Review

Ferroptosis in Cancer Immunotherapy—Implications for Hepatocellular Carcinoma

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Abstract: Ferroptosis is a recently recognized iron-dependent form of non-apoptotic regulated cell death (RCD) characterized by lipid peroxide accumulation to lethal levels. Cancer cells, which show an increased iron dependency to enable rapid growth, seem vulnerable to ferroptosis. There is also increasing evidence that ferroptosis might be immunogenic and therefore could synergize with immunotherapies. Hepatocellular carcinoma (HCC) is the most common primary liver tumor with a low survival rate due to frequent recurrence and limited efficacy of conventional chemotherapies, illustrating the urgent need for novel drug approaches or combinatorial strategies. Immunotherapy is a new treatment approach for advanced HCC patients. In this setting, ferroptosis inducers may have substantial clinical potential. However, there are still many questions to answer before the mystery of ferroptosis is fully unveiled. This review discusses the existing studies and our current understanding regarding the molecular mechanisms of ferroptosis with the goal of enhancing response to immunotherapy of liver cancer. In addition, challenges and opportunities in clinical applications of potential candidates for ferroptosis-driven therapeutic strategies will be summarized. Unraveling the role of ferroptosis in the immune response could benefit the development of promising anti-cancer therapies that overcome drug resistance and prevent tumor metastasis.

Keywords: ferroptosis; immunotherapy; HCC; cancer; cell death; lipid peroxidation; liver cancer

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1. Introduction

Cell death is crucial for normal development and homeostasis throughout an organism's lifetime [1]. Various forms of cell death have been identified, each having its respective modalities and features. Ferroptosis is one of the more recently described forms of non-apoptotic cell death. The term ferroptosis was first mentioned in 2012 by Dixon as a newly identified and unique iron-dependent cell death [2]. Ferroptotic cells display cytological changes that differ from morphological, biochemical, and genetic characteristics of other cell death forms, including decreased mitochondria cristae and ruptured mitochondrial membranes [3–5]. Compared to apoptosis, ferroptosis lacks rupture or blebbing of the plasma membrane, chromatin condensation, or rounding of the cell, and the execution of ferroptosis is not known to require specific pro-death protein expression [6]. As implied in its name, ferroptotic cell death is defined by the requirement of iron. Caused by excessive peroxidation of membrane phospholipids and production of reactive oxygen species (ROS), the plasma membrane loses its selective permeability, ultimately resulting in cell death. Regulated by multiple layers of metabolic signaling pathways including iron metabolism, lipid metabolism, and mitochondrial function, ferroptotic cell death is a complex process that will be further discussed in more detail. There is recent evidence suggesting that ferroptotic cell death plays an important role in mediating a wide variety of cellular processes in diseases, including immune activation. Whether ferroptotic cancer cells are immunogenic is currently unclear, but targeting ferroptosis in immunotherapeutic approaches is considered as a promising strategy.

Primary liver cancer is one of the world's most common causes of cancer-related death and represents a serious health and economic burden [7,8]. Hepatocellular carcinoma (HCC) is the most common primary liver tumor, comprising around 75% of all liver cancer cases and typically developing in the context of liver fibrosis or cirrhosis [9]. In patients with advanced cirrhosis and HCC, liver transplant often remains the only curative treatment option [10]. Classic chemotherapies such as cisplatin are not regularly used in HCC due to the rapid development of chemoresistance [11], toxicity to various organs, and limited efficacy [12]. The first effective systemic therapy in advanced HCC was achieved using multi-kinase inhibitors, such as sorafenib, lenvatinib, carbozantinib, or regorafenib, but all of these substances provide only a rather small improvement of overall patient survival [13–15]. Treatment options are usually limited, as surgical resection or organ transplantation is feasible only in a fraction of patients and systemic therapies are not curative [16].

Antitumor immunotherapy has emerged as standard therapy for cancer treatment for many tumors (including HCC), and great progress has been achieved in the past few decades. The most successful immunotherapy to date is the modulation of immune checkpoint pathways, e.g., by targeting the T-cell inhibitory molecules programmed cell death-1 protein (PD-1) and programmed death-ligand 1 (PDL1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that contribute to the tumor's escape from cytotoxic T-cell responses [17,18]. Recently, a phase III trial reported that combination of atezolizumab (anti-PD-L1 antibody) and bevacizumab (anti-vascular endothelial growth factor receptor (VEGFR)) resulted in increased overall survival of patients with advanced HCC in comparison to sorafenib, making atezolizumab/bevacizumab the new first-line treatment for unresectable HCC [19]. Two other PD-1 inhibitors (nivolumab and pembrolizumab) have already been approved as a second-line monotherapy in advanced HCC in the USA, but the response rates are still low (15–20%) [20,21]. It has become increasingly clear that the efficacy of cancer immunotherapy depends on several factors including the potential to induce an anti-tumor immune response to overcome resistance. Immunogenic cell death (ICD) is seen as a promising concept in achieving strong and long-lasting anti-cancer immunity by not only killing malignant cells but also activating the immune system [22], which has the potential to synergize with immune checkpoint blockade. Despite ongoing research, less is known about regulators of ferroptosis and its role in immune activation, but there is some evidence that ferroptotic cell death might be immunogenic. These considerations support the concept to develop ferroptosis-based approaches to enhance response to immunotherapy.

Here, we review existing studies and our current understanding regarding the molecular mechanisms of ferroptosis in cancer and immune cells, which may open the door to a new era of cancer immunotherapies targeting non-apoptotic RCD processes in primary liver cancer to improve its poor prognosis.

2. Molecular Basis of Ferroptosis

Dixon et al. [2] first proposed ferroptosis in 2012 as a recently recognized non-apoptotic form of cell death. The knowledge about various cell death modalities and especially our understanding of ferroptosis is incomplete. Further research is necessary in order to understand why certain stimuli trigger ferroptosis instead of apoptosis. However, it is clear that ferroptosis describes an oxidative, iron-dependent process characterized by massive lipid peroxidation-mediated membrane damage and accumulation of ROS to toxic levels [1,5]. Its morphology differs from other known cell death types, such as apoptosis, necrosis, autophagy, necroptosis, or pyroptosis, as ferroptotic cells have characteristic mitochondrial atrophy accompanied by a reduction or disappearance of mitochondrial cristae [4,23]. Previous studies concur that ferroptosis mostly resembles necrosis-like morphological changes but depends mainly on iron signaling [24]. The two main biochemical characteristics are lipid peroxidation and iron accumulation, which will be detailed separately in the following sections.

2.1. Lipid Peroxidation and Antioxidant Defense

The peroxidation of polyunsaturated fatty acids (PUFAs) in phospholipids and their incorporation into the cell membrane are the main characteristics of ferroptosis (Figure 1) [25]. Catalyzed by acyl-CoA synthetase long-chain family member 4 (ACSL4), PUFAs are incorporated into phospholipids to form polyunsaturated fatty acid-containing phospholipids (PUFA-PLs). Free PUFAs become a radical target after incorporation into the plasma membrane by ACSL4. Plasma membrane PUFAs in turn are vulnerable to free radical-initiated oxidation and trigger additionally self-amplified Fenton reaction, leading to destruction of the plasma membrane lipid bilayer and affecting membrane function [26]. The exact details remain unclear, but the peroxidation of phospholipid PUFAs is likely catalyzed by lipoxygenase (LOX) family enzymes [27]. There is recent evidence that the amount of PUFAs at the plasma membrane has an influence on the response to ferroptosis: *n*-3, but also, remarkably, *n*-6-long chain PUFAs showed cytotoxic effects proportional to the number of double bonds [28]. However, the antioxidant enzyme GPX4 is able to directly reduce phospholipid hydroperoxide to hydroxyphospholipid, which hinders the production of intracellular lipid hydroxyl radicals (Figure 1a). Glutathione (GSH) is considered as an essential cofactor for GPX4, as it can donate reducing equivalents for GPX4 opposing conversion of lipid hydroperoxides (L-OOH) to non-toxic lipid alcohols (L-OH) [29]. GSH synthesis is dependent upon cystine import into the cells by the cysteine/glutamate transporter (also known as system x_c^- , Figure 1b) which consists of two subunits, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2) [30]. The imported cystine can be oxidized to cysteine, which is then used to synthesize GSH required for GPX4 activity as a reducing cofactor. The GSH-GPX4 antioxidant system has an important role in protecting cells from ferroptosis as its activity determines the extent of ferroptosis by adjusting ROS accumulation and oxidative stress [31]. Pharmacological inhibition of the upstream regulator system x_c^- (see Section 3.1) or the downstream effector GPX4 (see Section 3.2) of GSH induces ferroptosis. The same can be achieved through depletion of system x_c^- (encoded by the SLC7A11 gene) or GPX4 genetic depletion [32].

Figure 1. Molecular basis of ferroptosis and strategies for therapeutic induction of ferroptosis. A key cause of ferroptosis is the lipid radical chain reaction leading to excessive production and failure of elimination of phospholipid hydroperoxides (PLOOH). Two major mechanisms mediated by (a) glutathione peroxidase 4 (GPX4) and (b) system x_c^- serve to prevent PLOOH accumulation. System x_c^- mediates the uptake of cystine for the production of cysteine and glutathione (GSH). GPX4 converts GSH to glutathione disulfide (GSSG), resulting in reduced PLOOH and inhibition of ferroptosis. (c)

Extracellular Fe^{3+} enters the cell through transferrin receptor 1 (TFR1) on the cell membrane, is reduced to Fe^{2+} , and is combined with reactive oxygen species (ROS) to participate in lethal iron-dependent peroxidation of polyunsaturated fatty acids (PUFAs), and finally induces ferroptosis. Multiple ferroptosis-inducing agents (system x_c^- , GPX4 inhibitors, nanoparticles) hold great promise for cancer therapy. Created with BioRender. Available online: <https://biorender.com> (accessed on 10 February 2022). Abbreviations: SAS: sulfasalazine, LDL-DHA: low-density lipoprotein docosahexaenoic acid, RSL3: Ras-selective lethal small molecule 3, FIN56: *N*2,*N*7-dicyclohexyl-9-(hydroxyimino)-9H-fluorene-2,7-disulfonamide, ML210: α -[(2-chloroacetyl)(3-chloro-4-methoxyphenyl)amino]-*N*-(2-phenylethyl)-2-thio-phenacetamide, ML162: α -[(2-chloroacetyl)(3-chloro-4-methoxy-phenyl)amino]-*N*-(2-phenylethyl)-2-thiophene-acetamide, HSPB1: heat shock protein beta-1.

2.2. Iron Accumulation

As an essential trace element, iron is necessary to maintain cell metabolism [33]. Extracellular iron is internalized into the cell bound to transferrin (TF) as a TF-Fe^{3+} complex (Figure 1c). Taken up by the transferrin receptor 1 (TFR1) at the plasma membrane through clathrin-mediated endocytosis, it is released into the cytosol as free Fe^{2+} [34]. During ferroptosis, Fe^{2+} participates in Fenton chemistry to generate hydroxyl radicals and initiates lipid peroxidation, thus leading to propagation of damage throughout the membrane as already discussed [31].

Therefore, elevated levels of iron and iron derivatives (heme or iron-sulfur clusters) can increase the vulnerability to ferroptosis, which plays an important role in tumor cells due to their extraordinary growth. Because of this increased susceptibility of cancer cells, ferroptosis might be a promising tool for anti-cancer treatment. Moreover, several proteins and genes that are involved in iron metabolism have been shown to modulate sensitivity to ferroptosis by changing cellular iron contents [35]. This emphasizes the association of cell death pathways with cell metabolism. Iron dependency also becomes apparent through blocking of TF-mediated iron import or recycling of iron-containing storage proteins, which leads to attenuation of ferroptosis [36]. Previous findings revealed involvement of other cell death types, contributing to ferroptosis and cell death fate: autophagic cell death requires the iron-carrier TF and the amino acid glutamine to trigger death [37]. Gao et al. demonstrated that ferroptosis is a form of autophagic cell death known as ferrotinophagy when autophagy is activated upon ferroptotic cell death induction. Autophagy is activated to degrade cellular ferritin and thereby increases iron levels and sensitizes to ferroptosis. The concentration of free ferrous ions in the cytoplasm determines whether it acts as a beneficial cofactor or as a toxic-free radical catalyst in the cell [38]. Growing evidence suggests that the autophagic machinery, at least under certain conditions and connected to human diseases, contributes to ferroptotic cell death and plays a dual role in tumorigenesis and, with it, cancer therapy [39].

Non-canonical ferroptosis induction by increasing intracellular Fe^{2+} refers to increasing iron intake through TFR1 expression or decreased expression of iron transporter, or excessive activation of heme oxygenase 1 (HMOX1) [40]. Heme metabolism influences iron contents, as it is a major source of dietary iron in mammals, derived primarily from hemoglobin and myoglobin. It plays an essential role in the liver, which is the principle organ that responds to changes in systemic iron signals in order to maintain body iron homeostasis [41]. As a central metabolic and signaling molecule, it is involved in diverse transcription and cell signaling processes, e.g., heme inhibits the proteasome, and binding to immunoglobulins [42,43]. HMOX1 catalyzes the catabolism of heme, which raises intracellular ferrous iron in the cytosol and is, therefore, crucial for maintaining cellular redox homeostasis. HMOX1 knockout mice showed iron accumulation in liver and kidney, and HMOX1 deficiency in humans is connected to several abnormalities, indicating an important role of HMOX1 in human health [44].

2.3. Regulatory Pathways

As described previously, ferroptosis is a regulated form of cell death driven by accumulation of membrane lipid peroxides from iron overload. Various genetic changes were considered important to contribute to this dynamic type of cell death. The major drivers of ferroptosis “decide” over life or death in response to ferroptotic stimuli. A predictive biomarker for monitoring ferroptosis appears to be the enzyme ACSL4 that is involved in fatty acid metabolism, contributing to accumulation of lipid intermediates (see Section 2.1.) [45]. Upregulation of ACSL4 enhances PUFAs content in phospholipids, which are susceptible to oxidation reactions. Expression levels of ACSL4 correlate with ferroptosis sensitivity—this was demonstrated by suppression of ACSL4 expression by RNA interference (RNAi) in HepG2 and HL60 cells, leading to an increase of ferroptosis resistance, while its overexpression restores ferroptosis sensitization [46]. As with factors involved in fatty acid metabolism, ferroptosis can also be regulated via signal transduction pathways of iron metabolism. Decreasing iron utilization may increase the sensitivity to ferroptosis whereas intracellular iron pools seem to promote ferroptosis [47]. Various genes and proteins related to iron metabolisms are generally upregulated during ferroptosis, including the iron responsive element binding protein 2 (IREB2). IREB2 is considered the core regulator of iron metabolism binding iron-responsive elements in the mRNA of target genes including the TF receptors and knockdown results in resistance to ferroptosis induction [2]. Additionally, overexpression of iron-storage proteins such as ferritin are supposed to play a wide anti-ferroptotic role [48].

GPX4 is the major lipid hydroperoxide (LOOH)-neutralizing enzyme, which in turn represents a target for several regulating proteins. Erastin and Ras-selective lethal small molecule 3 (RSL3) both inactivate GPX4 and are proposed as efficient ferroptosis inducers. Erastin does so indirectly by inhibiting cystine import, which then lacks as an essential cellular building block of GSH. Erastin is also a potential activator of mitochondrial voltage-dependent anion channel 2/3 (VDAC2/3), highlighting the participation of mitochondria dysfunction in erastin-induced ferroptosis [49]. RSL3 directly binds the active site of GPX4 and inhibits the phospholipid peroxidase activity [50].

Research on the mechanism of ferroptosis also required the identification of ferroptosis inhibitors in order to study the role of ferroptosis *in vivo* and discuss potential pharmacological approaches aiming to subvert lipid peroxidation. Ferrostatin-1 (Fer-1), liproxstatin-1, vitamin E, and zileuton have been identified and suppress ferroptosis as lipoxygenase inhibitors, preventing the propagation of oxidative damage within the membrane [51,52]. Other than that, iron chelators such as deferoxamine inhibit the accumulation of iron [2]. A full understanding of the regulatory network including ferroptosis inhibitors might provide potential health benefits to prevent symptoms of diseases that are related to ferroptosis [53].

In summary, many potent biomarker candidates have already been identified, which could be useful to further study the fundamental basis of lipid metabolism in ferroptosis and to define the interplay of involved regulators. However, further functional studies are required to unravel the critical role of certain biomarkers in order to find applications in ferroptosis-based cancer therapy.

2.4. Immunogenic Features of Ferroptosis (in Cancer Cells)

There is rising evidence supporting the notion that ferroptosis can play a significant role in mediating various functions during immune responses and immunotherapies. For early ferroptotic cancer cells, it has been proven that they release adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1), which can be recognized as damage-associated molecular patterns (DAMPs) by certain immune cells, triggering an inflammatory response [54]. At present, the most effective systemic therapy in advanced HCC is the combinational approach of atezolizumab and bevacizumab [19]. The response rate is largely dependent on the immune status of the tumor: inflamed (“hot”) tumors with high infiltration of tumor-reactive T cells respond significantly better to checkpoint inhibitors than “cold” or “excluded” tumors that are poorly infiltrated by immune cells including

antigen-presenting cells (APCs) [55,56]. Immune checkpoint inhibitors (such as PD-1 or CTLA4) mainly act by activating effective anti-cancer immune responses driven by cytotoxic T cells. Ferroptosis in cancer cells can be induced by CD8⁺ T cells that release INF- γ , which leads to system x_c^- downregulation and consequently lipid peroxidation [57].

Compared to other forms of cell death, ferroptosis is considered an immune-activating process. Although further research is still needed to clarify the interaction between ferroptotic cell death and the immune system, there is some preliminary evidence showing that ferroptosis plays a direct role for the immune system. This is not surprising since iron metabolism and lipid peroxidation were linked to regulation of immune functions, even before ferroptosis was discovered. Further determination of specific immune signals associated with ferroptosis would be useful in order to enhance the immune effect in tumor immunotherapy.

Typically for RCD, DAMPs are released as immune modulators, stimulating immune cells (e.g., macrophages or monocytes) (Figure 2) [58]. These are endogenous molecules that bind to pattern recognition receptors (PRRs), thus activating the production of immune stimulants, including a variety of cytokines and chemokines. A typical DAMP, which is released by ferroptotic cells in an autophagy-dependent manner, is the nuclear transcription factor HMGB1 able to bind chromosomal DNA involved in DNA recombination and repair processes [59]. It was shown to be actively secreted by innate immune cells in response to microbial infections but can also be passively released by somatic cells undergoing cytoplasmic membrane destruction during cell death [60]. HMGB1 acetylation induced by histone deacetylase inhibition is the trigger for HMGB1 release during ferroptosis [61]. Moreover, another study shows that HMGB1 is an essential factor for cancer cell immunity and plays paradoxical roles in tumor development by promoting both cell survival and death [62]. For a more complete investigation of the full immunogenic potential of this cell death pathway, it is important to discover the full repertoire of DAMPs released during ferroptosis. While the role of HMGB1 and ATP release has already been clarified in apoptosis and necroptosis [63], its involvement in ferroptotic cell death processes has remained elusive until now. Recent research highlights the involvement in the regulation of TFR mRNA levels and p38 phosphorylation in the rat sarcoma (RAS)-JNK/p38 pathway [64]. Additionally, Ye et al. found that knockdown of HMGB1 reduces erastin-mediated ferroptosis and the connected ROS generation [64]. Hence, HMGB1 is a critical regulator of ferroptosis.

Furthermore, other immune signals can be intermediate or final products of lipid peroxidation such as 4-hydroxynonenal (4-HNE) or malondialdehyde (MDA), driving inflammation by activating macrophages to produce pro-inflammatory cytokines [65]. They can trigger inflammation partly through activating TLR4 signaling [66]. However, thus far, there is no direct evidence that these toxic aldehydes are alone sufficient to induce ferroptosis. Taken together, despite the mechanisms reviewed here, current evidence is insufficient for a complete understanding of the relation between ferroptosis and the immune system. A more comprehensive understanding of DAMP release mechanisms and their regulation could enable development of potential new drugs for therapeutic interventions in cancer.

Figure 2. The immunogenic features of ferroptotic cancer cells. Ferroptotic cells release DAMPs, including HMGB1, ATP, and CRT (1), that act as immune modulators inducing maturation of antigen-loaded DCs (2). Activated DC release pro-inflammatory cytokines and present TAA to T cells (3). Activated CD8⁺ T cells release IFN- γ , activating IFNR, which, in turn, downregulates expression of glutamate-cystine antiporter system x_c^- (4), and consequently promotes tumor cell lipid peroxidation and ferroptosis (5). Immune-checkpoint blockade of PD-1 enhances antitumor responses against cancers (6). Simultaneously, ferroptotic cancer cells release PGE2 that suppresses cytotoxic T cell activity (7). Created with BioRender. Available online: <https://biorender.com> (accessed on 10 February 2022). Abbreviations: APC: antigen-presenting cell; ATP: adenosine triphosphate; CD: cluster of differentiation; CRT: calreticulin; DAMPs: damage-associated molecular patterns; DC: dendritic cell; GPX4: glutathione peroxidase 4; GSH: glutathione; HMGB1: high mobility group box 1; 4-HNE: 4-hydroxynonenal; IFN- γ : interferon- γ ; IFNR: interferon-receptor; IL: interleukin; MDA: malondialdehyde; MHC: major histocompatibility complex; PD-1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; PGE2: prostaglandin E2; TAA: tumor associated antigen; TCR: T-cell receptor; TLR: toll-like receptor; TNF α : tumor necrosis factor α .

2.5. Immune Response to DAMPs Released by Ferroptotic Cells

Until now, two ways for ferroptotic cells to influence inflammatory responses in immune cells have been characterized. On the one hand, the release of DAMPs by non-immune ferroptotic cells attract APCs and other immune cells. Secretion of DAMPs attracts and stimulates dendritic cells (DCs) for tumor-associated antigen (TAA) uptake and processing followed by MHC class I-restricted cross-presentation of these TAAs, which leads to the priming of T cells and clonal expansion of cancer-specific cytotoxic T lymphocytes (Figure 2) [67]. For example, HMGB1 is able to trigger an inflammatory response in peripheral macrophages via activation of the NF- κ B pathway [59]. Functioning as an adjuvant, HMGB1 contributes to activation of the innate and adaptive immune systems. Targeting HMGB1 release using neutralizing anti-HMGB1 antibodies limits inflammatory response in macrophages during ferroptosis [59]. On the other hand, ferroptosis in immune cells themselves might compromise immune responses. It has been shown that non-apoptotic cell death, including ferroptosis, is more likely to cause inflammation than apoptosis but

little is known about the recognition and clearance mechanisms for cells dying through non-apoptotic cell death pathways. Phagocytic clearance of dead cells plays a vital role in immune homeostasis, especially during cancer therapy, and the exact mechanistic of ferroptotic cell engulfment remains unclear. However, Luo et al. have demonstrated that different types of macrophages readily engulfed ferroptotic cells through a mechanism different from the engulfment of apoptotic cells. Ferroptotic cells can produce oxygenated phosphatidylethanolamines on the outer plasma membrane that can be recognized by TLR2 on macrophages, leading to clearance of ferroptotic cells [68]. Moreover, macrophages can be classified as pro-inflammatory or anti-inflammatory pro-carcinogenic macrophages, both functioning in eliminating pathogens in order to maintain immune homeostasis. Environmental signals decide on pro- and anti-inflammatory polarization, whereas an imbalance contributes to various diseases and tumor development [69], and previous studies indicated a relationship between macrophage polarization and ferroptosis. Dai et al. showed that ferroptotic cancer cells cause autophagy-dependent protein release, triggering pro-carcinogenic macrophage polarization via activation of several signaling pathways, which could restrict anti-tumor immunity [70]. Moreover, macrophage subgroups differ concerning their sensitivities to ferroptosis induction, with pro-inflammatory ones being more susceptible [71]. Regarding the adaptive immune system, T cells can also induce ferroptosis in tumor cells. It has been reported that CD8⁺ T cells release cytokines (e.g., TNF or IFN- γ), which significantly downregulate expression of system x_c^- components driving tumor cells into ferroptosis due to reduced cysteine uptake [57]. This might provide a direction for further bridging the gap between ferroptosis and immunotherapy in the treatment of malignant cancers.

Despite system x_c^- , the other key regulator of ferroptosis, GPX4, was also reported to play an essential role in the expansion of both antigen-specific CD4⁺ and CD8⁺ T cells [72]. A lack of GPX4 leads to ferroptotic cell death induction with an accumulation of lipid peroxides in T cells, thereby preventing their immune response to infection. Moreover, it was recently found that ferroptosis-resistant CD8⁺ T cells overexpressing GPX4 have normal immune functions, but deletion of ACSL4 impairs their immune response [73]. GPX4 also plays a pivotal role in preventing ferroptosis after T-cell activation by only expanding GPX4-expressing CD4⁺ and CD8⁺ T cells [72]. Additionally, the induction of ferroptosis in cancer cells has been shown to be associated with increased release of prostaglandin E2 (PGE2), an immunosuppressive agent directly suppressing cytotoxic T-cell activity (Figure 2 (7)). Therefore, PGE2 promotes the proliferation of tumor cells and plays a central role in impairing anti-tumor immunity, which could be a barrier to the induction of a potent immune response [74]. Thus, understanding the molecular mechanisms underlying the immune response of ferroptotic cell death would help guide the design of new immune-related treatments.

To do so, a more detailed investigation of the TME including activated immune cells that secrete proinflammatory chemokines and cytokines is necessary. Recent studies confirmed the role of ferroptotic cancer cells regulating tumor immunity in different ways. As other cell death types, ferroptotic cells release “find me” signals that attract APCs and other immune cells, leading to the engulfment by macrophages [75]. During maturation and cross-presentation by DCs, ferroptotic cancer cells interact further with the immune system through TLR4 [76].

In a very recent integrative study, a ferroptosis scoring model based on ferroptosis-related genes and their association with immune infiltration in cancer was constructed [77]. For this purpose, 30 genes were classified into ferroptosis driver and suppressor groups, and transcription factors and therapeutic agents targeting ferroptosis regulators in cancer were determined and validated. This score can be a potential biomarker of the immune response in the TME and the therapeutic response to immunotherapy. The described dual effects of ferroptosis on tumor immunity, and with it, patient’s prognosis score, differ from person to person, but the results showed that the ferroptosis score, as an independent prognostic factor, is feasible for predicting the prognosis of different cancers and immunotherapy

efficacy. Supporting previously obtained results, high ferroptosis scores had higher immune cell infiltration scores due to CD8⁺ T cell and T-helper cell activity [57]. This highlights the importance of the TME that should be considered in the design of therapeutic compounds and confirms the powerful combinatorial use of T cell-mediated ferroptosis induction with checkpoint blockade [78]. Despite some limitations of this study (more datasets for verification and combinatorial approaches would be needed), this ferroptosis score might have potential to help improve the survival rate of patients with HCC and predict tumor progression earlier as prognostic markers that currently find application in clinic.

3. Ferroptosis in Cancer Therapy

Ferroptosis has not only been investigated to expand the basic understanding regarding cell death modalities, but its connection to cancer and cancer treatment also exhibits extraordinary potential in tumor treatment [25]. Since iron is important for the regulation of redox homeostasis in normal cells, understanding the role of iron in cancer development is an active area of research. Moreover, there is epidemiological evidence that high intake of dietary iron increases the risk of several cancers including HCC [79]. Higher demand for iron and upregulation of TFR1 make cancer cells more susceptible to iron-dependent cell death modalities such as ferroptosis [71]. By changing cellular iron content, the sensitivity of cells towards ferroptosis can be altered in various processes, e.g., degradation of the iron storage protein ferritin or transferrin regulation [80], resulting in iron overload and inducing ferroptosis in cancer cells. Iron reduction therapy could also be promising; agents (e.g., deferoxamine) that chelate iron by forming a stable complex hindering its further chemical reaction [41] exert antiproliferative effects and antitumor activities in several cancers, including HCC, by depriving cancer cells of essential iron [81]. Prevention of ferroptosis can be, in this case, also applicable for treatment of iron-related disorders [82].

In leukemia cells, ferroptosis induced by iron loading exerts a therapeutic effect [82]. However, excessive iron was identified to be related to poor prognosis of leukemia due to reduced blood cell differentiation, which is why efforts to reduce the amount of iron are still preferable in this context [38]. In addition, systemic administration of iron modulating agents goes along with severe side effects because of their ubiquitous role [25]. A sufficient iron supply is required for the activity of many iron-containing or dependent enzymes involved in cell division, as well as for T-cell growth and expansion [83].

HMOX1 expression correlates with cancer progression, as well as poor prognosis by demonstrating that heme iron dysregulation actively promotes tumor development [84,85]. Li et al. showed in 2017 that excessive heme could directly induce neuronal ferroptosis [86]. However, as for other factors that control iron metabolism, the exact role of HMOX1 in ferroptosis is unclear and has a dual role depending on the context [42]. Several studies showed that heme sequestration could be an effective strategy to manage and treat an array of pathologic conditions, including cancer, by making heme unavailable for tumor cells [87,88]. Heme reduction in the tumor environment by heme-sequestering proteins affects oxygen levels and blood vessel growth limiting tumor hypoxia and normalizing tumor vasculature [89]. Although Sohoni et al. emphasize that it would not be a stand-alone treatment, their findings suggest a potential new path forward in treating cancer cells with heme-sequestering proteins in tandem with chemotherapy or other forms of cancer treatments.

Additionally, INF- γ was found to be involved in regulating tumor cell proliferation, and recently its role in ferroptosis was revealed. It downregulates system x_c^- protein levels via JAK/STAT signaling, which leads to enhanced glutathione depletion and increased lipid peroxidation in HCC cells. This identification provided new insights into the feasibility of using INF- γ in cancer treatment [90].

Due to its multifaceted role, ferroptosis inhibitors should be taken into account such as lipophilic antioxidants that prevent lipid peroxidation [32]. To the best of our knowledge, no clinical trials regarding ferroptosis inhibitors in liver diseases have been performed until now. However, in preclinical studies using mouse models of liver fibrosis or non-

alcoholic steatohepatitis (NASH), the use of the lipophilic antioxidants Fer-1 and Lip-1 prevents disease progression [91]. However, unsatisfactory pharmacokinetics preclude their clinical application [92]. Potent Fer-1 analogues were synthesized by introducing structural modifications to the Fer-1 scaffold to improve solubility and stability and showed *in vivo* efficiency [93].

3.1. System x_c^- Regulators

More suitable for therapeutic induction of ferroptosis in human cancers is targeting the major mediators with the help of inducers that can either act directly on GPX4 or indirectly by inhibiting system x_c^- (Figure 3, Table 1). Erastin and RSL3 were proven to trigger ferroptosis in highly proliferating cancer cell lines including head and neck, gastric, melanoma, and lung cancer [94]. Poor water solubility, renal toxicity, and unstable metabolism limit erastin's application *in vivo* [95], but this issue can be overcome by using drug delivery vehicles, such as exosomes, with low immunogenicity, high biocompatibility, and high efficiency [96]. Moreover, scientists have developed erastin derivatives, including piperazine erastin (PE) and imidazole ketone erastin (IKE), showing better water solubility and stability in experimental models of fibrosarcoma and diffuse large B-cell lymphoma [97].

Figure 3. Ferroptosis regulators with known potential relevance to cancer therapy. The initiation and execution of ferroptosis is a highly regulated process. Ferroptosis is activated by several classes of ferroptosis inducers including system x_c^- inhibitors, GPX4 inhibitors, other inducing components, and combinatorial approaches. Ferroptosis is also inhibited by several pharmacological and genetic agents that inhibit lipid metabolism, lipid peroxidation, and iron metabolism. At the same time, the

tumor suppressor protein p53 exhibits a dual regulatory role in the control of ferroptosis depending upon the stress conditions. These ferroptosis regulators could not only be used as potential therapeutic agents for cancer therapy, but also can provide a valuable tool for ferroptosis studies. The detailed mechanisms of action are shown in Table 1. Created with BioRender. Available online: <https://biorender.com> (accessed on 10 February 2022). Abbreviations: ARF6: ADP ribosylation factor 6; BCAT2: branched-chain amino acid aminotransferase 2; BECN1: Beclin 1; DPI: diphenylene iodonium; FIN56: N2,N7-dicyclohexyl-9-(hydroxyimino)-9H-fluorene-2,7-disulfonamide; FSP1: ferroptosis suppressor protein 1; GPX4: glutathione peroxidase 4; GSH: glutathione; HSPB1: heat shock protein beta-1; LDL-DHA: low-density lipoprotein-docosaheptaenoic acid; ML162: α -[(2-chloroacetyl) (3-chloro-4-methoxyphenyl)amino]-N-(2-phenylethyl)-2-thiopheneacetamide; ML210: ([4-[bis(4-chlorophenyl)methyl]-1-piperazinyl](5-methyl-4-nitro-3-isoxazolyl)-methanone; NRF2: nuclear factor erythroid 2-related factor 2; RSL3: Ras-selective lethal small molecule 3; QSOX1: quiescin sulphydryl oxidase 1; SAS: sulfasalazine; σ 1R: sigma 1 receptor.

Table 1. Selected drugs and compounds associated with targeting the ferroptotic pathway. This table lists compounds currently known to induce or promote ferroptosis, including their classification, mechanism, suitability for *in vivo* administration, and in which type of cancer they have already been studied.

Category	Drug/Compound	Mechanism	Cancer Research	
			Tumor Type	Effect on Ferroptosis
System x_c^- inhibition	Erastin	Inhibition of system x_c^- and VDAC2/3 [2]	human lung cancer cell line (A549, Calu-1) [98,99] human liver cancer cell line (HepG2) [100] human leukemia cell line (HL60) [95] human B cell lymphoma cell line (SU-DHL-8, WSU-DLCL-2) [50] human fibrosarcoma cell line (HT1080) [99] human kidney carcinoma cell line (Caki-1, 786-O) [99] human ovarian epithelial-endometroid carcinoma cell line (COV362) [101] human neuroblastoma cell line (SH-SY5Y) [102] lung cancer xenograft mice (N5CP) [103] high grade serous ovarian carcinoma (HGSOC) xenograft mice [101]	Induction
	Sulfasalazine	A prodrug of 5-aminosalicylic acid that inhibits cystine uptake [104]	human liver cancer cell lines (Huh-7, SK-Hep-1, HepG2, PLC/PRF/5, Hep3B) [105] human bladder cancer cell line (T24) [106] human cervix carcinoma cell line (HeLa) [98] human astrocytoma cell line (U373MG) [98] human glioblastoma cell line (T98G) [98] human embryonic kidney cell line (293T) [98] human colorectal cancer cell lines (HCT116, HT29, LOVO, DLD1) [107] human glioma cell line (D54-MG, STTG-1, U251-MG, U87-MG) [108] human breast cancer cell lines (MDA-MB-231, T47D, BT549 and MCF7) [109] human glioma xenograft mice [108]	Induction
	Sorafenib	Inhibits system x_c^- -mediated cystine import, leading to GSH depletion and iron-dependent accumulation of lipid-ROS [104,110]	human hepatocellular carcinoma cell line (Huh7, PLC/PRF5, Hep3B, HepG2) [111] human kidney carcinoma cell line (Caki-1, ACHN, 786-O) [111,112] human melanoma cell line (SK-MEL-3) [111] human lung carcinoma cell line (NCI-H460, N5CP, A549) [103] human pancreatic carcinoma cell lines (PANC-1) [111] human colon carcinoma cell lines (HCT116, HT-29) [111] human fibrosarcoma cell line (HT-1080) [113] patient-derived human hepatocellular carcinoma xenograft mice (HCC-PDX) [114] thyroid cancer xenograft mice acute myelogenous leukemia xenograft mice [115]	Induction

Table 1. Cont.

Category	Drug/ Compound	Mechanism	Cancer Research	
			Tumor Type	Effect on Ferroptosis
	DAZAP1	Maintains SLC7A11 mRNA stability [116]	human hepatocellular carcinoma cell lines (HepG2, SMMC-7721, Hep3B, Bel-7402, Huh7, L02) [116]	Inhibition
	BECN1	Blocks system x_c^- activity via binding of SLC7A11 [117]	human colorectal cancer cell lines (HCT116, CX-1, LoVo, SW48) [118] Human pancreatic cancer cell line (PANC1) [119] human fibrosarcoma cell line (HT1080) [119] human non-small-cell lung cancer cell line (Calu-1) [119] human synovial sarcoma cell line (SW982) [120] human colon cancer xenograft mice (HT-29) [121] human fibrosarcoma xenograft mice (HT1080) [119]	Induction
	NRF2	Key regulator of antioxidant response including system x_c^- -expression [122]	human lung cell lines (H460, H1975, H1299,95D, A549) [123] human hepatocellular carcinoma cell lines (Hep3B, Bel-7402, and HepG2) [124] human breast carcinoma cell line (MDA-MB-231) [125] human neuroblastoma cell line (SH-SY5Y) [125] human lung cancer xenograft mice [123]	Inhibition
	QSOX1	Inhibition of NRF2 activation [126]	human hepatocellular carcinoma cell lines (HCC-LM3, MHCC97L, MHCC97H, SMMC7721, SMMC-7402, HuH7, PLC, Hep3B, HepG2) [127] cervical carcinoma cell line (HeLa) [128] human breast carcinoma cell line (BT549, BT474) [129] human kidney carcinoma cell line (786-O) [130] human primary breast tumor clinical study [131] human kidney carcinoma xenograft mice [130]	Induction
	BCAT2	Regulates intracellular glutamate concentration [132]	murine hepatocellular carcinoma cell line (H22) [132] murine pancreatic carcinoma cell line (Panc02) [132] human pancreas carcinoma cell line (AsPC-1) [132] human hepatocellular carcinoma cell line (HepG2) [132] human fibrosarcoma cell line (HT1080) [132] human colon cancer cell line (SW-480) [132] patient-derived human breast carcinoma cell line (BCC, MCF-7) [133] human ovarian cancer cell line (HeLa) [134] human colon cancer cell line (HCT116) [134] hepatocellular carcinoma xenograft mice (H22) pancreatic carcinoma xenograft mice (SW1990) [135] pancreatic ductal carcinoma xenograft mice [136]	Inhibition
	Fenugreek (trigonelline)	Blocks NRF2	human hepatocellular carcinoma cell line (HepG2) [137] human epidermoid cancer cell line (HEp2) [138] human breast carcinoma cell line (MCF-7) [138]	Induction
	σ 1R	Regulates ROS accumulation via NRF2 [139]	human hepatocellular carcinoma cell line (HepG2, Huh-7, SMMC-7721, PLC/PRF/5) hepatocellular carcinoma xenograft mice (Huh-7) [139] human breast cancer cell lines (MCF-7, MCF-41) [140]	Inhibition
GPX4 Inhibition	RSL3	Inhibition of GPX4 phospholipid peroxidase activity [50]	human head and neck cancer cell line (AMC-HN2-11) [141] human lung cancer cell lines (H1650, HCC827, PC9, NCI-H1975) [142] human prostate cancer cell lines (DU145, PC-3, 22Rv1, LNCaP, NCI-H660) [143] human colon cancer cell lines (HCT116, LoVo, HT29) [144] human acute lymphoblastic leukemia cell line [145] rat glioma cell line (C6) [146] human glioma cell line (U373, U87, U251 cell lines) [146] human pancreatic carcinoma cell lines (PANC-1) [147] human ovarian carcinoma cell line (IGROV-1) [147] human bone rhabdomyosarcoma cell line (A-673) [147] human breast carcinoma cell line (MCF7) [147] human prostate carcinoma cell line (PC-3) [147] head and neck cancer xenograft mice [141] prostate cancer xenograft mice [143]	Induction

Table 1. Cont.

Category	Drug/ Compound	Mechanism	Cancer Research	
			Tumor Type	Effect on Ferroptosis
	FIN56	Degradation of GPX4 protein [147]	human lung carcinoma cell line (HT-1080, BJeLR, Calu-1) [147] human osteosarcoma cell line (143B) [147] human kidney carcinoma cell line (CAKI-1, UO-31, 786-O) [148] human bone rhabdomyosarcoma cell line (A-673) [147] human breast adenocarcinoma cell line (MCF7) [147] human prostate carcinoma cell line (PC-3) [147] human non-small cell lung cancer cell line (NCI-H1975) [147] human colorectal carcinoma cell line (LS411N) [147] human pancreatic adenocarcinoma cell lines (PANC-1) [147] human ovarian carcinoma cell line (IGROV-1) [147]	Induction
			lung carcinoma xenograft mice [50]	
	DPIs	Inhibition of GPX4 via GSH depletion [50]	human lung carcinoma cell line (BJeLR, Calu-1) [2,50] human renal cell carcinoma cell lines (A498, TK-10, SN12C, CAKI-1, RXF-393, ACHN, 786-0, UO-31) [50] human fibrosarcoma cell line (HT1080) [50]	Inhibition
			lung carcinoma xenograft mice [50]	
	Altretamine	Anticancer agent inhibiting GPX4 without depleting cells of GSH [149]	human B-cell lymphoma cell line (OCI-LY3, OCI-LY7, U-2932 DLBCL) [149] human osteosarcoma cell line U-2-OS [149] human breast cancer cell lines (MCF-7, MDA-MB-231) [149]	Induction

Ferroptosis inducers may also be useful in combination with common therapeutic agents. Sato et al. suggest a synergizing effect of pre-treatment of short exposure to erastin followed by cisplatin, which efficiently induces cell death in a variety of cancer cell lines [150]. Cisplatin was originally considered the most effective and widely used anti-cancer agent for the treatment of solid tumors [151]. However, cisplatin was also proven to be a ferroptosis inducer via GSH and GPX4 inactivation in different cell lines, and its synergistic effect with erastin on their anti-tumor activity opened up a new way for the utility of classic drugs [152]. The combination of erastin and radiation was shown to induce a significant inhibition of tumor growth in a cell model of lung tumor and in mouse model of sarcoma. Other studies confirmed a potentiating effect in models of glioma, melanoma, and breast cancer [153,154]. Another study confirmed a combined effect of erastin with irradiation in non-small cell lung cancer (NSCLC) by inducing ferroptosis [155]. Radiotherapy is still one of the most widely used cancer therapies, but radioresistance remains a major factor leading to the failure of radiotherapy [156]. Several studies revealed that ionizing radiation can enhance the anti-tumor effect by synergizing with ferroptosis triggers and immunotherapy by increasing the expression of ACSL4 and GPX4 [78,157].

An additional potent inhibitor of the x_c^- transporter, sulfasalazine (SAS), may induce eradication of chemotherapy/radiotherapy-resistant cancer cells by potentiating the cytotoxicity of cisplatin in some cancer types [158]. Nevertheless, it was shown that erastin is a substantially more potent inhibitor of system x_c^- function than SAS.

Sorafenib was the first multi-kinase inhibitor approved for HCC treatment, and there is evidence that sorafenib might induce ferroptosis in addition to its kinase inhibitory functions [159]. As for erastin and SAS, there have been approaches combining ferroptosis induction using sorafenib nanoparticles with the common anti-cancer drugs cisplatin or lactoferrin [160]. However, sorafenib's exact lethal mechanism of action in cells has not been clear until now, and there is disagreement about it. It may not involve a direct kinase inhibition and/or binding to an alternative target such as an unknown kinase whose activity is necessary for system x_c^- [161]. Besides inhibiting tumor proliferation through the RAF/MEK/ERK signaling pathway, sorafenib can block tumor angiogenesis via VEGFR and platelet-derived growth factor receptor (PDGFR) [162]. Sorafenib-induced ferroptotic anticancer activity can be enhanced by blocking another critical regulator named

metallothionein (MT)-1G that also plays an antioxidant role [163]. Upregulation of MT-1G was observed in sorafenib-resistant cancer cells and was shown to limit sorafenib action. It could be one regulator contributing to acquired resistance against sorafenib in human HCC cells, which is one reason for poor prognosis [164]. Thus, MT-1G pathway inhibition enhances the ferroptosis-inducing activity of sorafenib. Similarly, expression levels of the retinoblastoma (RB) protein that acts as a negative regulator of cell proliferation impacts the response of HCC cells to sorafenib and the regulation of ferroptosis [165]. Liver cancer cells with reduced RB levels show a 2~3 times higher cell mortality upon exposure to sorafenib than control cells with normal RB levels [166]. Therefore, evaluating the RB status before treatment application could be used to judge the drug resistance to sorafenib in HCC patients.

A combinatorial strategy using sorafenib together with the immune system modulator YIV-906 (PHY906, KD018) is currently under investigation in a phase II clinical study (NCT04000737). Preclinical research suggests that YIV-906 could act as an enhancer of the anti-tumor activity of sorafenib [167]. Combination therapy of sorafenib and the GPX4 inhibitor RSL3 may be a promising strategy in HCC treatment as studies confirmed synergic therapeutic effect on HCC progression in mice [168]. Another recent study aimed to verify the effect of recombinant human adenovirus type 5 (H101), which is an oncolytic adenovirus that can selectively replicate in tumor cells (NCT05113290). H101 showed anti-tumor effects on liver cancer and a synergistic effect with sorafenib on hepatoma cells *in vitro* [169]. The ongoing clinical study intends to verify this promising evidence to support clinical medication for advanced HCC patients.

An alternative strategy focuses on decreasing intracellular cysteine levels in cancer cells by inhibiting the import of cystine via system x_c^- . Depletion of extracellular cysteine and cystine can be achieved by treatment with cyst(e)inases and was shown to increase ROS and reduce GSH levels [170]. They may represent an effective therapeutic modality as cysteine depletion and the following inactivated antioxidant cellular response successfully suppresses tumor growth [171,172].

3.2. GPX4 Regulators

Independent of system x_c^- , several inhibitors target the other major ferroptosis mediator GPX4 or the metabolic pathways crucial for its action (Figure 3, Table 1). RSL3 ((1S,3R)-methyl 2-(2-chloroacetyl)-2,3,4,9-tetrahydro-1-[4-(methoxycarbonyl)phenyl]-1H-pyrido [3,4-b]indole-3-carboxylate) was first identified for inducing iron-dependent cell death by directly targeting selenocysteine residue of GPX4, thereby directly inhibiting PL-peroxidase activity [27,50]. As for erastin, the poor water solubility and metabolic instability of RSL3 precludes its systemic *in vivo* use yet it has to be further optimized for clinical application [144,173]. In contrast, systemic toxicity and pharmacodynamic analysis by Yang et al. [52] observed RSL3 to be well tolerated in animal studies. In mouse models, RSL3 was shown to block the activity of GPX4, leading to ferroptosis induction and thus inhibition of fibrosarcoma growth.

Additional GPX4 inhibitory agents are ML162 (α -[(2-chloroacetyl) (3-chloro-4-methoxyphenyl)amino]-N-(2-phenylethyl)-2-thiophene-acetamide; DPI7: diphenylene iodonium) and ML210 ([4-[bis(4-chlorophenyl)methyl]-1-piperazinyl](5-methyl-4-nitro-3-isoxazolyl)-methanone), both exhibiting similar ferroptosis-inducing activity as RSL3 [25].

A new and specific inducer of ferroptosis, N2,N7-dicyclohexyl-9-(hydroxyimino)-9H-fluorene-2,7-disulfonamide (FIN56), was discovered by Shimada et al. [125] from a systematic survey. Treatment of cells with FIN56 resulted in degradation of GPX4 protein and lipid ROS generation afterwards [174]. Another GPX4 inhibitor called altretamine (2,4,6-tris (dimethylamino)-1,3,5-triazine) was identified and experimentally confirmed to share the same mechanism of action as SAS as an inhibitor of GPX4 lipid repair activity [149]. However, the precise mechanism remains elusive. Pharmacokinetic and toxicity analysis revealed a great inter- and inpatient variability regarding bioavailability of oral altretamine [175]. Altretamine has been applied in ovarian cancer, including a phase II

study showing ferroptosis induction, and was generally well tolerated and associated with prolonged progression-free and overall survival [176,177].

Several studies that focus on identifying ferroptosis resistance genes revealed additional potent ferroptosis-regulating proteins and agents: the flavoprotein apoptosis-inducing factor mitochondria-associated 2 (AIF2), renamed as ferroptosis suppressor protein 1 (FSP1), is a potent ferroptosis suppressor by preventing lipid peroxidation through an ubiquinone (also known as coenzyme Q₁₀, CoQ₁₀)-mediated mechanism that is distinct from glutathione-dependent protective pathways [178,179].

3.3. Targeting SLC7A11

Attention was shifted to another factor that can influence the effect of ferroptosis: the most frequently mutated gene in human cancer, the TP53 tumor suppressor gene, might also play a role in ferroptosis and especially ferroptosis in tumor suppression [180]. P53 is involved in various cellular processes, including mediating cell cycle arrest, apoptosis, senescence, or differentiation [181]. Recent published studies confirmed the ability of p53 to suppress tumor development by making cells more sensitive to iron by inhibiting SLC7A11 expression, which is a key component of the system x_c^- [182–184]. Therefore, novel anticancer drugs that are able to reactivate the mutant p53 should have wide clinical applicability, which is much needed to combat tumors with mutant p53. However, at the same time, other studies showed a negative effect of p53 on ferroptosis in cancer cells different from the previously identified function as a positive regulator of ferroptosis. In this case, p53 seemed to play a role in inducing p21 expression to promote GSH synthesis, thus rendering cancer cells more resistant to ferroptosis [185]. Therefore, a clearer idea of this bidirectional control both as a positive and negative modulator of ferroptosis needs to be attained, and the exact role of p53 is in need of clarification. The pharmacological induction of ferroptosis may be a homeostatic mechanism to prevent tumor formation and an emerging anticancer strategy to combat tumors, but the impact of p53 expression on ferroptosis sensitivity is still poorly understood. Besides p53, there are many more proteins or agents that regulate SLC7A11 and represent an important therapeutic target for HCC patients. The RNA binding protein deleted in azoospermia-associated protein 1 (DAZAP1) was found to be an important oncogene in HCC and plays a significant role in ferroptosis by interacting with SLC7A11 mRNA [116]. Beclin 1 (BECN1) contributes to signaling pathways in ferroptosis by directly blocking system x_c^- activity via SLC7A11 binding [153]. Therefore, an AMP-activated protein kinase (AMPK)-mediated phosphorylation of BECN1 is required for formation of BECN1-SLC7A11 complex and subsequent ferroptosis initiation [186]. Recent results show that the system x_c^- inhibitors erastin and SAS promote BECN1 phosphorylation and AMPK-promoted cancer cell ferroptosis [119]. The aminotransferase branched-chain amino acid aminotransferase 2 (BCAT2) participates as a specific ferroptosis inhibitor being downregulated via the AMPK pathway. With its role in regulation of sulfur amino acid metabolism, it is involved in regulating intracellular glutamate levels and contributes to ferroptotic death in HCC cells [132]. Synergizing with sorafenib, SAS was demonstrated to downregulate BCAT2 levels to induce ferroptosis *in vitro*.

3.4. Targeting NRF

Interestingly, SLC7A11 is also a transcriptional target of the nuclear factor erythroid 2-related factor 2 (NRF2), which has been shown to play a central role in protecting HCC cells against ferroptosis through the p62-Kelch-like ECH-associated protein 1 (KEAP1)-mediated pathway [122]. NRF2 is involved in iron and ROS metabolism, where it protects HCC cells against ferroptosis through upregulation of multiple genes such as HMOX1 [122]. The common leucine zipper transcription factor NRF2 is essential for redox homeostasis and regulates the metabolism of multiple cancer cells, which makes it a promising target for tumor therapy [162]. Several studies have assessed the role of NRF2 in ferroptosis, and upregulation of iron and ROS by genetic or pharmacological inhibition of NRF2 expression

leads to an enhanced anti-tumor effect of erastin and sorafenib [153]. NRF2 is supposed to be the master transcriptional regulator of the expression of antioxidant molecules. It is typically located in the cytoplasm bound by KEAP1 and under stress activates antioxidant response elements (ARE). The sigma 1 receptor (σ 1R) acts as mediator of oxidative stress by regulating the key antioxidant pathway NRF2-KEAP1 [187]. σ 1R has emerged as a promising treatment for neurodegenerative diseases but was also shown to play a role in other diseases in which oxidative stress is implicated [188]. Bai et al. demonstrated in 2019 a regulatory role for σ 1R in protecting HCC cells against ferroptosis by preventing ROS accumulation [139]. Another NRF2-inhibiting protein is the quiescin sulphydryl oxidase 1 (QSOX1), which was identified as a tumor suppressor in HCC [127]. It was shown to have a cooperative effect *in vitro* and *in vivo* with sorafenib, resulting in increased ferroptosis in HCC cells, which makes it a novel candidate for sorafenib-based combination therapeutic strategies [126]. Withaferin A (WA) was identified as a natural ferroptosis-inducing agent by activating NRF2 pathway characterized by an increase in intracellular Fe^{2+} through HMOX1-mediated heme degradation [189]. In neuroblastoma cells, upregulated HMOX1 expression induces ferroptosis by iron ion enrichment [190].

The alkaloid fenugreek (trigonelline) has been reported to act against NRF2, which improved the pro-ferroptotic effect of sorafenib or erastin in HCC cells [191]. Overall, NRF2 plays a central role in defense against oxidative stress and for protection of HCC cells from ferroptotic cell death inhibition, which makes it a promising therapeutic target for HCC [122]. While inhibition of NRF2 enhances ferroptosis susceptibility, its activation leads to cellular resistance to ferroptosis.

3.5. Nanoparticles

Nanotechnology aimed at engineering small molecules inducing ferroptosis has also attracted substantial research interest. Specifically designed nanoparticles as carriers for iron ions have been reported to disrupt iron metabolism and induce ferroptosis in cancer cells. They are capable of precisely tuning physiochemical properties, for example, by rapidly and massively increasing cellular iron levels, and can be used to overcome tumor resistance in cancer therapy, having a relatively low risk of side effects compared with locally injected agents [192]. Nanoparticles exploit the enhanced permeability retention (EPR) effect to selectively target the tumor. The groundwork for further exploitation of ferroptosis-inducing nanoparticles is laid, but more efforts and *in vivo* studies are necessary to elucidate the real potential of nanoparticles for induction of ferroptosis [193]. Combinatorial nanodrugs can be constructed like a nanoparticle, consisting of iron-abundant protein ferritin, erastin, and rapamycin that is also an FDA-approved immunosuppressive drug [194]. Polyethylene glycol-coated ultra-small nanoparticles Coined Cornel dots (C'dots) were recently approved by the FDA, causing iron overload and thereby inducing ferroptotic cell death [195]. Nanoparticles containing low-density lipoprotein-docosahexaenoic acid (LDL-DHA) have been demonstrated to trigger ferroptosis in HCC cells by lethal lipid ROS accumulation and the depletion of GSH [196]. Studies confirmed the preventative role of fatty acids in hepatocarcinogenesis, and LDL-DHA nanoparticles could be included in the growing list of ferroptosis-inducing agents with anticancer effects [197,198]. However, there are still potential risks of ferroptosis-driving nanotherapeutics due to cytotoxic side-effects that should be taken into consideration for an efficient and safe nanotherapeutic design. Clinical investigations of nanoparticles in cancer treatment are needed, but clinical investigations are required to establish this [199].

3.6. Other Classes

Determining ferroptosis sensitivity by enriching PUFAs in cellular membranes that can be oxidized, ACSL4 could also be a potential target. One regulator is the ADP ribosylation factor 6 (ARF6), which was proven to sensitize cancer cells to RSL3-induced lipid peroxidation and ferroptosis [200]. Traditional medical plants as new therapeutic biochemical agents can target cancer cells as well [201]. Solasonine is a naturally occurring glycoalkaloid and

was shown to participate in ferroptosis by promotion of ferroptotic cell death of HCC cells via GPX4-induced destruction of the GSH redox system [202]. Overloading cells with iron can also induce ferroptosis, which was shown when treating cells with ammonium sulfate and iron chloride [25]. An iron-reducing combinatorial approach using the iron chelator and classic ferroptosis inhibitor deferoxamine, together with conventional transarterial chemoembolization (TACE), is currently under investigation in patients with unresectable HCC (NCT03652467). However, the short half-life of deferoxamine limits its clinical application [203]. Another critical regulator of ferroptotic cancer cell death was confirmed by Sun et al. [164] in 2015, who proved that the heat shock protein beta-1 (HSPB1) is involved in iron uptake and lipid oxidation but also enhances erastin-induced ferroptosis. However, there is also a higher risk of severe side effects because iron metabolism is essential in the regulation of redox hemostasis in normal cells. Artemisinins and its derivatives, including the anti-malaria drug artesunate, have been repurposed as anticancer drugs by ROS production and following cell death induction [204]. It accumulates in lysosomes, activates lysosome function, and promotes ferritin degradation and cellular iron accumulation [205]. Eling et al. [167] proved artesunate to be an effective and specific ferroptosis activator as well as a novel pathway for killing pancreatic cancer cells. In HCC cells, another derivative of artemisinin, dihydroartemisinin (DHA), was shown to induce ROS accumulation and thus ferroptosis, leading to reduced HCC development in a dose-dependent manner both *in vitro* and *in vivo* [206]. Recently, a synergistic mode of action was also proven for the combined treatment of sorafenib and artesunate in HCC cell lines *in vitro* and *in vivo* [207]. The well-tolerated artesunate enhanced the anticancer effects by inducing oxidative stress of low dose sorafenib, which makes it a preferred candidate to synergize with sorafenib to inhibit HCC [63].

3.7. Ferroptosis in Cancer Immunotherapy

Immunotherapy is currently one of the most promising anti-cancer treatment methods enhancing response rates through activation of the immune system, whereby tumor cells may be recognized and eliminated by the immune system with a high degree of specificity [208]. However, immunotherapy is effective in only about 30 percent of cancer patients because of (acquired) resistance as well as inadequate biomarkers for patient stratification [209]. Therefore, new insights from ferroptosis research may provide new opportunities to increase the response rates to cancer immunotherapy and explore the ways in which to make the immune system work for more patients. This could open up a promising direction in the future using combinatorial approaches of immunotherapy, e.g., anti-PD-1/PD-L1 immune checkpoint inhibitor with ferroptosis-promoting modalities.

T cells are critical mediators of antitumor immunity, and ferroptosis has recently been shown to contribute to cancer immunotherapy driven by cytotoxic T cell response [40,210]. INF- γ that is secreted from cytotoxic CD8⁺ T cells sensitizes tumors to ferroptosis through downregulation of system x_c[−] subunits SLC3A2 and SLC7A11 expression. This impairs cystine uptake by tumor cells, followed by enhanced lipid peroxidation and ferroptosis [57]. It was confirmed by correlation of reduced SLC3A2 expression with strong CD8⁺ T cell signature and INF- γ expression [211]. The immune checkpoint inhibitor anti-PD-L1 was proven to potentiate erastin- and RSL3-induced tumor growth inhibition *in vitro* and *in vivo*, stimulating CD8⁺ T cells to release INF- γ , ultimately increasing the sensitivity of cancer cells to ferroptosis [57].

A recent study from Xu et al. [174] reported a novel role of GPX4 in regulating immune homeostasis by preventing regulatory T cells (T_{regs}) from lipid peroxidation and further ferroptosis. This protects and sustains activated T_{reg} function and could be a potential target for new therapeutic strategies to improve cancer treatment as T_{regs} were shown beforehand to polarize tumor-associated macrophages (TAMs) to maintain a strong suppression of effector T cells within the TME [212].

Besides tumor growth inhibition, ferroptosis could also switch from antitumor to immunosuppressive activity through PGE2 release from ferroptotic cancer cells, which

is a key immunosuppressive factor that suppresses the antitumor functions of NK cells, DCs, and cytotoxic T cells [213]. There is no doubt that cancer cells greatly impact the microenvironment by releasing cell signaling molecules influencing the development and progression of cancer. The TME comprises not only cancer cells but also immune cells, stromal cells, extracellular matrix, and various secreted signaling molecules, which also shape the TME [214]. In recent years, with the deepening research in TME features, the role of immune cells in tumor development and progression has been emphasized [214–216]. It must be kept in mind that tumor and immune cells share similar growth signals and metabolic properties; therefore, cancer therapy often goes along with an impaired antitumor immunity. Appropriate T-cell functions may not be properly induced or maintained in tumors due to resource competition, emphasizing that balancing T-cell metabolic activity is a key factor when modulating anti-tumor immunity [217]. With better understanding of metabolic features of the TME and tumor-infiltrating lymphocytes (TILs), new opportunities to improve and promote appropriate immune cell function arise [215]. The importance of the TME as a limiting barrier to antitumor immunity and achieving greater treatment efficacy became increasingly clear through expanded research in recent years. One contributor of a protumor TME and promotor of immunotherapy resistance was recently identified by Jiang et al. [209]. The tyrosine-protein kinase receptor TYRO3 suppresses tumor cell ferroptosis by upregulation of key anti-ferroptosis genes such as SLC3A2 and facilitates the development of a pro-tumorigenic microenvironment by decreased levels of pro-inflammatory macrophages and enhanced macrophage polarization towards a pro-carcinogenic phenotype. TYRO3 inhibition promoted tumor ferroptosis and sensitized the tumors to anti-PD-1 therapy, which makes it promising as a therapeutic target to overcome resistance to immunotherapy [210]. However, the influence of TYRO3 on other immune cells remains unclear, and further research of the influence of ferroptosis to the overall TME is required to get a more complete overview about what effect tumor cell ferroptosis has on immune cells.

Two recent studies confirmed a crucial role of the fatty acid transporter CD36 in promoting T-cell ferroptosis: increased CD36 expression in CD8⁺ TILs promoted lipid peroxidation followed by ferroptosis, thereby resulting in CD8⁺ T-cell dysfunction by lower INF- γ production in the TME [218,219]. Therefore, blocking of CD36 could be useful to boost anti-tumor immunity.

Ferroptotic cancer cells can also enhance repolarization of macrophages to a pro-carcinogenic phenotype, which restricts anti-tumor immunity and induces higher resistance to ferroptosis induced by deletion of GPX4 [220]. Targeting these cells with ferroptosis inducers could be a promising therapeutic strategy to reverse immunosuppression in the TME [221].

Another potential target to enhance the anti-cancer activity of existing immunotherapies is iron modulation [222]. As previously mentioned, iron metabolism is fundamental for vital biological processes and has been identified as one of the pivotal regulators of ferroptosis by accumulating ROS through the Fenton reaction [41]. Cancer cells exhibit distinct iron metabolism characterized by iron addiction to enhance tumor growth and metastasis [223]. Cancer cells ensure their iron supply through multiple mechanisms, mainly through activation of iron uptake and parallel downregulation of export pathways [224]. In addition, dysregulated iron metabolism can alter the polarization of TAMs to an anti-inflammatory phenotype associated with enhanced release of iron, which helps to meet the metabolic demands of cancer cells [225]. Therefore, within the TME, tumor cells reconfigure immune cells such as TAMs to satisfy their enhanced demand of iron by altered polarization and resulting in enhanced tumorigenesis, as well as immunosuppression [226].

The glycoprotein ceruloplasmin (CP) has been discovered to play a suppressing role in HCC cells by regulating iron homeostasis [227]. Depletion of CP significantly increased the accumulation of intracellular ferrous iron (Fe²⁺) and lipid ROS, promoting erastin- and RSL3-induced ferroptosis [165].

Ruiz-de-Angulo et al. developed iron oxide-loaded nanovaccines (IONVs) that can enhance immunotherapy-promoted tumor ferroptosis [228]. They were able to demonstrate enhanced immunotherapeutic efficacy with the help of engineered nanocarriers that enable effective vaccine delivery to the tumor and downregulate tumor immune escape mechanisms [229]. This is a good example establishing the combination of ferroptosis and immunotherapy as a promising cancer treatment strategy.

As mentioned previously, scores derived from algorithms evaluating ferroptosis modification patterns and TME immune infiltration characterization could be applied in clinical practice to guide therapeutic regimens [77,230,231]. Ferroptosis-related gene signatures can be employed as prognostic or diagnostic tools and guide novel directions in cancer treatment. Very recently, Liu et al. developed a scoring system based on a defined ferroptosis related gene signature in HCC that considers individual heterogeneity of ferroptosis modification [230].

Collectively, it will be important to weigh the pros and cons of when to apply ferroptosis inducers or inhibitors and how this affects the efficacy of immune checkpoint inhibitors and other immunotherapies. Distinct tumors may require distinct therapeutic approaches, but overall ferroptosis-based immunotherapy may potentially reverse the immunosuppressive TME and therefore resistance. On the one hand, ferroptotic cancer cells secrete DAMPs that foster anti-tumor immunity through APC activation and lymphocyte recruitment. On the other hand, tumor-infiltrating immune cells, which could eventually undergo ferroptosis, release immunosuppressive molecules such as PGE₂, disrupting the anti-tumor immunity.

Further research on this complicated crosstalk between tumor and immune cells and the dual role of ferroptosis in tumor immunity is necessary in order to widen the perspective on targeting ferroptosis in cancer immunotherapy. Ferroptosis might be promoted by delivering iron into the TME as an adjuvant; however, hyperinflamed tumors already show an iron overdose, which improves cancer progression and immune evasion. Therefore, iron chelators could be promising, but iron toxicity should be taken into account [222].

3.8. Clinical Trials in HCC Treatment

A variety of clinical trials are ongoing worldwide concerning HCC treatment. Regarding ferroptosis-inducing agents, clinical trials are still limited to the use of sorafenib and mainly study combination therapy, with targeted therapy or immunotherapy. Although the relationship between ferroptosis and immunotherapy is not well understood, a synergistic mode of action of PD-L1 antibodies with ferroptosis inducers in tumor growth inhibition *in vitro* and *in vivo* was proven [57].

Preclinically demonstrated antiproliferative effects of sorafenib in liver cancer cell lines and mouse xenograft HCC models were confirmed in clinical trials with advanced HCC patients who showed a nearly 3 month median survival benefit, as compared with those who received placebo [13]. Sorafenib's mode of action targets two key pathways by inhibition of the Raf-MEK-ERK signaling pathways abrogating tumor growth, as well as VEGFR and PDGFR inhibiting neoangiogenesis [232]. However, further pharmacogenomic studies are required since several studies found that continuous sorafenib treatment was not beneficial and reduces patient's quality of life, leading to drug interruption or discontinuation [233,234]. This limitation of sorafenib's effectiveness might be overcome by optimizing the dosing schedule of sorafenib, e.g., by the "ramp-up" strategy starting with a reduced dose to improve patients' compliance and avoid treatment interruption [235].

Table 2 summarizes currently ongoing trials of sorafenib combination treatments in HCC, mainly focusing on combination strategies with immunotherapies, such as immune checkpoint inhibitors. Even though sorafenib was shown to induce ferroptosis *in vitro* as well as in mouse models, a recent study suggests that sorafenib does not seem to be a bona fide ferroptosis inducer, and erastin only triggers ferroptosis in certain cancer cells [99]. Therefore, there is significant controversy as to whether ferroptosis is really necessary for anti-tumor effect of sorafenib *in vivo* and to which extent ferroptosis is considered in clinical

trials investigating sorafenib-immunotherapy combinations. The ferroptosis-independent role in other types of cell death (apoptosis, pyroptosis, necroptosis) is still not well understood, and further studies are essential to unveil the underlying mechanisms [236]. Therefore, deeper understanding of sorafenib's mode of action in HCC is required to uncouple the kinase inhibitory function from the ferroptosis-inducing function to further improve efficacy in HCC systemic therapy.

Table 2. Ongoing clinical trials with ferroptosis-associated agents for HCC treatment. clinical studies are needed to determine the therapeutic value of ferroptosis induction in patients with HCC as preclinical data are not sufficient.

Trial Identifier	Phase	Drug	Target	N	Study Start	Estimated Study Completion
NCT03518502	IV	Sorafenib monotherapy vs TACE + sorafenib	VEGFR	130	1 March 2012	28 February 2022
NCT01624285	II	Sorafenib tosylate	VEGFR	356	16 July 2012	July 2023
NCT01730937	III	SBRT + sorafenib tosylate	radiation, VEGFR	193	April 2013	June 2025
NCT01840592	II	Sorafenib + doxorubicin (TACE)	cytostatica, VEGFR	30	April 2013	April 2022
NCT02733809	IV	Sorafenib	VEGFR	40	January 2014	December 2024
NCT02143401	I	Navitoclax + sorafenib tosylate	Bcl-2, VEGFR	44	7 November 2014	7 May 2022
NCT02576509	III	Nivolumab vs. sorafenib	PD-1, VEGFR	743	7 December 2015	30 June 2022
NCT02716012	I	MTL-CEBPA + sorafenib	VEGFR	51	1 March 2016	1 January 2022
NCT03037437	II	Sorafenib + HCQ	autophagy inhibition, VEGFR	68	16 February 2017	April 2023
NCT03298451	III	Durvalumab + tremelimumab vs. sorafenib or lenvatinib	PD-1 + CTLA-4, VEGFR	1504	11 October 2017	30 April 2023
NCT03211416	II	Sorafenib tosylate + pembrolizumab	PD-1, VEGFR	41	7 December 2017	7 December 2022
NCT03412773	III	Tislelizumab vs. sorafenib	PD-1, VEGFR	674	28 December 2017	May 2022
NCT03434379	III	Atezolizumab + bevacizumab vs. sorafenib	PD-L1 + VEGFR	558	15 March 2018	30 June 2022
NCT03439891	II	Nivolumab + sorafenib	PD-1, VEGFR	24	16 April 2018	30 September 2023
NCT03652467	I	Deferoxamine + conventional TACE	Ferric ions	100	1 September 2018	31 December 2023
NCT03645980	II	DKN-01 + sorafenib	DKK-1, VEGFR	70	10 October 2018	31 August 2022
NCT03755791	III	Cabozantinib + atezolizumab vs. sorafenib	VEGFR	740	10 December 2018	1 December 2021
NCT03775395	III	HAIC + sorafenib	chemotherapy, VEGFR	250	12 December 2018	1 December 2021
NCT02436902	III	TACE + sorafenib vs. monotherapy	VEGFR	240	1 February 2019	30 August 2022
NCT04926532	II	Toripalimab + sorafenib	PD-1, VEGFR	30	1 August 2019	31 December 2021

Table 2. Cont.

Trial Identifier	Phase	Drug	Target	N	Study Start	Estimated Study Completion
NCT03606590	II	NovoTTF-100L(P) + sorafenib	electrical field + VEGFR	25	15 February 2019	September 2021
NCT03965546	I	Sorafenib + ET140202-T cell	AFP/MHC complex, VEGFR	27	30 May 2019	June 2022
NCT04926532	II	Toripalimab + sorafenib	PD-1, VEGFR	30	1 August 2019	31 December 2021
NCT03971201	II	Surgery + sorafenib vs. sorafenib	VEGFR	200	6 September 2019	30 June 2023
NCT04143191	III	Sorafenib + TACE	VEGFR	158	15 September 2019	15 September 2023
NCT04039607	III	Nivolumab + ipilimumab vs. sorafenib or lenvatinib	PD-1 + CTLA-4, VEGFR	634	30 September 2019	4 January 2025
NCT04232722	II	Sorafenib + arsenical	VEGFR + apoptosis	43	1 January 2020	30 June 2022
NCT04000737	II	YIV-906 + sorafenib	VEGFR	125	10 January 2020	19 May 2023
NCT04387695	III	SBRT + TACE + sorafenib	radiation, VEGFR	54	30 April 2020	1 June 2023
NCT04518852	II	TACE + sorafenib and PD-1 mAb	PD-1, VEGFR	60	14 September 2020	31 January 2023
NCT04599777	II	TACE + sorafenib and tislelizumab	PD-1, VEGFR	30	1 October 2020	December 2022
NCT04687163	III	Sorafenib or lenvatinib + HAIC	chemotherapy, VEGFR	400	30 December 2020	1 December 2022
NCT04229355	III	DEB-TACE + sorafenib	VEGFR	90	2 February 2021	30 December 2022
NCT04763408	IV	Sorafenib, lenvatinib	VEGFR	1000	9 April 2021	31 July 2029
NCT04770896	III	Atezolizumab + lenvatinib or sorafenib	PD-L1 + VEGFR	554	26 April 2021	8 October 2024
NCT04967495	not applicable	TACE + sorafenib or lenvatinib + iodine-125 seeds brachytherapy (TACE-MKI-I)	radiotherapy, VEGFR	171	9 July 2021	8 January 2025
NCT04992143	II	TACE + tislelizumab and sorafenib	PD-1, VEGFR	20	20 August 2021	30 June 2023
NCT04465734	III	HLX10 mAb + HLX04 mAb vs. sorafenib	PD-1, VEGFR	477	15 November 2021	15 March 2024

Abbreviations: AFP: alpha-fetoprotein; Bcl-2: B-cell lymphoma 2; CEBPA: CCAAT enhancer-binding protein alpha; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; DEB-TACE: drug-eluting bead TACE; DKK-1: dickkopf-related protein 1; DKN-01: DKK1-neutralizing monoclonal antibody; H101: recombinant human type-5 adenovirus; HAIC: hepatic arterial infusion chemotherapy; HCQ: hydroxychloroquine; MHC: major histocompatibility complex; MKI: multikinase inhibitor; PD-1: programmed cell death protein 1; SBRT: stereotactic body radiation therapy; TACE: transarterial chemoembolization; TTF: tumor treating fields; VEGFR: vascular endothelial growth factor receptor.

Ongoing phase III trials targeting the anti-cancer immune response through ICI in first- and second-line treatment in combination with sorafenib and other tumor-targeting drugs will hopefully significantly improve the effectiveness of established cancer treatment. Nevertheless, the revolution of immune therapies that has changed the paradigm in HCC treatment also brings difficulties in developing effective drugs. With increasing numbers of effective agents, complexity of clinical decision making and design of clinical trials will also

increase [237]. Moreover, significant drug toxicity is still relevant, especially in cirrhotic patients, since liver dysfunction decreases the threshold for severe side effects [238].

Traditionally, new therapies were compared with standard of care or placebo to demonstrate greater efficacy of the new drug. To date, most systemic therapies tested in phase III trials for advanced HCC have failed to improve on or parallel the efficacy of sorafenib [239]. The way in which to sensitize therapy-resistant cancer cells to ferroptosis remains one of the most important questions that needs to be addressed. Very recently, Yao et al. showed that the loss of the tumor suppressor gene encoding leukemia inhibitory factor receptor (LIFR) confers resistance to ferroptosis-inducing drugs through upregulation of iron-sequestering cytokine lipocalin-2 (LCN2) [240]. Therefore, anti-LCN2 therapy using LCN2-neutralizing antibody could be used in combination with sorafenib to improve liver cancer treatment by targeting ferroptosis. Additionally, there are hints that this may have the potential to sensitize tumors to radiation, which requires further investigation [240].

The growing knowledge of ferroptosis regulators has not been translated into clinical benefits as most of them have been tested only on cell-line models, which are limited in translating therapy response to *in vivo* settings of patients [240]. Although ferroptosis research is mainly still ongoing in the preclinical phase, the future options are endless, and clinical trials may pave the way to become a prominent therapeutic utility in liver cancer treatment. One key development is targeted ferroptosis induction, e.g., by nanoparticles to increase specificity and reduce side effects [241].

Even though advanced liver cancer remains a significant unmet medical need, in particular for those patients who are resistant to front line systematic therapy, a number of effective drugs are already available to clinicians for the management of advanced HCC, and further investigation including future clinical trials are expected to be conducted in the future.

4. Conclusions and Perspectives

Since its discovery in 2012, extensive research on ferroptosis has helped us understand iron-dependent cell death mechanisms at molecular levels. With the proof of the anti-cancer potential of ferroptosis induction, it can be a powerful weapon to improve cancer prevention and treatment.

Liver cancer remains as one of the major challenges for basic and clinical oncology because of late diagnosis, limited treatment options, and high risk for recurrence. This is likely why the progress of innovative treatment options has lagged behind that of other tumors, and new treatments for advanced liver cancers are needed. A number of ferroptosis-inducing small molecules have been developed, which hold much potential, e.g., by lowering the threshold for activation of ferroptotic cell death [242]. Meanwhile, considering that iron deficiency and iron overload may have an impact on anti-tumor activity, studies are needed to uncover the suitable iron concentration and optimal dose of ferroptosis-related drugs to minimize tumor progression. However, some challenges remain to be overcome as knowledge about the exact role and the mechanism of ferroptosis in physiological and pathological processes, as well as its treatment application, is still limited. A common issue is that translating anti-cancer strategies to the clinical setting may be limited due to the heterogeneity and improved complexity of human cancers in comparison to animal models. In addition, conditions in culture are often non-physiologic, as the behavior and properties of cancer cells in tumors, as well as ferroptosis susceptibility, are influenced by multiple factors, including their *in vivo* microenvironment [243].

Overcoming the issue of therapy-resistant cancers, ferroptosis inducers create high expectations for the therapeutic potential of ferroptosis induction [244]. Chemotherapy alone or in combination with radiotherapy or immunotherapy showed limited clinical benefits in primary liver cancer patients because of the emergence of drug resistance [245]. To overcome resistance to apoptosis in particular, inducing non-apoptotic cell death such as ferroptosis may provide an alternative strategy. Several approaches have been tested either by direct or indirect inhibition of system x_c^- (e.g., sorafenib, erastin, SAS) or GPX4

(e.g., RSL3, ML210, WA). The specificity and optimal dose of ferroptosis inducers require further clarification, and there are still many questions to answer before the potential of ferroptosis is fully unveiled. The usage of ferroptosis-inducing small molecules may enable a precise and targeted drug delivery because of enhanced permeability and retention, but their poor bioavailability and residual cytotoxicity limits the success of nanomaterials in clinical settings [246].

The growing body of recent evidence supports the notion that ferroptosis can trigger immune responses, which could be leveraged to increase immunotherapy efficacy by increasing the immunogenicity of tumor cells or killing immunosuppressive cells. Ferroptosis has recently been suspected to be involved in T cell-mediated anti-tumor immunity and affects the efficacy of cancer immunotherapy [210]. Interactions with factors of the TME shape the vulnerability to ferroptosis but require further research to explore more factors in the TME and a better understanding of the complicated ferroptosis-based crosstalk between tumor cells and immune cells. This is important in order to combine ferroptosis-inducing regimens with immune checkpoint inhibitors, which have become the basis of treatment in many solid cancers.

With this, not only new and effective immunotherapeutic options but also combinatorial therapies with synergistic modes of action might be developed. The efficacy of ferroptosis-mediated cancer therapies could also be boosted with interdisciplinary cooperation of different approaches (e.g., local-ablative radiation) regulating multiple cell death pathways simultaneously.

Moreover, ferroptosis appears to play a dual role such as a “double-edged sword” in promoting and suppressing tumorigenesis. On the one hand, ferroptotic cells can suppress tumor growth by activation of anticancer immunity via stimulation of the immune system. On the other hand, ferroptotic cells can also initiate cancers and other diseases by suppression of an antitumor immune response, which hinders effectiveness of anti-cancer therapy [247]. This has to be kept in mind and considered in terms of dosage, timing, and type of treatment as the dual role of ferroptosis emphasizes the importance of compartmentalizing the induction of ferroptosis to specific cell types. Considering the full spectrum of the TME landscape will assist in determining conditions under which ferroptosis promotes versus inhibits tumor growth *in vivo* to evaluate the impact on stroma cells such as T-lymphocytes or tumor-associated macrophages.

Despite the ongoing clinical studies about ferroptosis in immunotherapy for cancer treatment, many details regarding the immunogenic potential of this non-apoptotic cell death modality remain elusive. The release mechanisms of DAMPs and their regulation have been a recent research focus, but an interesting question is whether these DAMPs are unique for ferroptosis or common across several cell death types [248]. Different cell death modalities lead to different immunogenicity outcomes, which highlights the importance of studying the hallmarks of immunogenic cell death such as kinetics and patterns of DAMP exposure for every cell death type individually [63,249]. Future studies should provide the necessary insights for a more complete understanding and validation of ferroptosis inducers in real clinic settings. Expectations are high, but many questions are waiting to be answered. Although several biomarkers have been proposed, it remains a major challenge to specifically and accurately quantify ferroptotic response, especially *in vivo* [250]. Notwithstanding these limitations, tremendous progress in gaining insights into ferroptosis and its role in cancer has been made during the last years, which opened the door for great strides toward an even better understanding. We can be optimistic about the future, which holds great promise for creating new translational anticancer strategies and the prospects of novel combination therapies based on ferroptosis induction, which will hopefully result in prolonged life expectancy and improved patient-reported outcomes in the forthcoming management of this devastating disease.

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