) is an enzyme 3 (E3) ubiquitin ligase, the activity of which depends on the formation of a [Fe–O–Fe] centre within its hemerythrin-binding domain. Upon iron-replete conditions, FBXL5 is activated, leading to the proteasomal degradation of its target IRP2 [66, 67]. Despite their similar RNA-binding affinity, several studies suggest that IRP2 responds better to varying iron concentrations, in comparison to IRP1 [68, 69]. This could be partly explained by the fact that the regulation of IRP2 is directly dependent on cytosolic iron and does not require ISC formation, like IRP1.

Another well characterized mechanism that orchestrates the expression of iron-related genes is the transcriptional regulation mediated by hypoxia inducible factors (HIFs) [70, 71]. The transcriptional activity of HIF1α and HIF2α is post-translationally regulated in an oxygen- and iron-dependent manner by specific hydroxylases, including prolyl-4-hydroxylase domain proteins (PHDs) [72] and the asparaginyl hydroxylase factor inhibiting HIF1 (FIH1) [73]. Upon iron- and oxygen-replete conditions, PHDs are fully functional and hydroxylate both HIF1α and HIF2α, leading to their proteasomal degradation. On the other hand, FIH1 directly inhibits HIFs' transcriptional activity by hydroxylating a conserved asparagine residue. Under hypoxic or iron-deprivation conditions, the function of both hydroxylases is

blocked, leading to the enhancement of HIFs' transcriptional activity. Genes encoding for Tf, TfR1, DMT1, FPN and CP, are among the HIF targets implicated in the regulation of iron homeostasis [70, 74]. In a very recent study, they identified endonuclease Regnase-1, as novel regulator for HIF- mediated regulation of iron uptake. Regnase-1 promotes HIF2α activation, by destabilizing PHD3 mRNA [75]. Interestingly HIF2α expression is negatively regulated by the IRP/IRE system, since its mRNA contains a 5′-IRE. This is suggested to be a negative-feedback mechanism to prevent hemoglobin production, upon conditions that both heme's building blocks (oxygen, and iron) are deprived [76].

Hepcidin, a peptide hormone mainly synthesized in hepatocytes, is also considered to be an important regulator of both systemic and cellular iron homeostasis. Hepcidin directly interacts with FPN and promotes its internalization and subsequent degradation, thus blocking iron export from hepatocytes, macrophages and intestinal cells. Hepcidin's expression seems to be mainly regulated by systemic iron status. Increased Tf-TfR signaling, resulting from high extracellular iron levels (in the plasma or in the intestine) promotes the transcriptional activation of Hepcidin through BMP signaling. Hepcidin is also positively regulated by inflammatory cytokines, including interleukin-6 [77, 78].

4 Mitochondrial Iron Homeostasis

Mitochondria have a pivotal role in the maintenance of cellular and systemic iron homeostasis (Fig. 2). They are the lone sites for heme biogenesis, a vital prosthetic group, highly required for oxygen transport and mitochondrial respiration [79]. Additionally, mitochondria are essential for the biogenesis of ISCs. ISCs serve as cofactors of numerous enzymes, participating in indispensable cellular processes, including DNA replication, DNA repair and mitochondrial respiration [80]. Although there has been identified a cytosolic ISC assembly machinery, disruption of the mitochondrial ISC generation pathway, strongly inhibits the maturation of cytosolic and nuclear ISC-binding proteins [81]. This highlights the importance of the mitochondrial involvement for cellular and organismal homeostasis.

4.1 Mitochondrial Iron Import

A significant portion of cytosolic iron is transported into mitochondrial matrix for the generation of both heme and ISCs. There have been proposed several mechanisms that could mediate cytosolic iron transfer into the mitochondrial intermembrane space (IMS) [82]. One of the first theories suggests that LIP-derived cytosolic iron can be directly imported into mitochondria in a membrane potential-dependent and an ATP-independent process [83]. A growing amount of evidence supports the existence of a "kiss-and-run" mechanism, in which Tf-containing endosomes get in

close proximity and fuse with the OMM, thus leading to the release of ferrus iron in the IMS [84, 85]. Alternatively, it has been suggested that cytosolic iron existed in a chelator-inaccessible state (likely bound in iron containing proteins) can be transferred across the OMM via protein-protein interactions with residual mitochondrial proteins. PCBPs, the cytosolic iron chaperones, could be involved in this process [53].

In all cases, the transport of IMS ferrous iron across the inner mitochondrial membrane (IMM) is mediated by mitoferrins (Mfrns), members of the mitochondrial solute carrier family. Mfrn2 is ubiquitously expressed in mammals, while the expression of its homolog Mfrn1 is restricted to hematopoietic tissues [86]. Mitochondrial iron import seems to be predominantly controlled by the post-translational regulation of Mfrns. Particularly, the protein levels of Mfrn1 (and not Mfrn2) are significantly increased in developing red blood cells, due to extension in the protein's half-life [87]. The increased stability of Mfrn1 is mediated by its physical interaction with ATP-binding cassette subfamily B member 10 (Abcb10), which is highly expressed during erythroid maturation. The specific interaction promotes mitochondrial iron uptake to support the increased demands for hemoglobin synthesis [88]. Concerning Mfrn2, there has not been reported any regulatory mechanism. Though, there must be a kind of regulation, since overexpression of Mfrn2 does not affect mitochondrial iron levels [87]. In addition to Mfrns, mitochondrial calcium uniporter is also involved in mitochondrial iron import [89, 90]. Once transported into the mitochondrial matrix, ferrous ions can be directly utilized for the biosynthesis of heme or ISCs, or they are stored for subsequent use.

4.2 Iron-Sulfur Cluster Biosynthesis

ISCs are essential cofactors, required for all living organisms, as they ensure the function of vital biological processes. These inorganic compounds consist of iron and sulfide ions, assembled in various combinations, the most common of which are the rhomboid [2Fe–2S] and the cubane [4Fe–4S] forms. Although it was initially believed that ISCs could be spontaneously formed in vivo, their biogenesis, as well as the maturation of ISC-containing proteins involve tightly regulated multistep processes, which are highly conserved from bacteria to yeast and humans [91].

In mammalian systems, as in almost all organisms, mitochondria are the major sites for ISC biogenesis. A critical initiation step for ISC formation is the abstraction of sulfur ions from cysteine. This is mediated by the homodimeric cysteine desulferase Nfs1 (Nitrogen Fixation 1 *S. Cerevisiae* Homolog), in complex with ISD11 which stabilizes and structurally supports Nfs1 activity [92]. The abstracted sulfur ions are subsequently transferred to the scaffold protein ISCU, which binds to each Nfs1 monomer. Frataxin, a mitochondrial matrix iron chaperone, likely donates ferrus ions into the ISCU complex, which serves as a platform for bringing ferrus and sulfur ions in close proximity [93]. Apart from its main role as a ferrus donor, frataxin enhances the reactivity of Nfs1 with cysteine's thiol side chains [94]. Ferrus

and sulfur ions react in a process involving ferredoxins and ferredoxin reductases, thus leading to the formation of [2Fe–2S] ISCs. The newly synthesized rhomboid ISCs are sequestered to a mitochondrial chaperone complex, comprising the Heat Shock Protein Family A (Hsp70) Member 9 (HSPA9) and the co-chaperone DnaJ Homolog Subfamily C Member 20 (HSC20). This chaperone/co-chaperone system delivers [2Fe–2S] either to recipient mitochondrial ISC-binding apo-proteins, or to specific carrier proteins, such as glutaredoxin 5 (Grx5), that mediate the downstream ISC trafficking [95]. Particularly, Grx5 has been found to interact with and transfer ISCs to the ATP Binding Cassette Subfamily B Member 7 (ABCB7), an IMM transporter that mediates the export of ISCs to IMS [96]. Grx5 seems to be also involved in the formation of cubane [4Fe–4S] ISCs, by providing the rhomboid cluster to specific ISC proteins [97, 98].

In addition to the well-characterized mitochondrial pathway for ISC biogenesis, eukaryotic cells have developed a cytosolic ISC assembly (CIA) mechanism for the maturation of cytosolic and nuclear ISC-binding proteins [99]. Although there are cytosolic ISCU variants that could mediate de novo synthesis of ISCs in the cytosol, the CIA machinery requires intact mitochondrial ISC biogenesis [96, 100]. Remarkably, it has been suggested that a yet-unidentified ISC intermediate is exported to the cytosol in an ABCB7-dependent manner, in order to support CIA. Mutations in ABCB7 gene are the genetic cause of X-linked sideroblastic anemia with ataxia (XLSA/A), characterized by deficiency in the maturation of cytosolic ISC proteins, mitochondrial iron overload and defects in heme synthesis [81].

4.3 Heme Biosynthesis

Heme biogenesis is a multistep process, which involves eight sequential enzymatic reactions in both mitochondrial and cytosolic compartments [79]. The initial step is mediated by aminolevulinic acid synthase (ALAS), which catalyzes the condensation of glycine and succinyl-CoA to δ-aminolevulinic acid (ALA). The resulted ALA is exported to the cytoplasm through still not well-defined mechanisms. Although it was initially believed that ABCB10 is the transporter involved in ALA transport [101], recent studies rule out its involvement in this process and suggest that it is rather implicated in the regulation of hemoglobinization [102]. Cytosolic ALA is transformed to coproporphyrinogen III, through a 4-step enzymatic process, involving porphobilinogen synthase (PBGS), hydroxymethylbilane synthase (HMBS), uroporphyrinogen synthase (UROS) and uroporphyrinogen decarboxylase (UROD). The newly formed coproporphyrinogen III is transported to IMS, where it is converted to protoporphyrinogen IX by coproporphyrinogen oxidase (CPO). Protoporphyrinogen IX is then imported through ABCB6 to the mitochondrial matrix, and transformed by protoporphyrinogen oxidase (PPO) into protoporhyrin IX. Ferrochelatase (Fech) catalyzes the final step, by incorporating ferrous ions into protoporphyrin IX, leading to heme formation. Interestingly, Fech is an ISC-binding protein, highlighting the

interconnection between heme and ISC biogenesis. The specific interplay explains the anemic phenotypes observed upon perturbation of ISC biogenesis [103].

4.4 Mitochondrial Iron Storage

Given that mitochondria are the principal sites for ROS production, increased levels of ferrous iron in mitochondrial matrix could lead to the formation of harmful hydroxyl radicals through Fenton reaction. To counteract iron toxicity, mammalian tissues with high metabolic demands express mitochondrial ferritins (FtMts) [104, 105]. FtMts are highly similar to cytosolic FTHs. They form shell-shaped oligomers in which they sequester ferrus ions and keep them in the less-redox active ferric form. Unlike cytosolic ferritins, FtMt expression is not regulated by the IRP/IRE system [106]. It was recently shown that its expression is controlled in the transcriptional level through a complex regulatory mechanism, involving multiple transcription factors and epigenetic changes [107, 108]. Interestingly, overexpression of FtMt in human lung cancer cell lines leads to a dose-dependent cytosolic iron depletion and concurrent iron overload in mitochondria [109].

Frataxin, the ferrous donor for the biosynthesis of ISCs is also suggested to act as an iron storage protein. Its yeast homolog Yfh1 is capable of forming 48-mers in which up to 2000 iron atoms can be stored [110]. The absence of frataxin multimers in mammalian systems could be explained by the redundant function of mitochondrial ferritins, which are not expressed in yeast [111]. In any case, independently of its capability to form multimers, frataxin can be classified as an iron storage protein, since it sequesters the redox-active ferrus ions in the matrix. Similar to Frataxin, Fech, the ferrous donor for heme biosynthesis can be also considered as an iron storage protein.

5 Iron Dyshomeostasis in Pathological Conditions and Ageing

Perturbations of systemic, cellular and subcellular iron levels can lead to life-threatening pathologies (summarized in Table 1) [80, 112–114]. Interestingly, iron dyshomeostasis is associated with ageing, the main risk factor for various diseases while iron accumulation appears to be a common feature during ageing [115–117]. However, how and whether loss of iron homeostasis influences normal ageing is not well known. Recent evidence shows that abundance of iron shortens the lifespan of animal models [117–120]. Knocking-down MCO-1, a *Drosophila melanogaster* ferroxidase, decreases iron content and protects from iron-induced toxicity [118]. Those flies live significantly longer compared to wild type animals in iron excess. Research on *C. elegans* has shown that iron influences longevity. Loss of ferritin

leads to increased labile iron pool, ROS formation and rapid ageing [117]. Frataxin lesion initiates a pro-longevity iron starvation response in a mechanism that appears to involve induction of an evolutionary conserved pathway for mitophagy induction [119]. Thus, it is reasonable to hypothesize a direct and bidirectional association between iron metabolism and ageing. The mechanisms of iron accumulation during ageing and their involvement in the pathogenesis of various age-related disorders are only beginning to be elucidated. Deeper understanding of the underlying mechanisms that control iron homeostasis is expected to provide novel strategies against age-associated diseases and promote healthy ageing. Below, we mention only some of the diseases associated to iron dyshomeostasis.

5.1 Friedrich's Ataxia

Iron is required for normal brain function, but its accumulation has been observed in various diseases of the brain. Friedrich's ataxia (FA) is one of the best-studied neurological disorders of iron metabolism [121]. FA is an autosomal recessive inherited disease that causes progressive degeneration of sensory neurons in the dorsal root ganglia and symptoms begin to emerge early in life. Patients suffer from impaired muscle coordination (ataxia) and cardiomyopathy among others, but cognition is not affected. FA is characterized by iron accumulation in mitochondria due to dysfunction of iron-sulphur cluster biogenesis, caused by mutations in the frataxin gene [113, 122]. The majority of patients carry an unstable GAA repeat expansion that markedly reduces the expression of frataxin, whereas fewer patients have point mutations. Experimental models suggest that frataxin plays important roles in iron-dependent increase of ROS that affect Fe–S cluster proteins, in mitochondrial energy conversion and oxidative phosphorylation, highlighting the role of oxidative stress and normal mitochondrial function in FA [123]. Furthermore, a recent study has demonstrated that frataxin deficiency causes mitochondrial biogenesis defect in vivo, in vitro and in whole blood of patients [124]. Intriguingly, iron supplementation limits cardiac hypertrophy in mouse models [125]. Current understanding of the involved mechanisms allowed the assessment of genetic and cell-based treatment of FA. Ongoing approaches include increasing cellular frataxin levels and activity, improving mitochondrial function and decreasing oxidative stress, however no successful treatment is available owing to the complexity and inadequate understanding of ISC biogenesis and trafficking [126].

5.2 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease of the central nervous system that affects predominantly dopaminergic neurons in the substantia nigra [127]. It is a progressive disorder that affects movement and early signs include tremors and trem-

Table 1 Pathologies associated with iron dyshomeostasis

Human disease	Involved gene	Encoded protein	Cellular localization	Relevant protein function	Relevance to iron homeostasis	References
Friedrich's ataxia	FXN/FRDA	Frataxin	Mitochondrial matrix (mMatrix)	Assembly of Fe–S cluster proteins	ISC biogenesis	[121–124]
Congenital sideroblastic anemias (CSAs):						
X-linked sideroblastic anemia (XLSA)	ALAS2	5′-Aminolevulinate synthase 2	Mitochondrial matrix	Generation of 5-aminolevulinic acid	Heme synthesis	[80]
XLSA with cerebellar ataxia	ABCB7	ATP binding cassette subfamily B member 7	Inner mitochondrial membrane (IMM)	Transporter	ISC biogenesis	[145–148]
Other types of CSAs	SLC25A38	Solute carrier family 25 member 38	IMM	Regulates mitochondrial glycine	Heme synthesis	[142]
	NDUFB11	NADH:Ubiquinone oxidoreductase subunit B11	mMatrix	Transfers e from NADH to Q10	Unclear	[143, 144]
	GLRX5	Glutaredoxin-related protein 5	mMatrix	Protein lipoylation	ISC and heme	[151, 152]
	ATP6	ATP synthase Fo subunit 6	mMatrix	ATP synthesis	Unclear	[153]
	TRNT1	tRNA-nucleotidyltransferase 1	mMatrix	tRNA Nucleotidyltransferase	Unclear	[155]

(continued)

Table 1 (continued)

Human disease	Involved gene	Encoded protein	Cellular localization	Relevant protein function	Relevance to iron homeostasis	References
	LARS2	Leucyl-tRNA synthetase	mMatrix	L-leucine to tRNA(Leu) catalysis	Unclear	[154]
	HSPA9	Mitochondrial 70 kDa heat shock protein	mMatrix	Chaperone	Unclear	[156]
Multiple respiratory chain complexes deficiency	LYRM4	ISD11	mMatrix	Forms a complex with the cysteine desulfurase NFS1	ISC biogenesis	[157]
Hereditary myopathy with lactic acidosis	ISCU	Iron-Sulfur cluster assembly enzyme	mMatrix	Scaffold protein for ISC assembly	ISC biogenesis	[158–160]
Multiple mitochondrial dysfunctions syndrome	NFU1	NFU1 iron–sulfur cluster scaffold	mMatrix	Scaffold proteins for ISC assembly	ISC biogenesis	[161, 162]
	BOLA3	BolA family member 3	mMatrix			

(continued)

Table 1 (continued)

Human disease	Involved gene	Encoded protein	Cellular localization	Relevant protein function	Relevance to iron homeostasis	References
Complex I deficiency	NUBPL	Nucleotide binding protein like	mMatrix	Assembly of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I)	ISC biogenesis	[163]
Wolfram syndrome type 2	CISD2	NAF-1/Miner 1	Outer mitochondrial membrane (OMM)	Fe–S cluster transport between mitochondria and cytosol (?)	ISC traffick-ing(?)	[164–166]
Parkinson's disease	SNCA	Alpha-synuclein	Cytoplasmic, found at tips of neuronal cells	Inhibit iron transporter recycling	Abnormal brain iron accumula-tion	[128–130]
Alzheimer's disease	APP	Amyloid precursor protein	Trans golgi netowork (TGN)	Physically interact with iron	Iron accu-mulation in senile plaques, neurofibril-lary tangles	[134–139]
	MAPT	Tau proteins	Cytoplasmic	Physically interact with iron		
Diabetes	Adipokine genes	Adipokines		Systemic effects	Iron overload	[167–169]
Cardiovascular disease					Iron deficiency	[170–172]
Cancer	SLC40A1 HAMP TFRC STEAPs etc.	Ferroportin/SLC40A1 Hepcidin Transferrin receptor protein 1 STEAP metalloreductases etc.	Cell membrane Circulation Cell membrane Cytosolic	Iron export Regulates ferroportin Iron import Iron reduction	Low iron export and high import	[174, 176, 177, 179, 180]

bling. PD is also characterized by abnormal iron accumulation and mitochondrial defects, but its etiology remains enigmatic [112]. A hallmark of PD is the presence of Lewy bodies in the brain, formed by the abnormal accumulation of alpha-synuclein leading to brain cell death [127]. Interestingly, iron directly binds to alpha-synuclein, and accelerates a-synuclein aggregation, suggesting an important relevance to PD pathology [128, 129]. Inversely, alpha-synuclein dysregulates iron homeostasis by inhibiting SNX-3-retromer-mediated retrogate recycling of iron transporters [130]. Accumulating evidence suggests that macroautophagy plays an important role in dopaminergic neuron iron-dependent cell death [131, 132]. Overexpression of DMT1 in SH-SY5Y neuronal cells dramatically enhances ferrous uptake and promotes cell death. Chronic exposure to iron enhances oxidative stress and autophagy-induced cytotoxicity, but not apoptosis-induced cell death [132]. Whereas, in another setting, iron deposition increases alpha-synuclein levels and ROS generation and decreases cell viability by a mechanism that involves autophagy inhibition [131]. These seemingly opposite findings may suggest the importance of fine-tuning iron homeostasis, and that protective mechanisms may act as a double-edged sword according to intrinsic and extrinsic cues (high, prolonged stress versus low, acute stress).

5.3 Alzheimer's Disease

Alzheimer's disease (AD) is the most pervasive chronic neurodegenerative disease and its prevalence is expected to increase owing to population ageing [133]. It is characterized by progressive dementia and although pharmacological treatment may ameliorate some of the symptoms, there is currently no cure for AD. It has been identified as a protein folding disease, caused by accumulation of amyloid beta-containing extracellular plaques, and of neurofibrillary tangles made of abnormally folded tau protein in the brain. Interestingly, it has been demonstrated that senile plaques and neurofibrillary tangles contain elevated iron concentrations. However it remains unclear whether this accumulation is simply a repercussion of the disease or a significant causative factor, leading to neurodegeneration. Amyloid beta increases the levels of iron content and oxidative stress in neuronal cells overexpressing the Swedish mutant form of human β-amyloid precursor protein and in *C. elegans* [134]. The iron homeostasis disruption is probably associated with elevated expression of the iron transporter DMT1, but not TfR. Furthermore, iron has been found to associate with beta-amyloid and tau proteins and induce plaque and tangle formation [112, 135]. Iron overload stimulates amyloidogenic processing and alters neuronal signaling to increase amyloid beta plaque formation, leading to cognitive deterioration in transgenic mouse model of Alzheimer's disease [136]. In a *D. melanogaster* model of Alzheimer's disease, iron appears to induce ROS formation via Fenton chemistry and the resulted oxidative stress is essential for amyloid beta toxicity. Furthermore, treatment with clioquinol, an iron chelator, increases the lifespan of flies expressing amyloid beta [137]. Administration of iron in an Alzheimer's disease mouse model enhances APP cleavage and amyloid beta aggregate deposition, and impairs spatial

learning and memory. Chelation of iron can prevent iron-induced amyloidogenic APP processing and reverse behavioral deficits [138]. Chelation of iron by deferoxamine can inhibit iron-induced hippocampal tau hyperphosphorylation in transgenic animals of Alzheimer disease, via CDK5 and GSK3β pathways [139]. Tau-knockout mice develop age-dependent brain atrophy, neuronal loss with concomitant cognitive deficits [140]. Iron is also elevated in brain regions, mediating age-dependent neurodegeneration. Oral supplementation of the iron chelator clioquinol is able to reverse these changes. Thus, we can surmise the importance of iron homeostasis in AD progression. Iron chelators may represent potential therapeutic agents for AD development. Further understanding of the role of iron in AD pathology might lead to novel pharmacological interventions.

5.4 Congenital Sideroblastic Anemias

The congenital sideroblastic anemias (CSAs) are a diverse group of inherited disorders characterized by pathological mitochondrial iron accumulation of erythroid precursors [141]. In previous years, the genetic basis of several distinctive forms of CSA has been elucidated. A common denominator is their involvement in subcellular iron metabolism. Most of them are associated with impaired heme synthesis or iron-sulfur cluster biogenesis.

X-linked sideroblastic anemia (XLSA) is the most common type of CSA. It is a genetic disorder associated with heme synthesis, due to a mutation in the erythroid-specific aminolevulinic acid synthase 2 (ALAS2) gene. Heme synthesis takes place in the mitochondrion with ALAS2 enzyme playing an important role in the initial step for the generation of ALA. Deficiency in ALAS2 results in disordered heme synthesis, iron-loaded mitochondria and potential death from hemochromatosis [80]. Mutation in mitochondrial carrier family transporter solute carrier 25A38 (SLC25A38) causes another form of CSA similar to XLSA [142]. SLC25A38 is involved in making glycine available for heme synthesis in the mitochondria suggesting its involvement in heme synthesis.

Two recent studies revealed a novel form of CSA caused by a mutation (p.F93del) in the respiratory complex 1 protein NDUFB11 [143, 144]. NDUFB11 is essential for mitochondrial oxidative phosphorylation, but its involvement in iron homeostasis is unclear. The p.F93del mutation causes respiratory insufficiency and impairs erythroid proliferation, suggesting the mechanism of anemia [143].

X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A) is another type of CSA caused by mutations in the ABCB7 transporter [145–147]. ABCB7 is present in the inner mitochondrial membrane. Its direct role in heme synthesis in humans is not clear. Studies suggest that ABCB7 is associated with the maturation of Fe–S cluster containing proteins [80]. In mice, *Abcb7* is an essential gene, and participates in ISC biogenesis [148]. In *C. elegans*, depletion of ABCB7/ABTM-1 is lethal [149]. ABTM-1 lesion shows accumulation of ferric iron. Intriguingly, silencing of *abtm-1* induces developmental arrest and apoptosis and increases oxidative stress and

lifespan. The paradoxical effects on lifespan may be attributed to the concept of hormesis, a phenomenon whereby favorable outcomes occur in response to low stress [150].

Mutation in the splice donor site of intron 1 of the mitochondrial glutaredoxin 5 (GLRX5) is the cause of another type of sideroplastic anemia [151, 152]. GLRX5 is required for ISC biogenesis and has an important role in erythropoiesis. More importantly, it was shown that deficiency of GLRX5 decreased proteins associated with heme, such as ferrochelatase, highlighting the tight association between heme and ISC synthesis, the two mitochondrial iron processing pathways that are merely thought to be independent [152].

Other mutations in various genes have been reported to be associated with sideroblastic anemia. These include mutations in the mitochondrial encoded ATP6 gene, the mitochondrial leucyl-tRNA synthetase LARS2 encoding gene, TRNT1 gene, which encodes a template-independent RNA polymerase for the maturation of cytosolic and mitochondrial tRNAs and the gene encoding for mitochondrial heat shock protein HSPA9 [153–156].

5.5 Human Diseases Related to Gene Mutations Associated to ISC Biogenesis

A homozygous mutation in LYRM4, encoding ISC biogenesis factor ISD11, was found to cause multiple respiratory chain complexes deficiency [157]. ISD11 forms a complex with the cysteine desulfurase NFS1. NFS1 is also important for the supply of inorganic sulfur for ISC biogenesis and its activity is lost in ISD1-deficient cells. Thus, the early step of ISC assembly is compromised in those patients. An intron mutation in ISCU gene, which results in a splicing defect, affecting both cytosolic and mitochondrial splice variants, causes hereditary myopathy with lactic acidosis [158–160]. Fatal mitochondrial diseases are associated with mutations in ISC scaffold genes NFU1 (alternative to ISCU) and BOLA3 [161, 162]. Mutations in nucleotide-binding protein-like (NUBPL), a Fe–S cluster protein that plays a critical role in the assembly of the mitochondrial membrane respiratory chain NADH dehydrogenase, cause complex I deficiency [163]. Recently, NEET proteins were described as a new class of iron–sulphur proteins with potential role in iron transport between mitochondria and the cytosol [164]. The class I NEET proteins are encoded by two genes, CISD1 and CISD2. Mutations in CISD2 gene have been linked to Wolfram syndrome type 2 [165, 166]. Treatment with the iron chelator Deferiprone is currently under evaluation. Development of appropriate models for these diseases and deeper investigation of the respective pathways and their exact role in ISC biogenesis may provide valuable information for the development of potential therapeutic strategies for multiple diseases associated to ISC biogenesis and trafficking.

5.6 *Other Human Pathologies Associated to Iron Dysregulation*

Human pathologies of the brain and different forms of anemia are the most well studied diseases related to iron. However, iron regulation has been proposed to be involved in far more maladies including cardiovascular disease (CVD), cancer, metabolic diseases and, as previously discussed, immunological and infectious disorders.

Diabetes is a group of metabolic diseases affecting an evergrowing proportion of world population. Iron overload has been associated with increased diabetes risk. However, the underlying mechanisms remain incompletely understood [167]. It is imperative to investigate the molecular mechanisms that are related to iron homeostasis and why it is a critical component of diabetes etiology. Recent studies show that iron levels may regulate adipokine expression and insulin sensitivity in adipocytes [168, 169]. Adipokines or adipose tissue-derived hormones are diverse proteins that may exert their effects in an autocrine, paracrine and most importantly in an endocrine fashion, affecting distal organs. The effect of iron on adipokine regulation may suggest a systemic effect of iron through release of adipokines from iron-sensing adipocytes. Potential molecular effectors for iron regulation in diabetes, apart from adipokines, include oxidative stress, ROS formation, hypoxia inducible factors, AMP-activated protein kinase (AMPK) and iron responsive elements [167].

Cardiovascular disease (CVD) is the leading cause of death in elderly and burdens the communities with high economic costs. Several studies have shown that both iron overload and iron deficiency can be detrimental in CVD patients [170–172]. For several years, the role of iron in CVD remains ambiguous and controversial, partially owing to the lack of reliable tools for monitoring systemic, cellular and subcellular iron levels and incomplete understanding of the relevant mechanisms. Future studies will aid in identifying the optimal treatment for patients, which should be determined according to particular cases.

Cancer is the second leading cause of death in developed countries and despite the recent advances in cancer research, a curative treatment with permanent results does not exist. Sustaining growth and proliferation are two of the hallmarks of cancer [173]. As already discussed, iron is vital for cell proliferation, metabolism and growth. Accumulating evidence implicates cellular iron changes as an imperative feature of cancer [113, 174, 175]. Cancer cells ingeniously utilize pathways of iron uptake, storage and efflux in order to increase intracellular metabolically-available iron for their survival and foster proliferation and growth. Most proteins implicated in iron metabolism, have now been associated to cancer development. FPN, the only iron efflux pump in vertebrates, is downregulated in cancer cells leading to elevated non-heme iron and accelerated proliferation, whereas FPN overexpression reduces breast tumor growth in mice [176]. Interestingly, the circulating levels of hepcidin are induced, potentially acting in a cell-non-autonomous fashion to induce FPN degradation and subsequent cellular iron levels [174]. Expression levels of TrF1 and some members of STEAP metalloreductases are also induced in various cancer types leading to increased iron uptake. A lot more proteins and pathways, associated to

iron homeostasis, have been mechanistically linked to cancer, while novel strategies are now emerging towards cancer treatment. Iron chelators and interventions that block iron uptake and induce iron efflux (i.e. TfR overexpression) are now under investigation [174, 175]. Ferroptosis, an iron-dependent form of apoptosis, distinct from other well-known forms of cell death (including apoptosis and necrosis) is also involved in cancer development. Targeting the ferroptosis-related pathways is considered a promising strategy for therapeutic interventions [177–179]. Two distinct mechanisms associated to Glutathione Peroxidase 4 (GPX4) inhibition, have recently been implicated in ferroptosis, rendering GPX4 an essential regulator of ferroptotic cell death [177, 180]. Inactivation of the p53-mediated tumour suppression pathway, is the most common cause of tumorigenesis [181]. High levels of ROS can also initialize a p53-mediated ferroptosis, independenty of cell-cycle arrest, senescence or apoptosis, gradually leading to tumor growth suppression [179]. Additionally, the clinically approved anti-cancer drug, sorafenib, can initiate ferroptosis, supporting the hypothesis for the use of iron-dependent cell death mechanisms against cancer.

6 Biochemical and Biophysical Methods for Iron Detection

Multiple pathological conditions, including cancer and neurodegeneration, are characterized by impaired iron homeostasis. To better understand the iron-dependent molecular pathways that lead to disease, it is of great importance to use efficient methods to detect iron ions in distinct biological samples. The fact that iron can be found in numerous different forms (ferrus, ferric, ISCs, heme, etc.) renders its quantification quite difficult. In the following section we are going to discuss several biochemical and biophysical approaches, commonly used for iron determination (summarized in Table 2).

6.1 Colorimetric Assays

Colorimetric iron detection is based on the ability of several compounds to form colored complexes when interacting with iron. The majority of those compounds act as chelators, since they are bidentate ligands to ferrous ions. The most common chromogens used for colorimetric iron determination are Ferene S, Ferrozine and 2,2′-Bipyridyl [182–185]. The general mechanism involves the ligation of three chromogens to one ferrous ion, giving rise to a stable colored complex. The particular method is supposed to detect free chelatable ferrous iron. However, the measurement is highly dependent on the binding affinity of the respective ligands. Increased binding strength could lead to the chelation of ferrous ions that are already bound to endogenous proteins, rendering the accuracy of this method quiet questionable. To avoid those limitations, additional steps are required for the treatment of biological samples, prior to the colorimetric iron quantification [186]. Initially, a digestion treat-

Table 2 Common biophysical and biochemical methods for iron determination

Methods	Compounds used	Detectable Fe form	References
Colorimetric methods	Ferene-S	Fe^{2+}	[182]
	Ferrozine	Fe^{2+}	[183]
	2,2′-Bipyridyl	Fe^{2+}	[184]
	Ag nanoparticles (NALC–Ag NPs)	Fe^{3+}	[188]
	gold nanoparticles (AuNPs)	Fe^{3+}	[189]
Atomic spectroscopy methods	Atomic absorption Spectroscopy (AAS)	Total Fe	[190]
	Inductively Coupled plasma (ICP)-AAS	Total Fe	[191–193]
	ICP-Mass spectrometry (ICP-MS)	Both Fe isotopes (^{56}Fe, ^{57}Fe)	[191, 194–196]
In situ analyses	Perls' Prussian Blue (PPB)—potassium ferrocyanide	Fe^{3+} (but also Fe^{2+})	[200–204]
	Turnbull's blue	Fe^{2+}	[205–207]
	Electron energy loss spectroscopy (EELS)	Total Fe	[209–211]
	Confocal Raman microscopy	Heme iron	[212–214]
	Calcein AM	Labile iron pool (LIP)	[215]
	Fluorescein coumarin iron probe (FlCFe1)	Labile iron pool (LIP)	[216, 217]
	iron-caged luciferin-1 (ICL-1)	Labile iron pool (LIP)	[218]
	Fe-TRACER	Iron-containing proteins	[219]
	Synchrotron X-ray fluorescence (SXRF)	Total Fe	[220–224]
Biophysical approaches	Electron paramagnetic resonance (EPR)	Heme, [2Fe–2S], [4Fe–4S]	[229–234]
	UV-vis spectroscopy	Heme, [2Fe–2S], [4Fe–4S]	[241–244]

ment in acidic environment (by using hydrogen chloride or nitric acid) is required for the release of complexed iron ions. Subsequent addition of reducing agents, like ammonium acetate and sodium ascorbate, maintains the pH and reduces the iron ions to the ferrous state [187]. Following those treatments, the colorimetric compound is added in the sample, and after a short incubation time, the resulting absorbance is measured by using a spectrophotometer. Absorbance values are analogous to the total (ferrus and ferric) iron content of the respective sample. Interestingly, recent studies introduced two novel colorimetric methods for the determination of ferric ions, based on silver and gold nanoparticles respectively [188, 189].

In general, colorimetric iron determination is a simple and quite inexpensive technique, in comparison with the complex analytical spectrometric methods, that are described below. Though, there are still limitations that need to be taken under consideration. First, some chromogens, including Ferene S, can interfere with other metals, like copper, thus leading to underestimation of iron levels in the examined sample. Additionally, the total iron content is indirectly determined, after addition of the reducer, and may not represent the real iron levels. Another limitation is that colorimetric assays cannot simultaneously detect multiple metals in the same sample.

6.2 Atomic Spectroscopy Methods

Atomic absorption spectroscopy (AAS) is a highly sensitive technique, used for detection and quantitative analysis of chemical elements in biological samples. The particular method is initiated with the atomization of a given sample to its atomic constituents through heating. This is mediated by flame or electrothermal atomizers. Subsequent optical radiation produces light, part of which is absorbed by the atomic constituents. The emitted radiation then passes through a monochromator, which separates the element-specific radiation from the non-specific one. Then, a detector receives the separated wavelengths, which are further analyzed by a processor. AAS is considered a low-cost method, and it can analyze multiple elements of a given sample in a short period of time [190]. The major drawback of this technique is that it requires large amount of material, which is not always possible in biological samples.

Inductively coupled plasma (ICP) is a multi-elemental analytical technique, which is also used for determination of trace metals. Similar to AAS, the initial step is the atomization of the sample molecules to their atomic constituents. In ICP, the atomization is conducted by extremely high tempreratures (ranging from 6000 to 10,000 K), compared with those of AAS. This is achieved by a plasma torch, a device that receives Argon gas and leads to its ionization through the generation of an electromagnetic field. The generated electrons by colliding with the neutral Argon, are continuously moving within the circular magnetic field, generating intense heating. Subsequently, the sample is introduced into the excitation area of the plasma, after it has been converted to gas through a nebulizer. In the plasma, the sample

molecules are atomized and become excited. A detector is then used to "translate" the signal into information concerning element-identity.

According to the method used for detection, ICP can be distinguished into ICP-Optical emission spectroscopy (ICP-AES) and ICP-Mass spectrometry (ICP-MS). In ICP-AES, the detector receives the light emitted by the sample and a photomultiplier is used to convert it to electrical signals. The resulted electrical pattern is compared with known electrical patterns, finally leading to the simultaneous determination of multiple elements (including iron) in the given biological sample [191–193]. In ICP-MS, the ions generated in the plasma enter a mass-spectrometer, by passing through a series of cones. There, the ions are separated based on their mass-to-charge ratio, and subsequently an analyzer is used to determine the concentration of each element. Although its maintenance cost is extremely high, one of the major advantages of ICP-MS is that it can distinguish between different isotopes for each element. Additionally, ICP-MS is a highly sensitive method (compared to colorimetric assays and AAS) and does not require large sample amounts, as in the AAS [194–196]. In the case of iron determination, ICP-MS analysis sometimes leads to false-negative results, due to the signals obtained by Argon-containing molecules (Ar–O), which have the same molecular weight as the most common iron isotope (^{56}Fe). For this reason, there have been used specific chelator agents [197] as well as other gases, instead of Argon, for the generation of the plasma (like helium, hydrogen and ammonia) [198, 199].

6.3 Methods for In Situ Iron Determination

Although colorimetric assays and atomic spectroscopic methods can detect iron with relatively high sensitivity, they do not give any information concerning iron distribution in tissues, cells, or subcellular compartments. The starting biological material used for these methods has been subjected to homogenization, chemical treatments and sequential dilutions, which may interfere with the distribution and chemical properties of iron ions.

During the past decades, specific iron probes have been used for in situ iron determination in histological samples. Perls' Prussian Blue (PPB) is a common staining for ferric detection. The general principle of PPB's function is the reaction of the soluble potassium ferro-cyanide with ferric ions of the sample, giving rise to an insoluble bright blue pigment. This method is commonly used for diagnostic procedures due to its easy application and its relatively low cost. Importantly, it has been shown that ferrocyanide can also react with ferrous iron, producing a white pigment that is gradually transformed to blue, upon oxidation. As such, PPB can generally detect non-heme iron (both ferrus and ferric forms) [200–204]. Turnbull blue is a similar histochemical method, more rarely used, which uses the ferrus detector ferri-cyanide [205–207]. The sensitivity of both techniques can be enhanced by the addition of diaminobenzidine (DAB) [208].

Histochemical staining is commonly combined with electron microscopy, the high resolution of which can provide elemental detection even in subcellular compartments. Electron energy loss spectroscopy (EELS) is a high sensitive electron microscopy method, which allows quantitative determination of element distribution within subcellular compartments. EELS is based on the inelastic scattering of laser-beam electrons, when they interact with the atomic electrons of the specimen. The resulted energy-loss is used for the detection of specific elements in the sample. One of the major drawbacks of this method is the need for using extremely thin tissue sections [209–211].

Confocal Raman microscopy has been also used for in situ analysis of bioinorganic molecules. Its function is based on the inelastic scattering of monochromatic light (Raman scattering) when it passes through the specimen. Heme-containing proteins (including cytochromes) have several absorption bands in the visible region of the electromagnetic spectrum, and thus, they can be easily monitored via Confocal Raman microscopy [212–214].

Recent studies have suggested interesting fluorescent-based techniques for in situ iron determination. Fluorescein coumarin iron probe (FlCFe1) is an analog of the LIP-specific calcein AM [215], and was recently used for ratiometric determination of LIP within tumour spheroids [216, 217]. Another powerful method involves the bioluminescent probe iron-caged luciferin-1 (ICL-1). ICL-1 selectively reacts with ferrus ions and can be used for in vivo bioluminescence imaging of LIP [218]. Jiang et al. introduced a fluorescent-based method for specifically tracking iron-containing proteins. Fe-TRACER can successfully interact with known iron-containing proteins (as evidenced by X-ray diffraction), and thus, it can be easily used for imaging or proteomic analyses [219].

In addition to electron- and fluorescent microscopy, X-ray- based techniques have been successfully used for in situ iron determination. Specifically, Synchrotron X-ray fluorescence (SXRF) permits metal imaging with high lateral spatial resolution at cellular and subcellular level, requiring minimal sample pre-treatments. The method is based on the intrinsic properties of specific elements (including iron) to fluoresce, upon excitation of an X-ray beam and as such, it does not involve the use of external iron-specific probes. Importantly, due to the weak interaction of X-rays with matter, there is no need for chemical fixation and sectioning of the tissue samples [220–224].

6.4 Biophysical Techniques for Determining Iron-Containing Complexes and the Transition of Iron Oxidative States

As previously mentioned, iron has the ability to rapidly change its oxidative status by gaining or losing electrons. This transition ability is required for its function as a cofactor in the form of ISCs or heme. During the last decades, several biophysical methods have been developed for the investigation of iron's oxidative state and the determination of iron-containing complexes under normal or pathological conditions.

Electron Paramagnetic Resonance (EPR), alternatively known as Electron Spin Resonance (ESR), is a powerful spectroscopic method which allows the structural analysis of systems with unpaired electrons (paramagnetic systems). The EPR principle of function is based on the energy difference (ΔE) between the two possible spin states of an electron ($Ms = \pm ½$), in the presence of an external oscillating electromagnetic field of resonant energy equal to ΔE. The energy that is absorbed gives rise to a signal, which is monitored by a detector [225]. In the case of iron determination, the EPR signals may vary (different system spins) according to the number of iron atoms in the respective system (protein, ISC, heme, etc.) [226]. Remarkably, EPR is capable of distinguishing different oxidative states of iron [227, 228] and iron-containing complexes, including heme [229–231], rhomboid [2Fe–2S] [232] and cubane [4Fe–4S] ISCs [233, 234]. For the better determination of the charge-transfer transitions, EPR can be combined with Circular Dichroism (CD) spectroscopy [235–238]. Generally, EPR is a highly sensitive technique since it can detect iron species at really low concentrations. However, it is mostly used for iron determination in purified proteins, as cells or subcellular organelles have multiple metals that are EPR-detectable [239, 240].

UV-vis spectroscopy (UV/VIS) is a biophysical method which relies on the excitation of certain species when they absorb light in the ultraviolet to visible range. In several studies, UV/VIS has been used for determining the oxidation state of both heme [241, 242] and ISCs [243, 244] in iron-containing proteins.

7 Concluding Remarks and Future Directions

While the biochemistry and genetic pathways involved in iron homeostasis have been investigated for several decades and our current understanding for the role of iron metabolism in biological systems has advanced enormously over the past years, plenty fundamental questions remain elusive. Generation of transgenic animal models has been proven invaluable for the elucidation of key proteins and mechanisms that regulate iron homeostasis at the systemic and cellular level, as described in the previous sections. Novel iron-related proteins and related mechanisms are expected to be discovered soon and answer central questions. At the systemic level the discovery of hepcidin has been revolutionary, however how organ specific regulation is achieved is unknown. Furthermore, how iron crosses the placenta or the blood brain barrier remains a mystery. The reciprocal interaction of the microbiome and iron remains vague. A regulatory mechanism for systemic iron excretion is also unknown. At the cellular level, a key question is how intracellular trafficking and homeostasis of iron is achieved. Detailed mechanisms on mitochondrial iron efflux and intake need to be defined. These are only some of the key unresolved questions. The development of powerful and sensitive methods for rigorous monitoring of organismal and cellular iron in health and disease will facilitate bigger advancements in the field for the years to come. Current clinical interventions, such as iron chelation need to be

evaluated further. Deeper understanding of the involved mechanisms will provide the possibility for new theurapeutic interventions for a plethora of iron-mediated diseases.

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