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A GUIDE TO...

A guide to ferroptosis, the biological rust of cellular membranes

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Unprotected iron can rust due to oxygen exposure. Similarly, in our body, oxidative stress can kill cells in an iron-dependent manner, which can give rise to devastating diseases. This type of cell death is referred to as ferroptosis. Generally, ferroptosis is defined as an iron-catalyzed form of regulated necrosis that occurs through excessive peroxidation of polyunsaturated fatty acids within cellular membranes. This review summarizes how ferroptosis is executed by a rather primitive biochemical process, under tight regulation of lipid, iron, and redox metabolic processes. An overview is given of major classes of ferroptosis inducers and inhibitors, and how to detect ferroptosis. Finally, its detrimental role in disease is briefly discussed.

Introduction

The past two decades of cell death research have revealed the existence of several modes of regulated necrosis [1]. Ferroptosis as a distinct form of regulated, iron-catalyzed cell death driven by excessive lipid peroxidation (LPO) within cellular membranes was first conceptualized by Stockwell's lab [2]. However, research studying the toxicity of compounds, toxins, and transition metals already demonstrated the existence of lipid peroxidation-driven cell death far before ferroptosis was discovered [3,4]. Furthermore, identification of the commonly used ferroptosis-inducing compounds, erastin and RLS3, as well as findings that genetic modulation of genes controlling redox metabolism drives non-apoptotic cell death, was also reported in the pre-ferroptosis era [5–7]. Cell membrane rupture during ferroptotic cell death is characterized by hydrogen abstraction and oxygenation of polyunsaturated fatty acids (PUFAs) of phospholipids (PLs), which is catalyzed by redox-active iron. This subsequently leads to cell death due to disruption of membrane stability and the accumulation of lipid hydroperoxides to lethal levels [8]. Although the process of lipid peroxidation

Abbreviations
4HNE, 4-hydroxy-2-nonenal; ACSL, acyl-CoA synthetase long-chain family member; BH4, tetrahydrobiopterin; CoA, coenzyme A; CoQ10, ubiquinone; CoQ10H2, ubiquinol; DMT1, divalent metal transporter 1; D-PUFA, deuterium-polyunsaturated fatty acid; Fe2+, ferrous iron; Fe3+, ferric iron; Fer-1, ferrostatin-1; FIN, ferroptosis inducing compound; FPN, ferroportin; FSP1, ferroptosis suppressor protein 1; GLN, glutamine; GPX4, glutathione peroxidase 4; GSH, glutathione; HMOX1, heme oxygenase 1; IKE, imidazole ketone erastin; IRI, ischemia–reperfusion injury; LIP, labile iron pool; Lip-1, liproxstatin-1; LOX, lipoxygenase; LPO, lipid peroxidation; MDA, malondialdehyde; MUFA, monounsaturated fatty acid; NCOA4, nuclear receptor coactivator 4; NRF2, nuclear factor erythroid 2-related factor 2; PL, phospholipid; PLOOH, phospholipid hydroperoxide; POR, cytochrome P450 oxidoreductase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RSL3, RAS-selective lethal molecule; RTA, radical trapping antioxidant; SFA, saturated fatty acid; TFR1, transferrin receptor 1.

has been linked to several regulated cell death modalities, ferroptosis is exclusively driven by excessive lipid peroxidation [9]. By this, ferroptosis-induced cell death relies on alterations of factors contributing to iron metabolism, antioxidant defense, and lipid metabolism.

In recent years, ferroptosis has been increasingly explored in diseases related to ischemia–reperfusion injury (IRI) or iron toxicity, including neurological disorders, single or multiorgan injury, infarction, and stroke [10]. Intriguingly, extensive studies also suggest a pivotal role of ferroptosis in tumor suppression [11]. As such, pharmacological modulation of ferroptosis either via inhibition or induction may hold great promise for the treatment of a multitude of diseases [12]. The detection of ferroptosis in pathophysiology remains challenging; however, a snapshot of the most important detection techniques is made [13]. In this review, we discuss the main regulatory mechanisms of ferroptosis, different classes of inhibitors and inducers, and current available detection tools. In addition, the role of ferroptosis in some generally accepted pathologies is briefly summarized. Essentially, this review provides a guide to ferroptosis as it exists today.

Ferroptosis execution

Excessive lipid peroxidation of PUFAs containing PLs within cellular membranes is the major executioner mechanism of ferroptosis [2]. Although LPO events also occur in other modes of regulated cell death, an extended oxidative lipidomic approach showed only excessive phospholipid peroxidation during ferroptosis [9]. PUFA-containing PLs have weak C–H bonds in between adjacent carbon–carbon double bonds, which makes them susceptible to LPO [14]. Furthermore, PUFA incorporation into cellular membranes PLs is required for the initiation of ferroptosis [15,16]. Oxidative damage of PUFA-PLs can be initiated either through non-enzymatic free-radical chain reactions involving Fenton chemistry [17] or enzyme-mediated processes catalyzed by iron-dependent lipoxygenases (LOXs) or cytochrome P450 oxidoreductase (POR) (Fig. 1, left panel) [8,18]. The subcellular membranes essential for ferroptosis and the sequence of their peroxidation remains a topic of debate and is likely dependent on how ferroptosis is induced. Different cellular organelles including endoplasmic reticulum [15,19], mitochondria [20], and lysosomes [21–23], all seem to be able to initiate ferroptosis.

Fenton chemistry refers to a series of iron-catalyzed reactions in which oxygen-centered radicals such as hydroxyl radicals are produced [24]. These highly reactive free radicals initiate LPO by abstracting labile hydrogen atoms from PUFAs, producing phospholipid radicals which subsequently react with molecular oxygen during the propagation phase. As a result, PL peroxyl radicals further attack adjacent PUFAs yielding new phospholipid radicals along PL hydroperoxides (PLOOH), which in the presence of redox-active labile iron is further converted to phospholipid alkoxyl radicals (Fig. 1, middle panel). All newly formed PL radicals as well as singlet oxygen molecules generated via the Russell mechanism can re-enter the chain reaction to further amplify PLOOH [25]. Enzymatic lipid peroxidation, which is mediated by LOXs or POR, directly catalyzes the deoxygenation of free and esterified PUFAs producing PLOOH (Fig. 1, left panel) [26,27]. The crucial role of LOXs during ferroptosis is still a matter of debate, due to lack of genetic evidence. Phosphatidylethanolamine binding protein 1 (PEBP1) was suggested to associate with 15LOX to acquire specificity for the phosphatidylethanolamine phospholipids that are key to ferroptosis [28]. Whereas radical-trapping antioxidants (RTAs) can rescue cells from ferroptosis by interfering with the autoxidation process, the inhibition of LOX cannot reverse ferroptotic cell death [17].

The propagation of this free radical chain reaction within cellular membranes eventually leads to the formation of secondary toxic aldehydes such as 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA) [4]. Both 4HNE and MDA can form adducts with proteins and DNA, which in turn results in biomolecular damage [29]. Finally, the membrane becomes thinner and forms curvatures followed by the increased accessibility to oxidants and eventually cell membrane rupture [30], likely involving mechanosensing channels [31] and osmotic processes [32,33].

The auto-amplifying chain reaction can be terminated when lipid hydroperoxides and lipid peroxyl radicals decompose into inactive non-radical products (Fig. 1, right panel). For this, the cell depends on endogenous RTAs including vitamin E [34], vitamin K [35], tetrahydrobiopterin (BH4) [36,37], ubiquinol ($CoQ_{10}H_2$) [38,39], hydropersulfides [40,41], vitamin A and its active derivates [42], 7-dehydrocholesterol [43] and squalene [44]. RTAs intervene directly in the chain reaction by scavenging unpaired electrons thereby counteracting LPO. Furthermore, the selenium-dependent glutathione peroxidase 4 (GPX4), an essential enzyme of the glutathione (GSH) system, impedes the execution of ferroptosis by detoxifying PLOOHs to their corresponding non-toxic PL alcohol forms [7].

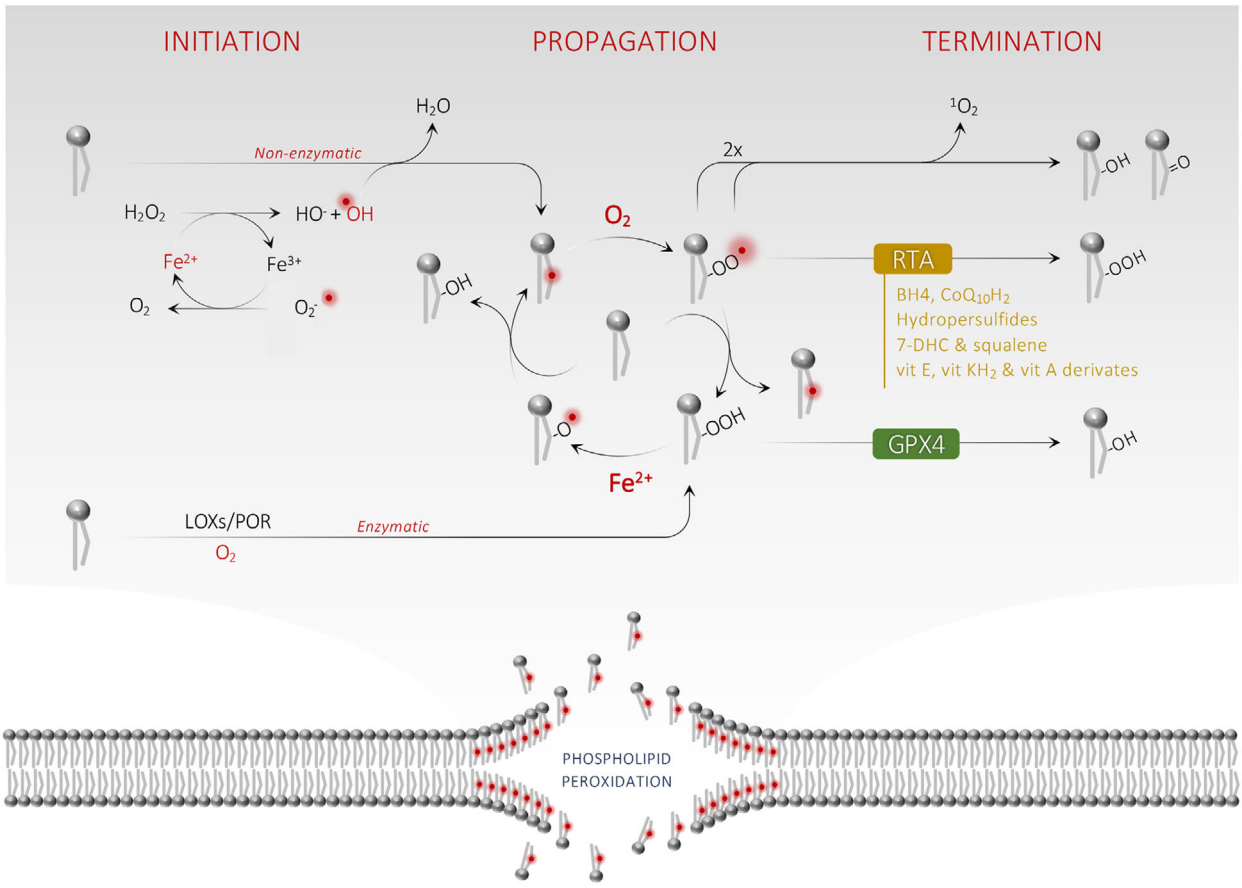


Fig. 1. Phospholipid peroxidation process. LPO initiation step includes the formation of phospholipid radicals catalyzed by redox-active labile iron generated during the Fenton reaction. Additionally, LOXs oxygenate PUFAs directly via an enzymatic process. In the subsequent propagation phase, phospholipid radicals react with molecular oxygen forming phospholipid peroxyl radicals which in turn form phospholipid hydroperoxide and new phospholipid radicals. In the presence of iron, phospholipid hydroperoxide decomposes into phospholipid alkoxyl radicals which refuel the chain reaction by attacking another PUFA. During the termination phase, damaging phospholipids can be neutralized either through the reaction between two phospholipid peroxyl radicals, endogenous RTAs, or peroxidase activity of GPX4. $^1O_2$, singlet oxygen; BH4, tetrahydrobiopterin; $CoQ_{10}H_2$, ubiquinol; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; GPX4, glutathione peroxidase 4; H_2O_2, hydrogen peroxide; HO^{-}, hydroxyl; LOX, lipoxygenase; LPO, lipid peroxidation; O_2, oxygen; $O_2^{\cdot-}$, superoxide radical anion; $OH^{\cdot}$, hydroxy radicals; PL, phospholipid; POR, cytochrome P450 oxidoreductase; PUFA, poly-unsaturated phospholipids; RTA, radical trapping antioxidants; Vit E, KH_2 or A, vitamin E, KH_2, or A.

Ferroptosis induction

Four major classes of ferroptosis-inducing compounds (FINs) have been described to modulate the sensitivity towards ferroptosis (Table 1). Class I, II, and III FINs induce ferroptosis by interfering with redox metabolism, whereas class IV FINs overrule these redox protective mechanisms by directly targeting iron metabolism.

Unleashing ferroptosis redox brakes

Class I ferroptosis inducers downregulate GSH required for the proper functioning of GPX4 by depleting directly or indirectly intracellular cysteine for example by inhibiting cystine/glutamate antiporter system X_C^-. Subsequent GPX4 inactivation results in PLOOH accumulation and ferroptotic cell death. Erastin was the first small molecule identified as a class I ferroptosis inducer [2]. Later, imidazole ketone erastin (IKE), piperazine erastin (PE), and the FDA-approved compounds sulfasalazine and sorafenib were added [45,46]. In addition, excessive concentration of glutamate is also classified as a class I inducer [47]. Noteworthy, PE, IKE, and sorafenib are the only compounds suitable for *in vivo* use due to their high inhibitory potential, stability, and pharmacokinetic profile [47]. Class II inducers directly inhibit GPX4 function by covalent interaction with

Table 1. Ferroptosis-inducing compounds. $(NH_4)_2Fe(SO_4)_2$, ferrous ammonium sulfate; DPI, diphenyleneiodonium; FIN, ferroptosis-inducing compound; FSP1, ferroptosis suppressor protein 1; GPX4, glutathione peroxidase 4; GSH, glutathione; HMOX1, heme oxygenase-1; IKE, imidazole ketone erastin; PE, piperazine erastin; SQS, squalene synthase.

Ferroptosis inducers			
Class	Target	Mechanism	Compounds
FIN I	GSH	Direct or indirect depletion of intracellular cysteine by inhibiting e.g. System Xc^-	Erastin, IKE, PE, sorafenib, sulfasalazine, glutamate
FIN II	GPX4	Covalently binding and inhibition of GPX4 activity	RSL3, ML162, withaferin A, DPI compounds
FIN III	CoQ_{10}	FSP1 inhibition or CoQ_{10} depletion via SQS activation	FIN56, iFSP1, icFSP1, Statins
FIN IV	Redox-active iron	Iron loading, iron oxidation and increase in LIP by HMOX1	FINO2, withaferin A, artemisinin, artesunate, hemin, hemoglobin, $(NH_4)_2Fe(SO_4)_2$

the nucleophilic active-site selenocysteine [48] and include RSL3, ML162, a variety of diphenyleneiodonium compounds [8], and the medicinal plant anticancer agent withaferin A [49]. Class III inducers essentially downregulate mevalonate-derived ubiquinone (CoQ_{10}), which acts as an endogenous lipophilic radical trap. Ferroptosis inducer 56 (FIN56) was the first compound discovered with this mode of action, along inactivation of GPX4 [50]. Recently, the discovery of first-generation (iFSP1) and second-generation (icFSP1) inhibitors of ferroptosis suppressor protein 1 (FSP1) showed the potential to suppress ferroptosis independently of GPX4 activity [38,51]. Finally, different statins have been classified as class III inducers since it inhibits HMG-CoA reductase enzyme which in turn blocks CoQ_{10} biosynthesis [52,53].

Overruling redox protective mechanisms

Class IV FINs are grouped for their ability to increase the levels of cytosolic non-chelatable redox-active iron, often referred to as the labile iron pool (LIP). Iron loading using hemin [54], hemoglobin [55] or ferrous ammonium sulfate ($(NH_4)_2Fe(SO_4)_2$) [49] have shown to trigger ferroptosis *in vitro* as well as in preclinical models of intracerebral hemorrhage. Furthermore, ferritinophagy induced by salinomycin, artemisinin, and its prodrug artesunate triggers ferroptosis in cancer cells by increasing the LIP [56,57]. Ferroptosis-inducing endoperoxides such as $FINO_2$ directly oxidize ferrous iron (Fe^{2+}) but also inactivate GPX4 indirectly in cells [58]. Similarly, withaferin A also induces ferroptosis by exerting dual effects involving LIP increase through heme oxygenase 1 (HMOX1)-mediated degradation of heme and GPX4 inactivation [49].

Ferroptosis regulation

Lipid metabolism

Peroxidation of specific membrane phospholipids and subsequent cell membrane damage ultimately drives cells to death. PLs acylated with PUFAs are the main target of lipid peroxidation since weak bis-allylic protons of PUFAs are more easily abstracted in comparison with the hydrogens in monounsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs) [14]. PUFAs containing the heavy hydrogen isotope deuterium (D-PUFA) are much less susceptible to ferroptosis. As such, treating cells with D-PUFAs suppresses ferroptosis sensitivity, underscoring the importance of PUFA peroxidation in the execution of ferroptosis [8]. In addition, the lipid composition of the cellular membrane as well as the abundance of PUFAs determine the extent of LPO and thus also ferroptosis sensitivity (Fig. 2, brown panel) [15,17]. Although free fatty acids are substrates for the synthesis of lipid signaling mediators, the incorporation of esterified PUFAs in cellular membranes is necessary to exert lethal effects upon oxidation [15,16]. Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) are two key enzymes involved in the biosynthesis and remodeling of PUFAs in cellular membranes. Thus, the deletion of both genes suppresses ferroptosis by depleting LPO substrates [59]. In contrast, supplementing cells with exogenous PUFAs enhanced erastin-induced ferroptosis [8]. Unlike PUFAs, MUFAs induce a ferroptosis resistance state in cells by blocking the formation of lipid reactive oxygen species (ROS) and displacing oxidizable PUFAs from the phospholipid membrane. The protective role of MUFAs relies on the activity of acyl-coenzyme A synthetase long-chain family member 3 (ACSL3) protein, which catalyzes the esterification

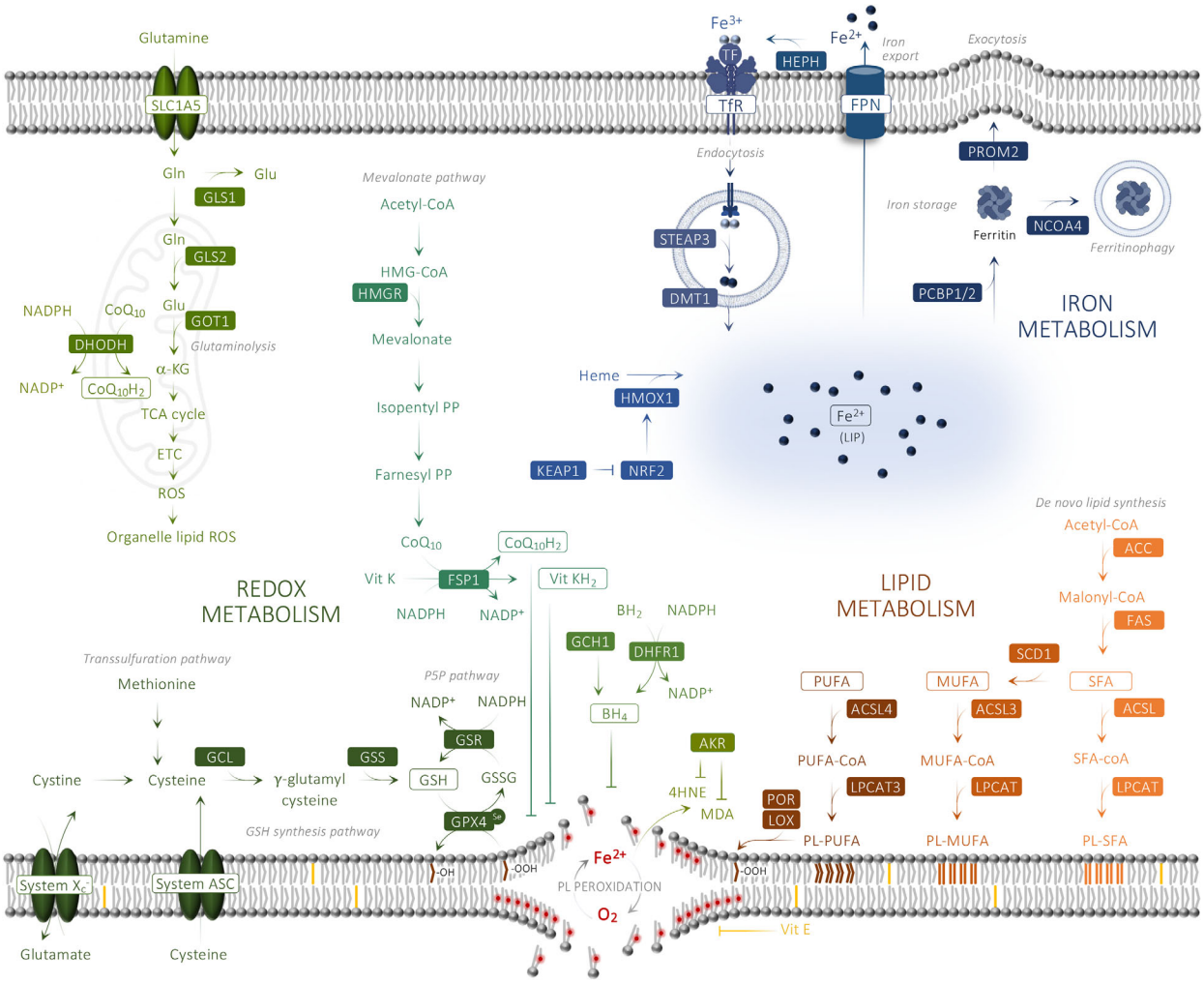


Fig. 2. Metabolic regulation of ferroptosis. Ferroptosis is tightly regulated by three key elements: redox, iron, and lipids. The different antioxidant defense mechanisms implicated in the ferroptosis pathway include the XC- GSH-GPX4 pathway, transsulfuration pathway, mevalonate pathway, FSP1-Vitamin K (vit K)/CoQ10 pathway, glutaminolysis, DHODH-CoQ10H2 pathway, GCH1-BH4 pathway, and aldo-keto reductases (redox metabolism displayed in green). Fluctuations in the labile iron pool (Fe^{2+}) are mainly controlled by TFR, FPN, DMT1, NCOA4, NRF2, and HMOX1 (iron metabolism displayed in blue). The peroxidation of PUFA-containing phospholipids (PUFA-PLs) within cellular membranes is mainly regulated by ACSL4, LPCAT3, LOX, POR and Vitamin E (vit E), (lipid metabolism displayed in orange). An imbalance between the production of endogenous oxidants and antioxidants and the presence of excess free labile iron and oxidizable phospholipids acylated with PUFAs are both required for ferroptosis execution. 4HNE, 4-hydroxy-2-nonenal; ACC, acetyl CoA-carboxylase; ACSL, acyl-CoA synthetase long-chain family member; AKR, aldo-keto reductases; BH2, dihydrobiopterin; BH4, tetrahydrobiopterin; CoQ10, ubiquinone; CoQ10H2, ubiquinol; DHFR1, dihydrofolate reductase; DHFR1, dihydrofolate reductase; DHODH, dihydroorotate dehydrogenase; DMT1, divalent metal transporter 1; ETC, electron transport chain; FAS, fatty acid synthase; Fe^{2+}, ferrous iron; FPN, ferroportin; FSP1, ferroptosis suppressor protein 1; GCH1, guanosine triphosphate cyclohydrolase 1; GCL, glutamate-cysteine ligase; Gln, glutamine; GLS1, glutaminases; Glu, glutamate; GOT1, glutamic-oxaloacetic transaminase 1; GPX4, glutathione peroxidase 4; GSH, glutathione; GSR, glutathione–disulfide reductase; GSS, glutathione synthetase; GSSG, oxidized glutathione; HEPH, hephaestin; HMOX1, heme oxygenase 1; KEAP1, Kelch-like ECH-associated protein 1; LOX, lipoxygenase; LPCAT, lysophosphatidylcholine acyltransferase; MDA, malondialdehyde; MUFA, monounsaturated fatty acid; NCOA4, nuclear receptor coactivator 4; NRF2, nuclear factor E2-related factor 2; O_2, oxygen; PCBP1/2, poly(rC)-binding protein 1/2; PL, phospholipid; POR, cytochrome P450 oxidoreductase; PP, pyrophosphate; PROM2, prominin2; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SCD1, stearoyl-CoA desaturase 1; SFA, saturated fatty acid; STEAP3, transmembrane epithelial antigen of the prostate 3; TCA cycle, tricarboxylic acid cycle; TFR1, transferrin receptor 1; α-KG, alpha-ketoglutarate.

of MUFAs with coenzyme A (CoA) [60]. Downregulating the expression of ACSL3 in cells treated with exogenous MUFAs showed less protection against ferroptosis [61]. In addition, stearoyl-CoA desaturase 1 enzyme, which converts SFA into MUFAs, also showed to sensitize cells to ferroptosis upon inhibition [62].

Iron metabolism

Iron homeostasis is kept under exquisite control by many cellular processes. Increasing levels of unbound redox-active iron or LIP triggered by dysregulation of either iron import, export, storage, or turnover impacts the sensitivity towards ferroptosis (Fig. 2, blue panel). Under physiological conditions, circulating ferric iron (Fe^{3+}) is internalized into cells as a transferrin-iron complex through the membrane-bound transferrin receptor 1 (TFR1) [63]. Once released in endosome compartments, Fe^{3+} is reduced to Fe^{2+} via the endosomal metalloreductase enzyme six-transmembrane epithelial antigen of the prostate 3 (STEAP3) [64]. Subsequently, Fe^{2+} fuels the LIP in the cytoplasm through divalent metal transporter 1 (DMT1) [65]. Genetic inactivation of TFR1 has previously been shown to prevent ferroptosis upon erastin treatment or cystine deprivation [6]. Conversely, treatment with transferrin enhanced erastin-induced cell death [66]. Excess of cellular Fe^{3+}, which is not needed for metabolic functions such as synthesis of iron-containing enzymes, is sequestered within the iron storage protein complex ferritin [67]. Not surprisingly, autophagy-mediated degradation of ferritin, referred to as ferritinophagy promotes accumulation of lipid ROS by increasing the LIP [68]. Consistently, the knockdown of selective cargo receptor nuclear receptor coactivator 4 (NCOA4), which recruits ferritin to autophagosomes, blocks ferroptosis [69]. Ferrous iron can also be released from heme by HMOX1, which is controlled by the nuclear factor E2-related factor 2 (NRF2). Under condition of oxidative stress, NRF2 unleashes from the Kelch-like ECH associated protein 1 (KEAP1) bond, allowing nuclear translocation and activation of target genes with mainly anti-ferroptotic function [70]. However, in certain contexts, excessive activation of HMOX1 along an insufficient buffer capacity of ferritin upon NRF2 upregulation has been shown to promote ferroptosis by increasing the LIP [49]. Cellular iron export can be mediated through the transmembrane protein ferroportin (FPN). Downregulation of FPN either genetically or pharmacologically increases ferroptosis sensitivity by limiting iron export [71,72]. Alternatively, upregulation of the prominin2-mediated ferritin exocytosis pathway promotes resistance to ferroptosis [28]. Thus, modulation of the intracellular redox-active iron levels by the cellular iron homeostatic network is key in regulating ferroptosis sensitivity.

Redox metabolism

The thiol-containing tripeptide glutathione is an essential intracellular antioxidant that is synthesized from cysteine via two ATP-dependent enzymes, glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS) [73]. The importance of cystine and cysteine to maintain GSH biosynthesis was previously shown by findings that cells supplemented with cystine-free medium die due to GSH depletion and this death could be prevented by the administration of lipophilic antioxidants such as alpha-tocopherol [74]. The intracellular cysteine pool mainly relies on the system X_c^- which takes up extracellular cystine, the oxidized form of cysteine, in exchange for intracellular glutamate (Fig. 2, green panel) [73]. Indeed, glutamate serves as a trigger for ferroptosis induction since high extracellular glutamate concentrations inhibit system X_c^- [2]. Cellular cysteine can also be provided via the alanine-serine-cysteine (ASC) system or synthesized from methionine via the transsulfuration pathway [75,76]. Conditions that hinder intracellular cysteine and consequently GSH levels [2,77] or selenium/selenocysteine uptake mechanisms via low-density lipoprotein receptor-related protein 8 (LRP8), directly impact the activity of the GPX4 enzyme. GPX4 converts GSH to oxidized GSSG, which is then recycled back by glutathione reductase (GSR) at the expense of $NADPH/H^+$ [78]. Obviously, pharmacological or genetic inactivation of GPX4 is an often-used strategy to induce ferroptosis [79–81].

Along the X_C^--GSH-GPX4 axis, cells can also counteract lipid peroxidation by promoting endogenous radical trapping antioxidant systems. FSP1, formerly known as AIFM2, maintains the reduction of CoQ_{10} using NADPH [38]. Upon activation, FSP1 is recruited to the plasma membrane where it generates $CoQ_{10}H_2$ which in turn traps PL peroxyl radicals and prevents subsequent phospholipid peroxidation [38,82]. Like the function of FSP1 in the extramitochondrial membrane, dihydroorotate dehydrogenase (DHODH) reduces mitochondrial CoQ_{10} to $CoQ_{10}H_2$ independently of GPX4 or FSP1 [83]. BH4 is another endogenous RTA that protects against ferroptosis independently from GPX4 [82]. Treating BH4-deficient cells with exogenous dihydrobiopterin (BH2), the dehydrogenated product of BH4 that is converted by dihydrofolate reductase (DHFR), protected against

RSL3- and ML162-induced cell death. Consistently, genetic deletion of the rate-limiting enzyme guanosine triphosphate cyclohydrolase 1 (GCH1) reduced intracellular levels of BH4 and decreased the antioxidant capacity of the cells [37]. Noteworthy, a class of aldo-keto reductases was also classified as endogenous RTA since it has been shown to detoxify oxidative lipid breakdown products such as 4HNE [4,84]. Finally, the ferroptotic pathway is also tightly linked to the glutamine (Gln) metabolism, known as glutaminolysis. The Gln transporter SLC1A5, glutaminases 2 (GLS2), and glutamic-oxaloacetic transaminase 1 (GOT1) are required for Gln import and the conversion to glutamate and α-ketoglutarate. Genetic knockdown of these genes showed to counteract ferroptosis [20,66].

Ferroptosis detection

Considering the high clinical relevance of ferroptosis, understanding its core molecular machinery is paramount for disease prevention, diagnosis, treatment, and prognosis. Since it remains challenging to detect (per) oxidized PLs and redox-active iron in biofluids, a variety of biomarkers related to lipid and iron metabolism are needed to differentiate ferroptosis [48,85–87]. Considering the central role of excessive LPO in ferroptosis, different tools have been described to study how (per) oxidized lipids are involved in the dying process by detecting and quantifying the extent of LPO or lipid ROS. Both C11-BODIPY and LiperFluo are widely used probes to measure lipid ROS. Upon oxidation, the fluorescent switch of these probes can easily be detected by fluorescent microscopy or flow cytometry [15,88]. Toxic lipid degradation products, such as MDA and 4HNE, can be determined by thiobarbituric acid reactive substances (TBARS) approach [89], western blotting, or staining procedures [90]. Another approach to detect oxidized phospholipids involves the use of an E06 antibody that specifically labels oxidized phosphatidylcholines [91,92]. The most sophisticated and specific approach for detection of PLOOHs is the use of liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis, referred to as oxidative lipidomics [15,93].

Furthermore, alterations in iron homeostasis have shown to control the sensitivity towards ferroptosis. For instance, increased expression of iron influx proteins TFR1 and DMT1, or contrary, decreased expression of efflux proteins FPN, ceruloplasmin, and hephaestin enhance ferroptosis induction [94]. Recently, TFR1 was proposed as a biomarker to detect ferroptosis *in vitro* and *in vivo* [95]. However, the use of TFR1 antibodies is dependent on cell type, tissue, and condition since iron influx may be orchestrated by several influx transporters in different cell types [94]. Additionally, altered expression levels of NCOA4 [96], HMOX1 [49], NRF2 [97,98], and heat shock protein beta-1 (HSPB1) [99] have also been linked to ferroptosis. However, many of these suggested biomarkers are often context-dependent and therefore considered as rather bystander effects of ferroptosis. Next to the analysis of protein and/or gene expression, several commercial assays are available to measure the iron content such as calcein-AM assay [100], FeRhoNox, FerroOrange, Mito-FerroGreen probes [49,101,102], and Perl's Prussian Blue staining with or without DAB-enhancement [103]. However, a more reliable technique to measure the total iron content, ferrous iron as well as ferric iron, is capillary electrophoresis coupled plasma mass spectrometry with dynamic reaction cell (CE-ICP-DRC-MS) [104–106].

Pharmacological ferroptosis inhibition

The high clinical relevance of ferroptosis in a variety of diseases has boosted the development of novel therapeutics (Table 2). Here, we will only give a snapshot of the most potent ferroptosis inhibitors.

Iron chelators

Considering the central role of iron in ferroptosis execution, iron-chelating therapies such as deferasirox

Table 2. Ferroptosis inhibiting compounds. $(NH_4)_2Fe(SO_4)_2$, ferrous ammonium sulfate; DPI, diphenyleneiodonium; FIN, ferroptosis-inducing compound; FSP1, ferroptosis suppressor protein 1; GPX4, glutathione peroxidase 4; GSH, glutathione; HMOX1, heme oxygenase-1; IKE, imidazole ketone erastin; PE, piperazine erastin.

Ferroptosis inhibitors			
Class	Characteristics	Mechanism of action	Compounds
I	Iron chelators	Remove excess iron	Deferasirox, deferiprone, deferoxamine, ciclopirox, CN128
II	Lipophilic radical traps	Trapping chain-carrying radicals in phospholipid bilayer	Vitamin E, BHT, Fer-1, Lip-1, XJB-5-131, CoQ_{10}, UAMC-3203
III	Deuterated PUFAs	Prevent initiation and propagation of lipid peroxidation	D4-arachidonic acid, D10-docosahexaenoic acid
IV	Lipoxygenase inhibitors	Prevent LOX-induced LPO	CDC, baicalein, PD-146176, AA-861, zileuton, FerroLoxin-1/2

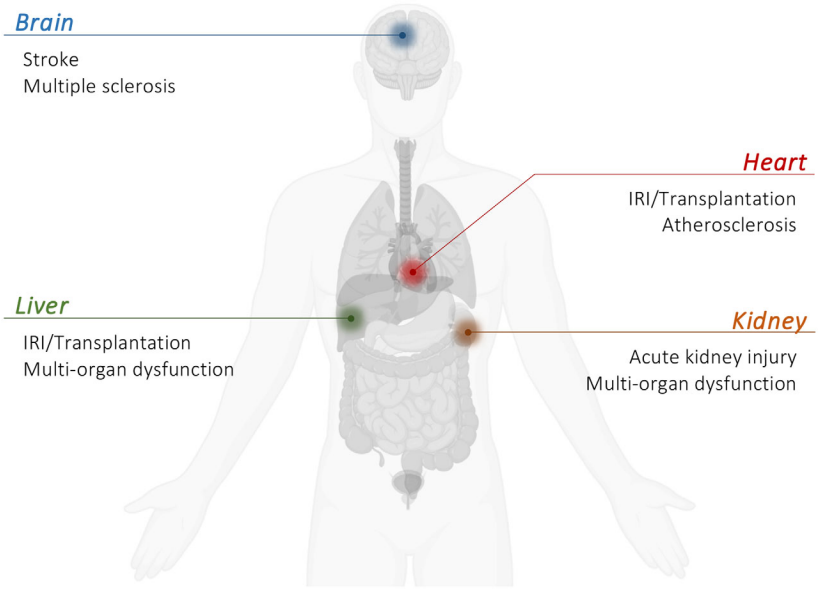


Fig. 3. Ferroptosis-driven pathologies and ferroptosis-associated therapeutic benefits in experimental rodent models. Although the number of ferroptosis-driven pathologies is increasing, only a few pathologies have shown a convincing ferroptosis signature in patients along with a therapeutic benefit in representative experimental preclinical models. To date, evidence of a ferroptosis signature in human disorders is based on the presence of elevated levels of redox-active iron, peroxidized phospholipids and lipid degradation products in either biofluids or injured tissue. Therapeutic targeting of ferroptosis using mainly lipophilic radical trapping agents in experimental rodent models highlights its therapeutic potential.

[107], deferiprone [108], deferoxamine [2], ciclopirox olamine [2] and CN128 [109] have been widely considered as potential therapeutic agents in the treatment of ferroptosis-driven diseases. Indeed, iron chelators have already been shown to mitigate ischemia–reperfusion injury (IRI) in a variety of experimental animal models [110–112], as well as the severity of several neurodegenerative diseases in both animal models and human clinical trials [113–116]. Although these compounds show different pharmacokinetic and metabolic properties, the mechanism of action relies on free iron chelation thereby avoiding the formation of highly reactive hydroxyl radicals [117]. However, side effects related to the essential role of iron in many metabolic processes are discouraging this treatment strategy.

Lipophilic radical traps

Since ferroptosis is driven by a radical chain reaction within cellular membranes, different strategies that halt this process have been developed. Small molecules that react with chain-carrying radicals, and thus inhibit phospholipid autooxidation are identified as lipophilic radical trapping antioxidants. Extensive high-throughput screenings using cell-based ferroptosis assays identified novel synthetic RTAs such as Ferrostatin-1 (Fer-1) and Liproxstatin-1 (Lip-1) [118–120]. These lipophilic RTAs scavenge unpaired electrons at the level of toxic phospholipid radicals [121]. Although Fer-1 has been shown to be a potent inhibitor of ferroptosis in multiple *in vitro* settings, it is not well-suited for *in vivo* use because it suffers from solubility and metabolic stability problems [122]. Consequently, different improved analogues were developed showing highly improved *in vivo* efficacy such as SRS16-86 [123] or UAMC-3203 [81,122]. Additionally, other lipophilic RTAs have been suggested as potential ferroptosis inhibitors for both *in vitro* (e.g. butylated hydroxytoluene (BHT) [2] and CoQ_{10} [38,39]) and *in vivo* use (e.g. XJB-5-131 [124,125]).

Other ferroptosis inhibitors

PUFAs containing the heavy hydrogen isotope deuterium at the peroxidation site have also been found to suppress ferroptosis induced by RLS3 and erastin [8]. Apart from agents interfering with iron metabolism or harboring anti-oxidant properties, a range of ferroptosis inhibitors directed to the lipoxygenase enzyme have been described [8,15,126–129]. However, since genetic targeting of LOXs failed to prevent ferroptotic cell death, this class of inhibitors is still controversial [17,121].

Ferroptosis in disease

Emerging evidence indicates the involvement of ferroptosis in many human disorders. Therefore, pharmacological modulation of ferroptosis, by either inhibiting or inducing it, may represent a possible avenue for treating multiple pathologies. Here, an overview of the main diseases in which a ferroptosis signature in humans as well as its therapeutic potential in preclinical models have been explored is briefly discussed (Fig. 3). How infectious agents regulate ferroptosis to

promote their replication, dissemination, and pathogenesis [130], and how ferroptosis induction might be a promising novel anti-cancer therapy [12] is reviewed elsewhere. The immune response to ferroptotic cells is still debated requiring further clarification and is out of the scope of this review. Essentially, current knowledge suggests that ferroptosis boosts innate immunity [49,80], in which M1, but not M2, macrophages seemed to survive this oxidative stress environment [131], while suppressing adaptive immunity, at least in vaccination anti-cancer setup [132].

Organ injury

Ischemia–reperfusion injury is a complex pathophysiological condition induced by an imbalance between oxygen/nutrient needs and supply upon vascular occlusion of the organ during the ischemic event. Paradoxically, subsequent reperfusion exacerbates the injury of the affected organ through destructive inflammatory responses and massive cell death [133,134]. Eventually, IRI can lead to devastating diseases ranging from single to multiple organ-injury. Mounting evidence suggests ferroptosis as a major contributor to IRI-associated cell death and multiple preclinical studies have already shown the beneficial effects of targeting ferroptosis during IRI [135]. For instance, kidney tubular cells are sensitive to ferroptosis in response to IRI, a leading cause of acute kidney injury (AKI). Consistently, different ferroptosis inhibitors were able to mitigate kidney tubular cell death using preclinical models of AKI induced by IRI, genetic deletion of GPX4, and folic acid [81,136]. Furthermore, increased levels of ferroptotic oxygenated PLs were found in urinary cell pellets obtained from patients with AKI who did not recover [28]. Beyond the kidney, the liver has also shown to be dependent on the functionality of GPX4, since inducible knockout of this enzyme leads to massive hepatocyte cell death [81]. Moreover, lipophilic RTAs protect liver parenchyma from IR-induced injury in a preclinical mouse model [80]. The importance of monitoring ferroptosis during hepatic IRI has also been investigated in a transplantation clinical setting since higher levels of circulating MDA were observed in non-surviving patients undergoing liver transplantation when compared to those who survived [137]. Pharmacological inhibition of ferroptosis significantly reduced myocardial infarct size using an *in vivo* mouse heart model mimicking IRI [138] as well as injury during heart transplantation in mice [139]. Note that a beneficial effect of inhibiting LPO for organ preservation during heart transplantation in dogs was already shown in the nineties [140]. Lastly, a case report revealed a ferroptosis signature in myocardial tissue derived from patients suffering from COVID-19. Although this highlights the importance of ferroptosis monitoring in COVID-19 cardiac damage, further research is required [141].

Ferroptosis has also been implicated in plaque destabilization during atherosclerosis. For example, erythrophagocytosis as a key feature of advanced human atherosclerosis, induced ferroptosis *in vitro* and was characterized by increased HMOX1 and ferritin expression. In line with this finding, ferroptosis inhibition decreased HMOX1 and ferritin expression observed in erythrocyte-rich plaque regions derived from a mouse model of advanced atherosclerosis [142]. Moreover, several studies reported upregulation of important ferroptosis-related genes [143–145] as well as increased levels of iron in atherosclerotic lesions from humans [146].

Interestingly, multiple organ dysfunction syndrome (MODS), which refers to the critical illness that causes 30% of deaths worldwide has been linked to ferroptosis [147,148]. A prospective cohort study involving plasma samples of 176 critically ill adult patients revealed that the extent of organ dysfunction, reflected in the patient's sequential organ failure assessment (SOFA) score, is positively correlated to increased levels of MDA and catalytic iron. Furthermore, an excess amount of iron sulfate proved to be sufficient to overrule the systemic buffer capacity and induce MODS in mice. In this experimental model, a rapid increase of MDA in plasma and tissue was observed. Additionally, the administration of a highly soluble lipophilic RTA UAMC-3203 attenuated ferroptosis-driven multi-organ injury and death [81]. These findings suggest ferroptosis inhibition as a possible strategy to prevent MODS in critical care settings.

Central nervous system

The high content of PUFAs, dependency on iron, and limited antioxidant defense, make the central nervous system (CNS) highly vulnerable to damage by lipid peroxidation [149]. Before the conceptualization of ferroptosis in 2012, oxytosis, glutamate toxicity, or excitotoxicity were described to be involved in neuronal cell death in the context of several neurological disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) [150]. Later, it was suggested that cell death by oxytosis and ferroptosis have overlapping pathways [151]. Iron chelation or therapeutic intervention with ferrostatins prevented disease development in preclinical models of AD [152] and PD

[153], while healthy neurons were rescued in HD [154]. Recently, the detrimental role of ferroptosis was revealed in multiple sclerosis, a chronic inflammatory disorder of the CNS. A prominent ferroptosis signature was reflected by the accumulation of ferrous iron and an increase of oxidized phospholipids in the lesions and cerebrospinal fluid of patients. In addition, therapeutic intervention with the lipophilic RTA UAMC-3203 delayed relapse and ameliorated disease progression in a preclinical model for relapsing–remitting multiple sclerosis [155]. Strokes, including ischemic and hemorrhagic stroke, are the second leading cause of death after coronary heart disease and are characterized by acute focal CNS injury [156]. Both iron accumulation, as well as lipid peroxidation, are involved in the pathogenesis of stroke, indicating a role for ferroptotic cell death. The neurotoxic effects of MDA and 4HNE have been reported extensively, and 4HNE has been studied as a potential biomarker for ischemic stroke [157]. Thereby, it was demonstrated that intracerebroventricular treatment with Fer-1 after hemorrhage exhibited marked brain protection and improved neurologic function in mice [55]. Consequently, intranasal delivery of Fer-1 and Lip-1, as an easy method to pass the blood–brain barrier, attenuated neurological deficits after ischemic stroke [158]. This is further underscored by the use of α-Tocotrienol, one of the eight fat-soluble chemicals in vitamin E, which decreases stroke size in animal models [159] and is currently validated in clinical trials as a neuroprotectant in stroke (NCT01578629). Signatures of altered lipid metabolism [160] and GPX4 depletion in post-mortem samples of ALS patients [161] highlight the importance of ferroptosis targeting. In line with these recent findings, neuron-specific delivery of GPX4 and Fer-1 treatment ameliorated motoric impairment in a classical preclinical model for ALS (SOD1G93A) [162], suggesting GPX4 as an interesting therapeutic target. Note that data related to the *in vivo* use of Fer-1 should be interpreted with caution considering its metabolic instability *in vivo*.

Conclusion and perspectives

Although ferroptosis has only been conceptualized in 2012, it was supposedly already studied since the 50s or earlier. Since its conceptualization, ferroptosis has been a flourishing field with cutting-edge discoveries of novel regulatory genes, its involvement in disease, and novel pharmacological intervention tools. It is tempting to speculate that ferroptosis might be a very ancient cell death process, which evolved 2.5 billion years ago during the great oxygenation event. To create life, one had to find ways to protect biological membranes against excessive lipid peroxidation catalyzed by oxygen and iron. The genetic pathways discovered to date, likely reflect these protective mechanisms, rather than proactive signaling towards cell death as is the case in apoptosis, necroptosis, or pyroptosis. The clinical relevance of ferroptosis as a detrimental factor in a multitude of diseases as well as its tumor-suppressing efficacy has boosted translational ferroptosis research. The *in vivo* effectiveness of novel small molecule ferroptosis inhibitors and/or inducers in experimental disease models gives hope for future novel treatment options related to targeting ferroptosis.

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Conflict of interest

TVB holds patents related to ferrostatin-1 analogs (US9862678, WO2016075330, EP3218357, WO2019 154795).

Author contributions

GV wrote the manuscripts. EVS and TVB revised the manuscript.

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