

Discovering ferroptosis in biliary epithelium during liver fibrosis

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ABSTRACT

Ferroptosis is an iron-dependent regulated cell death characterized by iron accumulation and lipid peroxidation. The liver represents the primary site of iron-overload injury due to its role in iron metabolism, but the potential role of ferroptosis in the biliary epithelium is yet unknown. The study investigated the recruitment of ferroptosis under control conditions and after bile duct ligation (BDL), highlighting the effects of ferroptosis inducers and inhibitors in a cell line of cholangiocytes. In addition, we identified iron deposits through Perls Prussian blue as well as the presence of proteins such as ferritin, transferrin receptor 1 (CD71), glutathione peroxidase-4 (GPX4) and acyl-CoA synthetase long-chain family member 4 (ACSL4). The results showed an increase in the expression of ferritin, CD71 and ACSL4 in the BDL model, whereas GPX4 was reduced in the cholestatic model in comparison to the control. In addition, sorafenib failed to trigger ferroptosis in cholangiocytes, whereas erastin was a potent inducer. Conversely, ferrostatin-1 (Fer-1) did not inhibit ferroptosis, while β -mercaptoethanol (β ME) could protect cholangiocytes from ferroptosis-induced cell death. All these findings are important for understanding the involvement of ferroptosis in cholangiocyte pathophysiology and may support additional studies regarding the possible application of ferroptosis modulators in the treatment of liver fibrosis.

Key words: biliary epithelium; ferroptosis; liver fibrosis.

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Introduction

Cell death represents a fundamental process in the physiology of every tissue. The different types of cell death can be classified in two main categories: i) accidental cell death (ACD), consisting in the instantaneous end of cell's life due to acute insults of chemical, physical or mechanical nature, and ii) regulated cell death (RCD) that can be modulated by pharmacological or genetic factors.^{1,2} Since 2005, the Nomenclature Committee on Cell Death (NCCD) has been working to create a permanently updated nomenclature based not only on morphological aspects but also on genetic, biochemical, pharmacological and functional criteria.^{3,4}

In 2012, a new cell death mechanism characterized by iron accumulation was discovered, based on alterations in the intracellular environment.⁵ It is characterized by intracellular excessive lipid peroxide accumulation, glutathione (GSH) deprivation, glutathione peroxidase 4 (GPX4) inactivation and iron deposition.^{6,7} Moreover, this mechanism is driven by a molecular machinery distinct from apoptosis and closer to necrosis, where caspases activation is almost absent, while vacuolisation and mitochondrial alterations (reduction in mitochondrial crests and electron-dense ultrastructure) are predominant.⁸

Liver fibrosis refers to an increase in connective tissue, specifically collagen fibers, in the liver parenchyma. The increase in collagen fibers can involve the space of Disse (perisinusoidal fibrosis), the portal space (portal fibrosis) or the central vein (pericentral fibrosis). The different patterns are specific to different human liver diseases, and the fibrogenesis process is influenced by the primary anatomical site affected. The cells involved in collagen fiber deposition are hepatic stellate cells in both the space of Disse and in the pericentral region, and portal myofibroblasts in the portal space. Activation of portal myofibroblasts is responsible for the portal/periportal pattern of fibrosis. Their activation is mediated by either portal macrophages and the proliferation of biliary epithelial cells in the portal space during liver disease.^{9,10}

Iron metabolism and storage is tightly linked to liver function.^{11,12} Indeed, iron overload is one of the key features of alcohol-associated liver disease (ALD), where it is mainly deposited in the hepatocytes.¹³ Altered iron homeostasis may induce ferroptosis, leading to cell death and activation of the inflammatory process, which is found to be at the base of many hepatic disorders.¹⁴ In this context, it is crucial to clarify the involvement and the role of ferroptosis in biliary epithelial cells, to understand whether it can represent a possible therapeutic target.

Iron metabolism is characterized by three main steps: iron uptake, storage and export. Firstly, transferrin (Tf) binds free iron Fe³⁺ in circulation, then the newly formed Tf-Fe complex binds to the transferrin receptor (TfR/CD71) on the plasma membrane. The Tf-Fe-TfR conjugate is then internalized via clathrin-dependent endocytosis. Cytoplasmic iron is stored by ferritin heteropolymers which consist of heavy (FTH1) and light (FTL) chains. In the terminal part of the process, excess iron is exported by ferroportin.¹⁵ Ferritin represents a crucial protein as it is responsible for iron sequestration, storage and release. In detail, FTL is responsible for mineral nucleation, due to its negative charge, while FTH1 also shows ferroxidase activity.¹⁶ Alterations of iron metabolism promote ferroptosis due to inactivation of a cellular antioxidant system, such as the system Xc⁻/glutathione (GSH)/glutathione peroxidase 4 (GPX4)-dependent antioxidant defence, which induces the accumulation of lipid hydroperoxides. The system Xc⁻-mediated cystine uptake and subsequent GSH production and GPX4 activation play a central role in protecting cells from ferroptosis. In addition, the GSH-dependent GPX4 enzyme has been identified as the

main endogenous inhibitor of ferroptosis, since it can inhibit lipid peroxidation, by catalysing the GSH-dependent reduction of lipid hydroperoxides to lipid alcohols.¹⁷ By contrast, acyl-CoA synthetase long-chain enzymes (ACSLs) can convert saturated and unsaturated fatty acids to fatty acid acyl-CoA esters,¹⁸ acting as ferroptosis inducers. Indeed, ACSL4 can catalyse PUFA into PUFAs-CoA (polyunsaturated fatty acid coenzyme A), determining lipid peroxides production, thus promoting ferroptosis.¹⁹

Nowadays, the idea of a ferroptosis-inducing therapy is becoming very interesting against liver fibrosis. Several compounds have been identified as ferroptosis inducers (FINs), acting through different mechanisms. Particularly, class I use mainly intracellular GSH, class II use inactive GPX4, class III use GPX4 and endogenous antioxidant CoQ10; class IV increase the labile iron pool (LIP) thereby promoting lipid peroxidation.²⁰ Erastin is among main class I FINs that reduces GSH level, blocks the system Xc⁻ or targets mitochondrial voltage-dependent anion channel (VDAC).²¹ In cancer treatment, erastin can increase the chemotherapy effects of traditional anti-cancer drugs.²² Similarly, sorafenib, a known anti-cancer drug for the treatment of hepatocarcinoma (HCC), acts as a class I FINs, through the inhibition of the system Xc⁻.²³

However, it is important to reduce ferroptosis in specific hepatic cells. Several natural or synthetic compounds have been also reported to counteract ferroptosis, by scavenging lipid-derived radicals or restoring antioxidant defences. Fer-1 was the first synthetic ferroptosis inhibitor identified; it inhibits the process in cancer cells, by preventing the erastin-induced accumulation of cytosolic and lipid ROS. Furthermore, Fer-1 can represent a lipid ROS scavenger, with the N-cyclohexyl moiety acting as a lipophilic anchor in biological membranes.^{24,25} Similarly, β ME has been shown to suppress erastin-induced ferroptosis by bypassing system Xc⁻.²⁶ β ME is a reducing substrate of GPX4, which can use different reducing compounds determining its anti-ferroptotic effect.²⁷

Altogether, this evidence strengthens our interest in elucidating the role of ferroptosis in biliary epithelial cells, the effects of established ferroptosis modulators, such as erastin, sorafenib, ferrostatin-1 and β -mercaptoethanol, and how the mechanisms underpinning ferroptosis during liver fibrogenesis may reveal new potential therapeutic targets for liver diseases.

Materials and Methods

Animal models

The study was performed in Fischer male rats (225-250 g, purchased from Charles River Laboratories (Wilmington, MA, USA) that were subjected to control (n=5) or BDL (n=5) for 1 week. The ligation of the extrahepatic bile duct was performed as previously described.²⁸ Before each procedure, animals were treated with Euthasol® (pentobarbital sodium and phenytoin sodium, 200-250 mg/kg BW). The animals were housed in a temperature-controlled environment (20-22°C) with 12:12-h light-dark cycles and free access to standard chow as well as drinking water. All studies were performed following the regulations of the protocols approved by Baylor Scott & White Institutional Animal Care and Use Committee (#2011-010).

Histology and immunohistochemistry (IHC)

After collection, rat liver specimens were fixed in 4% paraformaldehyde. Liver samples were then embedded in paraffin and cut into 3 μ m thick sections. Perls Prussian Blue stain was per-

formed for evaluating iron (Fe^{3+}) deposition.²⁹ The entire liver sections were scanned with the Aperio scanscope CS system (Aperio digital pathology; Leica Biosystems, Milan, Italy) and processed by the open software for bioimaging analysis QuPath v.0.4.4. An image analysis algorithm quantified the total cell numbers (by eosin counter-staining) and identified the number of Perls positive cells (iron blue deposition) on the total surface area of tissue. The data were represented as the number of Perls-positive cells/ mm^2 . For IHC after deparaffinization and rehydration, sections were pre-treated for 20 min at room temperature with 4% hydrogen peroxide (H_2O_2), to inactivate the endogenous peroxidase activity. Antigens were retrieved, as indicated by the manufacturer's instructions. Sections were washed three times for 5 min with 1 x PBS and incubated with primary antibodies overnight at 4°C. Negative controls with the omission of the primary antibody (replaced with control serum from the same species) were performed for all samples. The secondary antibody used for CD71/TfR staining was the ImmPRESS horse Anti goat IgG polymer kit (MP-7405, Vector laboratories, Newark, CA, USA). We used the Dako REAL EnVision Detection System peroxidase/DAB+, Rabbit/mouse (K5007, Agilent technologies, Inc., Dako, Glostrup, Denmark) as secondary antibodies for anti-mouse. Samples were then incubated with peroxidase substrate solution (diaminobenzidine [DAB, Dako Liquid DAB+ substrate chromogen system; K3468; Agilent technologies, Inc. Dako, Glostrup, Denmark]), and sections were counterstained with hematoxylin for 30 sec at room temperature. The slides were scanned using the Aperio Scanscope CS system (Aperio digital pathology, Leica Biosystems). The stained slides were then processed by ImageScope version 12.3.3 (Leica Biosystems) to identify positive staining cholangiocytes and then analysed with the following formula: (positive cells in bile duct/total area of bile duct) $\times 100$. The bile ducts were selected by their histomorphological characteristics.

Cell culture

Commercial C57BL/6 mouse primary cholangiocytes (Cell Biologics Company, Chicago, IL, USA) were isolated from bile duct tissue. Enrichment of tissue epithelial cells was obtained by magnetic beads coated with EpCAM-1 (CD326) antibody. The cells were grown in flasks coated with gelatine solution, kept in an incubator at 37°C and 5% CO_2 . The medium was completed with the cell medium supplement kit containing EGF, insulin, transferrin, sodium selenite, l-glutamine, antibiotic, antimycotic and 2% FBS (fetal bovine serum). Erastin and Fer-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while sorafenib and βME were obtained from Sigma-Aldrich (Darmstadt, Germany).

Crystal violet assay

The crystal violet assay was used for the evaluation of cell viability. Briefly, the culture medium was removed after stimulations of 96 wells of tissue cultures, and the crystal violet solution (0.5% crystal violet dissolved in 20% methanol) was added for 20 min at room temperature. After extensive washing with tap water and after air drying, 1% SDS was added to each well to dissolve crystal violet for 1 h on an orbital shaker. Absorbance was read at 570 nm by model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) with respect to blank and zero cells. Cell viability was calculated regarding 100% of the untreated cell control.

Lipid peroxidation analysis

For lipid peroxidation imaging analysis, cells were grown on a $\mu\text{-Slide}$ 8 well (Ibidi) with or without ferroptosis inducer such as erastin 5 μM . After 4 h of treatment, the cells were co-incubated with a 1.5 μM lipid peroxidation sensor, C11-BODIPY (581/591) (Thermo-Fisher Scientific, Waltham, MA, USA) at 37°C for 20 min. After two rounds of PBS washing, live cells were analysed for

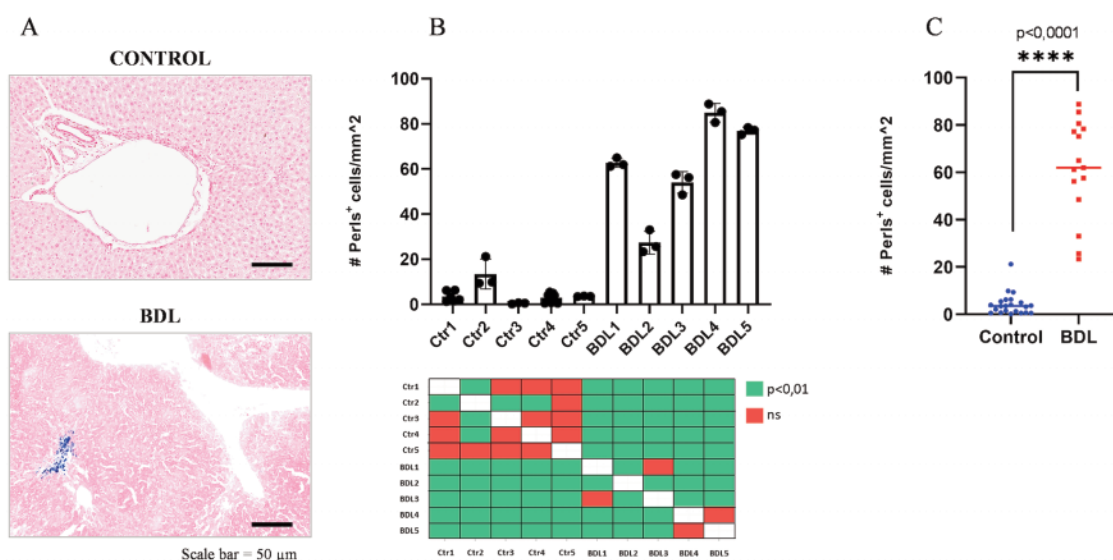


Figure 1. **A)** Histopathological examination of iron deposits conducted using Perls' Prussian Blue (PPB) staining, depicting positive blue staining in liver parenchyma of control and BDL mice. **B)** Statistical analysis revealed significant differences ($p < 0.01$) between most cases analysed of control vs cholestatic livers; the significance matrix depicted is a visual representation of all pairwise comparisons; green indicates significant differences; red indicates non-significant differences; diagonal cells are empty (no group compared to itself). **C)** The iron deposition was globally assessed in all cases together with a significant difference ($p < 0.0001$) between the two groups; each dot represents the number of Perls-positive cells per mm^2 for each section studied.

in situ detection and localization of the lipid peroxidation, using green (peroxidation form) and red (non-oxidate form) fluorescence with the aid of a Leica DM4500 B microscope.

Statistical analysis

GraphPad Prism 8.3.1 software (San Diego, CA, USA) was used to perform the statistical analyses. Continuous variables were tested for normality (Shapiro-Wilks test) and reported as means $\pm$ SD due to normality of their distribution. Differences between groups were evaluated by the Student's unpaired *t*-test when 2 groups were analysed or by 1-way analysis of variance when more than 2 groups were analysed followed by an appropriate *post-hoc* test. A *p*-value less than 0.05 was considered statistically significant. Multiple comparisons were evaluated by one-way ANOVA with Tukey's multiple comparisons test. The significance matrix was generated by StatsCalculators (2025) (<https://www.statscalculators.com/calculators/hypothesis-testing/tukeys-hsd-test-calculator>).

Results

Hepatic iron deposition

Blue iron-containing areas were observed in hepatic parenchyma of both control and bile duct ligated (BDL) livers using PPB staining. When compared to control animals, BDL groups showed more iron deposition in the hepatic cells (Figure 1A). The induction of cholestasis induced a significant ($p<0.01$) increase in iron deposition in comparison to the liver of control rats (Figure 1B). Moreover, the iron overload was globally assessed in all cases together with a significant difference ($p<0.0001$) between the two groups (Figure 1C).

BDL increased ferritin expression in the biliary epithelium

IHC detected that ferritin protein chains FTL and FTH1 were highly expressed in cholangiocytes of BDL livers when compared to controls (Figure 2 A,B). After ligation of the main bile duct, FTL and FTH1 expressions were significantly increased together with both the iron overload and the deposition of connective tissue, typically present in the BDL livers in comparison to control mice (Figure 2 A,B). A significant difference was found in the expression of FTL ($p=0.0212$) in biliary epithelial cells of BDL rats compared to control animals (Figure 2A). The immunoreaction of FTH1 ($p=0.0159$) followed a similar profile (Figure 2B) to that of FTL.

BDL enhanced CD71 expression in biliary epithelium

TfR1/CD71 is involved in the intracellular accumulation of iron, working as a critical element in the ferroptosis process.³⁰ This role, well-known in hepatocytes, has been less investigated in cholangiocytes, the other type of hepatic epithelial cell. A significant and specific enhancement of CD71 expression was found in biliary cells of BDL livers when compared to control samples (Figure 3). Quantification of CD71 immunoreactions in the different scanned slides confirmed the increase ($p=0.02$) in the biliary epithelium.

GPX4 and ACSL4 showed opposite actions in biliary disorder

The changes in GPX4 and ACSL4 expressions between the control and BDL groups are shown in Figure 4. Particularly, GPX4 protein expression was reduced in cholangiocytes of the cholestatic murine model ($p=0.026$) when compared to the control groups (Figure 4, upper panel). However, ACSL4 was elevated in bile duct

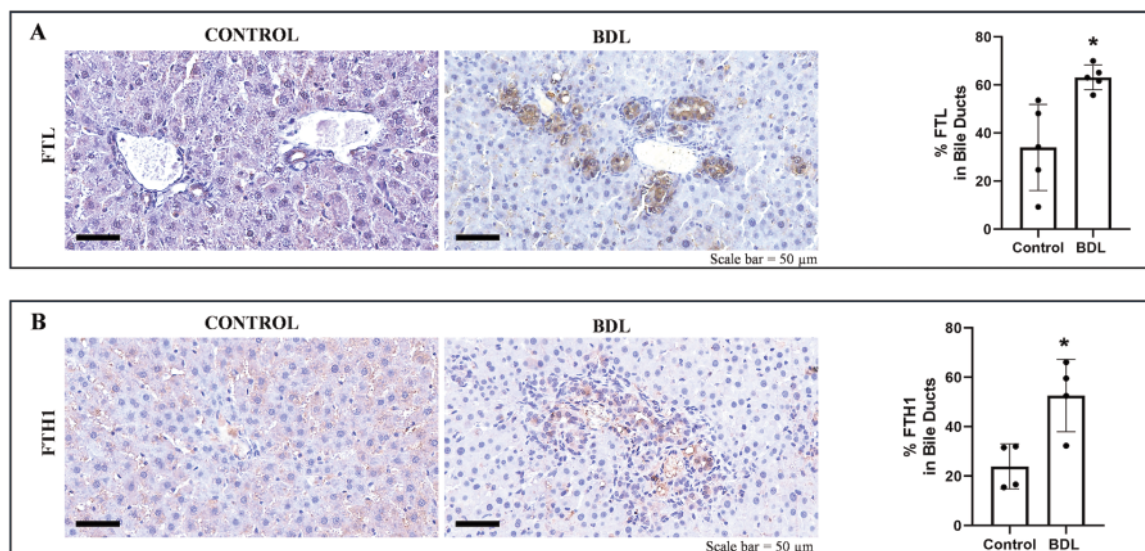


Figure 2. Iron absorbed into hepatocytes is strictly related to the cellular processes and in control conditions an excessive amount of iron is stored in ferritin. Cholestatic murine model showed elevated expression of FTL ($p=0.02$) (A) and FTH1 ($p=0.02$) in bile ducts compared to control groups (B). Scale bar: 50 μ m. Data are means $\pm$ SD of $n=5$ mice for FTL, $n=4$ for FTH1 per group. Each dot represents one liver sample analysed; * $p<0.05$ vs control group.

of BDL mice ($p=0.011$) if compared to control groups (Figure 4, lower panel). Both GPX4 and ACSL4 are important enzymes in ferroptosis, acting as antioxidant and lipid peroxidation enzymes, respectively. Overall, these data suggest that in cholestatic conditions cholangiocytes have an altered expression of both GPX4 and ACSL4, leading to ferroptosis.

Effect of erastin on the growth and viability of murine cholangiocytes

Erastin is a well-known small molecule that induces ferroptosis in a wide range of both control and transformed cell models,³¹

hepatocytes,³² and hepatocellular carcinoma.^{33,34} To verify the effectiveness of erastin on its ability to induce ferroptosis in cholangiocytes and in our model, we initially tested its toxicity at 24 h using a crystal violet growth assay. Figure 5A shows the effect of erastin on cell viability, which remains almost constant up to a concentration of 300 nM. We also observed an apparently proliferative effect of erastin at concentrations <300 nM, which returns to control values when extremely dilute concentrations around 2 nM are reached. Morphologically, the effect of vacuolisation, induced by the higher concentrations of erastin (Figure 5B) is also evident under phase-contrast microscopy, as already reported.³⁵

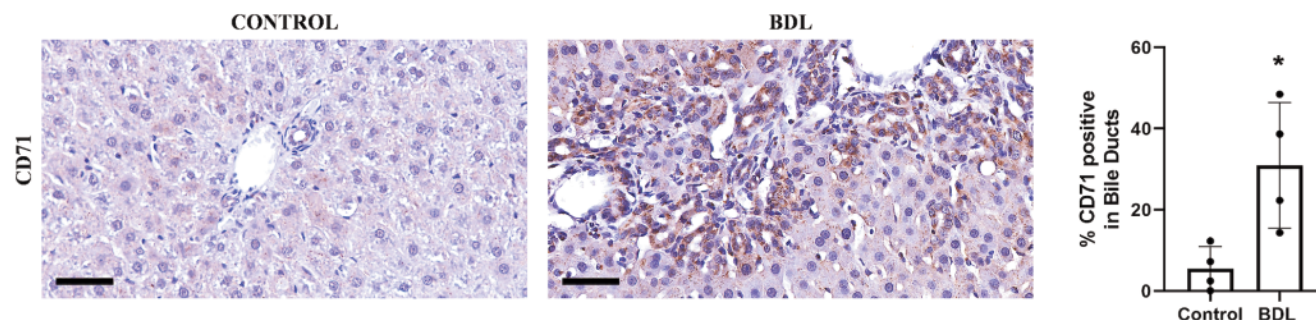


Figure 3. TfR1 or CD71, as responsible for iron absorption and metabolism, is fundamental in the process of ferroptosis. Bile ducts of BDL mice have significantly increased CD71 ($p=0.02$) when compared to control groups. Scale bars: 50 μ m. Data are means $\pm$ SD of $n=4$ mice per group. Each dot represents one liver sample analysed; * $p < 0.05$ vs control group.

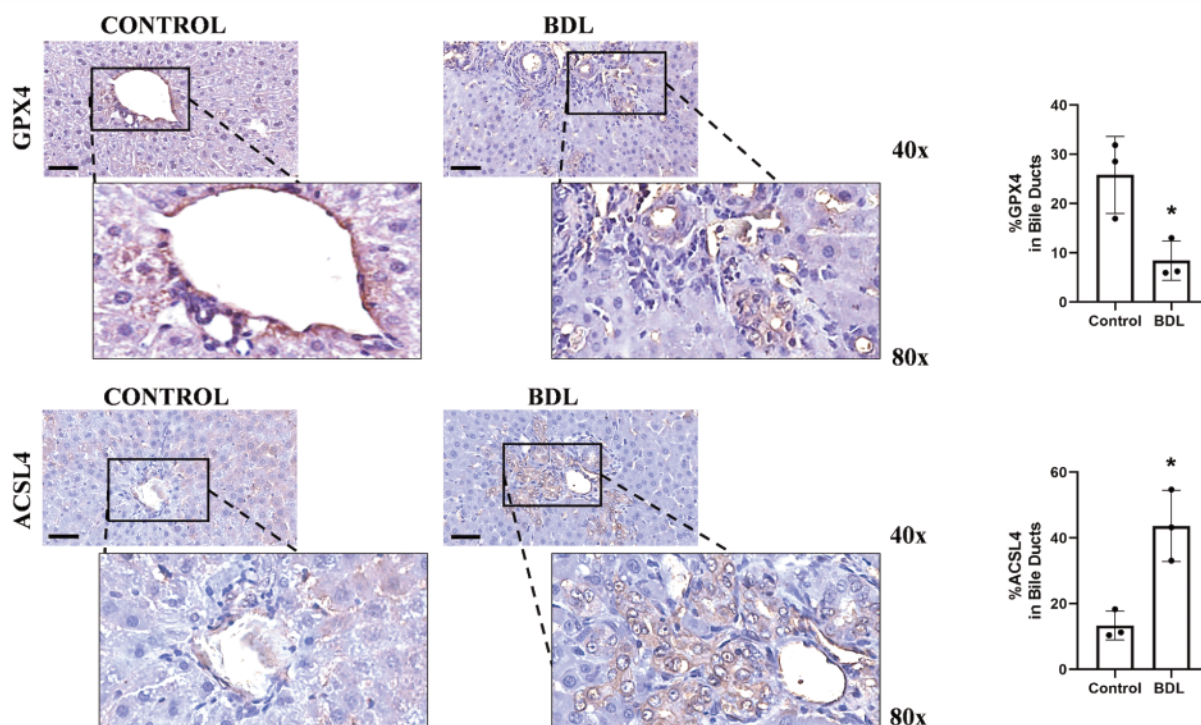


Figure 4. Immunoreactions for GPX4 and ACSL4 in control and BDL livers. The content of hepatic GPX4 was decreased in the cholestatic model ($p=0.026$) when compared to the control, whereas the expression of ACSL4 is increased ($p=0.011$) in biliary cells of BDL samples. Black boxes showed high magnification picture at 80X. Scale bars: 50 μ m. Data are means $\pm$ SD of $n=3$ mice per group. Each dot represents one liver sample analysed; * $p < 0.05$ vs control group.

Effect of sorafenib on the growth and viability of murine cholangiocytes

Sorafenib is a drug approved for the treatment of hepatocellular carcinoma, differentiated thyroid cancer, and renal cell carcinoma.^{36,37} In order to verify the effectiveness of sorafenib inducing ferroptosis in our model, its toxicity was tested at 72 h, identifying a working range between 6 and 0.3 μM (Figure 6A). Despite its high toxicity, no vacuolisation effect was observed in our cell line

at the concentrations investigated at 72 h (Figure 6B). Its failure to induce the ferroptosis process is also supported by another study.³⁸ β -mercaptoethanol, but not ferrostatin, is able to reverse erastin-induced ferroptosis in mouse cholangiocytes. Fer-1 is a synthetic ferroptosis inhibitor that acts by directly scavenging free radicals and inhibiting lipid peroxidation,³⁹ while βME acts as a reducing substrate for GPX4, a key enzyme in preventing ferroptosis.²⁷ Four concentrations of erastin were used as a single treatment and in combination with Fer-1 (1 μM) or βME (10 μM). Figure 7A shows

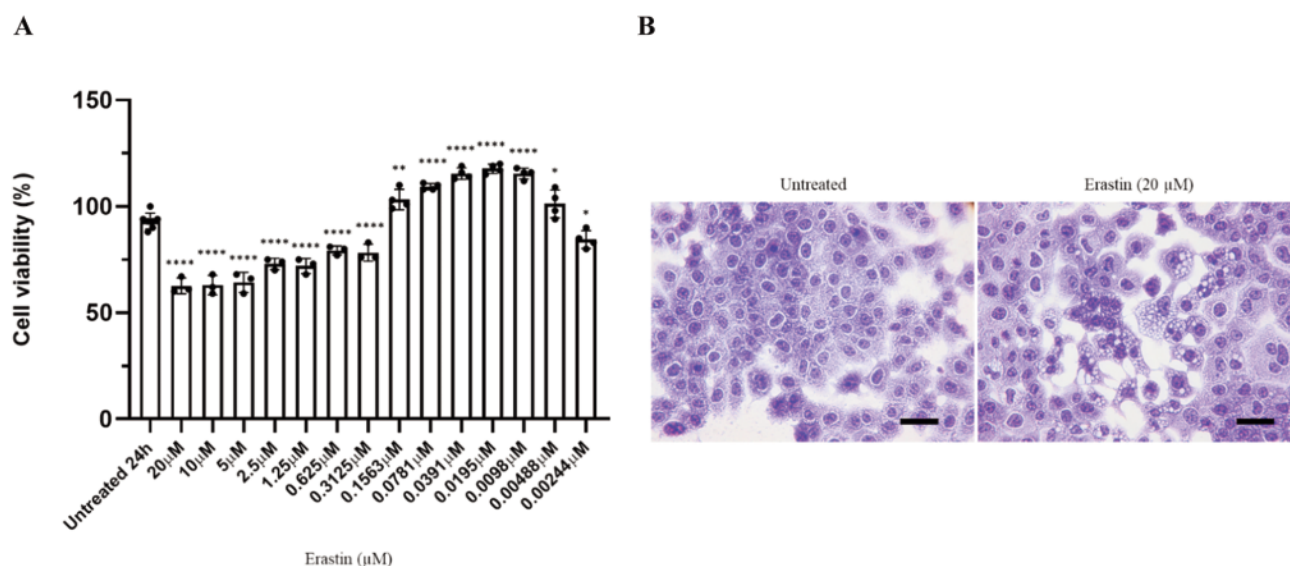


Figure 5. **A)** The effects of erastin on cell viability are maintained constantly up to a concentration of 300 nM. **B)** Phase-contrast microscopy showing the morphological changes with vacuolisation after higher concentrations of erastin, typically present in ferroptotic cells. Untreated group is used as reference for the statistical comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 20 μm .

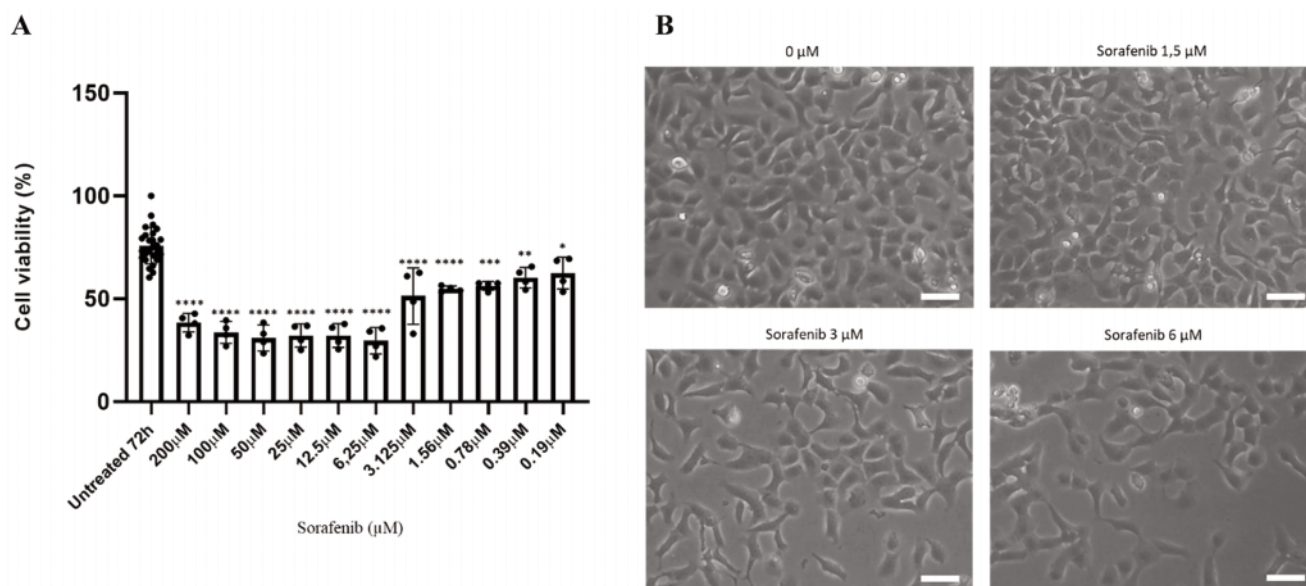


Figure 6. **A)** Sorafenib was unable to induce ferroptosis in our biliary model; we tested its toxicity at 72 h with a working range between 6 and 0.3 μM . **B)** A very high toxicity, without vacuolisation was observed in murine cholangiocytes. Untreated group is used as reference for the statistical comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bars: 20 μm .

the 24-h inhibition of erastin-induced growth, which is around a 50% reduction, as well as the non-toxicity of ferrostatin and β ME used at the indicated concentrations. The figure shows the ability of β ME to significantly reverse the effect of erastin even at the highest concentrations. However, no ability to inhibit ferroptosis was observed with combined treatment with Fer-1 at the concentrations indicated in the literature.⁴⁰ In Figure 7B, no vacuolisation in the combined erastin + β ME treatment was observed, while there was a recovery of cellular morphology comparable to the control.

Erastin induces ferroptosis by lipid peroxidation in mouse cholangiocytes

Ferroptosis is characterized by the lethal accumulation of lipid peroxides in cell membranes. In order to confirm that erastin is capable of reliably inducing ferroptosis in our model, cells were incubated with the BODIPY-C11 ratiometric probe (581/591 nm).⁴¹ Figure 8 clearly shows the fluorescence shift toward green in the sample treated with erastin (5 μ M) as early as 4 h after stimulation, confirming lipid peroxidation and ferroptosis induction.

Discussion

In this study, we investigated the role of ferroptosis in the biliary epithelium. The main findings are: i) an increase in iron deposition during cholestasis; ii) an increase in the expression of ferritin (both heavy and light chains) and CD71 (TfR1) in the BDL

model; iii) a reduction of GPX4, a crucial role player against membrane lipid peroxidation, in the BDL model compared to the control, in contrast to ACSL4, a protein regulator of lipid peroxidation, which significantly increased in BDL model; iv) erastin, a potent and specific inducer of ferroptosis, is also effective in primary cholangiocytes; v) Fer-1 was unable to inhibit ferroptosis and β ME could protect cholangiocytes from ferroptosis-induced cell death.

Several studies have indicated the involvement of ferroptosis in liver diseases such as acute hepatic injury, liver fibrosis, cirrhosis, hepatocellular carcinoma and non-alcoholic steatohepatitis (NASH), underlining its therapeutic potential.^{42,43} Indeed, patients with chronic liver disease may show hepatic, usually inside Kupffer cells, and splenic iron overloading.⁴⁴ Heme is taken up from damaged or senescent erythrocytes and broken down by the heme oxygenase-1 (HMOX1) enzyme into iron (Fe^{2+}), carbon monoxide (CO) and biliverdin.⁴⁵ Moreover, recent data showed that patients with metabolic dysfunction-associated steatohepatitis have excessive iron hepatic content with a significant relation with disease progression.⁴⁶ In this context, the role played by biliary cells in ferroptosis is still not well-known, but certainly this type of cell death could offer opposing roles depending on the cell types.

We used an experimental rat model of bile duct ligation (BDL), which represents a liver fibrosis model mediated by biliary obstruction. The progressive decrease of bile salt secretion can lead to the preservation of hydrophobic bile salts inside the hepatocytes, determining apoptosis, necrosis or other types of cell death.⁴⁷ In course of liver fibrosis induced by BDL, we showed a promotion in the expression of ferritin, CD71, ACSL4 and a parallel decrease of a ferroptosis-suppressing protein such as GPX4,

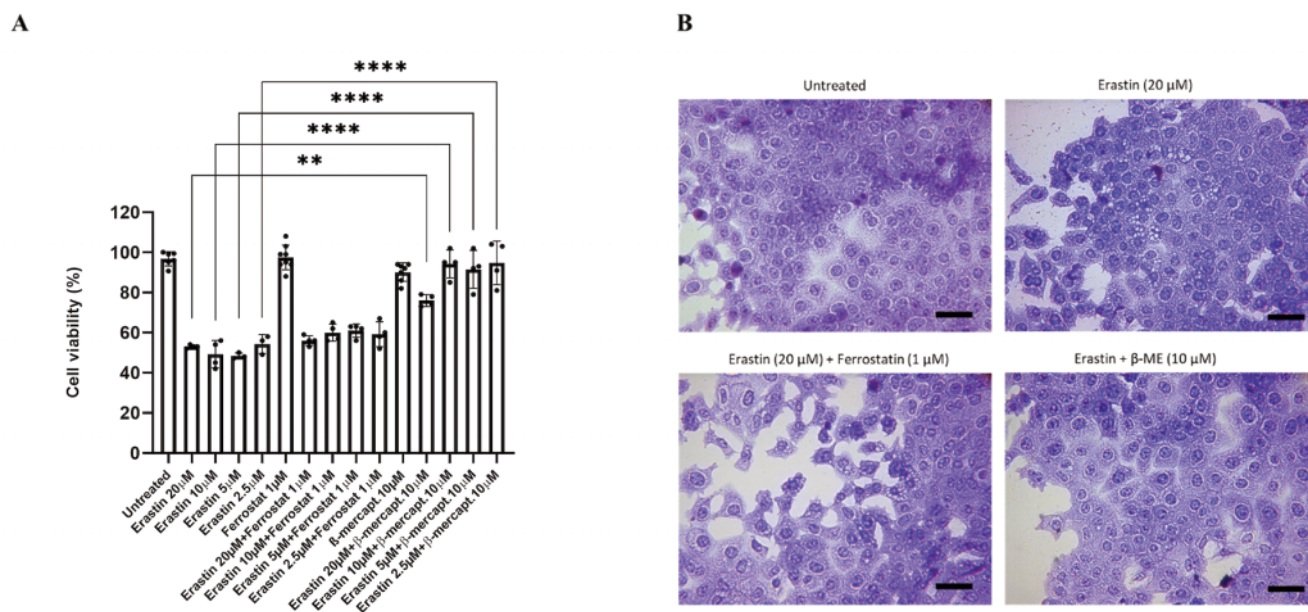


Figure 7. A) The effects of two ferroptosis inhibitors in the cell line. Four different concentrations of the inducer erastin were used in combination with Fer-1 (1 μ M) or β ME (10 μ M). **A)** We showed the 24-h inhibition of erastin-induced growth and the non-toxicity of the two inhibitors; furthermore, β ME, but not Fer-1, could significantly reverse the effect of erastin at all concentrations used (**** p <0.0001, ** p <0.01); the differences between the untreated control group and the combinations of erastin and β ME are not significant at concentrations of 10 μ M, 5 μ M, and 2.5 μ M, demonstrating β ME's ability to restore viability to the levels observed in the control group. **B)** To support these data from a morphological point of view, no vacuolisation was observed in the combination erastin + β ME. Scale bars: 20 μ m.

meaning that iron absorption, consumption and storage are affected with an increase on ferroptosis sensitivity. Indeed, CD71 transports extracellular iron into the cell, ferritin stores excess of iron, GPX4 and ACSL4 regulate lipid peroxidation and ROS generation.⁴⁸ Modifications in these proteins can negatively affect iron homeostasis inducing ferroptosis. Certainly, as an iron storage protein, FTH1 can decrease Fe^{2+} toxicity by exhibiting ferrous oxidase activity.⁴⁹ FTH1 levels are often higher in cancer cells and play an important role in iron uptake in cancer tissues. Furthermore, high levels of FTH1 expression appear to have real significance in terms of poorer prognosis in certain diseases such as acute myeloid leukemia (AML) and squamous cell carcinoma of the head and neck (HNSCC). FTH1 seems to be a protein involved in the process of metastasis, angiogenesis and cell survival.^{50,51} Therefore, it is essential to understand the mechanisms and modes of induction of the specific ferroptotic process in a pathological context. In a similar way, it may also be of significant clinical importance to comprehend the mechanism by which this process can be counteracted. Moreover, ferroptosis can be induced by erastin, which is able to inhibit the glutamate/cystine antiporter on the plasmatic membrane interfering with the synthesis of the antioxidant GSH and inducing oxidative stress.^{52,53} Erastin appears to be a promising molecule as both a radiosensitiser and

chemosensitiser. Several clinical trials are underway using an erastin analogue in combination therapy for multiple myeloma or ovarian cancer to overcome drug resistance.^{54,55} We found that erastin can induce *in vitro* ferroptosis in biliary epithelial cells supporting the concept that cholangiocytes, together with hepatocytes and hepatic stellate cells, may contribute to the progression of liver fibrosis with the activation of the ferroptotic process. This form of cell death has been demonstrated to be a process influencing drug resistance in HCC cells. Studying the mechanisms at the base of ferroptosis-mediated resistance to sorafenib in HCC cells has revealed potential target proteins and possible signaling pathways.⁵⁶ Sorafenib acts in the Raf/MEK/ERK mechanisms, blocking tumor proliferation, and inhibiting VEGF and PDGF signaling.^{57,58} Numerous data have also shown the ability of sorafenib to induce ferroptosis in several models.^{56,59,60} This was not the case in primary cholangiocytes, arguably because they can undergo ferroptosis using different pathways from those driven by the drug. On the other hand, the inhibition of ferroptosis is mainly obtained by Fer-1. In acute liver injury (ALI) mouse models, iron content was significantly increased. However, Fer-1 was able to suppress the pathological evolution by reducing hepatic iron accumulation.⁶¹ Another possible ferroptosis inhibitor is βME acting in the reduction of substrate for GPX4. It can directly decrease lipid

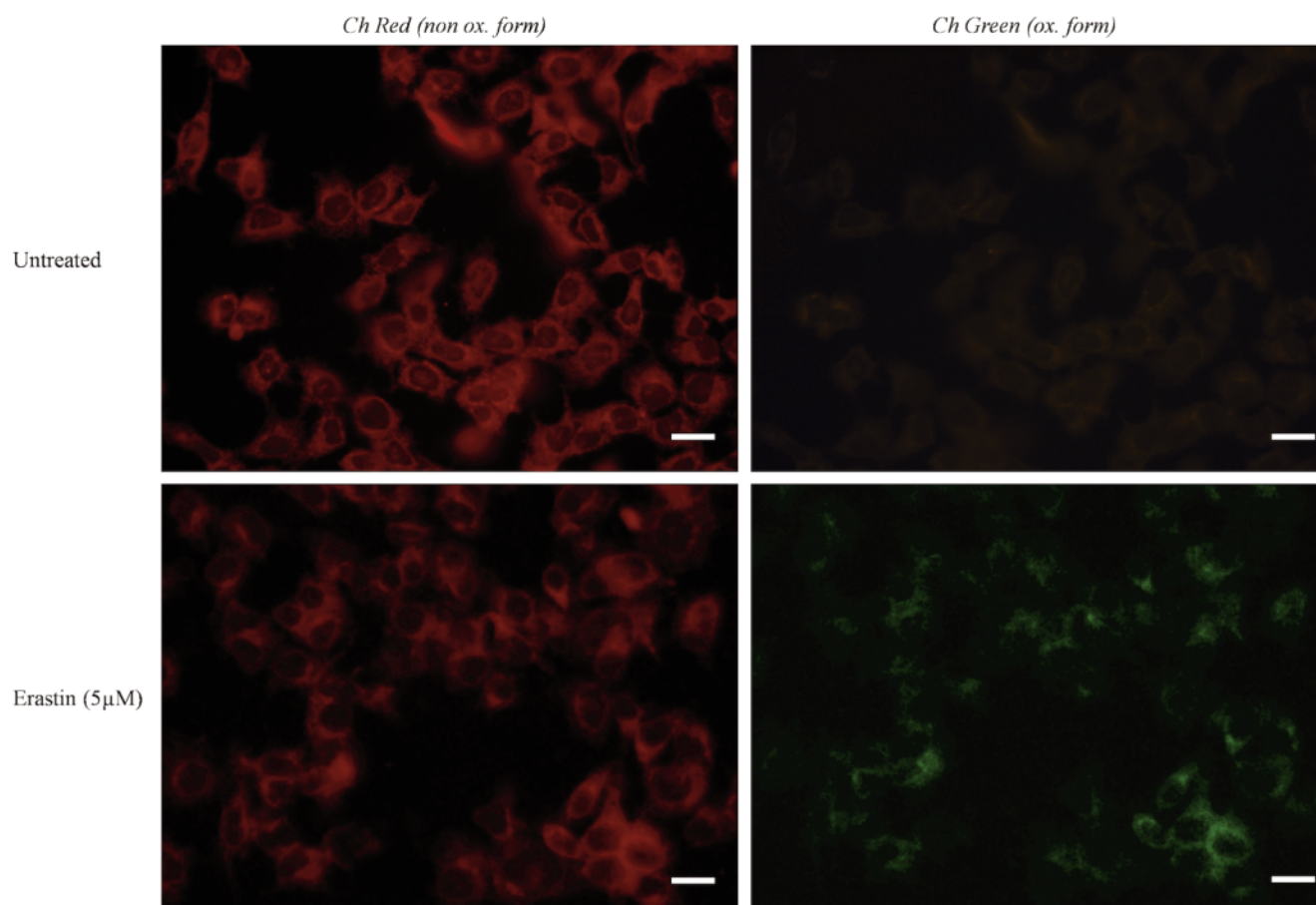


Figure 8. Representative and clear images of murine cholangiocytes showing the fluorescence shift toward green after treatment with erastin (5 μM), confirming lipid peroxidation and ferroptosis induction in our cell model. Scale bars: 20 μm .

hydroperoxides, protecting against ferroptosis-induced cell death.²⁷ Our data suggest that ferroptosis inhibition is not mediated by Fer-1 but by β ME, arguably acting on the GPX4 pathway and blocking its reduction. This process seems to be crucial in biliary cells for maintaining intracellular iron balance and preventing iron-dependent oxidative stress damage during liver fibrosis. Further investigations are warranted to substantiate these preliminary observations, as this study represents an initial step toward elucidating the biliary contribution to liver fibrosis progression through ferroptosis.

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