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Therapeutic exploitation of ferroptosis

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Abstract

Pathological breakdown of membrane lipids through excessive lipid peroxidation was first described in the mid-20th century and is now recognized as a form of regulated cell death, dubbed ferroptosis. Accumulating evidence unveils how metabolic regulation restrains peroxidation of phospholipids within cellular membranes, thereby impeding ferroptosis execution. Unleashing these metabolic breaks is currently therapeutically explored to sensitize cancers to ferroptosis inducing anti-cancer therapies. Reversely, these natural ferroptotic defense mechanisms can fail resulting in pathological conditions or diseases such as ischemia-reperfusion injury, multi-organ dysfunction, stroke, infarction, or neurodegenerative diseases. This minireview outlines current ferroptosis-inducing anti-cancer strategies and highlights the detection as well as the therapeutic targeting of ferroptosis in preclinical experimental settings. Herein, we also briefly summarize observations related to lipid peroxidation, iron and redox deregulation in patients that might hint towards ferroptosis as a contributing factor.

Keywords

Ferroptosis, iron, lipid, redox, metabolism, FSP1, GPX4, lipid peroxidation, radical trapping antioxidant, ischemia-reperfusion injury, stroke, neurodegenerative diseases, metabolic disorders

Conflict of Interest

TVB holds patents related to ferroptosis inhibitors (US9862678, WO2016075330, EP3218357, WO2019154795).

List of abbreviations

4-HNE	4-hydroxynonenal
ACSL3	long-chain-fatty-acid—CoA ligase 3
ACSL4	long-chain-fatty-acid—CoA ligase 4
AKI	acute renal injury
AKR10	aldo-keto reductases
ALS	amyotrophic lateral sclerosis
APAP	acetaminophen
BH4	tetrahydrobiopterin
CE	capillary electrophoresis
ICP- MS	inductively coupled plasma mass spectrometry
COPD	chronic obstructive pulmonary disease
CoQ10	co-enzym Q10, ubiquinol
ELISA	enzyme-linked immunosorbent assay
Fe2+	ferrous iron
Fe3+	ferric iron
Fer-1	ferrostatin-1
FPN	ferroportin
FSP1	ferroptosis suppressor protein 1
GCH1	guanosine triphosphate cyclohydrolase-1
GPX4	glutathione peroxidase 4
GSH	glutathione
HETE	hydroxyeicosatetraenoic acid
ICH	intracranial hemorrhage
IHC	immunohistochemical
IKE	imidazole ketone erastin
IRI	ischaemia/reperfusion injury
KO	gene knockout
LC-MS	liquid chromatography mass spectrometry
LIP	labile iron pool
Lip-1	liproxstatin
LOX	lipoxygenase
LPO	lipid peroxidation
MDA	malondialdehyde

MK4	menaquinone-4
MODS	multi-organ dysfunction syndrome
MS	multiple sclerosis
MUFA	mono-unsaturated fatty acids
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCOA4	nuclear receptor coactivator 4
NO	nitric oxide
Ox	oxidized
oxLipidomics	oxidative lipidomics
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
Prom2	prominin2
PUFA	polyunsaturated fatty acid
PUFA-PL	polyunsaturated fatty acid-containing phospholipids
ROS	reactive-oxygen species
RTA	radical trapping antioxidants
SAH	subarachnoid hemorrhage
SCD1	stearoyl-CoA desaturase 1
SSMD	Sedaghatian-type spondylometaphyseal dysplasia
TBARS	thiobarbituric acid reactive substance assay
TBI	raumatic Brain Injury
UVB	ultraviolet-B
WB	western blot

Introduction

Approximately 2,4 billion years ago, 'The Great Oxygenation event' changed the earth's atmospheric landscape. The significant increase in atmospheric oxygen levels introduced new opportunities, such as aerobic organisms, but also new challenges in resisting oxidative stress. As life evolved, organisms developed antioxidant defence mechanisms to safeguard them from the detrimental effects of oxidation. Despite its presence throughout life's evolutionary history on Earth, the term 'oxidative stress' itself is relatively recent (1). For instance, lipid peroxidation (LPO), or the oxidative degradation of membrane lipids, first emerged in a biological context during the mid-20th century (2). Improved detection methods allowed scientists to discover an increase in LPO degradation products, such as malondialdehyde (MDA), in aged individuals (2, 3). This observation was later extended towards disease conditions, observing a link between excessive LPO and diabetes (4, 5), atherosclerosis (6, 7), neurodegenerative diseases (8, 9), and toxin-induced tissue damage (10). More recently, massive phospholipid peroxidation has been implicated as the major execution mechanism of a cell death type coined ferroptosis (11). Ferroptosis occurs when the endogenous redox defence mechanisms are insufficient in detoxifying oxidized phospholipids and restoring equilibrium. This ultimately leads to the loss of membrane integrity (12, 13), pore formation due to activation of PIEZO and TRP channels, resulting in ionic flux, osmotic pressure buildup and eventual rupture of the cell membrane (14-16).

Ferroptosis, biological rust of all cellular membranes

Ferroptosis differs from other types of regulated necrosis, due to the lack of active signals triggering it. It typically prevails when ferroptosis preventive mechanisms fail. Although only coined in 2012, it is believed to be a primitive or ancient form of cell death solely driven by the biochemical process of membrane phospholipid peroxidation (17). Mechanistically, cell membrane rupture is a consequence of excessive peroxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids (PUFA-PLs) (11). This process is catalysed by redox-active iron, as it can initiate the formation of reactive hydroxyl radicals ($^{\bullet}$OH), as well as propagate the auto-oxidation of other PUFA-PLs (Figure 1). Additionally, iron can act as a cofactor for lipoxygenase (LOX) enzymes, a family of non-heme enzymes involved in the dioxygenation of free PUFAs. Yet, it was also suggested that this enzymatic LPO, facilitated by LOX, might simply contribute to initiation of the auto-oxidation (18), however its (patho)physiologic relevance is still lacking.

Given the potential toxicity of iron-catalysed peroxidation of PUFA-PLs, this process must be tightly regulated. Firstly, the lipid metabolism of the cell plays an important role, as the susceptibility of a cell towards ferroptosis depends highly on the availability of PUFAs in the cellular membranes. As reported in 2017, the Kagan lab showed that supplementation of free PUFAs, such as arachidonic acid, could increase cell death in response to ferroptosis induction (19). This effect was intrinsically connected to

the incorporation of these PUFAs into phospholipid (PL) species, as the downregulation of PL synthesis nullified this outcome (20, 21). Multiple independent lipidomics studies have pointed towards PUFA-PLs as the main target for ferroptosis-related LPO, mainly affecting phosphatidylcholine (PC), -ethanolamine (PE), -inositol (PI), -serine (PS) and -glycerol (PG) (19, 22, 23). Most recently, two independent labs testified for PE and PC species containing two PUFAs to be the primary targets for ferroptosis initiation, rather than the more abundant single-PUFA species (24, 25). Cellular supplementation with mono-unsaturated fatty acids (MUFAs) instead decreased ferroptotic response (26), while inactivation of enzymes involved in MUFA-PL synthesis, like stearoyl-CoA desaturase 1 (SCD1) and long-chain-fatty-acid—CoA ligase 3 (ACSL3) enhances ferroptosis (26, 27). This observation was reconstituted *in vivo* as supplementation of oleic acid significantly reduced iron-induced liver injury in mice (28). This indicates that MUFAs compete with PUFAs during PL synthesis, determining the membrane susceptibility for peroxidation.

Secondly, the ability of iron to participate in electron transfer reactions by switching between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) state lends iron to be an effective cofactor during essential cellular processes. Yet, this property also unleashes reactivity resulting in damage to biomolecules, as seen during the ferroptotic process. Therefore, the cell's iron metabolism acts as an intricate system to control the amount of free intracellular redox-active iron or the labile iron pool (LIP) (29). The transferrin receptor is responsible for the cellular uptake of ferric iron through endocytosis, while ferroportin (FPN; also known as SLC40A1) controls the release of ferrous iron. Additionally, ferrous iron is oxidized with oxygen to concentrate up to 4000 iron atoms as a solid oxo-mineral in the centre of ferritin, which serves as the main iron storage protein (30). When needed, iron is released in a process requiring the lysosomal degradation of ferritin, called 'ferritinophagy' (31, 32), mediated by the nuclear receptor coactivator 4 (NCOA4) (33). Furthermore, Brown et al. revealed that iron-bound ferritin can also be packaged into exosomes and released from the cell in a prominin2 (Prom2)-mediated process (34).

The third and last switch in the metabolic regulation of ferroptosis constitutes an interconnected network of cellular redox systems. In general, this network can be divided into three main systems. First, the GPX4-GSH-cysteine axis, in which the enzyme glutathione peroxidase 4 (GPX4) can reduce membrane-bound phospholipid hydroperoxides into less toxic lipid alcohols, using glutathione (GSH) as a cofactor (35-37). Interestingly, full body knockout of GPX4 is embryonically lethal in mice (38) and a very rare GPX4 mutation in humans results in Sedaghatian-type spondylometaphyseal dysplasia (SSMD) (39-42). This system is also highly dependent on the antiporter system x_c^-, which exchanges intracellular glutamate for extracellular cystine and supplies the necessary building block of GSH. Secondly, the FSP1-CoQ10-NAD(P)H axis serves as a backup system for GPX4, as the ferroptosis suppressor protein 1 (FSP1)

158 restores the levels of anti-oxidant co-enzym Q10 (CoQ10) as protection to ferroptosis (43, 44).
159 Additionally, new ferroptosis protection mechanisms continue to be discovered, including the
160 GCH1/BH4/PL pathway. GTP cyclohydrolase-1 (GCH1) suppresses ferroptosis through synthesis of anti-
161 oxidant tetrahydrobiopterin (BH4), also elevating the abundance of CoQ10 and protecting PLs with two
162 PUFA tails (45). Besides CoQ10 and BH4, the cell depends on other endogenous radical trapping
163 antioxidants (RTAs) to terminate the free radical-driven auto-oxidation, including vitamin E (35, 46, 47),
164 vitamin KH_2 (23), vitamine A (48), hydropersulfides (49) and squalene (50). Third, aldo-keto reductases
165 (AKRs) can detoxify oxidative lipid breakdown products such as 4-hydroxynonenal (4-HNE) (51).
166 In essence, lipids are continuously peroxidized through non-enzymatic iron-catalysed auto-oxidation or
167 LOX-mediated LPO, which is detoxified by GPX4 or other redox systems. Upon surpassing a certain
168 threshold, this self-propagating LPO triggers a fast-moving wave of death, destructing cells, tissues and
169 even whole organs (52, 53). As such, ferroptosis induction due to the absence of GPX4 was shown to
170 be detrimental in multiple tissues (37). It was only after the discovery of an *in vivo* ferroptosis inhibitor,
171 such as ferrostatin-analogues and liproxstatin 1 (Lip-1), that the (patho)physiological role of ferroptosis
172 could be fully explored (37). Recently, Kagan lab also discovered novel LOX targeting ferroptosis
173 inhibitors for *in vivo* use *viz.* FerroLOXIN -1/-2 (54).

174 <u>Rust away therapy resistant cancers</u>
175 Interestingly, ferroptosis was initially discovered in the context of cancer research (11). The Dixon lab
176 identified two small molecules, erastin and RSL3, capable of inducing ferroptotic cell death in RAS
177 mutated cancer cells. Since then, ferroptosis has been intensively investigated as a new strategy to
178 combat apoptosis resistance regardless of RAS mutational status (55-59).
179 Strategies to trigger ferroptosis can be subdivided into classes depending on their mode of action (56,
180 60). The most extensively investigated approach involves targeting the GPX4-GSH-cysteine axis (55, 59,
181 61). Small molecules like erastin and its analogue imidazole ketone erastin (IKE) inhibit the system x_c^-,
182 leading to a reduction in intracellular cystine and consequently a depletion of GSH (62, 63). FDA-
183 approved drugs such as sulfasalazine and sorafenib induce ferroptosis through this mechanism yet
184 displaying limited potency *in vivo* (62, 64). Extracellular degradation of cyst(e)ine also proved sufficient
185 in supressing tumour growth *in vivo* (65). In addition to this upstream targeting, compounds like RSL3,
186 ML162 and ML210 inhibit the catalytic activity of GPX4 directly and exhibit a potent anti-cancer effect
187 (36). Yet, their pharmacokinetic properties hinder *in vivo* applications, welcoming more stable
188 analogues, such as the ML210 metabolite JKE-1674 (66). Altretamine, an FDA-approved drug, also
189 appears to induce ferroptosis through GPX4 inactivation (67). Next, the FSP1-CoQ10-NAD(P)H axis holds
190 druggable targets in anti-cancer research, with inhibitors like iFSP1 offering an interesting new strategy
191 to enhance ferroptosis induction when combined with erastin or RSL3 (44). The more recently

developed inhibitor icFSP1 was shown to impair tumour growth in FSP1 overexpressing cell-derived xenograft mouse models (68). On a related note, the inducer FIN56 employs a dual strategy by depleting both GPX4 and CoQ10 (69). The GCH1/BH4/PL pathway remains largely unexplored. However, methotrexate, which inhibits a BH4 recycling enzyme, was shown to enhance the effect of other ferroptosis inducers (70).

An alternative method in inducing ferroptosis involves increasing the levels of intracellular redox-active iron (Fe^{2+}). Forcing iron overload with small iron oxide (C'dot) nanoparticles, originally developed for imaging and diagnosis, showed to induce ferroptosis in a tumour xenograft model (71). Furthermore, iron supplementation in the form of hemin or ferrous ammonium sulfate [$(NH_4)_2Fe(SO_4)_2$] proved sufficient in inducing ferroptosis in neuroblastoma cells (56). Additionally, Withaferin A, a natural compound, induced ferroptosis in therapy-resistant neuroblastoma through a dual mechanism. Alongside GPX4 inactivation, Withaferin A increased the LIP via HMOX1-mediated degradation of heme, producing biliverdin, carbon monoxide, and Fe^{2+}.(57). Similarly, the well-known IκBα inhibitor BAY 87-2243 causes HMOX1 upregulation resulting in iron accumulation and ferroptotic cell death (72). Concerning small molecules, FINO2 demonstrates the ability to oxidize iron and indirectly inhibit GPX4, thereby inducing ferroptosis (73). Lastly, recognizing the pivotal role of membrane lipid composition in ferroptosis-sensitivity, the third strategy aims to increase the availability of PUFA-PLs for oxidation and consequently enhance the occurrence of ferroptosis through PUFA lipid supplementation or diets (19, 74, 75).

Note that in context of ferroptosis as cancer therapy, identifying which tumours will respond to ferroptosis induction is vital as tumour heterogeneity may further delay treatment. Pre-treatments to sensitize tumours to ferroptosis anti-cancer treatment will be beneficial to ensure the full eradication of the tumour upon ferroptosis induction. Additionally, the indispensable role of ferroptotic breaks in healthy tissue calls for a tumour targeted approach to reduce side-effects of ferroptosis anti-cancer therapies (38).

Acute organ injury, blocking the synchronized wave of ferroptosis

In 2014, the Conrad lab was one of the first to show the pathophysiological role of ferroptosis in mice (37). With Lip-1 as the first tool to battle ferroptosis *in vivo*, they discovered that ferroptosis inhibition mitigated ischaemia/reperfusion injury (IRI) in the murine liver (37). This observation was backed up by a more recent paper using α-tocopherol (vitamin E), a natural lipophilic RTA (76). IRI refers to tissue damage that is caused when blood supply is returned to an ischemic region, for instance after an ischemic stroke, myocardial infarction, cross-clamping during surgery or upon transplantation. The reintroduction of oxygen-rich blood causes a "burst" of reactive-oxygen species (ROS) and iron, leading to LPO and ferroptotic cell death (77). In the context of transplantation, research involving human

subjects found a positive correlation between heightened serum ferritin levels in liver donors and the risk of liver damage in transplantation recipients (76). This indicates that high iron content in the graft, may lead to increased ferroptosis during reperfusion. On a related note, it was reported that acetaminophen (APAP)-induced liver injury could be inhibited by Fer-1 (78) or its more potent analogue UAMC-3203 (79). However, this evidence is still disputed (80).

In addition to the liver, ferroptotic cell death in IRI-induced acute renal injury (AKI) has also been extensively investigated over the past decade. The Linkermann lab elegantly showed in an *ex vivo* renal tubuli model that IRI-induced cell death happens in a synchronized manner (52), which could be inhibited by ferrostatin-1 (Fer-1). Additionally, a more stable analogue of Fer-1 could reduce IRI renal damage in mice. More recently, it was shown that ferroptosis inhibition may be beneficial in moderating AKI severity and preventing further progression to kidney failure (81). Ferroptosis inhibition has also proven beneficial in toxin-induced AKI, induced by folic acid (82) and oxalate-crystals (52).

Myocardial cells also seem to be particularly susceptible to ferroptotic cell death during myocardial infarction, as systemic treatment with Fer-1 in mice significantly reduced cardiac injury (83) and circulating levels of oxidized fatty acids/ arachidonic acid metabolites as measured by liquid chromatography mass spectrometry (LC-MS) (84). Iron chelation with deferoxamine improved IRI-related heart damage *ex vivo* (85). Moreover, IR-induced intestinal damage could be inhibited by Lip-1, which was shown to be dependent on long-chain-fatty-acid-CoA ligase 4 (ACSL4) (86). The susceptibility of pancreatic islets in the context of IRI remains elusive, despite their sensitivity to pharmacological ferroptosis induction (87).

It should be noted that prior to the coining of ferroptosis, several conditional GPX4 knockout models showed that the brain is particularly susceptible to ferroptosis induction (37). In the context of IRI, patients suffering from ischemic stroke showed increased plasma 4-HNE levels compared to healthy controls (88). In mouse models, genetic depletion of LOX12/15 (89) or cortical silencing of ACSL4 (90) improved ischemic stroke outcome compared to WT controls. Similarly, intranasal Lip-1 administration was also shown to be beneficial (91). Lastly, Edavarone, an FDA-approved drug identified as a potential ferroptosis inhibitor (92), proved to be beneficial in early treatment ischemic stroke in humans (93).

As the rupture of a blood vessel is also accompanied by a sudden local accumulation of iron, intracranial hemorrhage has been extensively researched in the context of ferroptosis. Similar to the observation made in ischemic stroke, LOX12/15 knockdown in mice subjected to subarachnoid hemorrhage (SAH) improved short-term neurological function (94). In addition, intracerebral selenium (95) or Fer-1 (95) administration could reduce neurological damage and improve functional recovery after an intracerebral hemorrhage (ICH) in mice. Increased levels of oxidized PE were also observed in cortical tissue of rats subjected to traumatic brain injury (96).

Ferroptosis not only continues in neighbouring cells resulting in wave of synchronised death (52), but might also induce remote injury due to release of cell permeable lipid degradation products (19). Therefore, it is postulated that ferroptosis may play a detrimental role in remote organ injury, namely in multi-organ dysfunction syndrome (MODS). MODS is described as the sequential and potentially reversible dysfunction of two or more organ systems and affects approximately half of all critically ill patients in the intensive care unit (97). Numerous case reports have shown that also iron intoxication results in multi-organ injury (98, 99). While predominantly treated with iron chelators, we showed that ferroptosis inhibition using UAMC-3203 is lifesaving against experimental MODS induced by iron overload (53). Additionally, we established a link between ferroptosis and MODS in two separate critical illness patient cohorts (100). It should be noted that organ dysfunction in the context of MODS is often attributed to sepsis, and it has been shown that experimental sepsis-induced organ injury cannot be mitigated by ferroptosis inhibition (52, 53). However, during septic shock, the occurrence of IRI due to dysregulation of vascular homeostasis may yet be an indication for the use of ferroptosis inhibitors in some patients. It is therefore of vital importance to develop better diagnostic tools to identify ferroptosis-related MODS in patients.

Chronic organ injury, restraining almost untraceable ferroptosis

The rich PUFA content, iron accumulation and GPX4 brain knockout models, indicate the potential involvement of ferroptosis in neurodegenerative diseases (101, 102). We reported a disrupted Fe^{2+}/Fe^{3+} ratio in spinal fluid and an excessive LPO signature in active and chronic lesions of multiple sclerosis (MS) patients. This would suggest that ferroptosis might be a detrimental factor in MS-associated demyelination (103). Indeed, ferroptosis inhibition using UAMC-3203, strongly delayed relapse in a preclinical model for relapse-remitting MS and ameliorated disease progression (103). An independent study on relapse-remitting MS corroborated this finding (104).

Ferroptosis has also been linked to amyotrophic lateral sclerosis (ALS) as GPX4 ablation in motor neurons leads to paralysis, delayed by a vitamin-E diet (105). In line with these findings, a recent paper showed that neuron-specific overexpression of GPX4 or intrathecal Fer-1 delivery ameliorates motoric deficits in a preclinical model for ALS (106). Furthermore, iron dyshomeostasis observed in Alzheimer's Disease suggests the involvement of ferroptosis (102). As Alzheimer's Disease is characterized by synapse degradation and eventual neural cell death in the learning and memory region of the brain, researchers developed a forebrain-specific GPX4 knockout model. This preclinical model showed increased LPO and cognitive impairment, exacerbated by a vitamin E deficient diet and improved by ferroptosis inhibitor Lip-1 (101). Parkinson's disease, associated with the loss of dopaminergic cells in the substantia nigra, also displayed signatures of LPO and ferroptosis in preclinical models (107, 108). Additionally, the impaired cysteine synthesis observed in Huntington's disease, a neurodegenerative

condition caused by a mutation in the gene encoding the huntingtin protein, might also hint to ferroptosis as a detrimental factor (109).

Next, ferroptosis may also be associated with several metabolic disorders. For example, the accumulation of oxidized PLs was clearly detected in human and mouse non-alcoholic steatohepatitis liver (NASH) tissue and serum (110). Endogenous expression of an antibody designed to target oxidized PLs, mitigated the adverse effects of this liver condition in a preclinical model (110, 111). Also, vitamin E was shown to be beneficial in patients suffering from NASH (112). Furthermore, a well-established connection exists between iron deregulation and other metabolic conditions, such as diabetes and obesity (113). However, there has yet to be any evidence on therapeutic application of ferroptosis modulators in these disorders. The application of ferroptosis therapy may also hold potential in respiratory conditions like asthma (114), chronic obstructive pulmonary disease (COPD) (115) and mucoviscidosis (116), yet substantial evidence remains elusive. In addition, high intracellular iron content, LPO and cell death typically observed in advanced atherosclerotic plaques hints towards ferroptosis (117). Indeed, erythrophagocytosis-induced ferroptosis during intraplaque angiogenesis leads to larger atherosclerotic plaques, an effect that can be prevented by ferroptosis inhibitor UAMC-3203 (118). Finally, a potential ferroptosis involvement is also suggested in age-related orthopedic diseases (119), skin damage due to UVB exposure (120) and psoriasis (121).

Ferroptosis detection in patients

In the past decade, investigations using pre-clinical models have unveiled the immense therapeutic possibilities associated with targeting ferroptosis, demonstrating efficacy in both acute (Table 1) and chronic (Table 2) diseases. Yet, as ferroptosis inhibitors are approaching clinical application, refining the clinical ferroptosis detection methods becomes imperative. Currently, diagnostic detection of ferroptosis primarily relies on colorimetric tests measuring elevated levels of Fe^{2+} or MDA. However, these tests are prone to variability. Furthermore, the ultimate evidence of ferroptotic cell death remains the detection of oxidized PLs e.g. by using immunodiagnostics, which require improved antibodies and optimizations, or oxidative lipidomics (oxLipidomics), with currently laborious data analysis hampering unbiased approaches (Table 3). Advancing these methodologies and discovering novel approaches to enable the reliable detection of a ferroptosis fingerprint in biofluids, instead of relying on invasive tissue biopsies, will be essential for transitioning ferroptosis to the clinical phase. This might prove particularly challenging in chronic disorders, where gradual disease progression is associated with almost untraceable LPO signatures. Major efforts are underway to enhance the identification of the ferroptosis fingerprint, aiming to accelerate research efforts and ensure accurate diagnosis for future ferroptosis therapies.

Perspectives

- Ferroptosis research is excelling and unveiling the great potential of ferroptosis treatments to rust away cancer, to block the wave of death in acute diseases or restrain ferroptosis in chronic diseases when it's still in its elusive stage.
- Ferroptosis targeting compounds are increasingly discovered, serving as tools to dissect ferroptosis biology or as drugs in preclinical development to target ferroptosis therapeutically.
- To ensure its practical application in the clinical settings, major efforts are still needed to improve the detection of a ferroptosis fingerprint in a diverse spectrum of diseases.

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Table 1 | Ferroptosis in acute diseases in mouse/rat

Disease	Ferroptosis signature	Observation	Refs.
IRI-Liver		Lip-1 reduced hepatocyte cell death and liver damage after experimental hepatic IRI	(37)
	4-HNE (IHC) MDA (TBARS)	IR-induced liver damage was associated with LPO and could be markedly prevented by Fer-1 or α-tocopherol	(76)
	4-HNE (IHC)	A high iron diet increased IR-induced liver damage and LPO which was attenuated by iron chelation treatment for 5 days prior to surgery	(76)
	4-HNE (IHC)	Pre-treatment with the ferroptosis inhibitor vitamin K (MK4) reduced IR-induced liver damage, LPO, hepatocyte cell death and diminished inflammatory cells infiltration	(23)
IRI-Kidney		Pre-treatment with Fer-1 analogue 16-86 attenuated IR-induced kidney damage	(52)
		Pre-treatment with the ferroptosis inhibitor vitamin K (MK4) reduced IR-induced kidney damage	(23)
IRI-Heart		Heart IRI could be prevented *ex vivo* by perfusion of the glutaminolysis inhibitor Compounds 698 or iron chelation	(85)
	oxFA (oxLipidomics)	Perfusion with Fer-1 upon IRI in an *ex vivo* heart model improved heart function, reduced infarct area and diminished circulating levels of oxidized fatty acids/ arachidonic metabolites as measured by LC-MS	(84)

	oxPE (oxLipidomics)	Systemic treatment of the transplant recipient mice with Fer-1 resulted in reduced cardiomyocyte death and circulating levels of oxidized PE species	(84)
	MDA (TBARS) [Fe^{2+}] (Colorimetric)	Fer-1 attenuated the IR-induced increase in tissue MDA and Fe^{2+} levels, reducing functional impairment and myocardial cell death	(83)
IRI-Intestine	MDA (TBARS) [Fe] (Colorimetric)	IR-induced intestinal damage was associated with LPO, iron accumulation, reduction in GPX4 activity and GSH depletion, reversed by oral administration of Lip-1. The ACSL4 inhibitor rosiglitazone reduced intestinal damage.	(86)
IRI-Pancreas		Pre-treatment of pancreatic islets with Fer-1 failed to improve islet graft function upon transplantation	(87)
IRI-Brain		LOX12/15 KO mice show less severe warfarin-induced hemorrhagic transformation after ischemic stroke compared to wildtype controls	(89)
		Mice treated with ML351, a LOX15 inhibitor, showed reduced infarct size and hemorrhagic transformation after ischemic stroke	(89)
	12-HETE (ELISA)	Intranasal Lip-1 administration in cortical ACSL4 overexpressing mice reduces infarct volume and neuronal cell death. This was associated with reduced LPO in brain tissue and preservation of GPX4 protein levels	(90)
		Cortical silencing of ACSL4 using RNAi reduced infarct size, neuronal death, microglia activation and alleviated neurological function	(90)
		Genetic inhibition of Tau-mediated iron export markedly protected against neuronal damage upon ischemic stroke	(91)
		Intranasal Lip-1 administration after cerebral IRI decreased infarct volume, improved cognitive impairment and enhanced spatial memory	(91)
Toxin-Kidney	4-HNE (IHC)	Single dose folic acid induced AKI is associated with LPO. Fer-1 improved renal function	(82)
		Daily Fer-1 administration improved renal function upon oxalate-induced renal damage	(52)
Toxin-Liver	MDA (TBARS) 4-HNE (IHC) oxFA (oxLipidomics)	(Single dose) APAP-induced hepatotoxicity was associated with LPO, GSH depletion and could be prevented by administration of iron chelators, Fer-1 and vitamin E	(78)
	4-HNE (IHC) MDA (TBARS)	Genetic inhibition of ACSL4 partially inhibited the development of APAP-induced hepatotoxicity linked to a reduced level of LPO and preservation of GSH levels.	(78)
	4-HNE (IHC) MDA (TBARS) Serum[Fe] (Colorimetric)	UAMC-3203 and iron chelation prevented single-dose APAP-induced hepatotoxicity, associated LPO and dampened APAP-related liver lipid composition changes	(79)
SAH		LOX12/15 knockdown in mice subjected to SAH showed decreased edema volume and improved short-term neurological function	(94)
ICH		Intracerebral selenium administration could reduce neurological damage and improve functional recovery after ICH in mice	(95)
	MDA (TBARS) 4-HNE (WB/ ELISA) [Fe] (Perl's stain)	Intracerebral administration of Fer-1 after ICH inhibited LPO, decreased iron accumulation and neuronal degradation and improved motoric function.	(122)
		IP Lip-1 administration after ICH resulted in decreased in iron accumulation, neuronal degradation and improved neuronal/ motoric function.	(122)

TBI	oxPE (oxLipidomics)	Affected cortical brain regions showed decreased GPX4 levels, associated with an increase in oxidized PE	(114)
MODS	C11-BODIPY staining	UAMC-3203 proved lifesaving against experimental iron-overload-induced MODS, associated with a reduction in tissue damage and decreased LPO in liver and kidney tissue	(53)
	MDA (TBARS)	Mice subjected to iron-overload exhibited overt liver damage driven by LPO. Dietary pre-treatment with oleic acid prior to iron intoxication mitigated these effects.	(28)

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Table 2 | Ferroptosis in chronic diseases in mouse/rat

Disease	Ferroptosis signature	Observation	Refs.
IRI–Kidney		Lip-1 treatment for 14days after severe IRI resulted in a significant reduction of fibrosis formation CKD development	(81)
MS		Early-phase disease in relapse remitting MS showed reduced GPX4 levels. UAMC-3203 administration started during the acute phase strongly delayed relapse and ameliorated early disease progression.	(103)
	[Fe^{2+}] (CE-ICP-MS) 4-HNE (IHC)	High iron and low GSH levels in chronic MS spinal tissue is associated with high LPO in the progressive stage. Treatment with UAMC-3203 starting at the peak of model paralysis, reduced disease severity and lesion size	(104)
ALS	MDA (TBARS) 4-HNE (WB)	Adenoviral-mediated overexpression of GPX4 or Fer-1 delivery in the spinal cord improved neuronal function and reduced disease-associated LPO and neuronal loss.	(106)
Parkinson	oxPE (oxLipidomics)	Elevated levels of oxidized PE were observed in brain tissue from a rat model for Parkinson's disease	(108)
Huntington		Cysteine supplementation delayed the onset of motor abnormalities, partly reversed the disease-related decrease in brain weight, and improved survival	(109)
NASH/ NAFLD		Neutralization of oxidized PLs through endogenous antibody expression improved steatosis, inflammation, fibrosis, hepatocyte death, and progression to hepatocellular carcinoma	(110)
	oxPC (oxLipidomics)	Inducible liver-specific expression of an antibody targeting oxidized PC prevented hepatic steatosis formation and accumulation of oxidized PC when initiated prior to model initiation and halted disease progression to hepatic fibrosis when induced after model establishment.	(111)
COPD	[Fe^{2+}][Fe^{3+}] (ICP-MS) [Fe] (Perl's stain) 4-HNE (IHC) oxPC, oxPE (oxLipidomics)	Chronic exposure to cigarette smoke resulted in increased pulmonary iron levels and LPO and an increased ratio of oxidized/ non oxidized PE and PC species.	(115)
	oxPC, oxPE (oxLipidomics) 4-HNE (IHC)	Cigarette smoke-induced damage was significantly increased in GPX4 KO mouse and reduced in mice overexpressing the GPX4 protein. This was associated with respective in- and decrease of oxidized PE and PC, LPO and cell death	(115)
	4-HNE (IHC)	Intratracheal NCOA silencing (siRNA) decreased bronchial LPO and cell death	(115)
Atherosclerosis	MDA (TBARS)	Atherosclerotic vascular tissue showed an increase in LPO and a decrease in GSH levels, reversed by Fer-1. This was	(117)

		associated with a seemingly improved lipid homeostasis and reduction of atherosclerotic lesion size.	
		Daily UAMC-3203 treatment decreased carotid plaque thickness	(118)
UVB	4-HNE (IHC)	Topical pre-treatment with Fer-1, decreased LPO within the epidermal layer 24h after UVB exposure and reduced immune myeloid cell infiltration and cytokine response.	(120)
Psoriasis	4-HNE (WB)	Daily topical Fer-1 application reduced experimental psoriasis and reduced psoriasis- associated LPO	(121).

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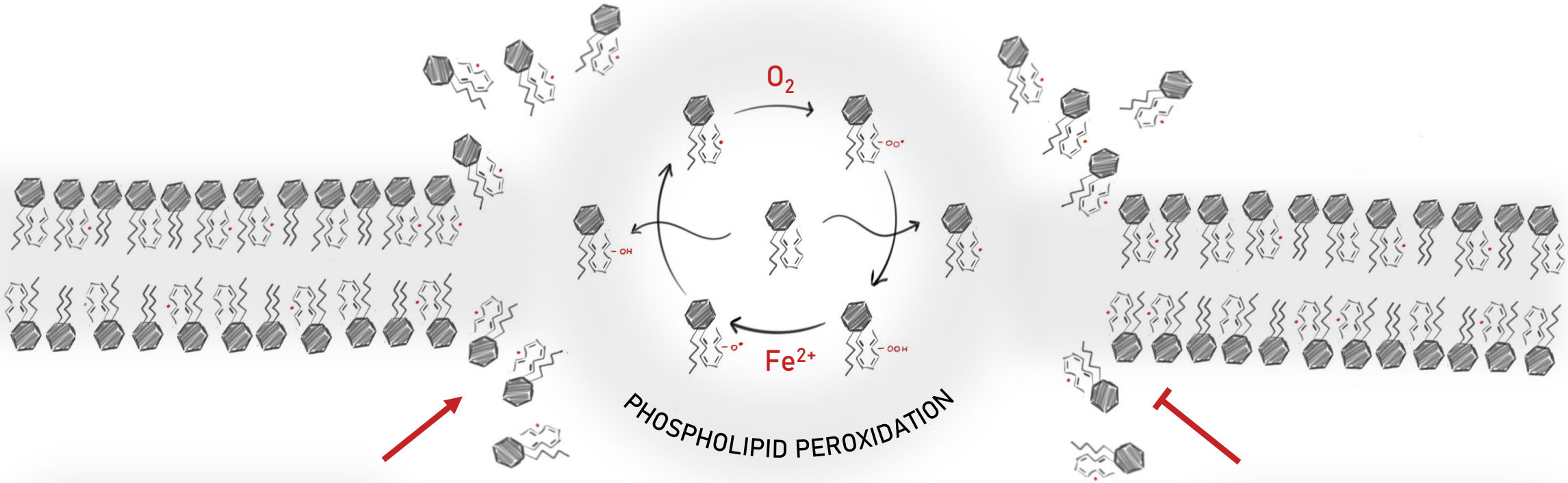
Table 3 | Ferroptosis signature observed in patients

Disease	Ferroptosis fingerprint	Observation	Refs.
AKI	oxPE (oxLipidomics)	Urine cell pellets collected at the start of dialysis in AKI patients showed significant higher levels of oxidized PE species in patients that did not recover kidney function regardless of AKI etiology	(114)
IRI-Liver		Retrospective analysis of clinical data of 202 pediatric living-donor liver transplantations (LT) and found that a high serum ferritin level of the donor is an independent risk factor for liver damage after LT.	(76)
Toxin-Liver	4-HNE (IHC)	Liver biopsies from patients suffering from drug-induced liver injury showed increased LPO, associated to the degree of liver damage.	(79)
NASH/ NAFLD	4-HNE (IHC)	Liver biopsies from patient suffering from alcoholic liver disease displayed increased LPO compared to controls with other disease indications	(79)
	oxPC (E06 IHC + ELISA)	Liver biopsy and plasma samples showed a high oxidized PL content compared to normal controls and patients suffering from NAFL without liver damage. This could be associated with the severity of liver fibrosis.	(110)
	4-HNE (IHC)	Liver biopsy samples showed increased LPO compared to healthy liver samples.	(123)
		Daily vitamin E treatment could improve liver function as evaluated up to 6 months after treatment initiation	(112)
IRI-Brain		Edavarone proved to be beneficial in early treatment of ischemic stroke	(93)
SSMD		Truncating mutations in GPX4 in two families affected with SSMD supports the pathogenic role of mutated GPX4. SSMD is a neonatally lethal and is characterised by severe mild limb shortening, cardiac conduction defects, and central nervous system abnormalities.	(42)
Iron overdose		Case report concerning iron-overdose in a 23-year-old female. Clinical signs indicated shock. Stabilization of vital signs after fluid resuscitation and treatment with iron chelators This patient presented with the gastrointestinal phase of iron overdose.	(98)
		Case report concerning an 18-year-old female after accidental ingestion of accidental iron overdose. Upon admission, the patient appeared conscious but lethargic and had persistent vomiting, abdominal pain, and acute gastrointestinal signs of toxicity, lethargy, altered mental status, and neurologic manifestations of toxicity. Treatment with fluid resuscitation and iron chelators stabilized the patient.	(99)

MODS	MDA (TBARS) [Fe] (Colorimetric)	In a patient cohort of 176 critically ill patients, the plasma MDA was associated with plasma Fe, the degree of organ dysfunction (SOFA-score), and 30-day survival	(53)
COVID -19	MDA (TBARS) [Fe] (Colorimetric)	In a cohort consisting of 120 critically ill COVID-19 patients, advanced clustering revealed a ferroptosis cluster with markedly elevated levels of plasma MDA, Fe, and myoglobin, associated with significantly higher in-hospital mortality compared to the other clusters	(100)
Astma		Airway epithelial cells from patients with controlled, non-exacerbating asthma, revealed co-localization of PEBP1 with LOX-15 which showed a weak correlation to the level of exhaled nitric oxide (FeNO).	(114)
COPD	oxPC, oxPE (oxLipidomics) 4-HNE (IHC) [Fe] (Perl's stain)	Single cell analysis from human lung tissue revealed a lower GPX4 expression and increased NCOA expression in bronchial cells derived from COPD patients compared to the other groups. GPX4 expression was positively correlated to lung function. Histological analysis revealed higher LPO and iron accumulation in COPD lung tissue	(115)
Psoriasis	4-HNE (WB)	GPX4 was shown to be significantly reduced in psoriasis lesions compared to normal skin, which was associated with an elevated level of LPO.	(121)

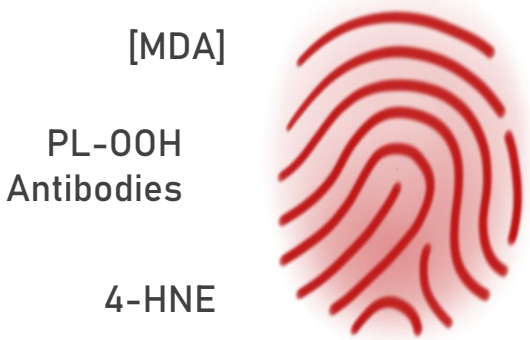
Figure legends

Figure 1: Ferroptosis in a nutshell. In essence, ferroptosis is a consequence of the self-amplifying cycle of excessive lipid peroxidation in cellular membranes. This process is catalyzed by the presence of oxygen and redox-active iron, resulting in membrane rupture and ultimately cell death. The cell can regulate ferroptosis by detoxifying oxidized phospholipids and restoring equilibrium, using multiple defence mechanisms that depend on the cell's lipid, iron and redox metabolism. In addition, the cell can counteract ferroptosis through presence of endogenous radical trapping mechanims. Also, synthetic radical trapping agents have been developed to block ferroptosis in disease settings. To apply ferroptosis treatments in the clinic, detection of a ferroptosis fingerprint at time of diagnosis will be critical.



REGULATION

- LIPID metabolism
- IRON metabolism
- REDOX metabolism



DETECTING FERROPTOSIS FINGERPRINT

INHIBITION

RADICAL TRAPPING AGENTS

NATURAL
- VIT. E
- VIT. A
- VIT. $K_{reduced}$
- BH4
- 7-DHC
- SQUALENE

SYNTHETIC
- LIPROXTATINS
- FERROSTATINS