# Emerging role of ferroptosis in metabolic dysfunction-associated steatotic liver disease: revisiting hepatic lipid peroxidation

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## Summary

**Metabolic dysfunction-associated steatohepatitis (MASH) is characterised by cell death of parenchymal liver cells which interact with their microenvironment to drive disease activity and liver fibrosis. The identification of the major death type could pave the way towards pharmacotherapy for MASH. To date, increasing evidence suggest a type of regulated cell death, named ferroptosis, which occurs through iron-catalysed peroxidation of polyunsaturated fatty acids (PUFA) in membrane phospholipids. Lipid peroxidation enjoys renewed interest in the light of ferroptosis, as druggable target in MASH. This review recapitulates the molecular mechanisms of ferroptosis in liver physiology, evidence for ferroptosis in human MASH and critically appraises the results of ferroptosis targeting in preclinical MASH models. Rewiring of redox, iron and PUFA metabolism in MASH creates a proferroptotic environment involved in MASH-related hepatocellular carcinoma (HCC) development. Ferroptosis induction might be a promising novel approach to eradicate HCC, while its inhibition might ameliorate MASH disease progression.**



Keywords: Metabolic dysfunction-associated steatohepatitis; Ferroptosis; Lipid hydroperoxides; Lipid peroxidation; Glutathione peroxidase; Labile iron; Polyunsaturated fatty acids; Phospholipids; Hepatocellular carcinoma

## Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), defined as the presence of steatosis in more than 5% of hepatocytes, is the most common liver disease worldwide affecting about 25–30% of the adult population worldwide.[1] This hepatic manifestation of the metabolic syndrome is often limited to isolated steatosis. However, a subset of MASLD patients (estimated at 1.5–6.5% of the global adult population) suffers from metabolic dysfunction-associated steatohepatitis (MASH), wherein steatosis is accompanied by hepatocyte ballooning and lobular inflammation which constitute the 'necroinflammatory' disease activity.[1,2] MASH can lead to advanced liver fibrosis which associates independently with overall mortality.[3] The field of clinical trials for MASH is a very vibrant one, but the need for more pharmacotherapeutic options persists and combination therapy is the future for MASH cure.[4]

Necroinflammation with hepatocyte cell death defines MASH and is a possible therapeutic target. Preclinical models of this chronic liver disease indicate that cell death, followed by inflammation and compensatory proliferation, is linked to the development of fibrosis, cirrhosis, and hepatocellular carcinoma.[5] The demise of hepatocytes, as evidenced by the presence of DNA strand breaks detected by terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL), is well documented in human MASH biopsy specimens but less prevalent in isolated steatosis.[6–9] Cell death was initially categorised as either apoptosis, i.e. caspase-dependent cellular demise without membrane permeabilisation, or the accidental necrosis with loss of membrane integrity and release of cellular content.[10] In the last decades several types of regulated necrosis have been discovered that use dedicated pathways to execute membrane permeabilisation, such as necroptosis, pyroptosis and ferroptosis.[11] Although initially thought to be specific for apoptosis, the TUNEL assay also detects these types of regulated necrosis because after plasma membrane rupture extracellular deoxyribonucleases enter to degrade DNA *in vivo*.[12–15] Several cell death modes occur in MASLD livers but their importance is under debate.[5] Reduced apoptosis due to caspase 3 deletion in mice protected against liver fibrosis in dietary-induced MASH.[16]

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Pan-caspase inhibitor emricasan, which targets apoptosis as well as pyroptosis, did not improve liver histology, but even tended to worsen lobular inflammation and ballooning in MASH with grade 1–3 fibrosis.[17–19] This might indicate that apoptosis and pyroptosis are less important in MASH; alternatively, inhibition of one type of cell death can induce a switch to another form, as demonstrated in mouse livers *in vivo*.[20] Indeed, the ongoing presence of high serum cytokeratin-18 fragments despite emricasan points towards persistent cell death.[19] In case of absence of caspase-8 activity, some cell types can switch to necroptosis, i.e. a necrotic cell death dependent on receptor interacting protein kinase (RIPK) 3-mediated phosphorylation of mixed lineage kinase like.[21] Part of the cell death machinery of necroptosis is activated in immune cells in MASH since RIPK proteins mediate inflammation, but primary mouse hepatocytes are quite resistant to necroptosis.[22,23] Essentially, it is difficult to discern the relative importance of the different cell death modes present in MASLD.

At present another subtype of regulated necrosis termed ferroptosis, executed by iron-catalysed peroxidation of polyunsaturated fatty acids (PUFA) in membrane phospholipids, is subject of intense research in many chronic diseases. Decades of research support the role of PUFA peroxidation in MASLD but the discovery of ferroptosis has led to new therapeutic options. In this review, we summarise the mechanisms of this necrotic cell death and evidence for hepatic ferroptosis in human MASLD. Ferroptosis targeting in preclinical models suggests that this cell death has a detrimental role in MASH, whereas ferroptosis induction in cancer cells could constitute a new therapeutic strategy for MASH-related hepatocellular carcinoma (HCC).

## Ferroptosis, dying through iron-catalysed lipid peroxidation

Metabolically active tissues such as the healthy liver inevitably form reactive oxygen species (ROS). In disease, an increase in oxidative stress typically results in Fenton reaction catalysed generation of hydroxyl radicals ($^{\bullet}OH$), which can overrun the cellular anti-oxidant capacity causing damage to biomolecules.[24] Polyunsaturated fatty acids in membrane phospholipids (PL-PUFA) are particularly prone to damage by $^{\bullet}OH$ because of their easily-extractable hydrogen atoms at the pentadienyl moiety. This will start a chain reaction of auto-(per)oxidation, catalysed by labile, ferrous iron ($Fe^{2+}$), called lipid peroxidation (LPO), with the formation of lipid hydroperoxides in phospholipids (PL-PUFA-OOH).[25–27] A small amount of unbound $Fe^{2+}$ is present in the cytosol of hepatocytes, estimated at some 5 μM.[28] Oxidised phospholipids (oxPL) destabilise cell membranes and break down into a myriad of oxidation products including toxic electrophiles, such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE) (Fig. 1).[25,26,29]

For a long time the cell death caused by excessive LPO was assumed to be apoptosis or accidental necrosis.[30] However, Marcus Conrad and colleagues discovered that deletion of selenoprotein glutathione peroxidase 4 (GPX4), the only peroxidase able to detoxify PL-PUFA-OOH into non-toxic lipid alcohols by means of the reductant glutathione (GSH), causes a distinct type of cell death.[31] The same cell death was observed in the lab of Brent Stockwell while screening for cytotoxic compounds in RAS-mutant cancer cells.[32] The small molecule erastin was found to inhibit the cystin-glutamate antiporter systemXc$^{-}$, leading to GSH depletion, resulting in necrotic cell death by lethal accumulation of iron-dependent PL-PUFA-OOH. This cell death was named ferroptosis and can be inhibited with iron chelators and lipophilic radical trapping anti-oxidants (RTAs), i.e. vitamin E and the synthetic ferrostatin-1 and liproxstatin-1, which reside in the lipid bilayer to dampen LPO.[33,34] GPX4 was identified as the downstream GSH-dependent protein whose inhibition also leads to ferroptosis.[35] Morphologically ferroptosis is characterised by rounding up of the cells, shrunken mitochondrial cristae and lysis of the plasma membrane which leaves undisturbed nuclei as remnants, but how LPO exactly leads to cell death through direct or indirect mechanisms is still disputed.[33,36–39] Next to the GPX4-GSH-systemXc$^{-}$ axis and vitamin E, other membrane-residing reductants were found to protect against ferroptosis. Ubiquinol (reduced vitamin CoQ10) and vitamin K are regenerated by ferroptosis-suppressor protein 1 (FSP1).[40,41] Another lipophilic reductant tetrahydrobiopterin (BH4) is synthesised by GTP cyclohydrolase 1 (GCH1) and regenerated by dihydrofolate reductase.[42,43] These ferroptosis defences act in concert with synergistic effects to counter lipid peroxidation in health and disease (Supplementary Fig. S1).[40,43] Ferroptosis is accompanied by the release of intracellular content, which does not occur in apoptosis where apoptotic bodies are formed. Due to its distinct cell death machinery, ferroptosis is morphologically and biochemically distinct from apoptosis and other types of regulated necrosis.[44]

Up till now LPO in ferroptosis was described as a random process which affects all classes of membrane phospholipids.[45,46] Some studies postulate that LPO is an enzymatic process, catalysed by the complex of 15-lipoxygenase and phosphatidylethanolamine binding protein 1 which oxidises specific regions of the omega-6 (n-6) PUFA arachidonic and adrenic acid incorporated into one species of phospholipids (phosphatidylethanolamine).[47,48] The nature of LPO during ferroptosis, i.e. enzymatic or not, remains under debate. Of note, GCH1 overexpression inhibited ferroptosis *in vitro* by preventing critical peroxidation of phosphatidylcholine (PC) with two PUFA chains. Such double PL-PUFA occur in

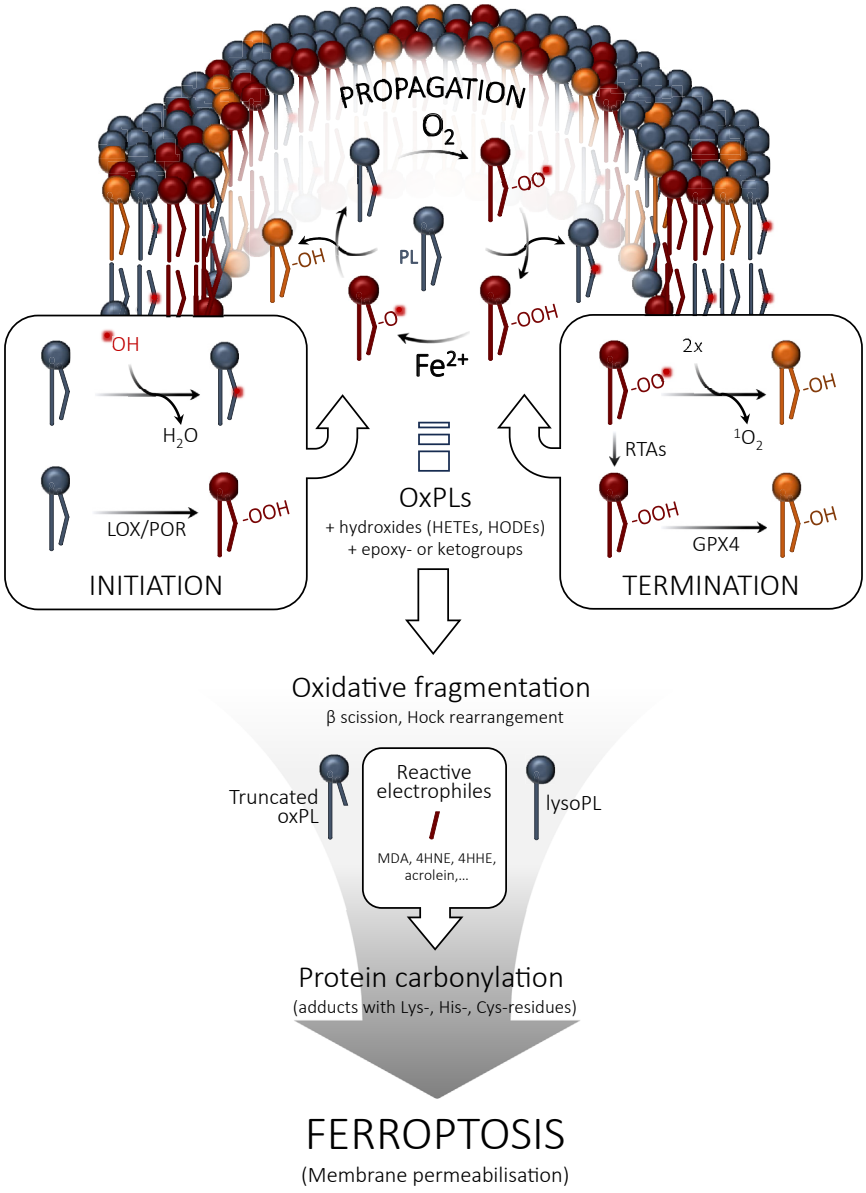


***Fig. 1:* Mechanism of lipid peroxidation leading to a myriad of oxidation products.** In polyunsaturated fatty acids (PUFA) the carbon atoms between two double bonds possess two weakly-bonded hydrogens atoms (bisallylic hydrogens). After the loss of 1 hydrogen atom and its electron to a radical the PUFA, attached as the second tail of membrane glycerophospholipids (sn-2 position) (PL-PUFA) in lipid bilayers, becomes a radical itself (PL-PUFA$^{\bullet}$). After a reaction with oxygen, the PUFA radical is transformed into a lipid peroxyl radical (PL-PUFA-OO$^{\bullet}$) which attacks other PL-PUFA to generate lipid hydroperoxides (PL-PUFA-OOH) and a new PUFA radical (PL-PUFA$^{\bullet}$). The latter will start the oxidation reaction anew in a positive feedback loop of auto-oxidation, called lipid peroxidation (LPO), which is a typical branched chain reaction as defined by the Nobel prize winning scientist Nikolay Semenov. Through Fenton reactions ferrous iron ($Fe^{2+}$) can transform the PL-PUFA-OOH into a radical, thereby amplifying the peroxidation of PL-PUFA. Evidently, higher numbers of double bonds in PUFA species would be predicted to enhance this non-enzymatic LPO. Alternatively, certain enzymes can oxidise PL-PUFA in specific locations. For example, after a shift in its substrate preference by phosphatidylethanolamine binding protein 1 (PEBP1), the enzyme 15-lipoxygenase (15-LOX) can oxidise arachidonic acid (AA) and adrenic acid (AdA) esterified to phosphatydilethanolamine (PE) to produce the ferroptosis death signal 15-hydroperoxy-eicosatetraenoic acid (15-HpETE)-PE. Likewise, the enzyme cytochrome P450 oxidoreductase (POR) from the endoplasmic reticulum facilitates ferroptosis as it releases hydrogen peroxide to drive LPO in the presence of $Fe^{2+}$. Regardless of how the LPO reaction starts, lipid hydroperoxides are unstable molecules and the addition of more oxygen can form PLs with combinations of hydroxides (e.g. hydroxy-octadecadienoic acids (HODEs) and hydroxy-eicosatetraenoic acids (HETEs)), keto- and epoxy-group. Oxidation-induced de-esterification of one of the fatty acid residues can lead to the formation of lysophospholipids (with only one fatty acyl tail) which are sensitive indicators of ferroptosis. Alternatively,

very minute amounts in living cells (including liver tissue) but might be the crucial drivers of ferroptosis.[42,49] In essence, ferroptosis is cell death due to 'biological rusting' of lipid membranes which ensues when LPO overrides the ferroptosis defenses.[50] Evidently, metabolism of PL-PUFA, cytosolic $Fe^{2+}$ and redox defences (including GSH stores) influences the propensity towards ferroptosis. For example, expansion of cytosolic $Fe^{2+}$ by excessive heme oxygenase-1 activity can lead to non-canonical ferroptosis, thereby turning this cytoprotective enzyme into a driver of ferroptosis.[45] Importantly, peroxidation of PUFA roaming freely in the cytosol or acylated to triglycerides is no trigger of ferroptosis. Interestingly, PL-PUFA-OOH were detected in tumour-associated neutrophils while these cells were not dead.[51] This suggests cells may survive *in vivo* with ferroptotic stress (at least some time) and approaches the boundary between survival with LPO and the execution of ferroptosis.

Most knowledge on ferroptosis was derived from *in vitro* experiments and requires translation to normal liver physiology. GPX4 is indispensable for the liver as hepatocyte-specific deletion of GPX4 results in death by 48 h after birth, but survival was prolonged by vitamin E supplementation.[13,52] Moreover, postnatal hepatocyte-specific knockout of GPX4 leads to spontaneous centrolobular hepatocyte ferroptosis and subsequent death, which is completely blocked by systemic application of ferrostatin-analogue UAMC-3203.[13,53] This indicates that normal centrolobular hepatocytes endure baseline levels of LPO which must not be allowed to accumulate to toxic levels. Of note, hepatocyte GPX4 is under transcriptional control of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). A PPARα agonist was able to restrain ferroptosis through GPX4 upregulation and reduced iron import.[54] Other ferroptosis defences seem redundant for normal liver physiology. Solute carrier family 7 member 11 (SLC7A11) is a subunit of the system$Xc^-$ whose whole body knockout (leading to GSH depletion) does not cause spontaneous hepatic ferroptosis, but sensitises mouse livers for iron overload-induced ferroptosis.[55] This implies that normal hepatocytes rely on different sources of GSH as the liver synthesises cysteine and GSH in the transsulfuration pathway.[56,57] Constitutional knockout of FSP1 has no phenotype in mice, but sensitises animals for poisoning with the vitamin K antagonist warfarin.[53] Deficiencies of the lipophilic BH4 are rare metabolic disorders which are not related to hepatocyte damage.[58] In healthy livers PL-PUFA integrity is mainly ensured by GPX4 under basal levels of LPO, but this steady-state may change in MASLD.

## Signatures of ferroptosis in MASLD patients

Decades of research into LPO provide evidence for ferroptosis in MASLD, but specific detection of this cell death *in vivo* can be challenging, as summarised in Supplementary Box 1. The majority of evidence comes from IHC and the TBARS assay, but mass spectrometry yielded interesting results (Table 1 and Supplementary Table S1). All studies agree that hepatic MDA (on the TBARS assay) is increased in MASH patients compared to lean controls, but this is not always the case for isolated steatosis.[59–61] In one study hepatic MDA correlated with higher histologic inflammation, ballooning and systemic insulin resistance.[61] The ferroptosis executor oxidised phosphatidylcholine (oxPC) was detected in cellular membranes of ballooned hepatocytes, the lipid droplet rim of macrovesicular steatotic hepatocytes and macrophages in MASH specimens.[62,73] The presence of oxPC in MASLD specimens was confirmed with another antibody.[63] Several oxPC positive hepatocytes display DNA strand breaks indicating ongoing cell death.[62] These findings interrogate our understanding of the ballooned morphology of hepatocytes, which could constitute undead hepatocytes damaged by LPO that contain small lipid droplets.[74,75] In fact, the accumulation of intracellular lipid vacuoles was observed in other cell types during ferroptosis *in vivo*, which originate from the endoplasmic reticulum.[36,76] Concerning IHC for 4HNE adducts, these markers of ferroptosis are increased in MASLD (hepatocytes and sinusoidal cells) compared to controls.[62,64–66] Regarding their topography, LPO breakdown products are mainly present in the pericentral region in MASH.[64,67]

To our knowledge, the gold standard for the detection of ferroptosis, i.e. oxidative phospholipidomics, was never used to examine MASLD liver tissue, but other mass spectrometry studies provide interesting clues. Ooi et al. found decreasing amounts of three PL-PUFA species and increases in their corresponding lysoPL at increasing steatosis grades. Isolated steatosis specimens displayed lower PL-PUFA compared to controls.[68] This points towards a signature of LPO (both isolated steatosis and MASH) and/or reduced PUFA formation in

oxidative fragmentation by beta-scission or Hock rearrangement can lead to truncated oxidised phospholipids (oxPL) and shortened carbon chains such as acrolein, malondialdehyde (MDA), 4-hydroxynonenal (4HNE) and 4-hydroxyhexenal (4HHE) among others. 4HNE is mainly derived from LPO of n-6 PUFAs, whereas 4HHE is a product of n-3 PUFAs. Importantly, due to multiple possible sites of oxidation, types of changes and breakdown mechanisms, as well as combinations thereof, one PL-PUFA species can give rise to a myriad of oxidation products, identifiable with mass spectrometry using in-house optimised workflows. MDA and 4HNE are the most well-studied breakdown products of lipid peroxidation with cytotoxic effects by binding to other biomolecules. 4HNE (and other reactive electrophiles) typically cause carbonylation of amino acids lysine, histidine and cysteine residues, thereby perturbing protein functions.

| Method | Number of patients | Study findings | Refs |
|---|---|---|---|
| TBARS | MASH: n = 35<br>Isolated steatosis: n = 15<br>Lean controls: n = 10 | Higher MDA in MASH and isolated steatosis compared to controls. Higher MDA in MASH than in isolated steatosis. | 59 |
| | MASH: n = 53<br>Isolated steatosis: n = 51<br>Controls: n = 88 | Significant increase in hepatic MDA in MASH compared to isolated steatosis. | 60 |
| | MASH: n = 34<br>Isolated steatosis: n = 18<br>Overweight controls: n = 16 | Higher MDA in MASH compared to controls. MDA correlates with histological inflammation, ballooning, waist circumference. | 61 |
| IHC | MASH: n = 32<br>Isolated steatosis: n = 15<br>Normal controls: n = 11 | Increased OxPC positivity and 4HNE adducts in isolated steatosis and MASH *versus* controls. oxPC in membranes of ballooned hepatocytes correlates with necroinflammatory disease activity and fibrosis. | 62 |
| | MASLD: n = 25<br>MASLD with F4: n = 3<br>Controls: n = 25 | oxPC positivity in MASH and cirrhosis, but not in isolated steatosis or controls. Higher oxPC positive area in F1,2 and 4 livers, compared to F0 livers. | 63 |
| | MASH: n = 17<br>Isolated steatosis: n = 23<br>Overweight controls: n = 7 | 4HNE adducts in hepatocyte cytoplasm and sinusoidal cells in MASH. 4HNE adducts occur mostly in centrolobular region and correlate with fibrosis and necroinflammation. | 64 |
| | MASLD: n = 21<br>Controls: n = 5 | Hepatocyte intracytoplasmic 4HNE adducts in MASLD, absent in normal livers. No correlation with MASH disease activity, fibrosis, ALT or BMI. Oral vitamin E reduced 4HNE adducts on paired biopsies. | 65 |
| | MASLD: n = 90<br>Controls: n = 13 | Increased hepatic 4HNE adducts in MASLD compared to controls. 4HNE adducts associate with histological ballooning, lobular inflammation and fibrosis in MASLD. | 66 |
| | MASLD: n = 24<br>Controls: n = 6 | MDA adducts in centrolobular hepatocytes in MASLD but not in controls. MDA adducts correlate with steatosis, inflammation and fibrosis. MDA adducts are present in periportal sinusoidal cells when hepatic iron is increased. | 67 |
| MS | MASH: n = 16<br>Isolated steatosis: n = 110<br>Obese controls: n = 50 | Decreasing PL-PUFA (but increasing PL-SFA and PL-MUFA) with increasing steatosis. Lower PC-, PE- and PI-PUFA in isolated steatosis compared to controls. Increasing lysoPC/-PE/-PI with increasing steatosis. | 68 |
| | MASH: n = 9<br>Isolated steatosis: n = 9<br>Controls: n = 9 | Increased absolute amounts of lysoPC in MASH compared to controls. Decreased PC-AA in MASH compared to controls. | 69 |
| | Cirrhotic: n = 20<br>MASH: n = 20<br>Isolated steatosis: n = 17<br>Controls: n = 31 | Increased lysoPE in isolated steatosis compared to controls. Increased 15-HETrE and 12-HETE in isolated steatosis compared to controls. | 70 |
| | MASLD: n = 23<br>Controls: n = 23 | lysoPC and lysoPE increased in MASLD compared to controls. | 71 |
| | MASH: n = 9<br>Isolated steatosis: n = 11<br>Controls: n = 2 | PL zonation disturbed and 13- & 9-HODE increased in MASH compared to isolated steatosis. 13-HODE associates with histological inflammation. | 72 |

4HNE: 4-hydroxy-2-nonenal; 9-HODE: 9-hydroxyoctadecadienoic acid; 12-HETE: 12- hydroxyeicosatetraenoic acid; 13-HODE: 13-hydroxyoctadecadienoic acid; 15-HETrE: 15- hydroxyeicosatrienoic acid; AA: arachidonic acid; ALT: alanine aminotransferase; BM: body mass index; MDA: malondialdehyde; MS: mass spectrometry; oxPC: oxidised phosphatidylcholine; lysoPLs: lysophospholipids; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; SFA: saturated fatty acid.

***Table 1:*** **Hepatic ferroptosis signatures in human MASLD.**

this chronic liver disease to avoid ferroptosis. The increase in hepatic lysoPL species in MASLD was also reported by others.[69–71]

Another hint towards the role of hepatic ferroptosis in MASLD is the efficacy of nature's premier RTA in this disease, i.e. vitamin E. A total of 15 randomised clinical trials (RCT) were conducted to test the effect of vitamin E on MASH, as summarised elsewhere.[77] Most cohorts were relatively small, with the largest (PIVENS) trial reporting that once daily oral 800 IU of vitamin E for 96 weeks reduced transaminases and histological abnormalities on follow-up liver biopsies, but only 43% of patients reached the criteria of histological response.[78] The beneficial effect of vitamin E could stem from its RTA activity. Indeed, four weeks of vitamin E treatment reduced the amount of 4HNE-adducts on follow-up liver biopsies assessed by IHC.[65] Collectively, data from RCTs indicate that vitamin E reduces transaminases and ballooning, less so in diabetic patients, but lacks anti-fibrotic capacities.[79] Given that vitamin E is a relatively weak ferroptosis inhibitor, more pronounced effects on MASH histology could be expected from stronger lipophilic RTAs.[80]

## Ferroptosis in MASLD microenvironment and gut–liver axis

Upon membrane permeabilization, ferroptotic cells release different danger-associated molecular patterns (DAMPs), such as oxPL and truncated oxidised lipids.[81] Ferroptosis can propagate *in vitro* to neighbouring cells, as well as in an *ex vivo* kidney tubule model and in the

tailfin of zebrafish, possibly through release of LPO products.[39,82,83] The role of oxPL and their breakdown products is well known in the MASH microenvironment. The lab of Joseph Witztum showed that oxPC induced mitochondrial dysfunction (with ROS production) in primary mouse hepatocytes accompanied by a drop in the cytosolic reductant NADH.[63] OxPC can hyperactivate dendritic cells and macrophages via their CD14 receptor.[84] In the setting of viral infections oxPL induce pro-inflammatory cytokine production by macrophages via toll-like receptor 4 signalling.[85] Ether-linked oxPL released by neutrophils can mediate neutrophil extracellular trap (NET) formation, thereby promoting more inflammation.[86] Likewise, the oxidised PUFA linoleic acid impaired mitochondria in hepatocytes leading to apoptosis.[87] In a MASH mouse model, truncated oxPC species inhibited mitochondrial oxygen consumption leading to lipid droplet formation in hepatocytes.[88] As ligands of PPARα, the LPO products can influence lipid metabolism of neighbouring cells.[89] These studies illustrate how oxPL can promote steatosis and cell death of hepatocytes, but they can also rewire the bioenergetics of hepatic stellate cells to commence extracellular matrix synthesis.[63,88,90] As remnants of the LPO process, lysoPL from ferroptotic cells may damage neighbouring cells since these toxic messengers cause ER stress and cell death of primary human hepatocytes.[91,92]

The electrophiles 4-HNE and MDA, released during ferroptosis, are known to have detrimental effects in MASH. Chen et al. confirmed that electrophiles in ferroptotic cells can modify over 400 endogenous proteins, possibly impairing their function.[93] Endogenous defences against electrophiles exist such as AKR1C1, which can be induced by 4HNE in hepatocytes.[94] Alternatively, cells can detoxify electrophiles through conjugation with GSH but this route is impaired during ferroptosis.[95] High 4HNE levels cause disturbances in calcium homeostasis and acute cytoskeletal damage in primary hepatocytes.[29] Compared to other cell types, hepatocytes are relatively resistant to 4HNE, but it is unknown whether this is also true for steatotic, damaged hepatocytes.[29,66] Indeed, 4HNE induces stress response pathways such as c-Jun N-terminal kinase (JNK) activation[96] and Akt signalling in hepatocytes, subsequently resulting in lipid droplet accumulation and insulin resistance.[96–98] Hence, electrophiles could act as toxic second messengers of ferroptosis that induce steatosis and cell death in neighbouring hepatocytes. Moreover, 4HNE avidly forms adducts with nucleic acids and interferes with DNA repair mechanisms thereby promoting HCC carcinogenesis.[99,100] The same electrophile activates quiescent hepatic stellate cells via direct interaction with JNK isoforms, as does MDA via the proliferation inducer c-myb.[101,102] Conversely, in preclinical liver fibrosis models a subset of ceroid macrophages was found which takes up MDA and suppresses fibrogenesis.[103] This suggests that the liver has endogenous mechanisms to counter the fibrotic effect of electrophiles.

Evidence is mounting for the release of cyclooxygenase (COX) and lipoxygenase (LOX) products by ferroptotic cells. Depletion of GPX4 and GSH increases the available hydrogen peroxide, resulting in higher COX and LOX activities which explain the increased prostaglandin E2 levels in the skin of mice after keratinocyte-specific GPX4 deletion.[35,104,105] Prostaglandin E2 is elevated in MASLD livers and has an immunosuppressive effect.[106] This illustrates that ferroptosis may not have a straightforward pro-inflammatory effect. Indeed, in classical vaccination studies with immunocompetent mice ferroptotic cells failed to elicit T lymphocyte-driven adaptive immunity, meaning that ferroptosis cannot be considered an immunogenic cell death.[107,108] In-depth studies with cells in early or late stages of ferroptosis indicate that the ferroptotic cell corpses themselves diminish cytotoxic CD8+ T cell proliferation, despite the release of proinflammatory ATP and high-mobility group box 1 (HMGB1).[108] Perhaps ferroptosis does not have to meet the criteria of immunogenic cell death to be detrimental in MASH. Dudek and colleagues recently described a subset of 'auto-aggressive' cytotoxic T cells that can be activated by metabolic DAMPs such as acetate and ATP from dying hepatocytes.[109] It would be conceivable that the DAMPs from ferroptotic hepatocytes hyperactive this subset of CD8+ T cells. The relation of ferroptosis and necroinflammation continues to be debated, as reviewed elsewhere.[110] For instance, during neuroinflammation activation of epigenetic regulator C9a repressed anti-ferroptotic genes and triggered ferroptosis in neurons.[111] It is unknown whether inflammation also promotes ferroptosis in a positive feedback loop in MASLD. Overall, the findings above suggest that recurring bouts of ferroptosis with DAMPs release promote steatohepatitis and fibrogenesis in this chronic liver disease (Fig. 2).

The liver microenvironment cannot be studied without taking into account the gut–liver axis. Multiple preclinical models have demonstrated that MASLD initiation and progression are driven by intestinal dysbiosis and gut-derived mediators such as lipopolysaccharide (LPS) and ethanol.[112,113] Moreover, the gut microbiome can promote or inhibit ferroptosis in the intestine and liver. For instance, LPS induced hepatic ferroptosis in mouse models of acute liver injury, in part through promotion of the 15-lipoxygenase/phosphatidylethanolamine binding protein 1 complexes.[48,114,115] However, ferroptosis inhibition did not improve acute liver injury in a polymicrobial sepsis model.[13] Through their metabolites gamma amino-butyric acid and capsiate, certain species of intestinal bacteria can inhibit ferroptosis in ischemia-reperfusion injury of the liver and gut, respectively.[116,117] The microbiome regulates the gut–liver axis through effects on bile salt metabolism

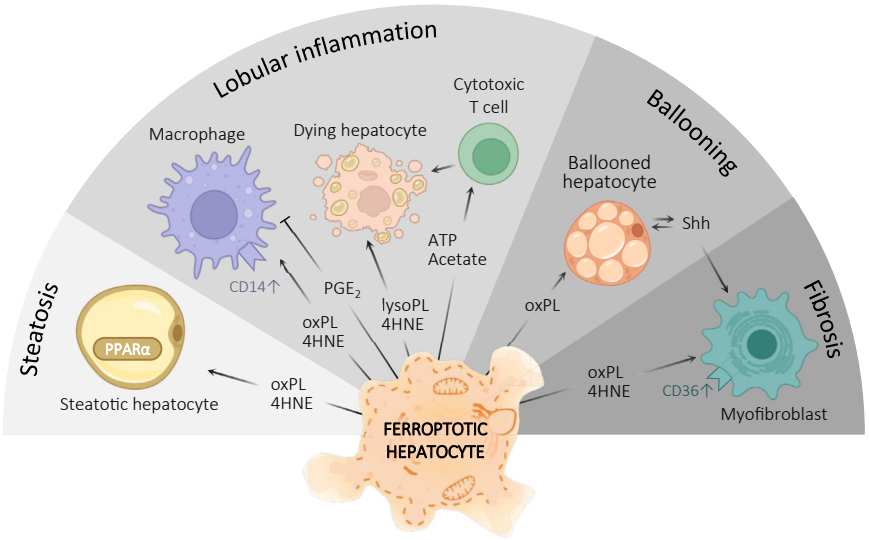


***Fig. 2:* Proposed model how ferroptosis drives the components of MASLD.** Ferroptotic hepatocytes release a multitude of danger-associated molecular patterns (DAMPs) in the MASLD microenvironment. The breakdown products of lipid peroxidation, oxidised phospholipids (oxPL) affect the mitochondrial bioenergetics of neighbouring hepatocytes, possibly via their agonistic effect on peroxisome proliferator-activated receptor α (PPARα). The truncated oxPL are responsible for lipid droplet induction and, hence steatosis and ballooning in other hepatocytes. In fact, ballooned hepatocytes are loaded with oxPL in their membranes, but their fate is unclear. Some ballooned hepatocytes produce Sonic Hedgehog protein (Shh) which acts as an autocrine pro-survival factor for undead hepatocytes and promotes fibrogenesis via induction of myofibroblasts. The latter also display the scavenger receptor CD36 which leads to fibrogenesis after binding with oxPL. Oxidised forms of the PUFA linoleic acid induce hepatocyte apoptosis, while certain species of oxidised phosphatidylcholine (oxPC) hyperactivate macrophages via their CD14 receptor. Oxidised phosphatidylethanolamine on ferroptotic cells serves as an eat-me signal for macrophages, but it is uncertain whether this happens in MASLD. Self-propagating waves of ferroptosis have been observed which pass from one cell to its neighbours. It is unclear whether this also occurs in MASLD livers. Next, lysophospholipids (lysoPL) can induce ER stress and apoptosis in hepatocytes in a concentration-dependent manner. The group of reactive electrophiles includes 4HNE which forms adducts with proteins from other MASLD players. Hepatocytes under metabolic pressure from overnutrition are more sensitive for the detrimental effects of 4HNE, which includes hepatocyte insulin resistance and more steatogenesis. The same electrophile can activate hepatic stellate cells via c-Jun NH2-terminal kinase activation, while MDA can be stored in macrophages as a cytoplasmic autofluorescent ceroid pigment. Waves of hepatic ferroptosis with DAMPs release could promote all histological aspects of MASLD, including disease activity and fibrosis, even though this cell death is not immunogenic and pro-inflammatory in every context. Indeed, metabolites such as ATP and acetate can activate the subset of auto-aggressive T cells recently discovered in MASLD. The latter induce cell death of hepatocytes in an in a major histocompatibility complex-class-I-independent fashion.

and glycochenodeoxycholate was shown to promote ferroptosis in MASLD.[118] The effect of intestinal dysbiosis on ferroptosis in MASLD merits further research and may depend on the precise nature of the alterations in gut microbiota.

## Ferroptosis targeting in preclinical MASLD models

Ferroptosis modulation in preclinical *in vivo* models helps elucidate the role of this cell death type in MASLD livers (Table 2 and Supplementary Table S2). The most used ferroptosis inhibitors are lipophilic RTAs, such as vitamin E, ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1). The latter compete with phospholipids and yield a radical that does not propagate the chain reaction. Importantly, natural phenols (including vitamin E) confer little protection against ferroptosis, while the synthetic Fer-1 and Lip-1 are more potent ferroptosis inhibitors due to advantageous dynamics in the lipid bilayer.[131,132] Most studies report about the effects of Fer-1 and Lip-1, although Fer-1 is hardly suitable for *in vivo* studies due to rapid inactivation in plasma.[80,82] Nevertheless, hepatic MDA decreased after chronic treatment with Fer-1 or Lip-1, leading to reductions in steatosis, lobular inflammation and fibrosis in choline-deficient models as well as dietary models wherein animals exhibit features of the metabolic syndrome.[119–122] In addition, ferroptosis inhibition reduced hepatic expression of pro-inflammatory cytokines such as interleukin-1 beta, tumour necrosis factor alpha and monocyte chemoattractant protein-1.[119,120,133] Importantly, oxidative phospholipidomics revealed the presence of lipid hydroperoxides in steatotic livers of mice after only 6 weeks of high-cholesterol high-fat diet, which is a strong indication for hepatic ferroptosis in these models.[134] Moreover, global GPX4 haploinsufficiency aggravated steatosis in a high-fat high-sucrose model of metabolic syndrome alongside increased hepatic 4HNE adducts.[135] More in-depth studies employed endogenous

| Intervention | Rodent model | Study findings | Refs |
|---|---|---|---|
| RTA | 3w MCD diet | 3w preventive Fer-1 (daily ip 5 mg/kg) or Lip-1 (daily ip 5 mg/kg) reduce hepatic LPO and prevent steatohepatitis and fibrosis. | 119 |
| | 10d MCD diet | 10d RSL3 (daily ip 10 mg/kg) increases histological abnormalities of MCD, while preventive Lip-1 (daily ip 10 mg/kg) prevents it. Preventive deferoxamine (daily ip 100 mg/kg) prevents histological abnormalities of MCD and RSL3-mediated exacerbation in MCD. | 120 |
| | 16w high-fat high-fructose diet | Increased hepatic GPX4, FSP1, GSH and TfR1 in mice on high-fat high-fructose diet. Therapeutic Lip-1 (daily ip 10 mg/kg) during last 4 weeks reduces bodyweight gain, dyslipidaemia, hepatic MDA, steatosis and MASLD activity score in mice. | 121 |
| | 18w high-fat high-fructose diet | Therapeutic Lip-1 (daily ip 10 mg/kg) reduces hepatic MDA and 4HNE adducts, coinciding with reduced steatosis, improved insulin resistance and liver fibrosis. Therapeutic deferiprone (daily ip 100 mg/kg) only mildly reduces hepatic inflammation. | 122 |
| oxPC scavenging | 30/48w AMLN model<br>Streptozotocin + 4w high-fat diet 4w CCl4 model | CCl4 mice model and Ldlr$^{-/-}$ mice on AMLN or streptozotocin-high fat diet display increased hepatic and serum oxPC. Neutralisation of oxPC reduces hepatic oxPC, TUNEL positivity, ALT, steatosis and MASH activity score, fibrosis, as well as number and size of MASH-related HCC. | 63 |
| | 16w high-chol. diet | Steatotic livers from hyperlipaemic Ldlr$^{-/-}$ mice on high cholesterol diet contain oxPC. Mice expressing Endogenous variable fragments of E06 reduce hepatic oxPC and steatosis. | 123 |
| | 6/20w FPC +4.2% sugar water | Hepatocyte-specific expression of variable fragments of E06 in mice on FPC for 6w reduce steatosis, ALT and plasma truncated and full-length oxPC species. Hepatocyte expression of E06 variable fragments for 14w in mice on FPC for 20w reduces ALT, hepatic lipid droplet size and liver fibrosis, without effect on insulin resistance. | 88 |
| | 8w atherogenic diet | IgM antibodies against oxPC reduce hepatic inflammation after 8w of atherogenic diet, without effect on steatosis and fibrosis. | 124 |
| | 3w high-fat high-chol. Diet | IgM antibodies against oxPC reduce hepatic inflammation after 3w of high-fat high-chol. diet, without effect on steatosis and fibrosis. | 125 |
| HSC ferroptosis | BDL | Preventive erastin and sorafenib for 2w reduce liver fibrosis in mice with BDL. Erastin and sorafenib induced ferroptosis in HSC cell lines via increased ferritinophagy by ZFP36 dependent mechanism. | 126 |
| | BDL | Erastin-induced ferroptosis of HSC cell lines partly depends on the BRD7-p53-SLC25A28 axis. Erastin *in vivo* reduces fibrosis. | 127 |
| | CCl4 | Erastin-induced ferroptosis in HSC attenuated liver fibrosis and depends on post-translational modifier m$^6$A. | 128 |
| | CCl4 and BDL | In an acute CCl4 liver fibrosis model, erastin induces HSC ferroptosis and reduces fibrosis. In the acute setting primary hepatocytes are resistant to erastin. In chronic liver fibrosis models erastin exacerbates liver fibrosis due to the induction of ferroptosis. | 129 |
| | Iron dextran | RSL3-induced ferroptosis in primary HSC induced fibrogenic gene expression. Chronic iron overload induced ferroptosis in hepatocytes and HSC which initiate fibrogenesis. | 130 |

ALT: alanine aminotransferase; AMLN, Amylin liver MASH model (high-fat, high-fructose, high-cholesterol); atherogenic diet, 21% milk fat and 0.2% cholesterol; BDL, common bile duct ligation model; CCl4: carbon tetrachloride; d, days; Fer-1: ferrostatin-1; FPC, Fructose palmitate cholesterol diet; FSP1: ferroptosis-suppressor protein 1; GPX4: glutathione peroxidase 4; GSH: reduced glutathione; HCC: hepatocellular carcinoma; ip: intraperitoneal; Ldlr$^{-/-}$: low-density lipoprotein receptor; Lip-1: liproxstatin-1; MCD: methionine- and choline-deficient diet; RSL3: RAS-selective lethal 3; TfR1: transferrin receptor 1; TUNEL: terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labelling; w: weeks.

***Table 2*: Ferroptosis targeting in experimental MASLD.**

production of an IgM antibody (clone E06) which scavenges oxPC in MASLD models. The variable fragments of E06 reduced steatosis, MASH disease activity and liver fibrosis, without altering the upstream metabolic drivers of MASLD.[63,88,123] It is unknown whether such an antibody inhibits ferroptosis or merely counters the spreading of oxPC.

With regards to (MASLD-induced) liver fibrosis, the induction of ferroptosis in hepatic stellate cells was hypothesised to be a new anti-fibrotic strategy since their trans differentiation into myofibroblasts is crucial for fibrogenesis.[136] Several studies present conflicting results on this topic. Remarkably, erastin and sorafenib *in vivo* had a very specific ferroptosis-inducing effect on HSCs (but not on hepatocytes) in carbon tetrachloride and bile duct ligation models of fibrosis, contrary to another study where erastin exacerbated diet-induced MASH.[120,126–128] Indeed, Du and colleagues found that inhibition of systemXc$^-$ in a chronic liver fibrosis model caused ferroptosis of HSC and hepatocytes leading to exacerbated fibrosis.[129] The selective induction of ferroptosis in one cell type of the liver might prove challenging given the overall detrimental role of this cell death in MASLD. Therefore, caution is warranted when attempting to induce ferroptosis in HSC since that may temporarily increase their fibrogenic gene expression, while ferroptosis in HSC and primary hepatocytes promoted liver fibrosis during chronic iron overload.[130] Moreover, the studies mentioned above did not study liver fibrosis in the context of MASLD.

## Metabolism links ferroptosis to MASLD

Ferroptosis is fundamentally intertwined with redox metabolism and availability of $Fe^{2+}$ and PL-PUFA. Alterations in these three metabolic pathways in MASLD explain the increased ferroptosis sensitivity.

### Redox metabolism in MASLD

Increased ROS production by certain subcellular organelles in MASLD could be the starting point for hepatic LPO, which can propagate in endoplasmic reticulum (ER) membranes and eventually reach the

plasma membrane.[137,138] For example, *ex vivo* high-resolution respirometry revealed that dysfunctional hepatic mitochondria in human MASH have lower maximal respiration compared to isolated steatosis, leading to an increased $H_2O_2$ leakage and LPO.[139] Under chronic metabolic stress, the unfolded protein response in the ER becomes maladaptive. Indeed, *in vivo* accumulation of unfolded proteins in hepatocyte ER causes oxidative stress mediated hepatocyte cell death in mouse livers.[140] Ferroptosis in MASLD will be facilitated by decreased activity of the GSH-GPX4 axis or a drop in lipophilic antioxidants. In a small Japanese study hepatic vitamin E levels were higher in a steatotic livers (from many aetiologies) compared to normal livers, possibly through sequestration of this lipophilic vitamin in lipid droplets.[141] This remains to be confirmed in larger cohorts. Moreover, changes occur in the central GPX4-GSH axis with lower hepatic GPX4 levels in a small cohort of MASH patients compared to controls with normal liver histology.[142] The activity of the GPX4 protein depends on the availability of hepatic GSH, which is lowered in human MASH and isolated steatosis compared to controls.[59,143] Mass spectrometry revealed lower hepatic concentrations of reduced and oxidised glutathione with a relative deficiency of reduced GSH in a modestly sized cohort of steatotic livers compared to controls.[71] However, some researchers have doubts about the lack of GSH in MASLD livers, since the liver is well endowed with GSH via the transsulfuration pathway.[56] Metabolomics studies argue that hepatic GSH is only diminished in a subset of MASLD patients, together with decreased S-adenosyl methionine and impaired VLDL secretion.[144,145] To our knowledge no efforts were made to measure the hepatic levels of other lipophilic antioxidants, i.e. CoQ10, vitamin K and BH4, in MASLD.

### Iron deregulation in MASLD

Free cytosolic $Fe^{2+}$ is essential for the ferroptosis since it catalyses Fenton mediated hydroxyl radical production as well as LPO. The amount of $Fe^{2+}$ can rise through the degradation of ferritin complexes in hepatocytes, directed by nuclear receptor coactivator 4 (NCOA4), in the process termed ferritinophagy.[146] To our knowledge, hepatocyte $Fe^{2+}$ levels have not been assessed in human MASLD liver specimens by mass spectrometry, but iron deregulation is present in this chronic liver disease.[147] Increases in hepatic iron stores, assessed with the Prussian blue stain (mostly ferritin iron), were found in 25–35% of European and North American MASLD patients.[148–150] Iron accumulation usually displayed a mixed pattern with deposition in hepatocytes and sinusoidal cells.[150] In the largest cohort macrophage iron overload correlated with the presence of MASH and advanced fibrosis, although others contested this.[149,151] Recently, the term 'dysmetabolic iron overload' was coined for high hepatic iron accumulation in MASLD, which is caused by inappropriately high hepcidin levels during chronic inflammation.[152] Enlarged adipose tissue in obesity can become an ectopic source of this hormone, while hepatocyte hepcidin secretion is induced by ER stress.[153,154] MASLD patients with macrophage iron accumulation displayed higher MASLD activity score and more TUNEL positive foci in their livers compared to the parenchymal pattern or no iron overload. In addition, MASLD patients with some form of hepatic iron overload displayed a signature of some hepatocyte necrotic cell death in their serum, although the identity of this cell death was not elucidated.[8] It is conceivable that ferroptosis plays a role in the subset of MASLD patients with hepatic iron overload.

These observations raise questions about the interaction of the iron-handling macrophages and ferroptosis. As reviewed by Yang et al., iron storage could make macrophages prone to ferroptosis and they could be activated by mediators from ferroptotic cells via their advanced glycosylation end-product specific receptor and toll-like receptor 2.[155] In MASH, resident Kupffer cells are depleted through an unspecified cell death while monocyte-derived macrophages infiltrate the liver to form phagocytosing lipid-associated macrophages in crown-like structures around steatotic hepatocytes.[156–158] Macrophages can phagocytose dying ferroptotic cells that express species of oxidised phosphatidylethanolamine as eat-me signals, possibly explaining the presence of oxPC in macrophages in human MASH.[62,159] Alternatively, macrophages themselves can commit to non-canonical ferroptosis due to erythrophagocytosis with haem degradation.[45,160] However, *in vitro* polarisation of macrophages to a pro-inflammatory phenotype renders these cells insensitive to GPX4 loss.[161] Increased inducible nitric oxide synthase in pro-inflammatory macrophages interferes with the pro-ferroptotic effect of 15-lipoxygenase, thereby explaining why these cells are unlikely to succumb to ferroptosis pressure.[162] It remains to be elucidated how precisely the different macrophage subtypes relate to ferroptosis in MASH with iron overload.

The question remains whether the mild-to-modest hepatic iron accumulation in MASLD could enhance ferroptosis. Several recent reports shed light on this question. Hepatocyte-specific deficiency of the iron chaperone protein poly (rC) binding protein 1 (PCBP1) increased hepatocyte labile iron (through increased ferritinophagy) and PL-PUFA-OOH on oxidative phospholipidomics. This illustrates that the loss of control over the hepatocyte free $Fe^{2+}$ leads to ferroptosis, which promotes the steatosis and portal inflammation in PCBP1-deficient mice (without obesogenic diet).[163] Under pressure from saturated fatty acid palmitate, hepatocytes increase their free $Fe^{2+}$, which is a cofactor to incorporate this toxic lipid in inert lipid droplets. Thus, the influx of palmitate, highly abundant in the plasma of MASLD patients, led to steatotic hepatocytes that are

more prone to ferroptosis through increased ferritinophagy.[164] In spite of these preclinical findings, not much is known about ferritinophagy in human MASLD, except for a reported increase in hepatic NCOA4 expression at the transcriptional level in obese patients.[165] Interestingly, agonism of PPARα and overexpression of its target gene FGF-21 protect hepatocytes from iron-overload induced ferroptosis in mice.[54,166]

### PUFA-phospholipid metabolism in MASLD

Membrane lipid bilayers consist of PL that contain two fatty acid tails.[167] PL-PUFA are formed through continuous removal of acyl tails and replacement with PUFA in the so-called Lands' cycle.[168] Mammals do not possess the enzymatic machinery for *de novo* synthesis of PUFA meaning that dietary PUFA intake is important for PL-PUFA which constitute the 'fuel' for ferroptosis.[167] Increased PL-PUFA content in membranes sensitises cells for this cell death, whereas increased PL-MUFA prevents ferroptosis.[169,170] One enzyme from the Lands' cycle, lysophosphatidylcholine acyltransferase 3 (LPCAT3), has great affinity for the incorporation of PUFA into PL after their activation by acyl-CoA synthase long-chain 4 (ACSL4).[171,172] Hepatic ACSL4 expression is elevated in MASH and isolated steatosis compared to controls, thereby promoting PUFA esterification into cell membranes and ferroptosis.[173–176] Hepatocyte-specific deletion ACSL4 and pharmacological inhibition reduced steatosis and liver fibrosis in preclinical MASLD due to increased mitochondrial respiration, and rendered hepatocytes resistant to LPO.[174] However, acute adenoviral-mediated knockdown of hepatocyte ACSL4 in mice on high-fat diet caused decreased hepatocyte lipid export and aggravated insulin resistance.[177] Of note, thiazolidinediones (such as pioglitazone) can inhibit ACLS4 independent from their PPARγ agonistic effect.[178] It would be tempting to speculate that part of the effect of pioglitazone on MASLD stems from an anti-ferroptotic effect.[79] Hepatocyte-specific deletion of LPCAT3 has unwanted effects as it leads to decreased VLDL secretion and (limited) steatosis.[179] Interestingly, ACSL4 and LPCAT3 are (in part) regulated by the nuclear receptor PPARδ,[180] while PUFA themselves are ligands of all three isotypes of PPAR, albeit with a varying affinity.[181] In this way, PUFA form a feedback control loop by regulating lipogenesis and beta oxidation and PL-PUFA are intertwined with the entire liver lipid metabolism.

Regardless of how the regulatory enzymes change, lipidomics studies report a decreased proportion of n-6 on omega-3 (n-3) PUFA (on the total amount of hepatic fatty acids), an increased n-6/n-3 ratio and deficit in very long-chain PUFA.[61,68,69,182–185] This suggests that cells lower the substrate for ferroptosis to avert this cell death or could indicate that long-chain PUFA are consumed in LPO. For instance, during CCl4-induced liver fibrosis, high levels of MDA occur in the centrolobular area accompanied by a gradual drop in PUFA content of PC. This consumption of PL-PUFA reduced membrane fluidity of centrolobular hepatocytes (leading to ER stress and cell death).[186] The concept that increased PL-PUFA in hepatocytes would sensitise for detrimental ferroptosis raises questions since dietary n-3 PUFA supplementation has been tested as a treatment for MASH.[187,188] Indeed, it is commonly assumed that n-3 PUFAs are beneficial since they are transformed into anti-inflammatory mediators while the opposite is true for n-6 PUFAs.[189] This apparent paradox could be solved by considering the relation between dietary PUFA intake and PL-PUFA content of hepatocytes. Elegant dietary studies in rats showed that intake of different PUFA does not greatly influence the types of fatty acids esterified to hepatocyte membrane PL, as opposed to the lipid composition of plasma and adipose tissue. The only exception is the balance of n-6/n-3 PUFA in hepatocyte PLs which correlates well with the dietary ratio.[167,190,191] Hence, dietary PUFA intake could improve MASLD without increasing PL-PUFA content of hepatocytes and their propensity towards ferroptosis. In the end, the balance between MUFA and PUFA in cell membranes might not be so important for hepatic ferroptosis, as a critical mass of (double) PL-PUFA may suffice for ferroptosis.[192]

## Ferroptosis induction in MASH-related HCC

Recently, immunotherapy has become first-line therapy for HCC, but the MASH-related form of this cancer may respond less favourably to this treatment, mandating the search for other therapeutic options.[193] Ferroptosis induction is a promising new strategy for cancer treatment as some tumours are sensitive to synthetic ferroptosis inducers.[194] Indeed, many cancer cells upregulate systemXc$^-$ for GSH supply to avoid spontaneous ferroptosis, while this membrane antiporter is dispensable for normal cells.[195] Sun et al. showed that hepatoma cell lines and xenografts are susceptible to ferroptosis due to systemXc$^-$ inhibition.[196] The multikinase inhibitor sorafenib, used for HCC, induces ferroptosis via systemXc$^-$ inhibition, although others could not confirm this.[197–199] SLC7A11 (a subunit of systemXc$^-$) is upregulated in human HCC tumours compared to adjacent non-tumour tissue, especially in advanced HCC, and associates with increased HCC metastasis.[200,201] 4HNE levels are lower in HCC specimens compared to surrounding non-tumour tissue, especially in HCC of Barcelona Clinic Liver Cancer class C.[202] 4HNE staining was even weaker in radiotherapy-resistant HCC lesions because of reduced ferrous iron and increased SLC7A11 and GPX4.[203,204] More evidence suggests human HCC enhance their ferroptosis defences such as FSP1.[205] The mitochondrial translocator protein, upregulated in HCC with adverse prognosis, protects against ferroptosis through upregulation of the NRF2 pathways.[206] Human HCC samples downregulate

tumour-suppressor glutamine synthase 2 (a promotor of ferroptosis in HCC cell lines) which allows for more HCC tumour growth.[207] Likewise, HCC with a poor prognosis display higher levels of TAK1, an activator of NF-κB and JNK signalling, to promote ferroptosis avoidance.[208,209] Moreover, heightened tumoral lactate induces ferroptosis resistance in an HCC xenograft model due to lipid bilayer remodelling (PL-PUFA reduction).[210] Indeed, most HCC tumours display a reduction in PL-PUFA of several classes and an increase in lysophospholipids.[211,212] Of note, these findings relate to HCC in general without differentiation for the subclass of MASH-related HCC.

Based on their dependence of systemXc$^-$ and GPX4, HCC cancer cells are prone to elimination by synthetic ferroptosis inducers. Their effect can be amplified by PUFA or ferrous iron supplementation to the cancer. As proof of concept, transarterial or intratumoral administration of nanoparticles containing PUFA docosahexaenoic acid induced ferroptosis in syngeneic and xenograft HCC models leading to marked tumour regression.[213,214] Optimisation of the coating of nanoparticles enabled the delivery of iron and induction of ferroptosis in a panel of cancer cells.[215] However, the induction of ferroptosis in cancer will affect both the cancers cells as well as the tumour microenvironment. For example, hepatocyte-specific deletion of GPX4 caused cell death of malignant hepatocytes in a mouse model of transposon-mediated HCC, but failed to attenuate tumour growth. Instead, ferroptosis in the HCC lesions induced infiltration of CD8+ T cells and myeloid-derived suppressor cells. The combination of ferroptosis inducer withaferin A with an inhibitor of myeloid cell recruitment or anti-PD-1 antibodies reduced tumour growth in this HCC model and improved survival.[216] It remains to be seen how HCC embedded in the unique MASH microenvironment would respond to ferroptosis inducers. In transposon-mediated HCC in steatotic livers the compound FSP inhibitor selectively induced ferroptosis in tumour cells, leading to an influx of M1 macrophages, dendritic cells and T cells through chemoattractants, with improved survival. This animal model greatly resembles a subset of human HCC, although other mouse models of MASH-related HCC are available.[205,217] The presence of ferroptosis was detected in two other MASH-related HCC models, i.e. streptozotocin and high-fat diet and ER stress-prone MUP-uPA mice on high-fat diet.[218,219]

The complex relation between ferroptosis and inflammation in the tumour microenvironment was studied in other cancer types. For example, CD8+ T cells activated by immune checkpoint inhibitors orchestrate cancer cell ferroptosis through the release of interferon gamma.[220,221] Importantly, unselective ferroptosis induction in cancer cells and immune cells could compromise the anti-tumour immune response of the tumour microenvironment. Uptake of AA by tumour-associated CD8$^+$ T cells via CD36 predisposes them to ferroptosis and dampens immune surveillance.[222] The development of compounds that selectively degrade GPX4 in cancer cells, but not in the tumour microenvironment, released HMGB1 to attract CD8$^+$ T cells and enhance anti-tumour response.[223] Interleukin-9 renders subsets of CD8$^+$ T cells resistant to ferroptosis and helps selective ferroptosis induction in cancer cells.[224] Conversely, caution is warranted as immunosuppressive ferroptotic neutrophils were found in the tumour microenvironment of several human cancer types. In the same study ferroptosis inhibition (instead of induction) synergised with immune checkpoint inhibition to limit tumour growth (Fig. 3).[51] The interplay between ferroptosis and inflammation will influence whether ferroptosis induction would be beneficial for HCC. Different HCC models can be used to induce ferroptosis in tumour cells, but studies with immunocompetent mice are more appropriate to elucidate the interplay with inflammation.

In the search for biomarkers to guide HCC therapy, several studies have explored the prognostic value of ferroptosis-related genes at the mRNA level and relation to immune cell infiltration. For instance, based on genes related to metal binding, haem processing and redox balance, a high risk groups were identified in patient samples from The Cancer Genome Atlas and International Cancer Genome Consortium with reduced overall survival and increased tumour mutational burden.[225–227] This subgroup of HCC tumours could be susceptible to immune checkpoint inhibition, and perhaps ferroptosis induction. The prognostic and therapeutic value of these multigene signatures in HCC needs to be validated in prospective studies, considering the aetiology of HCC.

On the other hand, ferroptosis in MASH might also have a procarcinogenic role. Grube et al. observed a reduction in HCC tumour growth in hepatocyte-specific ACSL4 knockout animals subjected to hepatocarcinogens. Since ACSL4 deletion is expected to reduce hepatocyte ferroptosis, this suggests that ferroptosis drives HCC progression.[218] Similarly, an effector of ER stress ATF4 reduced ferroptosis in MASH as well as HCC development.[219] Moreover, scavenging of oxPC with endogenous E06 led to a reduction of HCC lesions in mice exposed to the Amylin Liver NASH diet.[63] These studies (summarized in Supplementary Table S3) indicate that ferroptosis in MASH promotes MASH-related HCC, albeit evidence is mostly indirect, while these tumours avoid ferroptosis once they are established. This cancer type might be amenable to ferroptosis induction and more research is needed to explore ferroptosis induction in MASH-related HCC.

## Outstanding questions

- The ultimate proof of ferroptosis occurrence in MASLD is still lacking. The detection of PL-PUFA-OOH using oxidative lipidomics or spatial

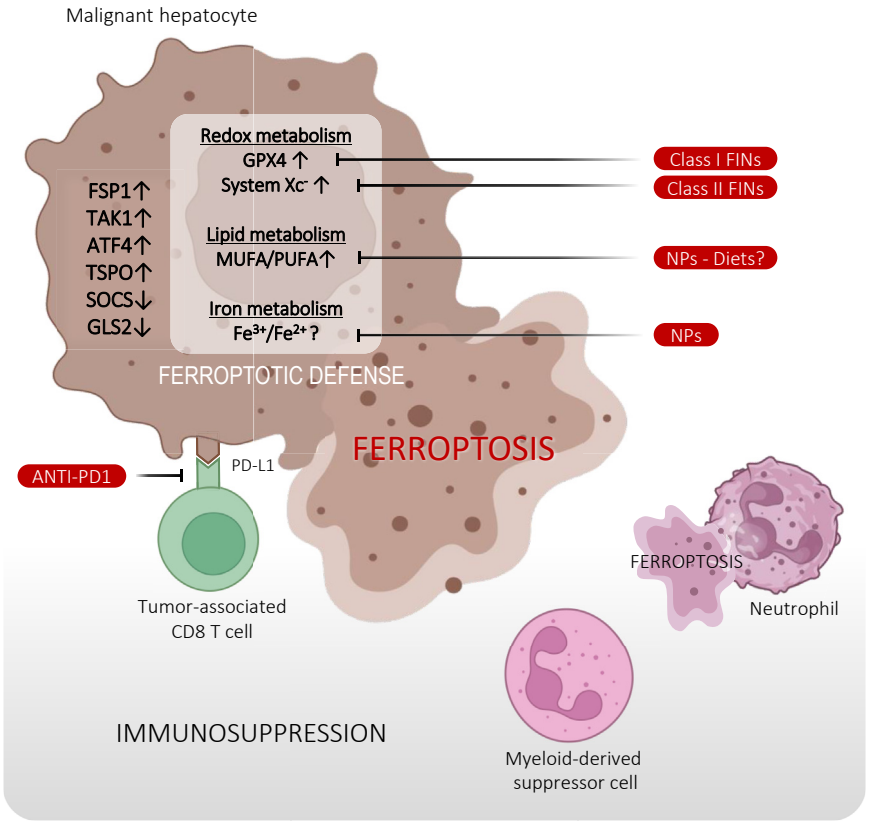


***Fig. 3:* Strategies to unleash ferroptosis on metabolic dysfunction-associated steatohepatitis-related hepatocellular carcinoma.** After their malignant transformation, hepatocellular carcinoma (HCC) cancer cells establish multiple mechanism to avoid spontaneous ferroptosis. Upregulation of the SLC7A11 subunit of system$Xc^-$, partly under the control of NRF2, provides cancer cells with a proliferation advantage due to greater GSH reserve. Reduced labile iron and increased SLC7A11 and GPX4, mediated by reduced suppressor of cytokine signalling 2 (SOCS2), confer resistance against radiotherapy-induced ferroptosis. An effector of ER stress activating transcription factor 4 (ATF4), upregulated in MASH and HCC tissue, was found to promote SLC7A11. Likewise, the mitochondrial translocator protein (TSPO), upregulated in HCC cases with poor prognosis, reduces ferroptosis sensitivity in HCC mouse through upregulation of the NRF2 pathways. Human HCC samples increase FSP1 expression and downregulate tumour-suppressor glutamine synthase 2 (GLS2) (a promotor of ferroptosis in HCC cell lines) which allows for more HCC tumour growth. HCC with an adverse outcome display higher levels of mitogen-activated protein kinase kinase 7 (TAK1), an activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun NH2-terminal kinase (JNK) signalling, which would lead to less hepatocyte ferroptosis. Moreover, heightened tumoral lactate, present in many cancers due to aerobic glycolysis, induces ferroptosis resistance in an HCC xenograft model due to lipid bilayer remodelling (more PL-MUFA, less PL-PUFA). Despite their inherent ferroptosis resistance, HCC xenografts in rodents can be sensitised through supplementation of PL-PUFA or $Fe^{2+}$ via nanoparticles (NPs) which promote more lipid peroxidation (LPO). After this sensitisation step, ferroptosis inducers (FINs) from different classes may efficiently induce lethal cell death through iron-catalysed LPO. However, elimination of HCC cancer cells by ferroptosis may not lead to straight-forward tumour regression, since these dying tumour cells interact with the immune cells in of the tumour-microenvironment. For example, the ferroptosis inducer withaferin A reduced tumour growth in an HCC model, but induced the expression of programmed death-ligand 1 (PD-L1) on other HCC cells and attracted myeloid derived suppressor cells. These changes in tumour immunology countered the tumour regression by ferroptosis induction. However, combination of the ferroptosis inducers with anti-PD-1 immunotherapy enhanced the anti-tumour effect. More research is needed in the context of MASLD, where the presence of auto-aggressive CD8 T cells may pose extra challenges. Indeed, caution is warranted as immunosuppressive neutrophils with a gene signature of ferroptosis were found in several human cancer types. In mouse models such tumour-associated neutrophils contained PL-PUFA-OOH, indicating the commitment to ferroptosis, and secreted prostaglandin E2 (PGE2) and oxidised phosphatidylethanolamine (oxPE) that downregulated the anti-tumour T cell response. Ferroptosis inhibition (rather than induction) therefore synergised with immune checkpoint inhibition to limit tumour growth in those models.

lipidomics in human MASLD liver tissue could clarify this.[228,229]

- Which cell type(s) undergo ferroptosis in MASLD livers. Advances in intravital liver microscopy will help monitor ferroptotic cells in preclinical MASLD models, identify the exact cell type to which they belong, and elucidate their interaction with the neighbouring cells.[230]

### Search strategy and selection criteria

A literature search was performed on PubMed with the following search terms from 1990 until November 2023: "Steatosis", "metabolic dysfunction-associated steatohepatitis", "metabolic dysfunction-associated steatotic liver disease", "ferroptosis", "lipid hydroperoxides", "lipid peroxidation", "glutathione peroxidase", "Non-transferrin bound iron", "labile iron", "ferrous iron", "redox active iron", "catalytic iron", "glutathione", "cysteine", "cystine", "systemXc$^-$", "polyunsaturated fatty acids", "phospholipids", "hepatocellular carcinoma". Only original research papers published in English were reviewed. The final reference list was selected based on originality and relevance to the scope of this review.

- Treatment directed at inhibition of hepatic ferroptosis might be insufficient when upstream drivers, such as adipose tissue dysfunction and gut dysbiosis, are not addressed.[231] A possible role for ferroptosis in the metabolic syndrome *per se* remains to be investigated. This differs from ischemia-reperfusion injury for example, where ferroptosis inhibitors help hepatocytes survive the acute insult, while no continuous disease drivers are present.
- Ferroptosis is a promising anti-cancer treatment, but the effect of ferroptosis induction on HCC in the MASH microenvironment needs to be carefully examined as this will determine the net effect of ferroptosis modulation.

## Conclusion

The presence of different types of hepatocyte cell death is a hallmark of human MASH, but their (relative) importance as therapeutic targets remains to be explored. The evidence summarised here pinpoints an important role for ferroptosis in MASLD pathogenesis. In normal liver physiology, the GPX4-GSH axis (fed by the transsulfuration pathway) is indispensable to prevent spontaneous hepatocyte ferroptosis. In MASLD ferroptosis defences are altered and eventually overridden by LPO, as evidenced by the presence of executors of ferroptosis (oxPC) in hepatocytes and sinusoidal cells, the drop in PL-PUFA and rise in lysoPC, as well as the rise in ferroptosis breakdown products.

Hepatic ferroptosis seems to have a detrimental role in MASLD as the DAMPs released can drive all components of MASLD. More ferroptosis inhibitors continue to be developed with improved pharmacokinetic and–dynamic properties, often based on the backbone of the lipophilic RTAs such as Fer-1 and Lip-1.[80] Chronic administration of improved inhibitors in MASLD models will shed more light on the therapeutic potential of ferroptosis in MASLD.

Lastly, ferroptosis induction has been explored as a possible new treatment strategy for HCC. These tumours rewire their metabolism to avoid ferroptosis. Finding exactly which ferroptotic brake mechanisms are upregulated in each patient with HCC will provide targeted treatment strategies and pave the way for precision oncology.

#### Contributors

CP, SF, TVB contributed to the concept and design of this review. CP performed the literature search and reviewed and selected the papers for inclusion. CP, SF and TVB wrote and edited the manuscript. CP and TVB created the figures. SF and TV critically revised the manuscript, the figures and tables. All authors read and approved the final version of the manuscript.

#### Declaration of interests

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105088.

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