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Ferroptosis and metabolic dysfunction-associated fatty liver disease: is there a link?

Short Title: Ferroptosis in MAFLD

Authors:

Gong Feng[1], Christopher D. Byrne[2], Giovanni Targher[3], Fudi Wang[4,5*], Ming-Hua Zheng[6,7,8*]

Affiliations:

[1]Xi'an Medical University, Xi'an, China;

[2]Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, Southampton General Hospital, Southampton, UK;

[3]Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy;

[4]The Fourth Affiliated Hospital, School of Public Health, Zhejiang University School of Medicine, Hangzhou 310058, China;

[5]The First Affiliated Hospital, Basic Medical Sciences, School of Public Health, Hengyang Medical School, University of South China, Hengyang 421001, China;

[6]NAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[7]Institute of Hepatology, Wenzhou Medical University, Wenzhou, China;

[8]Key Laboratory of Diagnosis and Treatment for The Development of Chronic Liver Disease in Zhejiang Province, Wenzhou, China.

***Corresponding authors:**

Ming-Hua Zheng, MD, PhD

NAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University; No. 2 Fuxue Lane, Wenzhou 325000, China.

E-mail: zhengmh@wmu.edu.cn; fax: (86) 577-55578522; tel: (86) 577-55579622

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Fudi Wang, MD, PhD
The First Affiliated Hospital, School of Public Health, Institute of Translational Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China.
E-mail: fwang@zju.edu.cn.

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Abbreviation list

GPX4 = glutathione peroxidase, GSH = glutathione, 4-HNE = 4-hydroxynonenal, HSC = hepatic stellate cells, IR = Insulin resistance, MAFLD = metabolic dysfunction-associated fatty liver disease, NAFL = nonalcoholic fatty liver, NAFLD = non-alcoholic fatty liver disease, NAS = NAFLD activity score, NASH = non-alcoholic steatohepatitis, OR = odds ratio, PDFF = proton density fat fraction, ROS = reactive oxygen species, T2DM = type 2 diabetes mellitus

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Conflicts of interest

All authors have nothing to declare.

Authors' contributions

Gong Feng and Ming-Hua Zheng designed the review outline. Gong Feng collected, information and data from literature and online, summarized and analyzed the data and drafted the manuscript. Ming-Hua Zheng, Christopher D. Byrne, Giovanni Targher and Fudi Wang advised on the structure and content of the manuscript, and revised the manuscript.

Data availability statement

Some or all data, during the review are available from the corresponding author by request.

Abstract

Non-alcoholic fatty liver disease (NAFLD), recently re-defined and re-classified as metabolic dysfunction-associated fatty liver disease (MAFLD), has become increasingly prevalent and emerged as a public health problem worldwide. To date, the precise pathogenic mechanisms underpinning MAFLD are not entirely understood, and there is no effective pharmacological therapy for NAFLD/MAFLD. As a newly discovered form of iron-dependent programmed cell death, ferroptosis can be involved in the development and progression of various chronic diseases, but the pathogenic connections and mechanisms that link MAFLD and ferroptosis have not been fully elucidated. The main characteristics of ferroptosis are the accumulation of lipid peroxides and reactive oxygen species. In this brief narrative review, the mechanisms of ferroptosis and its putative pathogenic role in MAFLD are discussed to highlight potential new research directions and ideas for the prevention and treatment of MAFLD.

Key-words: ferroptosis; MAFLD; pathogenesis, type 2 diabetes, metabolic syndrome

Introduction

In the past, two main mechanisms of programmed cell death have been recognized, namely apoptosis and necrosis. However, these two mechanisms do not explain all forms of cell death. Other forms of programmed cell death have recently been recognized, such as pyroptosis, autophagy and ferroptosis. Of these newly discovered forms of programmed cell death, ferroptosis has been widely studied in recent years, because it involves accumulating lipid-active oxygen species and appears to play a key role in the development of some types of tumors, neurodegenerative diseases, or endocrine diseases.[1-3]

Non-alcoholic fatty liver disease (NAFLD), recently named as metabolic dysfunction-associated fatty liver disease (MAFLD)[4,5,6], has reached epidemic proportions, becoming the most common cause of chronic liver diseases worldwide (affecting up to ~30% of world's adults).[7,8] Growing evidence supports the notion that MAFLD is a "multisystem" disease, in addition to causing severe liver damage (i.e., MAFLD-related cirrhosis)[9], affecting the vasculature and other organ systems that requires a multidisciplinary and holistic approach.[10]

To date, the role of ferroptosis in the development and progression of MAFLD has not been fully elucidated. Therefore, in this review we briefly discuss the role of ferroptosis in MAFLD in order to highlight new areas for potential research into the prevention and treatment of this common and burdensome liver disease.

1. Overview of ferroptosis

1.1 The concept and characteristics of ferroptosis

In 2012, Dixon et al. first coined the term ferroptosis as a new form of regulated cell death.[11] This form of cell death results from glutathione depletion and glutathione peroxidase inactivation (**Figure 1**).[10,12] As an iron-dependent form of non-apoptotic regulated cell death, iron plays a key role in the occurrence of ferroptosis. Experimentally, it has been shown that iron-chelating agents may inhibit ferroptosis.[13] At the same time, imbalance of iron metabolism induces lipid

peroxidation and production of reactive oxygen species (ROS), thereby triggering ferroptosis. The regulatory mechanisms of ferroptosis are closely related to reactive oxygen clusters, and accumulation of reactive oxygen clusters triggered by the Fenton-like reaction, nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation, and glutathione depletion.[1] Thus, as an atypical oxidative form of regulated cell death, ferroptosis may induce cell death by increasing ROS production, thereby affecting development of disease.

1.2 Comparison of ferroptosis with other forms of programmed cell death

From a morphological point of view, apoptosis is characterized by the occurrence of typical apoptotic cellular bodies and by no rupture of cell membranes.[14] Cell necrosis is characterized by cell swelling, nucleus concentration, fragmentation, and dissolution, as well as chromatin staining and flocculent, and organelle enlargement or fragmentation. In contrast, ferroptosis does not show any typical morphological features of both apoptosis and necrosis. Ferroptosis is typically characterized by cell membrane rupture and vesiculation, reduced mitochondrial cristae, mitochondrial atrophy, as well as lack of chromatin agglutination in the nucleus.[14]

In terms of biochemical characteristics and regulatory mechanisms, apoptosis is mainly dependent on cysteinyl aspartate-specific proteinase. During cell apoptosis, Ca^{2+} and pH levels of cytoplasm increase, and endonuclease is activated, leading to nuclear DNA fragmentation. Cell necrosis induces a severe local inflammatory response that is associated with activation of signaling pathways, such as receptor-interacting protein kinase 3. In contrast, in ferroptosis, intracellular Fe^{2+} accumulation occurs, and levels of lipid peroxidation increase significantly.[15] At the same time, ROS production increases, cellular cystine/cysteine uptake decreases, glutathione (GSH) is reduced, and some mediating factors, such as arachidonic acid, are released.[15] The essential nature of ferroptosis is intracellular Fe^{2+} accumulation that is a typical disorder of cell redox metabolism. Thus, intracellular antioxidant capacity decreases, and ROS and lipid peroxidation products accumulate in large quantities, inducing cell death. There is no overlap between the regulatory

mechanisms of ferroptosis and those implicated in cell apoptosis or necrosis, and small molecules that inhibit cell apoptosis and necrosis do not have any inhibitory effect on ferroptosis.[16]

2. Effects of regulatory mechanisms of ferroptosis on MAFLD

MAFLD comprises a histological spectrum of progressive liver conditions, ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis. The pathophysiology underlying MAFLD involves a multitude of interlinked processes, including insulin resistance (IR), lipotoxicity attributable to the accumulation of toxic lipid species, infiltration of proinflammatory cells causing hepatic injury and ultimately leading to hepatic stellate cell (HSC) activation and increased liver fibrogenesis.

2.1 Lipid peroxidation

Ferroptosis was first detected after stimulation of RAS-mutant cells by a small molecular substance (i.e., erastin).[17] Subsequent studies have shown that lipid peroxidation is one of the major drivers of ferroptosis.[18] Lipids are widely found in biofilms and lipoproteins. When lipid peroxidation occurs, lipids become the major target of peroxidation by increasing ROS. In turn, the increased production of ROS leads to increased oxidative stress, and oxidative stress-induced lipid peroxidation could play a key role in the development and progression of MAFLD.[19] Convincing evidence supports the importance of different lipid peroxidation products in the development of MAFLD. Among the different aldehydes that can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are the two most extensively studied and both are associated with different stages of MAFLD. A significant association between hepatic 4-HNE adducts and increasing stage of fibrosis has been described, and increased mitochondrial 4-HNE‐protein adducts during MAFLD development have been reported.[20,21] Studies also reported higher circulating levels of MDA, as measured by the 2-thiobarbituric acid reaction assay, and higher levels of low-density lipoprotein (LDL) oxidation in patients with MAFLD than in control subjects.[22,23]

2.2 Iron overload

Not only is abnormal lipid metabolism involved in the development of MAFLD, but also imbalance of iron metabolism may affect the occurrence of MAFLD.[24] Because iron has two different valence states, iron participates in intracellular redox reactions *in vivo*, enabling iron to produce oxidative free radicals. Iron overload induces oxidative stress by producing ROS. The liver is one of the most critical organs for iron storage. Approximately 25-30% of total iron in the body is stored in ferritin in the liver, and the intrahepatic contents of iron and ROS are greater in diseased liver than in normal liver, suggesting that ferroptosis may be associated with chronic liver diseases.[25]

A cross-sectional study, involving 5445 Chinese individuals, showed that there was a dose-response relationship between dietary iron intake and the prevalence of MAFLD.[26] In a case-control study in Southeast China, Pan et al. also found that elevated serum ferritin levels were associated with a higher risk of MAFLD (adjusted-odds ratio 1.62, 95% CI 1.16-2.27), and the hepcidin-to-ferritin ratio was significantly associated with a lower risk of MAFLD (adjusted-odds ratio 0.70, 95% CI 0.50-0.98). [27] In a proof-of-concept study, Rostoker et al. prospectively analyzed the association between intra-hepatic iron content and magnetic resonance imaging-proton density fat fraction (MRI-PDFF) in 68 patients on chronic dialysis.[28] Among these dialysis patients, 17 patients were followed-up during the period of iron therapy. The results of this pilot study showed that liver fat content (assessed by MRI-PDFF) of patients with moderate or severe iron overload was higher than that of normal iron load patients or mild iron overload patients [median (interquartile range) MRI-PDFF-assessed liver fat content: 7.9% (0.5-14.8%) vs. 5.0% (0.27-11%) vs. 5.0% (0.30-11.6%), respectively, $P<0.05$]. In 7 patients who received iron treatment, both liver iron and fat contents increased concomitantly, whereas in 10 patients with iron overload, liver fat content decreased after parenteral iron withdrawal, thereby suggesting that liver iron load may influence liver fat content in these dialysis patients.[28] Barrera et al. also demonstrated that in rats, iron overload significantly increased hepatic fat content, serum transaminase levels, and induced a disruption in the desaturation capacity leading to

polyunsaturated fatty acid (PUFA) depletion, all of which were diminished by antioxidant intervention.[29] Wang et al. showed that high iron levels served as a driving factor in the induction of ferroptosis, and the ferroptosis could damage liver mitochondria associated with elevated serum ALT levels.[30] It is known that inflammation and fibrosis are two critical stages in the pathophysiology of MAFLD. Meanwhile, iron is known to increase the respiratory burst activity of Kupffer cells, which may have a proinflammatory impact through the activation of nuclear factor (NF)-kB, thereby triggering the hepatic production of multiple pro-inflammatory and fibrogenic mediators.[31] Finally, in a small intervention study, Yamamoto et al. showed that dietary restriction of calories, fat and iron improved the grade of hepatic iron accumulation and oxidative stress in patients with MAFLD. In addition, the levels of serum ALT and ferritin were significantly decreased.[32]

It is known that IR is a pathogenic factor in the development and progression of MAFLD.[33] Previous studies found that excessive iron accumulation may adversely affect insulin secretion from pancreatic β-cells and, at the same time, may interfere with expression of insulin receptors, thus resulting in greater IR. Pancreatic β-cells are highly sensitive to levels of iron ions and can express hepcidin, which relieves iron overload.[34] Moreover, excessive iron accumulation also induces oxidative stress and mitochondrial damage, thereby further impairing pancreatic β-cell function. Hepatic iron overload may also exacerbate hepatic IR by directly damaging liver cells.[34,35]

2.3 Glutathione (GSH)

As discussed above, ferroptosis is a modulated form of cell death that is characterized by the iron-dependent accumulation of lipid peroxidation to lethal levels. When cystine transport proteins are inhibited (e.g., erastin), intracellular GSH is depleted, resulting in inactivation of glutathione peroxidase (GPX4) and accumulation of lipid peroxidation products that induce cell death.[36,37] Koruk et al. showed that serum GSH levels were higher in patients with MAFLD than in controls, suggesting that GSH might play a key role in MAFLD pathogenesis and disease progression.[38]

3. Impact of ferroptosis on MAFLD

3.1 Impact of ferroptosis on MAFLD-related risk factors

3.1.1 Obesity

Previous studies have shown that MAFLD is closely related to obesity, and ferroptosis might further promote the development of MAFLD by affecting obesity.[39,40] Experimentally, it has been reported that iron-chelating agents (e.g. deferoxamine, deferiprone or deferasirox) have the potential to treat obesity. For instance, Yan et al. reported that deferoxamine could reduce the expression of fat-generating genes in adipose tissue and reduce the expression of genes related to mitochondrial biosynthesis, thereby achieving the effect of treating obesity.[41] In another experimental model of polygenic obese mice, Ma et al. found that increased iron concentration is associated with adipose tissue remodeling and increased adipose tissue IR.[42] However, further experimental research is required in this field.

3.1.2 Type 2 diabetes

Apart from well-studied IR in peripheral cells, impaired insulin secretion from pancreatic β cells has been acknowledged as the core defect in the development of T2DM, because of exhaustion of pancreatic β cells and consequent failure of insulin secretion.[43] Pancreatic β cell death, involving the modulation of both pancreatic β cell mass and function, is believed to be the final pathogenic event in the progression of T2DM, leading to rapid deterioration of glycaemic control, if not treated properly.[44] Li et al. reported that ferroptosis may contribute to pancreatic β cell loss and dysfunction, and insulin secretion is worsened by ferroptosis-inducing erastin or RAS-selective lethal compounds.[45] Quercetin is a potential glucose-lowering supplement that might also exert some beneficial effects on ferroptosis.[45] A recent study showed that that daily quercetin intake was associated with a lower prevalence of T2DM in Chinese individuals, thus supporting a potetnial protective effect of quercetin in the development of T2DM.[46] The possible anti-diabetic effects of quercetin, which have been replicated both *in vivo* and *in vitro,* are linked mainly to the anti-oxidant and anti-inflammatory actions of quercetin on pancreatic β cells.[47,48] Recent

experimental data also suggested that quercetin might exert some beneficial effects on risk of T2DM, possibly by inhibiting pancreatic β cell iron deposition and ferroptosis.[45]

3.1.3 Other metabolic risk factors

The newly proposed diagnostic criteria of MAFLD are based on the evidence of hepatic steatosis (as assessed by histology, imaging techniques, or blood biomarkers), combined with one of the following three conditions: overweight/obesity, T2DM, or presence of metabolic dysregulation (i.e. defined by the presence of at least two of the following seven metabolic risk abnormalities that are often also present with metabolic syndrome: i.e. increased waist circumference, raised blood pressure, high triglycerides, low HDL-cholesterol, increased plasma glucose concentration, IR or elevated plasma C-reactive protein concentrations).[4,49] Epidemiological studies have also shown that elevated serum ferritin or iron overload are associated with higher levels of plasma glucose, diastolic blood pressure, uric acid, and IR.[50,51] Thus, as schematically summarized in **Figure 1**, it is reasonable to hypothesize that ferroptosis is closely related to obesity, T2DM or other coexisting metabolic disorders **t**hat occur with MAFLD.

3.2 Impact of ferroptosis on MAFLD severity

3.2.1 Hepatic inflammation or impaired liver function

Iron overload may aggravate MAFLD/NAFLD by increasing the risk of hepatocyte swelling, inflammation and fibrosis, thereby promoting the progression from simple steatosis to steatohepatitis (NASH).[52,53]

Recently, Tsurusaki et al. reported that ferroptosis is implicated in the development of liver fat accumulation into NASH.[54] These authors also reported that inhibition of ferroptosis prevents the development of NASH.[54] With development of NASH, ferroptosis may lead to liver injury and inflammatory response, thereby providing a new potential target for NASH treatment.[55]

3.2.2 Liver fibrosis

Liver fibrosis is a complex pathophysiological process and an intermediate pathogenic link in a variety of chronic liver diseases. Activation of HSC is a critical step in liver fibrogenesis (**Figure 1**). Currently, liver transplantation is the only effective treatment for patients with late-stage of liver fibrosis. However, the shortage of liver donors and the risks of follow-up transplantation limit the treatment of patients with advanced liver fibrosis. Therefore, it is urgent to find new treatment strategies for advanced liver fibrosis.[56] The activation, proliferation, and transformation of HSCs are key drivers in liver fibrogenesis. Therefore, focusing on HSCs as a target in MAFLD is important in treatment strategies targeting liver fibrosis.

Iron is abundant in HSCs that is a pre-requisite condition for ferroptosis. From the current evidence in animal studies (as discussed above), it appears that ferroptosis might act as a two-edged mechanism in the development and progression of liver fibrosis. Some studies showed that ferroptosis may exacerbate liver fibrosis and liver injury.[57,58] However, some experimental studies have recently suggested that induction of ferroptosis could be also considered a new strategy to improve liver fibrosis (as summarized in **Table 1**).[56-62] An animal study showed that artesunate inhibits liver fibrogenesis through ferroptosis.[59] Magnesium isoglycyrrhizate may regulate iron transport processes (mostly by up-regulating the expression of heme oxygenase-1), and promote accumulation of Fe^{2+} and lipid peroxides RTMN induce ferroptosis in HSC, thereby ameliorating liver fibrosis.[60] In addition, artemether up-regulates the gene expression of P53, inhibits the SLC7A11 recombinant protein, and inactivates GPX4 (eventually inducing ferroptosis of HSC), thereby inhibiting liver fibrogenesis.[61] Thus, ferroptosis might also inhibit the activation of HSC and thus ameliorate liver fibrosis.

Conclusions

Ferroptosis is a newly discovered form of regulated cell death. Iron-dependent lipid peroxidation is a major driver of ferroptosis and ferroptosis may also occur in MAFLD. The concept of ferroptosis-inducing treatment regulating liver fibrosis is of increasing interest in MAFLD. However, we suggest further mechanistic studies are needed to better understand the role of

ferroptosis in liver fibrogenesis. Experimental evidence suggests that altered iron metabolism and lipid peroxidation are pathophysiologically involved in the link between ferroptosis and MAFLD. However, the role of ferroptosis in the pathophysiology of MAFLD is complex and needs further investigation. On the one hand, ferroptosis can lead to the occurrence of MAFLD and development of liver inflammation; whilst on the other hand, some studies have shown that ferroptosis of HSC could inhibit the progression of liver fibrosis. Further mechanistic studies of ferroptosis are required to better elucidate whether altering this new form of regulated cell death has merit in the prevention and treatment of MAFLD.

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Table Legend

Table 1. Experimental studies examining the role of ferroptosis in liver fibrosis.

Figure Legend

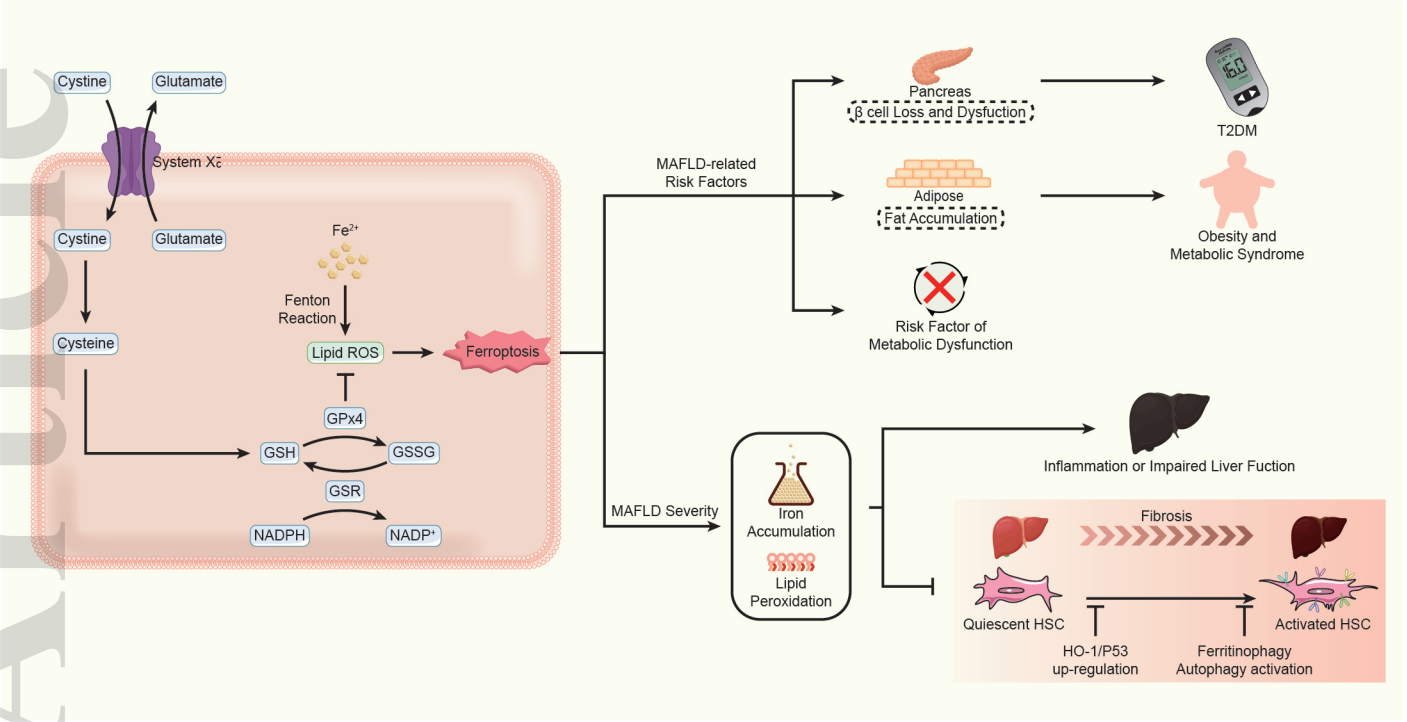
Figure 1. Potential regulatory mechanisms of ferroptosis in MAFLD.

Ferroptosis is closely related to obesity, type 2 diabetes mellitus (T2DM) and other metabolic risk factors, all of which occur in MAFLD. The main regulatory mechanisms of ferroptosis in MAFLD include increased lipid peroxidation and iron overload. Induction of ferroptosis of hepatocytes may also inhibit fibrogenesis and serve as potential anti-fibrotic treatment. Moreover, ferritinophagy-mediated hepatic stellate cells (HSC)-ferroptosis may be also responsible for its anti-fibrotic efficacy.

Table 1. Experimental studies examining the role of ferroptosis in liver fibrosis.

Author(s)	Year	Model	Fibrogenic marker(s)	Compound/target	Effect(s)
Yu et al[57]	2020	CCL4-induced fibrosis	Masson's trichrome	Slc39a14	Induction of HSC ferroptosis
Sui et al.[60]	2018	CCL4-induced fibrosis	α-SMA, collagen type 1	MgIG	Induction of HSC ferroptosis
Zhang et al.[63]	2018	BDL-treated fibrosis	ACTA2, collagen type 1	ELAVL1	Activation of HSC ferritinophagy/ferroptosis
Kong et al.[59]	2019	CCL4-induced fibrosis	α-SMA, collagen type 1, fibronectin	Artesunate	Activation of HSC ferritinophagy/ferroptosis
Wang et al.[61]	2019	CCL4-induced fibrosis	α-SMA, collagen type 1, fibronectin	Artemether	Induction of HSC ferroptosis
Zhang et al.[62]	2020	BDL-treated fibrosis	ACTA2, collagen type 1	ZFP36	Inhibition of HSC autophagy/ferroptosis
Zhang et al.[64]	2020	BDL-treated fibrosis	HA, LN, PC III, IV-C	BRD7	Induction of HSC ferroptosis

Abbreviations: ACTA2, actin alpha 2; CCL4, carbon tetrachloride; MgIG, magnesium isoglycyrrhizinate; HSC, hepatic stellate cells; α-SMA, alpha-smooth muscle actin; BDL, bile ductligation; ELAVL1, ELAV like RNA-binding protein 1; HA, hyaluronic acid; LN, laminin; PC III, procollagen type III; IV-C, IV- collagen; IRP2, iron regulatory protein 2; BRD7, bromodomain-containing protein 7.



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