Ferroptosis : biological rust of lipid membranes

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Forum Review Article

Ferroptosis: biological rust of lipid membranes

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Abstract

Significance: Iron is an essential element required for growth and proper functioning of the body. However, an excess of labile ferrous iron increases the risk of oxidative stress-induced injury due to the high reactivity of the unpaired reactive electrons of both ferrous iron and oxygen. This high reactivity can be exemplified in the outside world by one of its consequences, rust formation. In cells, this redox-active iron is involved in the formation of lipid radicals.

Recent Advances: Defect or insufficient membrane-protective mechanisms can result in iron-catalyzed excessive lipid peroxidation and subsequent cell death, now conceptualized as ferroptosis. Growing reports propose the detrimental role of iron and ferroptosis in many experimental disease models such as ischemia-reperfusion, acute and chronic organ injuries.

Critical Issues: This review first provides a snapshot of iron metabolism, followed by a brief introduction of the molecular mechanisms of ferroptosis, as an iron-dependent lipid peroxidation-driven mode of cell death. Upon describing how iron dysbiosis affects ferroptosis induction, we elaborate on the detrimental role of the iron-ferroptosis axis in several diseases.

Future Directions:

Despite compelling findings suggesting a role for ferroptosis in experimental animal models, the exact contribution of ferroptosis in human contexts still needs further investigation. Development of reliable ferroptosis biomarkers will be an important step in characterizing ferroptosis in human disease. This can provide therapeutic opportunities aiming at targeting ferroptosis in human diseases.

Keywords: iron, ferroptosis, ischemia/reperfusion, neurodegeneration, disease

Introduction

Iron: a double-edged sword

Iron is the fourth most abundant element and the second most abundant metal in the Earth's crust (34). It is an ideal redox active cofactor as its reactivity can be modulated easily by biological ligands/complexation (i.e. Heme). As a functional component of enzymes such as ribonucleotide reductase and cytochrome P450, iron is involved in cell proliferation and metabolism (148). In the form of heme in hemoglobin or as a cofactor of proteins of the citric acid cycle and electron transport chain, iron plays an essential role in oxygen transport and cellular energy metabolism. However, the redox properties of iron also make it vulnerable for oxidation. The intracellular non-protein bound redox-active iron, namely the cytosolic and mitochondrial labile iron pool (LIP), feeds iron-catalyzed reactive oxygen species (ROS) production. Ferrous iron (Fe^{2+}) catalyzes the formation of highly reactive hydroxyl radicals ($OH^{\bullet}$) upon oxidization to ferric iron (Fe^{3+}) through Fenton reaction. Fe^{3+} can also react with superoxide radicals ($O_2^{\bullet-}$) and be reduced back to Fe^{2+} forming an iron-redox cycle, via the Harber-Weiss reaction, which can ultimately result in generation of more $OH^{\bullet}$. Iron and iron containing complexes, such as iron-sulfur [Fe-S] clusters and heme, are essential for the function of various enzymes that are able to produce ROS (45). During high oxidative stress, iron can also catalyze the formation of lipid ROS, promoting lipid peroxidation of cellular membranes. Therefore, cells, through tight regulation of iron homeostasis, on the one hand sustain adequate amount of iron for proper functioning and on the other hand, control the size and toxicity of LIP (16).

Regulation of iron homeostasis

In mammals, iron metabolism, including iron absorption, recycling and storage, is tightly regulated at the cellular level, as well as systemically.

Systemic iron homeostasis

Systemic iron homeostasis depends on the intricate control of iron levels in plasma, where iron mainly traverses bound to transferrin. The glycoprotein transferrin not only serves as a principle cargo for iron delivery into cells but also, by scavenging iron, limits the generation of toxic radicals. Duodenal enterocytes absorbing dietary iron, macrophages

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which recycle hemoglobin iron from senescent erythrocytes and finally hepatocytes, which store iron, constitute the major suppliers of plasma iron (Fig. 1A) (72). Absorption of dietary non-heme iron, e.g. inorganic dietary iron that is mostly Fe^{3+}, requires the concerted functions of divalent metal transporter 1 (DMT1), also known as solute carrier family 11 member 2 (SLC11A2), and ferrireductase duodenal cytochrome B (DCYTB) (65,86). At the apical membrane of duodenal enterocytes, DCYTB reduces the insoluble Fe^{3+} to the soluble and absorbable Fe^{2+} before iron enters into enterocytes by DMT1. The enterocytic Fe^{2+} is exported into plasma by the action of basolateral iron exporter ferroportin, also known as SLC40A1, where it is oxidized back to Fe^{3+} by the multicopper ferroxidase hephaestin (68,189). The delivery of iron to ferroportin is suggested to be mediated by poly (rC) binding protein 2 (PCBP2) (208). The mechanism of dietary heme iron uptake is not fully characterized. The heme carrier protein 1 (HCP1) was initially proposed as a heme transporter, however, further studies identified it as a proton-coupled folate transporter (145,163). Within the enterocyte, heme oxygenase 1 (HMOX1) degrades the heme and the released iron is believed to be handled in the same manner as the absorbed inorganic iron (86) (Fig. 1A).

Macrophages can obtain iron via different routes such as transferrin receptor (TfR1) mediated uptake of TF-Fe^{3+}, CD163 mediated uptake of haptoglobin-hemoglobin, and LDL receptor-related protein (LRP/CD91) mediated uptake of free heme (199). Furthermore, during erythrophagocytosis, macrophages of the reticuloendothelial system recycle heme iron from senescent or damaged erythrocytes. The heme released from hemoglobin seems to be transported via heme-responsive gene 1 (HRG1) across the phagolysosomal membrane into the cytosol, where it is catabolized by HMOX1 to release iron. Macrophages supply iron to the plasma via ferroportin, supported by the ferroxidase enzyme ceruloplasmin that oxidizes Fe^{2+} to Fe^{3} (199). Hepatocytes take up iron mainly via TfR1-mediated uptake of TF-Fe^{3+}. However, in case of iron overload or excessive iron hemolysis they can also take up non-transferrin-bound iron, hemoglobin and free heme (7). The non-transferrin-bound iron can be absorbed by ZRT/IRT-like proteins 8/14 (ZIP8/14) (16). As for macrophages, the export of iron from hepatocyte into plasma is achieved through the cooperation of ferroportin and ceruloplasmin (7).

Systemic iron homeostasis is regulated by the liver-secreted hormone hepcidin, the expression of which is positively regulated by iron availability and inflammation and negatively by increased erythropoietic activity. Iron availability, defined by liver and plasma iron levels, controls hepcidin expression via bone morphogenetic protein (BMP)/SMAD signalling pathway (192). In the liver, iron overload is sensed by liver sinusoidal endothelial cells (LSECs), which produce BMP6. As a proposed mechanism, iron-induced ROS in LSECs activates nuclear factor erythroid 2-related factor 2 (NRF2), which subsequently promotes MBP6 production (110). The BMP6 further associates with BMP receptors (BMPRs) and hemojuvelin (HJV) complexes on the surface of hepatocytes and triggers the expression of hepcidin via SMADs (192). High concentrations of plasma iron-loaded transferrin, promotes the association of iron sensor protein HFE, located on the plasma membrane of hepatocytes, with transferrin receptor 2 (TfR2). The HFE-TfR2 complex is suggested to form a complex with HJV, which ultimately promotes BMP signalling (166). Inflammation, increases the expression of hepcidin predominantly through interleukin 6 (IL6), IL6 receptor, the janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway (192). Hepcidin produced by hepatocytes is released into the plasma, binds to cell-surface ferroportin, and triggers its internalization and lysosomal degradation (132). Thus, hepcidin lowers systemic iron by limiting the release of iron from duodenal enterocytes, macrophages and hepatocytes (Fig. 1A).

Intracellular iron homeostasis

Intracellular iron homeostasis involves regulation of cellular iron uptake, utilization, storage and export, which is controlled by post-transcriptional regulation of different iron metabolism genes (16). Mammalian cells obtain iron through different uptake mechanisms. As a major system, cells entrap plasma iron through TfR1-mediated endocytosis of TF-Fe^{3+} (Fig. 1B). Due to the acidic environment of endosomes, iron dissociates from TF and is reduced to Fe^{2+} by endosomal ferrireductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3) before entering the cytosol via DMT1 (134). The absorbed iron becomes part of an accessible transitory LIP, which serves as a crossroad of cellular iron metabolism. A fraction of LIP is incorporated into iron containing proteins or is exported into mitochondria by mitoferrin, where heme and iron-sulfur

cluster are synthesized. Non-used LIP can be either exported via ferroportin or stored in a redox-inactive form in ferritin complexes (72). Ferritin is the main protein responsible for iron storage and is composed of a 24-subunit multimer of ferritin heavy (FTH) and ferritin light (FTL) polypeptide chains. This large multimeric protein is able to accommodate up to 4500 iron atoms in its core (166). To store iron, PCBP1/2 act as chaperons to direct cytosolic Fe^{2+} to ferritin, where it is stored after being oxidized by the ferroxidase activity of FTH (96). Besides cytosolic ferritin, some cells appear to encode a mitochondrial ferritin to handle the large flux of iron into mitochondria (100).

To avoid iron-driven cytotoxicity, the abundance and availability of the LIP defined by the rate of iron uptake, utilization, storage, and export must be closely monitored. Cellular iron homeostasis is mainly controlled post-transcriptionally by the iron regulatory protein (IRP)/iron responsive element (IRE) system. IRP1 and IRP2 regulate stability and translation of iron-metabolism related mRNAs by binding to the hairpin structure of IREs in 5'- or 3'-UTR of these mRNA strands (166). During iron deficiency, on the one hand, IRPs bind to 5'-UTR IRE of mRNAs encoding FTH, FTL and ferroportin resulting in prevention of ribosome assembly and mRNA translation (Fig. 1B). On the other hand, IRPs also bind to 3'-UTR IRE of TfR1 and DMT1 mRNA leading to the increase of their stability and translation (6). Consequently, the increase of iron uptake, the decrease of iron storage/export boost the LIP levels. In condition of elevated LIP levels, IRP2 is degraded by F box and leucine-rich repeat protein 5 (FBXL5)-mediated ubiquitin proteasome pathway (152,188). Furthermore, free iron levels cause the assembly of an iron-sulfur cluster into IRP1, switching its conformation to a cytosolic-aconitase (c-aconitase) that prevents binding of IRP1 to IREs (191). The structure of IREs can also contribute to post-transcriptional control of gene expression. Fe^{2+} can bind directly to the 5'-UTR IRE and induces a conformational change in the IRE stem-loop structure, which decreases the affinity of IRE for IRPs while it increases its affinity for ribosomes (118). In conclusion, during iron-overload IRPs do not bind IREs, resulting in the translation of FTH, FTL, FPN, and degradation of TfR1 and DMT1 mRNA (Fig. 1B).

In addition to the IRP/IRE system, other mechanisms have been identified that regulate intracellular iron metabolism. PCBP chaperones can putatively affect iron homeostasis by

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delivery of iron to ferritin and non-heme containing protein (16). Utilization of iron stored in ferritin requires the release of iron by lysosomal degradation of ferritin (83). The nuclear receptor coactivator 4 (NCOA4), which is highly enriched in autophagosomes, binds to ferritin and act as a selective cargo receptor delivering ferritin to lysosomes for autophagic degradation, a process coined ferritinophagy (121). Therefore, lysosomal degradation of ferritin by releasing iron can also regulate LIP.

Iron dysbiosis

Considering the need of iron to preserve essential functions as well as its high reactivity, plasma and cellular iron levels need to be tightly regulated in order to prevent deleterious iron disbalances causing disease. Generally, three types of iron disorders are distinguished relating to iron absorption, transport and secondary iron imbalances caused by a non-iron related primary disease (109). Iron deficiency-induced anemia belongs to the iron absorption defects and is the most common iron disorder worldwide (109). Acquired iron deficiency due to malnutrition and acute or chronic bleeding are its primary causes. A particularly common comorbidity in hospitalized patients however is so-called anemia of chronic disease, in which chronic inflammation stimulates increased hepcidin production. Subsequent ferroportin degradation prevents iron delivery to developing erythrocytes, causing anemia (54). Additionally, a rare hereditary disorder named iron-refractory iron deficiency anemia (IRIDA) exists in which a genetic defect in the transmembrane serine protease 6 (TMPRSS6) gene induces continuous upregulation of hepcidin (71). The opposite is seen for classical hereditary hemochromatosis, a group of diseases characterized by mutations in either the gene encoding for hepcidin itself (hepcidin antimicrobial peptide or *HAMP*), or its major expression inducers (*HFE, HJV, TfR2*) or mutations of ferroportin at the hepcidin binding site (22). Inappropriately low levels of hepcidin result in a deregulation of iron absorption and consequent iron accumulation (22).

Many disorders caused by iron transport defects exist, such as aceruloplasminemia. This disease is caused by mutations in the gene encoding for ceruloplasmin (68). Aceruloplasminemia was the first iron protein disorder to be recognized as a neurodegenerative disease and can, because of its characteristic iron accumulation in the

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brain, be classified as neurodegeneration with brain iron accumulation (NBIA) (101). NBIAs represent a heterogenous group of genetic diseases characterized by abnormal accumulation of iron in the basal ganglia causing neuromuscular symptoms such as a progressive movement disorder, dystonia and parkinsonism, but also neuropsychiatric abnormalities, and optic atrophy or retinal degeneration (101). From the 15 genes found to associate to NBIAs however, only two, encoding ceruloplasmin and ferritin light chain, are directly associated to iron metabolism while the other genes seem to be involved in unrelated pathways (101). Next to disorders related to iron absorption and transport defects, various secondary iron disorders exist, including chronic inflammation-associated anemia and alcohol-induced iron abnormalities (109).

Ferroptosis: an iron-catalyzed mode of regulated cell death

An excess amount of improperly shielded labile iron can catalyze the formation of ROS, and in particular lipid ROS ensuing cell death (45). In 2012, a type of iron-dependent cell death coined ferroptosis was described (43). Ferroptosis is characterized by iron-dependent excessive accumulation of lipid hydroperoxides in cellular membranes leading to a necrotic cell death. This type of cell death can be inhibited specifically by lipophilic radical traps, such as vitamin E, Ferrostatin-1 (Fer-1), Liproxstatin-1 (Lip-1), and iron chelators. Furthermore, ferroptosis does not display the hallmarks of apoptosis and is biochemically and genetically distinct from necroptosis (43,55). An iron-catalyzed excessive lipid peroxidation reaction is considered to be the main detrimental executioner mechanism leading to ferroptosis (210). During excessive lipid peroxidation, oxygenation of polyunsaturated fatty acids (PUFAs) of membranes results in accumulation of toxic reactive aldehydes and subsequent membrane rupture (32,48,82). Among PUFAs of membranes, linoleic (C18:2), arachidonic (C20:4) and docosahexaenoic (C22:6) acids represent the main targets for lipid peroxidation (59). Lipid peroxidation is suggested to be initiated by either a non-enzymatic or enzymatic process (Fig. 2). During non-enzymatic lipid peroxidation, pro-oxidants, such as hydroxyl radicals ($OH^{\bullet}$) produced by iron-catalyzed Fenton reactions, attack PUFAs resulting in formation of phospholipid radicals ($PL^{\bullet}$). In presence of molecular oxygen, phospholipid radicals are converted to phospholipid peroxyl radicals ($PL\text{-}OO^{\bullet}$) which can further attack adjacent PUFAs yielding

the formation of phospholipid hydroperoxides (PL-OOH) and new phospholipid radicals. Under conditions where lipid peroxidation is not terminated, the new phospholipid radicals refuel the chain reaction and result in accumulation of phospholipid hydroperoxides. Phospholipid hydroperoxide, in turn, through an iron-catalyzed reaction, can be converted to alkoxyl radicals (PL-O$^{\bullet}$), which can further oxygenate other lipids (8). In enzymatic lipid peroxidation, non-heme iron-containing lipoxygenases (LOXs) initiate the dioxygenation of linoleic and arachidonic acids to various hydroperoxides and hydroperoxyeicosatetraenoic acid (HPETE) (59). Accordingly, overexpression of LOXs sensitizes cells to ferroptosis, presumably due to initial increase in the concentration of lipid hydroperoxides which can initiate ferroptosis (161). Oxidative lipidomic studies propose that oxidized PUFAs of phospholipids, such as arachidonic fatty acid and its elongation product adrenoyl (C22:4) fatty acids of phosphatidylethanolamine (PE) are one of the main consequences of ferroptosis (82). *In vivo*, also other PLs were found to be oxidized during ferroptosis in high-risk neuroblastoma xenografts (70).

The continued oxidation of PUFAs is hypothesized to be followed by thinning and increased curvature of membrane, thereby stimulating oxidative micellization and pore formation (1). Furthermore, lipid hydroperoxides can decompose to reactive toxic aldehydes such as 4-hydroxy-2-nonenals (4-HNE) or malondialdehydes (MDA), which can form covalent adducts with protein and damage essential proteins (8). Several mechanisms contribute to the detoxification of lipid hydroperoxides either by terminating peroxidation chain reaction via antioxidants, or by inactivating chemically reactive groups generated by oxidation (Fig. 2). The selenium-dependent glutathione peroxidases 4 (GPX4), an essential enzyme of the glutathione (GSH) system, detoxifies phospholipid hydroperoxides to their non-toxic corresponding hydroxide, viz. alcohol, and water, thereby protecting membranes from peroxidation damage (18,186). Detoxification of secondary reactive products containing aldehyde and keto groups, such as 4-HNE, can be achieved by a class of aldo-keto reductases (AKR), which reduces carbonyl groups of these products to respective hydroxyl groups. Lipid peroxidation can also be terminated by lipophilic radical traps, which neutralize lipid radicals by donating electrons (8). Accordingly, upregulation of *AKR1C1-3* genes are associated with resistance to ferroptosis (44). Recent findings propose that cells, in parallel to the GPX4 system, can also counteract

lipid peroxidation through promoting endogenous radical trapping antioxidant systems (14,47,88). The ferroptosis suppressor protein 1 (FSP1), formerly known as apoptosis inducing factor mitochondrial 2 (AIFM2), once recruited to the membrane reduces ubiquinone-10 (CoQ_{10}) to ubiquinol ($CoQ_{10}H_2$), which traps lipid peroxyl radicals and prevents subsequent lipid peroxidation (14,47). The GTP cyclohydrolase-1 (GCH1) protects cells from ferroptosis through controlling the production of the antioxidant tetrahydrobiopterin (BH4) and the levels of CoQ_{10} (88). Collectively, excessive lipid peroxidation results in depletion of PUFAs from membrane and alters membrane fluidity and integrity, which likely contributes to cell death (209).

Mechanism of ferroptosis induction

Dysfunction of the glutathione (GSH) system due to loss of activity of the lipid repair enzyme GPX4 represents the canonical pathway of ferroptosis induction (Fig. 3) (210). GPX4 reduces lipid hydroperoxides at the expense of its essential cofactor tripeptide GSH, as a reductant (18). Synthesis of GSH is dependent on the availability of intracellular cysteine, which can be provided by one of its main suppliers, system X_C^-. System X_C^- is a cell surface disulfide-linked heterodimer of SLC7A11 (xCT) and SLC3A2 (4F2hc), which imports cystine, the extracellular oxidized form of cysteine, in exchange for intracellular glutamate (154). The repression of system X_C^- function, by compounds such as erastin and sulfasalazine (SAS), inhibits the import of cystine, which is followed by a massive reduction of intracellular cysteine and GSH levels (44). Depletion of GSH indirectly inactivates GPX4, leading to accumulation of toxic lipid ROS and subsequent lipid peroxidation (43,44). In addition to system X_C^-, direct inhibition of GSH synthesis, e.g. repression of glutamate-cysteine ligase (GCL) by buthionine sulfoximine (BSO), also induces ferroptosis in some cellular contexts (210). Analysis of the mechanism of compound Ras-selective lethal small molecule 3 (RSL3) proposed direct loss of function of GPX4 as a ferroptosis inducing mechanism (210). RSL3 binds moderately to cysteines of GPX4, however its covalent binding to the selenocysteine (Sec) at the active site of GPX4, directly inhibits the phospholipid peroxidase activity of GPX4 (209). Consistently, loss of GPX4 leads to the rapid accumulation of lipid ROS and ferroptosis (55,210). In addition to RSL3, other compounds such as ML162, FINO2, withaferin A induce ferroptosis through GPX4

inactivation (24,58,70). Ferroptosis can also be induced via the non-canonical pathway, by elevated levels of LIP (Fig. 3). Pure iron overload using iron chloride, hemoglobin, hemin or ferrous ammonium sulfate triggers ferroptosis in neuronal contexts (70,103). Holo-transferrin as an iron carrier protein, induces ferroptosis upon cysteine deprivation (57). Furthermore, the increase of LIP through iron overload caused by iron oxide nanoparticles, excessive activation of HMOX1, or altered iron transport trigger ferroptosis in cancer cells (27,70,85,119).

A similar cell death process has been described in neurons. The high levels of extracellular glutamate, which acts as excitatory transmitter in the central nervous system (CNS), can lead to neuronal cell death (128). Excessive excitation of glutamate N-methyl-D-aspartate (NMDA) receptors, especially in ischemic brain damage, leads to Ca^{2+} influx and an oxidative stress induced cell death coined excitotoxicity (149). Furthermore, system X_C^- is inhibited by high extracellular concentration of glutamate which can result in the blockage of cystine uptake and reduced GSH levels causing oxidative glutamate-induced toxicity known as oxytosis (128,176). In this regard, glutamate by targeting system X_C^- acts as the classical ferroptosis inducers. In accordance, oxytosis and ferroptosis share many common features including GSH depletion, lipid ROS production, LOX activation and similar pharmacological profiles. Analysis of the cell death pathway downstream of system X_C^-, indicates that oxytosis is also inhibited by iron chelators and lipophilic antioxidants. Oxytosis is preceded by a large increase in the intracellular Ca^{2+} level and it can be prevented by calcium chelation and calcium channel blockers suggesting calcium entry as an essential step for this type of cell death (176). Although the role of calcium in ferroptosis is not as elaborate as for oxytosis, pharmacological decrease of calcium influx can protect against ferroptosis (102). Moreover, the loss or inhibition of BH3-interacting domain death agonist (Bid), which mediates mitochondrial release of apoptosis-inducing factor (AIF), protects from ferroptosis and oxytosis in neuronal HT22 cells (102,131). Therefore, oxytosis and ferroptosis can likely be considered two names for the same cell death pathway, at least in a neuronal context (102).

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Role of iron metabolism in ferroptosis

The antioxidant defense system, energy stress, lipid and iron metabolism are able to modulate ferroptosis (14,47,69,88,93). Accordingly, many inhibitors have been reported to inhibit ferroptosis (Supplementary Table 1). The elevated levels of LIP by catalyzing the formation of hydroxyl radicals and lipid peroxidation can promote ferroptosis (Fig. 2). Furthermore, inhibition of ferroptosis by iron chelators further illustrates this catalyzing role of iron in cell death. In support of role of iron in ferroptosis, modulators of iron homeostasis, including iron uptake, export, and storage are found to regulate ferroptosis (Fig. 4). Knockdown of TfR1 or depletion of transferrin abrogates ferroptosis induced by cystine deprivation or erastin (56,57,211). The TfR1 accumulates on the cell surface of ferroptotic cells (53). In hepatocellular carcinoma cells, depletion of ceruloplasmin promotes ferroptosis and results in the accumulation of intracellular ferrous iron, a process which can be counteracted by TfR1 depletion (162). The expression levels of ferritin heavy chain 1 (FTH1) impact sensitivity to ferroptosis (171,211). Silencing of *FTH1* enhances erastin-induced ferroptosis (171). Ferroptosis is accompanied by an increase of mitochondrial labile iron and could be promoted by repressing the transcription of ferritin and ferroportin genes (133). Silencing of *IREB2*, encoding IRP2, suppresses ferroptosis (43). Reduced expression of ferroportin promotes erastin-induced ferroptosis (60). Phosphorylated heat shock protein family B (HSPB1, also known as HSP27) acts as a negative regulator of ferroptosis, triggered by erastin (172). HSPB1, apparently, confers resistance to ferroptosis through inhibiting cytoskeleton-mediated iron uptake (172). Hyperactivation of HMOX1, which catalyzes the degradation of heme to ferrous iron, promotes ferroptosis by augmenting the levels of LIP (27,70).

The iron-sulfur cluster (ISC) synthesis is linked to ferroptosis sensitivity. Nitrogen fixation 1 (NFS1), a cysteine desulfurase that supplies sulfur from cysteine for the synthesis of ICSs, modulates ferroptosis. The suppression of NFS1 activates an iron starvation response, by increasing the expression of TfR1 and decreasing the expression of ferritin, which makes cancer cells susceptible to ferroptosis (4). Depletion of Frataxin, also involved in Fe–S cluster synthesis, accelerates erastin-induced ferroptosis by enhancing free iron accumulation (50). The iron-sulfur containing proteins CDGSH iron sulfur domain 1 (CISD1,

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also called mitoNEET), and CISD2 (also called nutrient-deprivation autophagy factor-1 (NAF-1)) negatively modulate ferroptosis through influencing iron homeostasis (84,216). The suppression of CISD1 is associated with mitochondrial ferrous iron accumulation and sensitization to ferroptosis induced by erastin (216). The overexpression of CISD2 confers resistance to ferroptosis induced by class I FINs sulfasalazine. Conversely, silencing of CISD2 leads to the increase of mitochondrial ferrous iron and susceptibility to ferroptosis (84). Lysosomes can accumulate large amounts of ferrous iron by degrading iron-containing macromolecules and can serve as an important source of iron for ferroptosis (179). The pharmacological inhibition of lysosome function suppresses ferroptosis (181). The iron chelator deferoxamine (DFO), a known inhibitor of ferroptosis, accumulates in lysosomes proposing that chelation of lysosomal iron is critical in the prevention of lysosomal permeabilization and ferroptosis (24). In accordance, lysosomal degradation of ferritin in autophagosome during ferritinophagy, is shown to be required for accumulation of LIP and ferroptosis. Silencing the cargo receptor of ferritin, NCOA4, suppresses lysosomal release of iron and ferroptosis (56,75). Finally, transporting iron out of cells can protect against ferroptosis. Cells become resistant to ferroptosis by upregulating Prominin2 protein that stimulates an iron export pathway via formation of multivesicular bodies and the release of ferritin containing exosomes (19). In addition to iron, other transition metals including copper have the potential to induce lipid peroxidation *in vivo* (8). In neuronal context, copper sensitizes cells to erastin-induced ferroptosis (120). However, the exact contribution of copper in other conditions still needs further investigation.

Detrimental role of iron-ferroptosis axis in disease

Based on mouse models of human disease and *Gpx4*-deficient mouse models, a picture emerges in which pathological conditions engage ferroptosis in tissue demise. It seems clear that ischemia reperfusion injury (IRI) in kidney (111), liver (55), brain (184), intestine (108), and heart (52,104) will benefit from anti-ferroptotic approaches. Accumulating evidence suggests also a crucial role for ferroptosis in tumor suppression, which highlights ferroptosis as a novel therapeutic strategy to eradicate cancers. Because this falls outside

the scope of this review, we refer the reader elsewhere on the role of targeting ferroptosis as novel anti-cancer treatment (69).

GPX4 and tissue homeostasis

Mutations in human *GPX4* cause a rare neonatal lethal disorder called sedaghatian-type spondylometaphyseal dysplasia (SSMD). Truncated forms of *GPX4* protein account for cardiac, nervous and skeletal system defects in SSMD (168). Knockout studies in mice indicate that GPX4 is essential for development and maintenance of tissue homeostasis. Systemic *Gpx4*-deficient mice die around embryonic day E7.5 and E8.5 (212). Not only during development, but also in adult mice depletion of GPX4 is detrimental. *Gpx4* ablation in adult mice is lethal and is associated with neurodegeneration in the hippocampus region of brain indicating the importance of GPX4 in survival of neurons (214). Tamoxifen-induced systemic *Gpx4* depletion causes early death accompanied by acute kidney injury (AKI) and neurodegeneration in mice (55,159,214). Conditional ablation of *Gpx4* in neurons of adult mice leads to degeneration of motor neurons in the spinal cord accompanied by paralysis and death (30). Specific deletion of *Gpx4* in forebrain neurons in mice causes cognitive impairment and hippocampal neurodegeneration (66). Selenocysteine incorporation into Gpx4 is essential for the survival of parvalbumin-positive (PV^+) GABAergic inhibitory interneurons and Gpx4 deficiency can impair neuronal inhibition leading to hyperexcitability phenotype (80,159). Accordingly, $GPX4^{cys/cys}$ mice that express GPX4 in which the essential catalytic selenocysteine is substituted with cysteine, show severe spontaneous seizures and hyperexcitability and die by three weeks after birth (80). A similar hyperexcitable phenotype is observed in cerebral cortex and hippocampus specific GPX4-deficient mice (159). Both mitochondrial- and spermatocyte-specific *Gpx4*-deficient mice have impaired sperm quality and structural abnormalities leading to infertility, despite normal embryogenesis and postnatal development (78,157). Similarly, heterozygous expression of a catalytically inactive mutant form of *Gpx4*, where the selenocysteine is mutated to serine impairs spermatogenesis and confers subfertility in males (79). GPX4 deficiency in T cells results in abrogation of T cell expansion and failure of immune response in response to viral and parasitic infection in mice (124). Keratinocyte-specific *Gpx4*-deficient mice have epidermal hyperplasia, dermal inflammatory infiltrate,

dysmorphic hair follicles, and alopecia in perinatal period (160). Absence of GPX4 in photoreceptors causes rapid and massive retinal degeneration in mice (185). Conversely, increased expression of GPX4 in photoreceptors reduces loss of retinal function after injection of paraquat or exposure to hyperoxia (115). Liver specific *Gpx4*-deficient mice die shortly after birth and present extensive hepatocyte degeneration, which could be compensated by vitamin E enriched diet (25). Loss of Gpx4 in hematopoietic cells causes anemia and liver iron overload in mice which is worsened in combination with vitamin E depleted diet (3,23). Finally, specific deletion of *Gpx4* in endothelium combined with vitamin E deficient diet dramatically impairs vascular homeostasis and leads to platelet aggregation and thrombus formation in various organs (200). The effect of vitamin E diet on lethality and phenotype of Gpx4-depleted mice underpins the noticeable interrelationship between vitamin E, as a natural lipophilic radical trapping antioxidant, and Gpx4.

Ischemia-reperfusion injury

Ischemia reperfusion injury (IRI) represents the collective cellular damage caused by initial ischemia and consequent reperfusion (I/R) of an organ. Ischemia results from restriction of the blood supply to the organ, often caused by an embolic event occluding the artery, and is characterized by hypoxia and metabolic imbalances in the cell. Counterintuitively, reoxygenation by restoration of the blood flow, reperfusion, often exacerbates the cellular damage and initiates an inflammatory response (51). The mechanistic details of IRI remain to be elucidated but dysfunction of the electron transport chain in mitochondria and subsequent anaerobic metabolism are believed to sensitize the cell to excessive ROS formation upon oxygen restoration in the reperfusion stage (33). The oxidative stress caused by ROS promotes cellular damage, endothelial dysfunction, inflammation and eventually cell death (202). Although the pathological processes involved in different I/R syndromes vary, IRI contributes to numerous disease states ranging from single-organ IRI such as induced by acute myocardial infarct (AMI) and stroke, to multi-organ and surgery-related IRI such as seen after trauma and organ transplantation, respectively (51). Already 30 years ago, the role for iron and lipid peroxidation was described in several experimental models of IRI as iron chelation therapy reduced lipid peroxidation and/or ameliorated

organ injury in experimental animal models of heart, brain, kidney and intestinal IRI (62,99,116,138). Ferroptosis was found to be critically involved in various pathologies characterized by IRI.

Acute organ injury

Acute kidney injury (AKI), formerly known as acute renal failure, is a common syndrome in hospitalized patients especially critically ill patients, which is characterized by tubular cell death and inflammation leading to the rapid loss or decrease of renal function (13). Three individual studies on patients with acute coronary syndrome undergoing contrast exposure, patients undergoing cardiac surgery, or critically ill patients found the level of plasma catalytic iron to be positively correlated with both the onset of AKI and mortality (91,92,97). Accordingly, higher catalytic iron levels as well as lower hepcidin concentrations in the plasma of critically ill patients requiring renal replacement therapy associate with an increased mortality risk (90). Catalytic iron is elevated and critically involved in various different animal models of AKI including IRI (137), cisplatin-induced AKI (11) and rhabdomyolysis-induced AKI (136), as evidenced by the protective effect of iron chelation by DFO in each of these models. More recently, the protective effect of Fer-1 on a model of iron-induced cell death in freshly isolated mouse kidney tubules demonstrated the role for ferroptosis in this context (167). In line with these findings, ferroptosis causes synchronized necrosis of renal tubules in models of IRI and oxalate crystal-induced AKI, for which inhibition of ferroptosis markedly improves the histological outcome and lowers serum damage marker levels (111). Treatment with Fer-1 reduces cell death and lipid peroxidation, which results in less tissue injury and lower serum damage marker levels in a model of rhabdomyolysis-induced AKI (63). Similarly, a role for ferroptosis was established in cisplatin-induced AKI, which would be promoted via ferritinophagy (37). Ferroptosis also plays a key role in AKI induced by folic acid (FA) overdose. In this model, Fer-1 mitigates tissue injury, tubular cell death, lipid peroxidation and inflammation (122). In addition, renal oxidized polyunsaturated phosphatidylethanolamines (PEox) are increased upon FA injection in mice, just as in the urinary pellet of humans requiring persistent dialysis (198). As mentioned before, induction of ferroptosis in adult mice by genetic inactivation of *Gpx4* causes AKI and early death in mice. Consistently, Lip-1 delays ferroptosis in tubular cells

and extends the survival of *Gpx4*-deficient mice (55). In addition to its role in kidney IRI, ferroptosis contributes to hepatic and intestinal IRI and other acute pathologies (Table 1, Fig.5).

Acute myocardial injury

Cardiomyopathy caused by iron accumulation in the heart is a well-known problem in hemochromatosis patients (64). Recently however, patients with IRI due to an acute myocardial infarct (AMI) were found to have iron residuals in the infarcted area of the heart (21). *Ex vivo* experiments with perfused hearts show that iron chelation therapy with deferoxamine successfully reduces the infarct size upon IRI and improves functional recovery (57). Before the term ferroptosis was coined, Dabkowski et al. found that transgenic overexpression of mitochondrial GPX4 in *ex vivo* hearts undergoing IRI improves the functionality of the heart, reduces the release of creatine kinase, a marker of heart damage, and lowers mitochondrial lipid peroxidation (35). The accumulation of iron following I/R was confirmed in an *in vivo* mouse model and *in vitro* findings showed that Fe^{3+} overload induces ferroptosis in primary cardiomyocytes, since cell death could be blocked by addition of Fer-1 (10). The transcription factor BACH1 involved in oxidative stress response as well as heme and iron homeostasis, was suggested to promote ferroptosis in a model of AMI (133). GPX4 overexpression also reduces cell death and ameliorates cardiac output in an *in vivo* mouse model of chemotherapeutic agent doxorubicin-induced cardiomyopathy, while GPX4 downregulation exacerbates the outcome (175). The Wang lab showed that doxorubicin stimulates HMOX1-mediated heme degradation, which leads to the release of iron causing cardiac lipid peroxidation in mice. Consistently, Fer-1 improves cardiac functioning and survival of the mice (52). Similarly, they and others showed the protective effect of Fer-1 in IRI-induced heart injury, as pre-treatment with the inhibitor leads to reduced infarct size, lower levels of myocardial damage markers in the serum and improved functional recovery (52,104). In addition, Fer-1 inhibits neutrophil recruitment, indicating that ferroptosis stimulates an inflammatory response in this setting (104).

Stroke, intracerebral hemorrhage and traumatic brain injury

Stroke is a devastating neurological condition, representing a leading cause of disability and death worldwide. Two main subtypes of strokes are ischemic, accounting for approximately 85%, and hemorrhagic, accounting for 15% of all stroke cases (127). Ischemic stroke is driven by vessel occlusion leading to interruption of blood supply to the brain. Restoration of blood flow after ischemia in spite of the beneficial effect of re-establishing the oxygen supply, can cause detrimental harm to brain function, resulting in reperfusion injury (89). Ischemia appears to be associated with the increase of iron in affected regions. Considering the cytotoxicity of iron, accumulation of iron can evoke ferroptotic cell death and exacerbate brain damage after reperfusion. In line with this reasoning, iron-fed animals have greater brain infarctions than control animals after middle cerebral artery occlusion (MCAO) (26). Moreover, iron chelation reduces the brain damage in animal models of stroke (67,141). Reperfusion injury of brain is suggested to suppress tau, a protein that normally facilitates iron export by trafficking amyloid precursor protein (APP) to stabilize ferroportin, which causes iron accumulation and a predisposition to ferroptosis (184). The ferroptosis inhibitors Lip-1 and Fer-1 markedly attenuate MCAO-induced functional deficits and reduce brain damage, which further reflects the involvement of ferroptosis in ischemic stroke (184).

Intracerebral hemorrhage (ICH) is the consequence of blood vessel rupture and bleeding into the surrounding brain (135). Despite the low percentage of onset, IHC causes high mortality and morbidity (135). The release of hemoglobin from lysed erythrocytes and its degradation product, iron containing heme, after ICH contribute to brain damage (203). ICH leads to upregulation of HMOX1 and iron overload in the brain (201). HMOX1 exacerbates early brain injury after intracerebral hemorrhage and its inhibition mitigates brain injury in ICH animal models (87,190,194). In addition to hemoglobin-bound iron, transferrin bound iron also contributes to ICH-induced brain injury (130). Iron chelators attenuate brain edema and neurological deficits in rat models of ICH (129). Both preliminary experimental and clinical trials have implicated improved efficacy with DFO for treatment of ICH (218). Recently, ferroptosis was shown to contribute to tissue damage in ICH models (28,103,221). Inhibition of ferroptosis by use of Fer-1 and Lip-1 protects the brain in a collagenase-induced model of ICH. Mice treated with Fer-1 after ICH exhibit

marked brain protection, less iron deposition and improved neurologic function (103). ICH is accompanied with reduced levels of GPX4 protein in brain tissue of rats. Treatment with Fer-1 ameliorates inflammation, neuronal loss and brain injury in rats after ICH (221). Total iron, ferrous iron and MDA concentrations are increased shortly after ICH in hippocampal and perihematomal tissues of mice. Accordingly, Fer-1 treatment mitigates neurological defects, brain atrophy and restores brain functions (28). In support of hemoglobin-mediated iron accumulation, organotypic hippocampal slice cultures (OHSCs) treated with hemoglobin accumulate iron, which is reduced by Fer-1 (103). As an adaptive response, GPX4 expression is induced in the ipsilateral striatum in a mouse model of ICH (2). Delivery of selenium, needed for synthesis of selenocysteine and optimal activity of GPX4, in form of a brain-penetrant seleno-peptide further drives the expression of GPX4 leading to neuronal protection and behavior improvement after ICH (2).

Traumatic brain injury (TBI) resulting from impact, penetrating and closed-head injuries is a major cause of mortality and morbidity worldwide (15). TBIs are associated with cerebral edema, neuronal damage, iron accumulation, intracranial hemorrhage and inflammation, which collectively lead to brain damage (15). Elevated levels of PEox, are detected in the cortex and hippocampus of a pediatric rat controlled cortical impact (CCI) model (198). Accordingly, TBI in a mouse CCI model is followed by dysfunctional iron metabolism and iron accumulation, neuronal degeneration, reduced GPX activity, and the accumulation of lipid ROS. The administration of Fer-1 by cerebral ventricular injection significantly reduces iron deposition and neuronal degeneration, injury lesions volume and improves cognitive and motor function (204).

Chronic neurodegenerative diseases

A growing body of reports has proposed the involvement of ferroptosis in chronic diseases (Table 2, Fig. 6). Among them, the role of iron is best characterized in neurodegenerative diseases. Dysregulation of iron homeostasis is associated with aging and is considered an important risk factor contributing to neurodegeneration. Several factors such as increased blood–brain barrier permeability, increased pro-inflammatory state of brain and changes in distribution of iron within the brain are involved in iron accumulation during aging (195). In many neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, iron

abnormally accumulates in a specific region of the brain which can cause neurotoxicity (195).

Alzheimer's disease (AD) is the most common type of dementia and neurodegenerative disease, associated with formation of neurofibrillary tangles, resulting from aggregation of the tau protein, and amyloid-β (Aβ) plaques in the brain (123). Growing evidence suggests that iron accumulation plays an important role in the pathology of AD. Elevated levels of iron are found in the often heavily damaged hippocampus of Alzheimer's patients (147), and associate with pathological lesions including neurofibrillary tangles and Aβ plaques in the entorhinal cortex and hippocampus of AD patients (125,169). In line with the findings that increased iron deposition in the brain coincides with early plaque formation and cognitive deficits in animal models of AD (98,158), increased iron levels detected through quantitative susceptibility mapping correlate with the progression of AD in humans (9). Ferrous iron is directly associated with amyloid pathology and diffuse amyloid deposits comprise an iron-amyloid complex in a mouse model of AD (178). It is suggested that the neurotoxicity of Aβ is mediated, at least partly, by iron that participates in lipid peroxidation and cellular oxidative stress (150). Although the acute induction of HMOX1 is cytoprotective, excessive and sustained activity of HMOX1, as seen in non-canonical ferroptosis, is suggested to contribute to neurodegeneration in AD (156). Increased expression of HMOX1 is a common phenomenon in AD. The prolonged overexpression of HMOX1 in mice promotes excessive iron production, tau phosphorylation and aggregation in mouse brain (77). Lipid peroxidation is thought to occur early in the pathogenesis of AD (17), and lipid hydroperoxide byproduct 4-HNE, found in Aβ plaques of AD patients, is suggested to covalently modify Aβ, thereby triggering its aggregation (165). A decreased expression of brain GPX4 and guanine-rich sequence-binding factor (GRSF1), which controls GPX4 synthesis, are found in a doxycycline-inducible mouse model of AD (213). Treatment with deuterated PUFAs, shown to inhibit ferroptosis *in vitro* (209), reduces lipid peroxidation and hippocampal Aβ levels in a mouse model of AD (146). Inhibition of 12/15 LOX activity, predicted to contribute to ferroptosis (161), mitigates neuropathology and also reduces Aβ levels *in vivo* (42). In addition, α-lipoic acid, which was validated to stabilize the cognitive function in Alzheimer's patients, inhibits lipid peroxidation,

inflammation and Tau-induced iron overload in an AD mouse model (220). Collectively, these lines of evidence converge on the potential involvement of iron and ferroptosis as the pathogenic mechanism of AD.

Parkinson's disease (PD) is the second most common neurodegenerative disease, characterized by irreversible dopaminergic neuronal loss in the substantia nigra (SN), which causes striatal dopamine deficiency. The intracellular protein inclusions, Lewy bodies and Lewy neurites, containing aggregates of α-synuclein in dopaminergic neurons of the SN are also a hallmark of PD (140). In PD, high levels of iron are observed in the degenerative dopaminergic neurons and are associated with microglia in the SN (61,197). Several studies have implicated colocalization of iron and α-synuclein protein in Lewy bodies, where it apparently promotes synuclein aggregation and accumulation (217). Iron chelation treatment with deferiprone has neuroprotective potential in early-stage PD patients (40). The neurotoxins that are commonly used in cellular models of PD are able to induce ferroptosis in differentiated lund human mesencephalic (LUHMES) cells (46). Moreover, Fer-1 inhibits neuronal loss in a mouse model of PD, proposing the involvement of ferroptosis in dopaminergic cell death (46).

Huntington’s disease (HD) is a progressive neurodegenerative disorder characterized by a combination of motor, cognitive and psychiatric deficits. The disease is caused by an expanded CAG trinucleotide repeat in the huntingtin gene, resulting in a mutant huntingtin protein which contains an abnormally long polyglutamine and causes neuronal dysfunction and eventually cell death (12). Iron accumulates in the brains of HD patients and mouse disease models (29,49). This is believed to contribute to the pathology since deferoxamine treatment improves the motor phenotype in a mouse model of HD (29). Not only oxidative stress but also lipid peroxidation is well-documented in HD (81) as exemplified by the marked increase of MDA and/or 4-HNE adducts found in brain and striatum tissue of both human HD patients and a HD mouse model (20,94). Lastly, Fer-1 protects from neuronal cell death in a brain slice model of HD (167).

Perspectives and concluding remarks

Ferroptosis represents an iron-catalyzed mode of regulated cell death executed by excessive lipid peroxidation. In recent years, our knowledge on the molecular mechanism

and regulation of ferroptosis has greatly advanced, which led to the re-evaluation of the ferroptosis signature in pathological conditions. Remark that although there is plenty of data showing a detrimental role for ferroptosis in many experimental disease models, the evidence for ferroptosis in humans is still sparse. Often signs of iron accumulation and lipid peroxidation degradation products such as 4-HNE and MDA along with a protective effect of iron chelation are used to hint towards a role of ferroptosis in human disease. More targeted approaches detecting specific types of oxidized phospholipids might provide better insight into human pathology and may help in detecting ferroptosis (139). For example, in active human MS brain plaques (144), plasma and liver samples from NASH patients (173), and human atherosclerotic lesions (187) oxidized phosphatidylcholine was detected using immunodiagnostics. Note that one needs to be cautious in relation to the specificity of these antibodies. In fact, the real evidence for ferroptosis is provided in AKI patients showing enhanced levels of oxPE in the urinary pellet detected through oxidative lipidomics (198). This class of oxPL is thought to be critically involved in the process of ferroptosis, highlighting its potential as a clinical marker for ferroptosis (82). Considering the central role of ferroptosis in IRI, its therapeutic targeting might be useful in the context of e.g. organ transplantation, trauma or multi organ failure in critically ill patients. In this respect, we recommend using analogues of Fer-1 and Lip-1 with an improved plasma half-life to verify the therapeutic potential of ferroptosis in experimental disease models (38,73,111,164). The harmful effect of iron released during IRI is well-known and iron chelation therapy markedly improves graft viability and function in animal models of liver, kidney and heart transplantation (155). Recently, Li et al reported that in a model of heart transplantation, treatment of the recipient animal with ferroptosis inhibitor Fer-1 at the time of transplantation reduced cell death and even blocked neutrophil recruitment (104). As for iron chelation therapy (155), treatment of the organ with ferroptosis inhibitors might reduce the damage caused by I/R during transplantation. Therapeutic targeting of ferroptosis in organ transplantation is therefore envisioned to become the first step to clinical implementation of ferroptosis inhibitors. In general, pharmaceuticals inhibiting ferroptosis other than iron chelators could potentially offer a better treatment option, while reducing the side-effects. Although compelling findings in experimental animal models suggest a role for ferroptosis in a plethora of diseases, the exact contribution of

ferroptosis in a human context still needs further investigation. Reliable ferroptosis biomarkers, along oxidative lipidomics approaches, will be an important step in characterizing ferroptosis in human disease.

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Antioxidants and Redox Signaling
Ferroptosis: biological rust of lipid membranes (DOI: 10.1089/ars.2020.8175)

Abbreviations Used

4-HNE = 4-hydroxy-2-nonenals

ACC = acetyl-CoA carboxylase

ACSL4 = acyl-CoA synthetase long-chain family member 4

AD = Alzheimer's disease

AIF = apoptosis-inducing factor

AIFM2 = apoptosis inducing factor mitochondrial 2

AKI = acute kidney injury

AKR = aldo-keto reductases

ALD = alcoholic liver disease

ALS = amyotrophic lateral sclerosis

AMD = age-related macular degeneration

AMI = acute myocardial infarct

AMNL = amylin liver NASH

AMPK = AMP-activated protein kinase

APP = amyloid precursor protein

ARNTL = arylhydrocarbon receptor nuclear translocator-like

BATCH1= BTB and CNC homology 1

BH4 = tetrahydrobiopterin

BMP = bone morphogenetic protein

BMPR = BMP receptor

BSO = buthionine sulfoximine

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c-ACO1 = cytosolic aconitase

CCI = controlled cortical impact

CISD = CDGSH iron sulfur domain

CNS = central nervous system

COPD = chronic obstructive pulmonary disease

CoQ_{10} = ubiquinone-10

$CoQ_{10}H_2$ = ubiquinol

CP = ceruloplasmin

CR = cystine reductase

DCYTB = duodenal cytochrome B

DFO = deferoxamine

DMT1 = divalent metal transporter 1

FA = folic acid

FBXL5 = F box and leucine-rich repeat protein 5

Fer-1 = ferrostatin-1

FIN = ferroptosis inducing compound

FPN = ferroportin

FPP = farnesyl pyro-phosphate

FSP1 = ferroptosis suppressor protein 1

FTH = ferritin heavy chain

FTL = ferritin light chain

GCH1 = GTP cyclohydrolase-1

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GCL = glutamate-cysteine ligase

GLS = glutaminase

GOT1 = glutamic-oxaloacetic transaminase 1

GPX4 = glutathione peroxidases 4

GRSF = guanine-rich sequence-binding factor

GSH = glutathione

GSS = glutathione synthase

GSSG = glutathione disulfide

HAMP = hepcidin antimicrobial peptide

HCP1 = heme carrier protein 1

HD = Huntington's disease

HEPH = hephaestin

HJV = hemojuvelin

HMGCR = 3-hydroxy-3-methylglutaryl-CoA reductase

HMOX1 = heme oxygenase 1

HPETE = hydroperoxyeicosatetraenoic acid

HRG1 = heme-responsive gene 1

HSPB1 = heat shock protein family B

ICH = intracerebral hemorrhage

IPP = isopentenyl pyrophosphate

IRE = iron responsive element

IRI = ischemia reperfusion injury

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IRIDA = iron-refractory iron deficiency anemia

ISC = iron-sulfur cluster

JAK = janus kinase

LAMP2 = lysosome-associated membrane protein-2

LDLR = low density lipoprotein receptor

Lip-1 = liproxstatin-1

LOX = lipoxygenase

LPCAT3 = lysophosphatidylcholine acyltransferase 3

LPO = lipid peroxidation

LPS = lipopolysaccharide

LRP = LDL receptor-related protein

LSEC = liver sinusoidal endothelial cells

LUHMES = lund human mesencephalic

MCAO = middle cerebral artery occlusion

MDA = malondialdehyde

Met = methionine

NAF-1 = nutrient-deprivation autophagy factor-1

NASH = non-alcoholic steatohepatitis

NBIA = neurodegeneration with brain iron accumulation

NCOA4 = nuclear receptor coactivator 4

NFS1 = nitrogen fixation 1

NMDA = N-methyl-D-aspartate

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NRF2 = nuclear factor erythroid 2-related factor 2

NTBI = non-transferrin-bound iron

OHSC = organotypic hippocampal slice culture

OSA = obstructive sleep apnea

PCBP = poly (rC) binding protein

PD = Parkinson's disease

PE = phosphatidylethanolamine

PL = phospholipid

PUFA = polyunsaturated fatty acids

PV^+ = parvalbumin-positive

ROS = reactive oxygen species

RSL3 = ras-selective lethal small molecule 3

RT = radial trap

SAS = sulfasalazine

Sec-tRNA = selenocysteine-tRNA

Ser = serine

SLC11A2 = solute carrier family 11 member 2

SN = substantia nigra

SQS = squalene synthase

SSMD = sedaghatian-type spondylometaphyseal dysplasia

STAT = signal transducer and activator of transcription

STEAP3 = six-transmembrane epithelial antigen of the prostate 3

tBH = tert- butyl hydroperoxide

TBI = traumatic brain injury

TF = transferrin

TfR = transferrin receptor

TMPRSS6 = transmembrane serine protease 6

ZIP8/14 = ZRT/IRT-like proteins 8/14

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Table 1. Acute organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
Liver	Ischemia reperfusion	Treatment with Fer-1, Lip1, DFO or α-tocopherol lowers lipid peroxidation and liver injury in mice.	(55,207)
	Acetaminophen	Fer-1 treatment reduces liver injury, lipid peroxidation, glutathione depletion, and prevents mortality *in vivo*.	(206)
Lung	LPS	Fer-1 treatment lowers lipid peroxidation and iron content both *in vitro* and *in vivo* and ameliorates lung injury and inflammation *in vivo*.	(113)
	Acute radiation	Lip-1 partially reduces lung injury, serum inflammatory cytokine levels and GPX4 downregulation in lung tissue in an *in vivo* model of thoracic radiation.	(105,106)
	Oleic acid	Oleic acid induces acute lung injury *in vivo* characterized by lipid peroxidation, iron overload, glutathione depletion and shrunken mitochondria.	(222)

Table 1. Acute organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
Intestine	Ischemia reperfusion	Lip-1 reduces lipid peroxidation, GPX4 depletion, serum inflammation markers and intestinal injury as well as secondary lung and liver injury *in vivo*. Secondary lung injury is also characterized by increased lipid peroxidation and iron content and decreased glutathione levels.	(107,108)
Pancreas	L-arginine	Lip-1 attenuates pancreatic injury and mortality upon knockdown of the circadian rhythm by arylhydrocarbon receptor nuclear translocator-like (*Arntl)* in L-arginine-induced pancreatitis.	(114)
Kidney	Severe acute pancreatitis	Lip-1 reduces renal lipid peroxidation and glutathione depletion, lowers serum inflammatory markers and ameliorates both pancreatic and renal cell injury.	(117)
Multi-organ injury	Total body irradiation	Ferroptosis inhibitor baicalein increases 30-day survival in an *in vivo* mouse model.	(180)

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Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
Liver	Alcoholic liver disease (ALD)	Fer-1 ameliorates liver injury and lowers lipid peroxidation both *in vitro* and *in vivo.*	(112)
	Nonalcoholic steatohepatitis (NASH)	Ferroptosis inhibitor Trolox and Lip-1 reduce liver injury, inflammation and lipid peroxidation in a choline-deficient, ethionine-supplemented and a methionine/choline deficient NASH model diet, respectively. Neutralization of increased oxidized phospholipids improves liver injury and function in hyperlipidemic low density lipoprotein receptor (Ldlr)-deficient mice fed an amylin liver NASH (AMNL) diet.	(143,173,183)
	Iron overload	Fer-1 treatment lowers hepatic lipid peroxidation and tissue injury in two mouse models of severe hemochromatosis as well as in an *in vitro* iron overload model.	(193)

Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
	Iron dysfunction	Mice with a hepatocyte specific Poly(RC) Binding Protein 1 (PCBP1) knockdown develop inflammatory steatosis characterized by increased lipid peroxidation, GPX4 levels and labile iron content. Dietary iron restriction and vitamin E supplementation ameliorate liver injury.	(142)
	Obstructive sleep apnea (OSA)	Elevated liver and serum lipid peroxidation and changes in ferroptotic markers GPX4 and ACSL4 in an *in vivo* model of chronic intermittent hypoxia suggest a role for ferroptosis.	(31)
Lung	Cigarette smoke	*In vivo*, heterogeneous GPX4 deletion leads to increased lipid peroxidation and cell death in lung homogenates in chronic obstructive pulmonary disease (COPD) model, which is attenuated in GPX4 overexpressing mice.	(215)

Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
	M. tuberculosis	Therapeutic Fer-1 treatment reduces lipid peroxidation, cell death, lung pathology and bacterial load in a severe *in vivo* model of tuberculosis.	(5)
	P. aeruginosa	*In vitro*, *P. aeruginosa* triggers ferroptosis by expressing lipoxygenase pLOXA, which oxidizes host PUFAs of lung epithelium cells. *P. aeruginosa* infected cystic fibrosis patients are characterized by elevated levels of oxidized PE.	(36)
Intestine	Ulcerative colitis	Fer-1 treatment improves the disease score, colon length and histological damage score and lowers colonic MDA and iron levels in a dextran sulfate sodium mouse model.	(205)
Eyes	N-Methyl-d-aspartate	Iron chelation reduces retinal iron content, lipid peroxidation and cell death *in vivo*.	(151)

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Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
	Age-related macular degeneration (AMD)	Both depletion of glutathione and stimulation with tert- butyl hydroperoxide (tBH) as *in vitro* models for AMD induce cell death of retinal pigment epithelial cells that can be blocked by Fer-1 and DFO.	(174,182)
	Danon disease	Knockdown of lysosome-associated membrane protein-2 (LAMP2) in retinal pigment epithelial cells causes increased sensitivity towards tBH induced ferroptosis.	(95)
Pancreas	Arsenic induced pancreatic dysfunction	Elevated lipid peroxidation and changes in ferroptotic markers GPX4 and COX-2 both *in vitro* and *in vivo*, suggest a role for ferroptosis. *In vitro*, Fer-1 attenuates these effects and partly restores insulin secretion.	(196)
Brain	Amyotrophic lateral sclerosis (ALS)	Two compounds, one in clinical trials and the other already approved, block ferroptosis *in vitro*. Iron chelation ameliorates ALS score in a clinical trial and ferroptotic marker 4-HNE and ferritin associate with clinical decline.	(39,41,74,170)

Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
	Multiple sclerosis (MS)	Increased levels of lipid peroxidation combined with reduced glutathione, GPX4, γ-glutamylcysteine ligase and cysteine/glutamate antiporter X_C^- expression in spinal cord of an *in vivo* model of MS suggest a role for ferroptosis.	(76)
	Periventricular leukomalacia	Fer-1 protects against cell death in an *in vitro* model	(167)
	Arsenic	*In vivo*, the cerebral cortex shows increased lipid peroxidation and iron content, reduced glutathione levels and inhibition of the cysteine/glutamate antiporter. In vitro, iron chelation inhibits cell death and mitochondrial ROS production.	(177)
Heart and cardiovascular system	Cigarette smoke induced aortic dysfunction	Fer-1 reduces lipid peroxidation, cell death, cytotoxicity and inflammation *in vitro*, and partially restores medial vascular smooth muscle cell loss *ex vivo*.	(153)

Table 2. Chronic organ injury with ferroptosis signature (not described in the main text)

Affected	Trigger/Disease	Evidence	Reference
Reproductive organs	Pre-eclampsia	Fer-1 helps restore lipid peroxidation and other features of ferroptosis both *in vitro* and *in vivo*, and lowers blood pressure and increases fetal survival *in vivo*. Placental patient material shows increased lipid peroxidation and iron content, and decreased glutathione and GPX activity.	(219)
	Arsenic	*In vivo*, the testes show increased lipid peroxidation and iron content, reduced glutathione levels and inhibition of the cysteine/glutamate antiporter. *In vitro*, Fer-1 mildly limits cell death and lipid peroxidation.	(126)

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Figure Legends

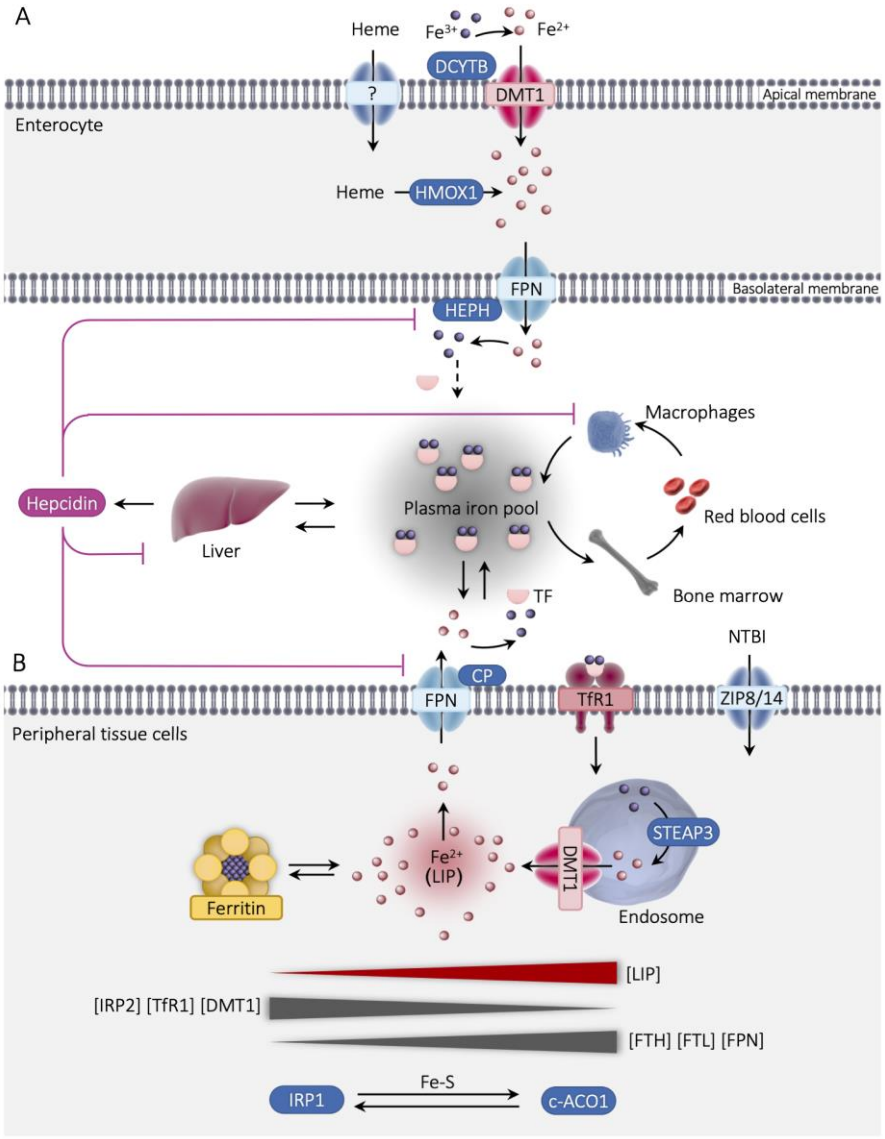

FIG. 1. Key players in the regulation of iron homeostasis. Duodenal enterocytes, macrophages and hepatocytes constitute the major suppliers of plasma iron. A) Systemic iron homeostasis. Dietary iron absorbed by duodenal enterocytes is exported through the concerted action of ferroportin and oxidase hephaestin. In plasma, Fe^{3+} binds transferrin ($TF\text{-}Fe^{3+}$) and circulates throughout the bloodstream to transfer iron to tissue cells. Macrophages and hepatocytes fuel iron plasma by exporting iron via the action of ferroportin and oxidase ceruloplasmin. Hepcidin produced by the liver is the main regulator of systemic iron homeostasis. Elevated levels of iron stimulate the synthesis of hepcidin in hepatocyte and its subsequent release into the plasma, which leads to

degradation of ferroportin. B) Intracellular iron homeostasis. Peripheral tissue cells obtain iron from plasma mostly through TfR1-mediated endocytosis of TF-Fe^{3+}. After being reduced to Fe^{2+}, iron is released to cytosolic labile iron pool (LIP) by DMT1. LIP supplies iron for synthesis of iron-containing proteins. Non-used LIP is either exported via ferroportin or stored in a redox-inactive form in ferritin complexes. Intracellular iron homeostasis is mainly achieved by IRP proteins that bind to IREs of 3' or 5'-UTR of mRNAs. Binding of IRPs to 5'-UTR impedes translation while binding to 3'-UTR stabilizes the mRNA. In condition of iron overload, IRP2 is degraded and IRP1 is transformed to a cytosolic aconitase (c-ACO1) and cannot bind further to IREs. Therefore, translation of ferroportin, ferritin heavy (FTH) and low chain (FTL) is proceeded and mRNA of TfR1 and DMT1 is degraded. In iron-deficient conditions, IRP1 and IRP2 can bind to IREs leading to translational repression of ferroportin, FTH and FTL and stabilization of TfR1 and DMT1 mRNAs. *DCYTB* duodenal cytochrome B; *DMT1* divalent metal transporter 1; *CP* ceruloplasmin; *FPN* ferroportin; *HEPH* hephaestin; *HMOX1* heme oxygenase 1; *LIP* labile iron pool; *NTBI* non-transferrin-bound iron; *STEAP3* six-transmembrane epithelial antigen of the prostate 3; *TfR1* transferrin receptor 1; *ZIP8/14* ZRT/IRT-like proteins 8/14.

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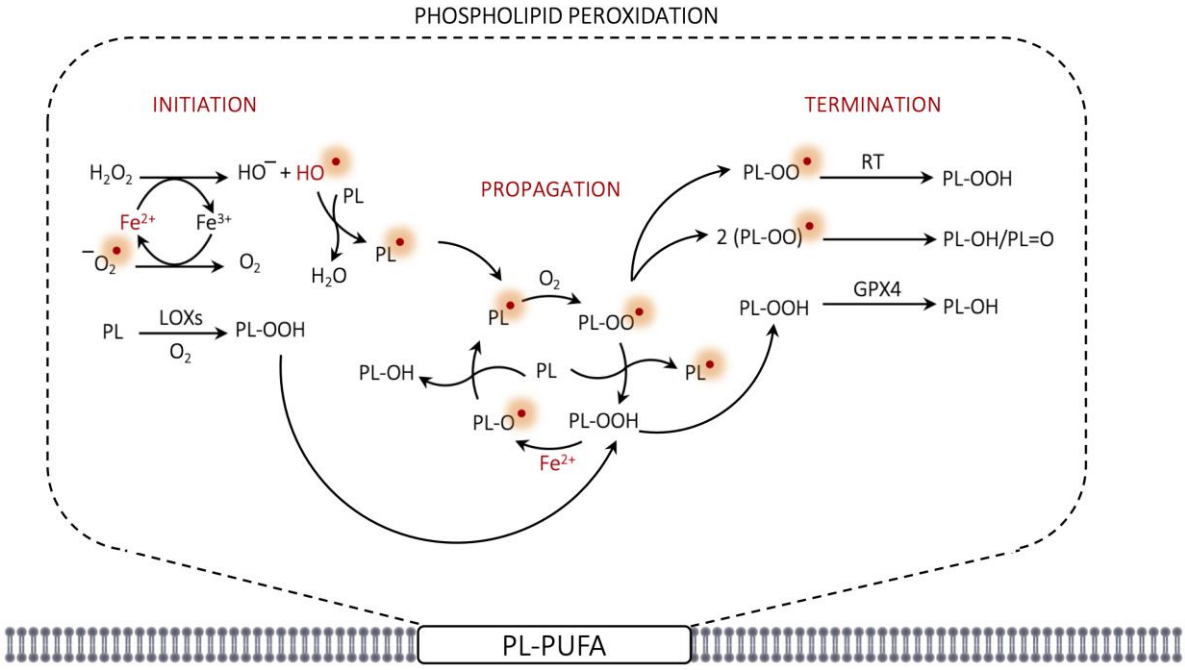

FIG. 2 Lipid peroxidation process. In the first step of lipid peroxidation (LPO), hydroxyl radicals ($OH^\bullet$), produced through Fenton and Haber-Weiss reactions, attack the PUFAs of phospholipids and generate phospholipid radicals ($PL^\bullet$). In addition, lipoxygenases can catalyze directly oxygenation of PUFAs through an enzymatic process. In the propagation phase, $PL^\bullet$ react with oxygen to generate a lipid peroxyl radical ($PL\text{-}OO^\bullet$) along another $PL^\bullet$, which forms an auto-amplifying cycle catalyzed by iron and oxygen. In the termination step, lipid peroxidation can be neutralized by lipophilic radial traps (RT), reaction of two lipid radicals with each other, or peroxidase activity of GPX4. *PUFA* polyunsaturated fatty acid.

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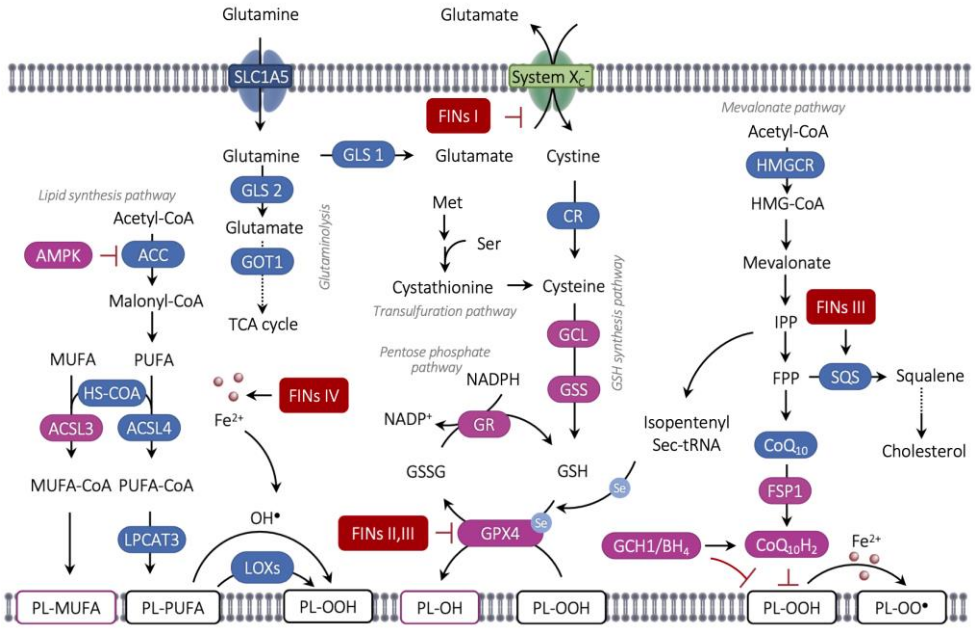

FIG. 3 Mechanism of ferroptosis induction and modulation. Ferroptosis inducing compounds (FINs) induce an iron-catalyzed excessive peroxidation of phospholipids containing polyunsaturated fatty acids (PUFA-PL) leading to cell death. Class I FINs trigger ferroptosis by depleting intracellular glutathione (GSH), which results in GPX4 inactivation. GSH depletion can occur as a result of inhibition of system X_C^- which imports cystine required for GSH synthesis. Inhibition of glutamate cysteine ligase (GCL) can also result in GSH drop and ferroptosis in some cells. Class II FINs trigger ferroptosis by direct inhibition and inactivation of GPX4. Class III FINs activate squalene synthase (SQS), an enzyme involved in cholesterol synthesis. Furthermore, they lead to GPX4 depletion. Class IV FINs promote ferroptosis by increasing the labile iron pool (LIP), or iron oxidation. Different pathways can modulate ferroptosis. The transsulfuration pathway can compensate the drop in cytosolic cysteine levels by supplying cysteine from cystathionine for glutathione synthesis. The pentose phosphate pathway that restores the cytosolic NAPH levels, needed for replenishment of GSH levels, can also impact on ferroptosis response in some cells. The mevalonate pathway by supplying endogenous antioxidant ubiquinone-10 (CoQ_{10}), and isopentenyl pyrophosphate (IPP) can modulate ferroptosis. IPP is needed for maturation of selenocysteine-tRNA (Sec-tRNA) needed for biosynthesis of GPX4. Glutaminolysis regulates sensitivity to ferroptosis induced by class I FINs. Inhibition of glutaminolysis by impeding glutamine uptake, mitochondrial glutaminase (GLS2) and

finally blocking the glutamic-oxaloacetic transaminase 1 (GOT1), blocks ferroptosis. Energy stress is proposed to inhibit ferroptosis partly through AMP-activated protein kinase (AMPK), which regulates phosphorylation of acetyl-CoA carboxylase (ACC) and polyunsaturated fatty acid biosynthesis. Incorporation of PUFA in membranes by concerted action of acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) sensitizes cells to ferroptosis, while incorporation of MUFA by ACSL3 inhibits ferroptosis. The ferroptosis suppressor protein 1 (FSP1) regulates ferroptosis by reducing CoQ_{10} to ubiquinol ($CoQ_{10}H_2$), which traps lipid peroxyl radicals and prevents subsequent lipid peroxidation. The GTP cyclohydrolase-1 (GCH1) protects against ferroptosis via its downstream metabolite BH4 and controlling the levels of CoQ_{10}. The fuchsia color indicates proteins/molecules which have inhibitory effect on ferroptosis. *CR* Cystine reductase; *FPP* farnesyl pyro-phosphate; *GSS* glutathione synthase, *GSSG* glutathione disulfide, *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase; *Met* Methionine; *Ser* serine.

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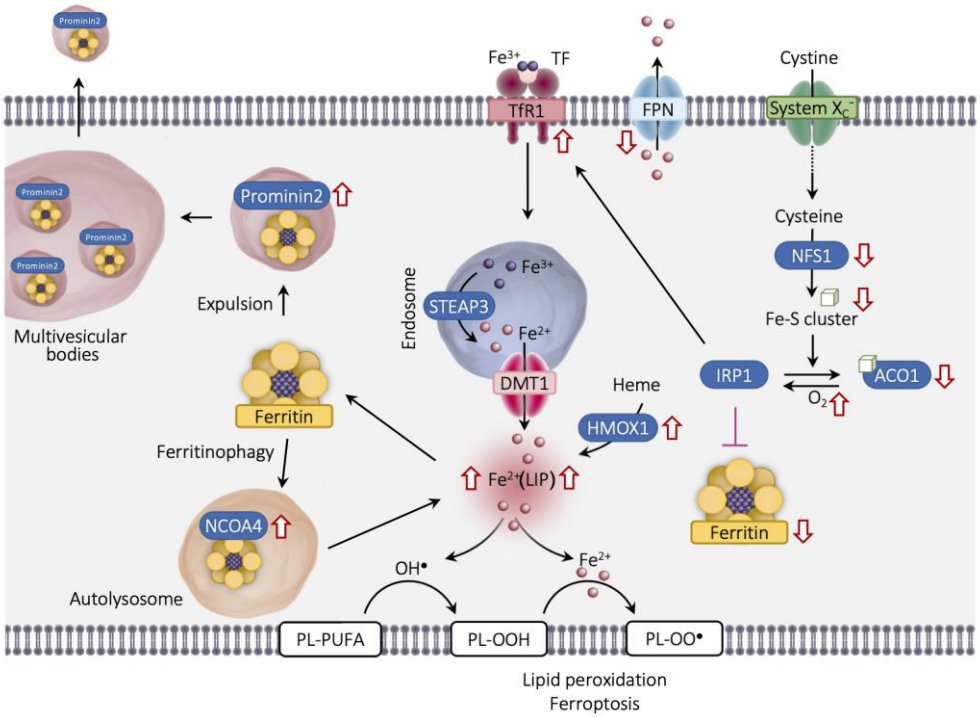

FIG. 4. Ferroptosis regulation by iron. Availability and the levels of labile iron pool (LIP) regulate sensitivity to ferroptosis. The iron uptake by transferrin receptor (TfR1) and transferrin (TF), the buffering capacity of ferritin and iron export by ferroportin (FPN) define sensitivity to ferroptosis in some cellular contexts. Suppression of nitrogen fixation 1 (NFS1), involved in synthesis of iron-sulfur (Fe-S) clusters, sensitizes to ferroptosis in high O_2 environments by activation of iron starvation response, promoting iron influx by increasing the expression of TfR1 and decreasing the expression of ferritin. Depletion of nuclear receptor coactivator 4 (NCOA4), a cargo receptor of ferritin in ferritinophagy, inhibits lysosomal release of iron and ferroptosis. Hyperactivation of HMOX1 leads to elevated levels of LIP and ferroptosis in some cells. Upregulation of prominin2 confers resistance to ferroptosis by promoting the release of ferritin containing exosomes. *c-ACO1* cytosolic-aconitase; *CP* ceruloplasmin; *DCYTB* duodenal cytochrome B; *DMT1* divalent metal transporter 1; *FPN* ferroportin, *HMOX1* heme oxygenase 1; *STEAP3* six-transmembrane epithelial antigen of the prostate 3; *TfR1* transferrin receptor 1.

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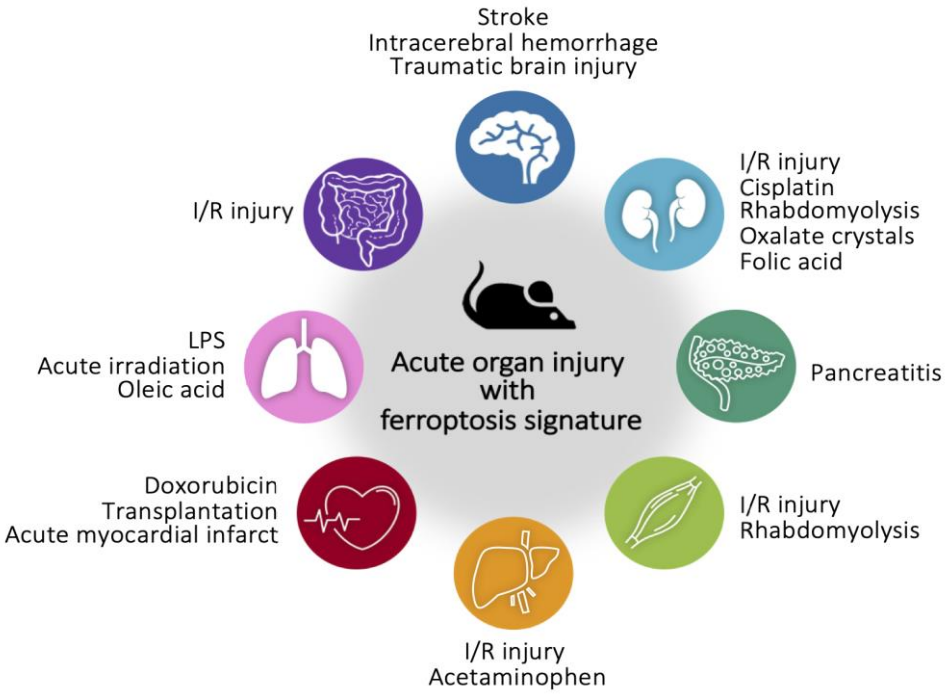

FIG. 5. Acute organ injuries with ferroptosis signature in experimental animal models. Pre-clinical experimental evidence suggests the contribution of ferroptosis to acute injuries in the brain, kidney, liver, pancreas, muscle, heart, lung and intestine. *I/R* ischemia reperfusion; *LPS* lipopolysaccharide.

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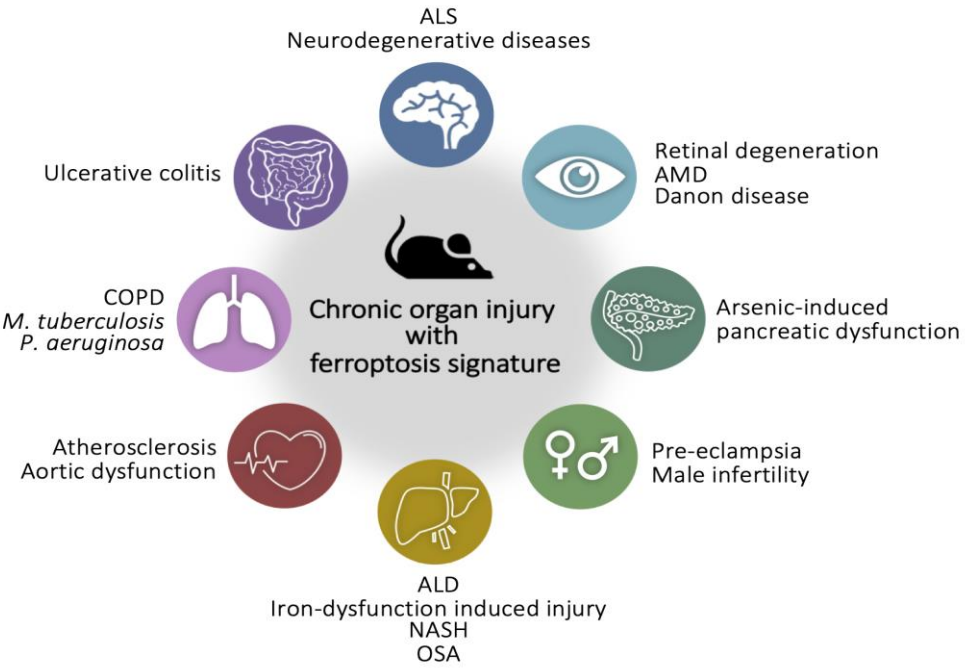

FIG. 6. Chronic organ injuries with ferroptosis signature in experimental animal models. Pre-clinical experimental evidence suggests the contribution of ferroptosis to chronic injuries in the brain, eye, reproductive organs, pancreas, liver, heart, lung and intestine. *ALS* amyotrophic lateral sclerosis; *AMD* age-related macular degeneration; *ALD* alcoholic liver disease; *COPD* chronic obstructive pulmonary disease; *NASH* non-alcoholic steatohepatitis; *OSA* obstructive sleep apnea.