

MASTER
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The Role of the Heme-Exporter FLVCR1 in Hepatocellular Carcinoma

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The Role of the Heme-Exporter FLVCR1 in Hepatocellular Carcinoma

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“The people who are crazy enough to think they can change the world are the ones who
do”

Steve Jobs

Part of this project was presented as a poster in IJUP'24 – Investigação Jovem da Universidade do Porto, 17.^a edição. This event was held in Faculdade de Economia da Universidade do Porto between the 8th and the 10th of May, 2024 (submitted abstract in Appendix II). This work was also selected for an oral presentation at Innovate Competition 16.0, part of the iMed Conference Lisbon 2024.

Part of the literature review was included in a mini review published in the journal *Frontiers in Physiology*: Duarte TL, Viveiros N, Godinho C and Duarte D (2024), Heme (dys)homeostasis and liver disease (more information in Appendix III).

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Resumo

Introdução: O cancro do fígado primário é a sexta neoplasia maligna mais comum a nível mundial e a terceira causa de morte relacionada com o cancro. O carcinoma hepatocelular (HCC) é o subtipo mais comum e compreende 75-85% de todos os casos, pelo que é essencial desenvolver novas terapias para melhorar a qualidade de vida e sobrevivência global dos pacientes. O fígado é o principal local de armazenamento de ferro, elemento que tem um duplo papel na progressão do cancro. O ferro é essencial para o crescimento e proliferação das células tumorais mas em excesso pode gerar espécies reativas de oxigénio, causando dano às células e promovendo a morte celular por ferroptose. No corpo humano, cerca de 95% do ferro está na forma de heme, o que requer que a sua concentração intracelular seja altamente controlada. O feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) é um exportador de heme que se provou estar sobreexpresso no HCC e associado a um pior prognóstico, no entanto o seu papel nesta doença continua inexplorado. Temos evidência de que a supressão genética do *FLVCR1* especificamente no fígado diminui a incidência do HCC em murganhos tratados com dietilnitrosamina e CCl₄. Em paralelo, o *FLVCR1* foi silenciado (*FLVCR1*-KO) em células de HCC humanas (Huh-7) por CRISPR/Cas9. A perda de função do FLVCR1 diminuiu a proliferação e migração celular in vitro e a capacidade de invadir a membrana corioalantóica. A nossa hipótese é que o *FLVCR1* terá um papel pro-tumorigénico no HCC. Assim, o nosso objetivo é avaliar o impacto da perda de *FLVCR1* no HCC.

Métodos: O ensaio Seahorse foi utilizado para medir o metabolismo energético de células Huh-7 *FLVCR1*-WT e *FLVCR1*-KO. Induzimos expressão de luciferase em células Huh-7 *FLVCR1*-WT e *FLVCR1*-KO através de transdução com lentivírus. As células Huh-7 foram injetadas intrahepaticamente no fígado de murganhos severamente imunodeficientes (NSG-SGM3) e os tumores foram monitorizados com imagem de bioluminescência e ecografia. Transfetámos células Huh-7 com siRNAs específicos para o *FLVCR1* e o knockdown foi validado por Western Blot e RT-qPCR. O impacto do knockdown do *FLVCR1* foi avaliado pelo ensaio de Single-Cell Migration.

Resultados: O *FLVCR1*-KO alterou o perfil metabólico glicolítico habitual das células *FLVCR1*-WT para um mais dependente da fosforilação oxidativa. As células Huh-7 *FLVCR1*-WT originaram tumores de grandes dimensões no fígado de NSG-SGM3, enquanto que as células Huh-7 *FLVCR1*-KO não tiveram capacidade de formação e crescimento do tumor. O knockdown do *FLVCR1* causado pelos siRNAs

reduziu a capacidade migratória das células Huh-7.

Conclusão: O *FLVCR1* tem um papel pro-tumorigénico no HCC, constituindo um potencial alvo terapêutico para melhorar a qualidade de vida dos pacientes.

Abstract

Background: Primary liver cancer is the sixth most common malignancy worldwide and the third cause of cancer-related deaths. Hepatocellular carcinoma (HCC) comprises 75-85% of all cases, so it is crucial to develop new effective therapies to improve patients' quality of life and expected lifespan. The liver constitutes the main iron storage site, an element that has a dual role in cancer progression. Iron is essential for growth and proliferation of tumor cells but in excess can generate reactive oxygen species, causing cell damage and promoting cell death by ferroptosis. In the human body, around 95% of iron is in the form of heme, which requires its intracellular concentration to be tightly controlled. Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) is a heme exporter that was proven to be overexpressed in HCC and associated to a worse prognosis, although its role in this disease remains unexplored. We have evidence that liver-specific *FLVCR1*-KO decreases the incidence of HCC in mice treated with diethylnitrosamine and CCl₄. In parallel, we generated *FLVCR1*-KO in human HCC cells (Huh-7) by CRISPR/Cas9. The loss of FLVCR1 function decreased cell proliferation and migration *in vitro* and the ability to invade the chorioallantoic membrane model. We hypothesize that FLVCR1 plays a pro-tumorigenic role in HCC. Herein, our aim is to evaluate the impact of *FLVCR1* loss in HCC.

Methods: Seahorse assay was used to measure energy metabolism in Huh-7 *FLVCR1*-WT and *FLVCR1*-KO cells. We established Huh-7 *FLVCR1*-WT and *FLVCR1*-KO luciferase-expressing cells through lentiviral transduction. Huh-7 were injected intrahepatically into the liver of severe immunodeficient mice (NSG-SGM3) and tumors were monitored by bioluminescence imaging and ultrasound. We transfected Huh-7 cells with *FLVCR1*-specific siRNAs and the knockdown was validated by Western Blot and RT-qPCR. *FLVCR1* knockdown impact was assessed by Single-Cell Migration assay.

Results: *FLVCR1*-KO caused a shift in the metabolism of *FLVCR1*-WT cells from a glycolytic to a profile more dependent in oxidative phosphorylation. *FLVCR1*-WT Huh-7 cells originated large tumors in the liver of NSG-SGM3 mice, whereas *FLVCR1*-KO Huh-7 cells did not have the capacity for tumor formation and growth. *FLVCR1* siRNA knockdown reduced the migratory capacity of Huh-7 cells.

Conclusions: *FLVCR1* has a pro-tumorigenic role in HCC and might constitute a relevant therapeutic target to improve the quality of life of patients.

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List of abbreviations

- AASLD** – American Association for the Study of the Liver Disease
- ABCG2** – ATP Binding Cassette subfamily G member 2
- ACD** – Anemia of Chronic Disease
- AFP** – Alpha-Fetoprotein
- ALA** – 5-Aminolevulinic Acid
- ALAS** – ALA Synthase
- ALP** – Alkaline Phosphatase
- ALT** – Alanine Aminotransferase
- AST** – Aspartate Aminotransferase
- ATP** – Adenosine Triphosphate
- BCLC** – Barcelona Clinic Liver Cancer
- B-Mode** – Brightness Mode
- CCl₄** – Carbon Tetrachloride
- cDNA** – Complementary DNA
- CRISPR** – Clustered Regularly Interspaced Short Palindromic Repeats
- CT** – Computed Tomography
- Ct** – Threshold Cycle
- CO** – Carbon Monoxide
- CO₂** – Carbon Dioxide
- DBA** – Diamond Blackfan Anemia
- DEN** – Diethylnitrosamine
- DFO** – Deferoxamine
- DMEM** – Dulbecco's Modified Eagle Medium
- DMT1** – Divalent Metal Transporter 1
- DsiRNAs** – Dicer-Substrate Short Interfering RNAs
- EASL** – European Association for the Study of the Liver
- ECAR** – Extracellular Acidification Rate
- EGFP** – Enhanced Green Fluorescent Protein
- FBS** – Fetal Bovine Serum
- FeLV-C** – Feline Leukemia Virus Subgroup C
- FLVCR1** – Feline Leukemia Virus Subgroup C Receptor-related protein 1
- FLVCR1a** – Feline Leukemia Virus Subgroup C Receptor-related protein 1a
- FLVCR1b** – Feline Leukemia Virus Subgroup C Receptor-related protein 1b
- FLVCR2** – Feline Leukemia Virus Subgroup C Receptor-related protein 2
- FPN1** – Ferroportin 1

GFP – Green Fluorescent Protein
GFP+ - GFP-Positive
GP73 – Golgi Protein 73
HBV – Hepatitis B Virus
HCC – Hepatocellular Carcinoma
HCV – Hepatitis C Virus
H&E - Haematoxylin and Eosin
HH – Hereditary Hemochromatosis
HIFs – Hypoxia Inducible Factors
HO – Heme Oxygenase
HO[•] - Hydroxyl Radical
H₂O₂ – Hydrogen Peroxide
HSAN – Hereditary Sensory and Autonomic Neuropathy
IL-6 – Interleukin 6
IREs – Iron Responsive Elements
IRIDA – Iron-Refractory Iron Deficient Anemia
IRPs – Iron Regulatory Proteins
KO – Knockout
MAFLD – Metabolic dysfunction-associated Fatty Liver Disease
MFS – Major Facilitator Superfamily
MRI – Magnetic Resonance Imaging
MTT – 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium Bromide
NADPH – Nicotinamide Adenine Dinucleotide Phosphate
NASH – Non-alcoholic Steatohepatitis
OCR – Oxygen Consumption Rate
PBS – Phosphate-buffered Saline
PCARP – Posterior Column Ataxia and Retinitis Pigmentosa
PET – Positron Emission Tomography
PIC – Protease Inhibitor Cocktail
PIVKA-II – Protein Induced by Vitamin K Absence or Antagonist-II
PS – Physical Status
PUFAs – Polyunsaturated Fatty Acids
ROS – Reactive Oxygen Species
RT-qPCR – Reverse Transcription-Quantitative Polymerase Chain Reaction
shRNAs – Short Hairpin RNAs
siRNAs – Small Interfering RNAs
SLC46A1 – Solute Carrier family 46 member 1

SLC48A1 – Solute Carrier family 48 member 1

TACE – Transarterial Chemoembolization

TBS – Tris-Buffered Salin

Tf – Transferrin

TfR1 – Transferrin Receptor protein 1

TNF- α – Tumor Necrosis Factor- α

US – Ultrasonography

WT – Wild-Type

1. Introduction

1.1. Hepatocellular Carcinoma

1.1.1. Epidemiology

Primary liver cancer is the sixth most common neoplasm worldwide, with approximately 865,269 new cases in 2022, and the third cause of cancer-related death, with 757,948 deaths in the same year, according to GLOBOCAN¹. There is a significant difference between the incidence and death rates in men (12.7 and 10.9) and women (4.8 and 4.1), being about 3 times higher in the former per 100,000. Asia and Africa have the highest incidence rates, with Mongolia being the most affected country with a rate of 96.1 per 100,000 (**Figure 1**). Portugal has an incidence and mortality rates of 6.7 and 5.8 per 100,000, respectively¹.

Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer, comprising 75%-85% of all cases¹. Studies have estimated that in 2025 more than 1 million individuals will have the disease and that the incidence and death rates are set to rise in the coming decades as the population grows^{2,3}. For this reason, there is a need to avoid cases that are preventable due to being directly associated with the main risk factors^{3,4}.

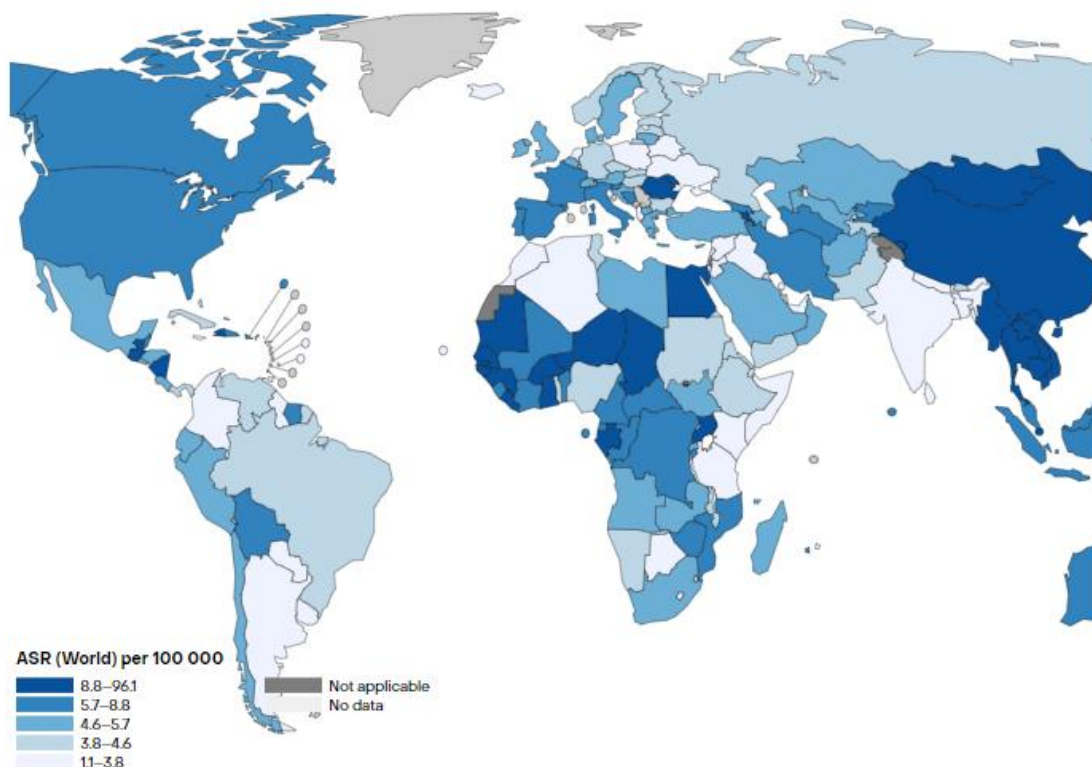


Figure 1 - Worldwide incidence of liver and intrahepatic bile ducts cancer. Age-standardized world map with incidence rates per 100,000 of liver and intrahepatic bile ducts cancer in 2022, including population of all ages and both genders. The color intensity is proportional to the magnitude of incidence. Adapted from GLOBOCAN 2022¹

1.1.2. Etiology

Hepatitis B virus (HBV) is responsible for more than 50% of all HCC cases⁵. It is possible to develop the disease in the presence or absence of cirrhosis, however most patients with HBV-induced HCC initially present with cirrhosis^{2,4}. The viral genetic material infiltrates normal hepatocytes and integrates into the host genome, originating mutations that lead to the activation of oncogenes or to the expression of viral oncoproteins, initiating the carcinogenic process^{2,4,5}. Similar to HBV, hepatitis C virus (HCV) replicates in hepatocytes, with the liver being its target organ⁶. However, HCV is an RNA virus, therefore incapable of genome integration. Through interactions mediated by viral proteins, inflammation, fibrosis and ultimately cirrhosis are promoted, which can result in HCC. In addition, these viral proteins also mediate oncogenic effects, including genetic and epigenetic alterations^{2,4,7}.

Excessive alcohol consumption, a known risk factor for HCC development, is defined as ingestion of more than 50-70 g per day for long periods of time⁷. Alcohol intake is mainly related to the development of cirrhosis, with a synergistic effect with HBV or HCV infection^{2,7}. The effect of alcohol on HCC development is also increased when combined with other risk factors such as smoking, diabetes, and obesity⁸.

Some cases of cirrhosis that later progress to HCC arise from more severe forms of metabolic dysfunction-associated fatty liver disease (MAFLD), namely non-alcoholic steatohepatitis (NASH), which are often cryptogenic forms since, once established, it is difficult to identify their cause^{2,7-9}. NASH is a chronic liver disease characterized by the accumulation of fat in the liver (i.e. hepatic steatosis), typical of MAFLD, accompanied by inflammation and damage of this organ¹⁰. This disease is often associated with the presence of Type 2 diabetes and obesity^{2,11}.

Hereditary hemochromatosis (HH), characterized by excessive iron accumulation and discussed further below, is associated with a 200-fold increased risk of developing HCC^{8,12}. Furthermore, there is evidence that iron overload can cause HCC independently of cirrhosis and can act as a cofactor for other risk factors such as HBV, HCV and alcohol^{8,13}. Therefore, it is essential that the amount of intracellular iron is strictly regulated as a means of protection against HCC development¹⁴.

Other factors that increase the risk for chronic liver damage and subsequent development of HCC include exposure to aflatoxins, primary biliary cholangitis, and α 1-antitrypsin deficiency^{2,14}.

1.1.3. Hepatocarcinogenesis

Hepatocarcinogenesis is a complex process that involves cellular and molecular alterations at the hepatocyte level culminating in a cell with malignant potential, capable of leading HCC progression¹⁵.

The risk factors previously mentioned can be a source of chronic liver injury and inflammation causing fibrosis, which is characterized by an abnormal wound healing response and accumulation of extracellular matrix proteins that replace the damaged tissue^{16–18}. This accumulation is responsible for the formation of fibrous scar, compromising the normal liver function. This process can be reversed, unless it progresses to cirrhosis, which is characterized by severe and impaired function of the organ due to increased intrahepatic resistance to blood flow^{16,17}. It should be noted that cirrhosis is a step in the development of HCC in 80-90% of patients¹⁹. The constant process of necrosis and subsequent cellular regeneration that occurs in cirrhotic or non-cirrhotic livers, in a state of chronic liver injury, leads to different alterations that give rise to precancerous nodules. These may be dysplastic nodules (≥ 1 mm) classified as low or high-grade based on cytological and architectural atypia^{19–22}. They consist of small nodules of hepatocytes that have aberrant metabolism, all of which have the potential to transform into malignant tumors, although high-grade nodules present a higher risk^{19,21}. At this stage, accumulation of genetic and molecular alterations drives the malignant transformation that characterizes HCC. High-grade dysplastic nodules differ from early-stage HCC due to the stromal invasion that is verified in the latter and several studies defend that there is a progression from one state to the other^{19,22}. Early-stage HCC refers to small well-differentiated tumors, typically < 2 cm, considered to have a good prognosis with the possibility of treatment and no significant symptoms. On the other hand, progressed HCCs are larger tumors containing cells capable of vascular invasion and metastasis, for which there are fewer therapeutic options¹⁹.

At the histological level, hepatocarcinogenesis is most often a multi-step process, although there are HCCs whose development occurs *de novo* (**Figure 2**)²³. In the first situation, the development of HCC follows the sequence of stages described above, with more aggressive poorly differentiated nodules appearing within well-differentiated ones, eventually replacing them, called a nodule-in-nodule appearance that contributes to intratumor heterogeneity^{20,23–25}. *De novo* hepatocarcinogenesis is assumed to be an alternative pathway in which the malignant tumor develops directly without precursor nodular lesions²⁰.

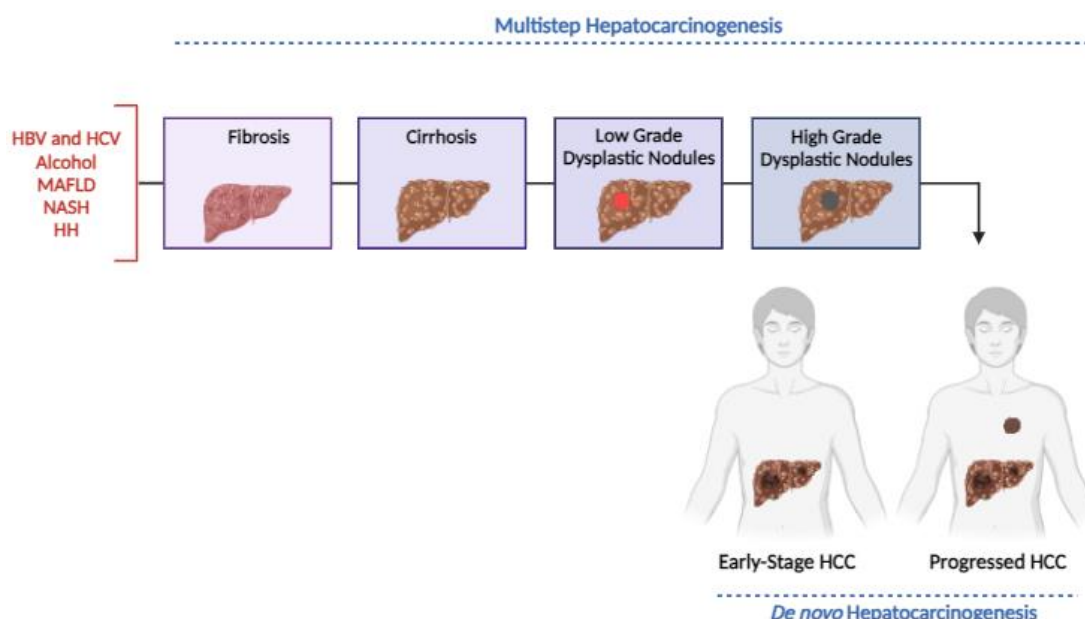


Figure 2 – Schematic representation of hepatocarcinogenesis. Presentation of the main risk factors for HCC and the consequent disease development comprising the stages of fibrosis, cirrhosis, low-grade dysplastic nodules and high-grade dysplastic nodules, in a multistep hepatocarcinogenesis, or direct development of HCC in de novo hepatocarcinogenesis. Abbreviations: HBV – hepatitis B virus; HCC – hepatocellular carcinoma; HCV – hepatitis C virus; HH – hereditary hemochromatosis; MAFLD – metabolic dysfunction-associated fatty liver disease; NASH – non-alcoholic steatohepatitis. Image created with Biorender.com.

At the molecular level, HCC tumorigenesis is always a multi-step process, which is due to a progressive accumulation of alterations with continuous selection and clonal expansion that transforms normal hepatocytes in cells presenting malignant properties¹⁵. Aberrant in more than 70% of HCC cases, the *TERT* gene is known to be the most frequently altered gene in this type of cancer. *TERT* is an oncogene not expressed in normal hepatocytes, however, when activated, increases the expression of telomerase, the enzyme responsible for maintaining telomeres, allowing cells to overcome senescence and proliferate uncontrollably²⁶. This alteration occurs early during hepatocarcinogenesis (preneoplastic lesions and early-stage HCC), either through mutation in the promoter region, genome integration of HBV in cases where the infection is verified, among other causes^{24,26}. In this disease, the *TP53* and *CTNNB1* genes are the most frequently mutated in their coding region, with frequencies between 18-50% and 18-40%, depending on the etiology, respectively^{19,24}. *TP53* is a tumor suppressor gene that encodes the p53 protein that has a recognized role in promoting apoptosis and cell cycle arrest and whose inactivation contributes to aberrant cell proliferation¹⁹. The *CTNNB1* gene, which encodes β -catenin, is involved in the Wnt/ β -catenin signaling pathway related to cell-cell adhesion and whose activation leads to cell motility and dedifferentiation. In contrast with *TERT*, *TP53* and *CTNNB1* mutations are more frequent in advanced stages of the disease²⁶. Other genetic and epigenetic alterations are also relevant to HCC tumorigenesis, namely

copy number variations, gene rearrangements, DNA methylation, histone modification and chromatin remodeling¹⁹.

1.1.4. Surveillance and Diagnosis

Most patients with HCC are asymptomatic during early stages of the disease, which contributes to the fact that 80% of patients are only diagnosed at more advanced stages²⁷. The most common symptoms include right upper quadrant pain, weight loss and the presence of a nodular, irregular, augmented liver. Furthermore, signs of cirrhosis, such as jaundice, and signs of portal hypertension, such as ascites, can also be verified, as well as hepatic bruits^{27,28}. Therefore, to detect tumors as early as possible, regular surveillance of patients at risk (cirrhotic and high-risk chronic HBV patients) is necessary²⁹.

The most used surveillance methods are ultrasonography (US) and the alpha-fetoprotein (AFP) tumor marker test, ideally carried out every 6 months³⁰. Liver US is a method that involves imaging technology that is more affordable and less invasive than other methods, such as computed tomography (CT) and magnetic resonance imaging (MRI), since it does not require the use of contrast agents^{30,31}. However, this technique has some limitations, such as the low sensitivity to detect early-stage HCC (about 47%), and to detect dysplastic nodules in the livers of cirrhotic patients. To overcome this limitation, liver US is frequently combined with the analysis of serum AFP levels^{28,30,32}. AFP is a glycoprotein produced mainly by the fetal liver and has been widely used as an HCC biomarker, usually indicating the existence of a nodule when its serum concentration is greater than 20 ng/mL. This analysis has been criticized due to low sensitivity and specificity, as AFP is not only elevated in HCC but also in other non-cancerous conditions such as pregnancy and chronic liver disease. Sensitivity and specificity of AFP as a diagnostic marker also depends on the cut-off values, patient characteristics and test design³³. Furthermore, not all early-stage HCC patients present high levels of AFP³⁴. Total AFP can be divided into AFP-L1, AFP-L2 and AFP-L3 glycoforms. AFP-L1 and AFP-L2 generally correlate with chronic hepatitis and pregnancy, respectively, whilst increased serum AFP-L3 levels are more specific to HCC patients³⁵. This finding made it possible to combine total AFP and AFP-L3, and more recently described biomarkers such as protein induced by vitamin K absence or antagonist-II (PIVKA-II) and golgi protein 73 (GP73), increasing the screening specificity of this methodology^{33,34}.

When nodules larger than 1 cm in diameter are detected on liver US, more sensitive techniques are needed for diagnosis³⁰, based on the guidelines established

by the American Association for the Study of the Liver Disease (AASLD) and the European Association for the Study of the Liver (EASL) (**Figure 3**)²⁹. In this situation, contrast-enhanced imaging is sufficient to diagnose cirrhotic patients unless the result is inconclusive, in which case a biopsy is required, despite the risks involved. In the case of non-cirrhotic patients, these imaging techniques alone are not sufficient to diagnose HCC, and a biopsy is necessary to provide histological information³⁶.

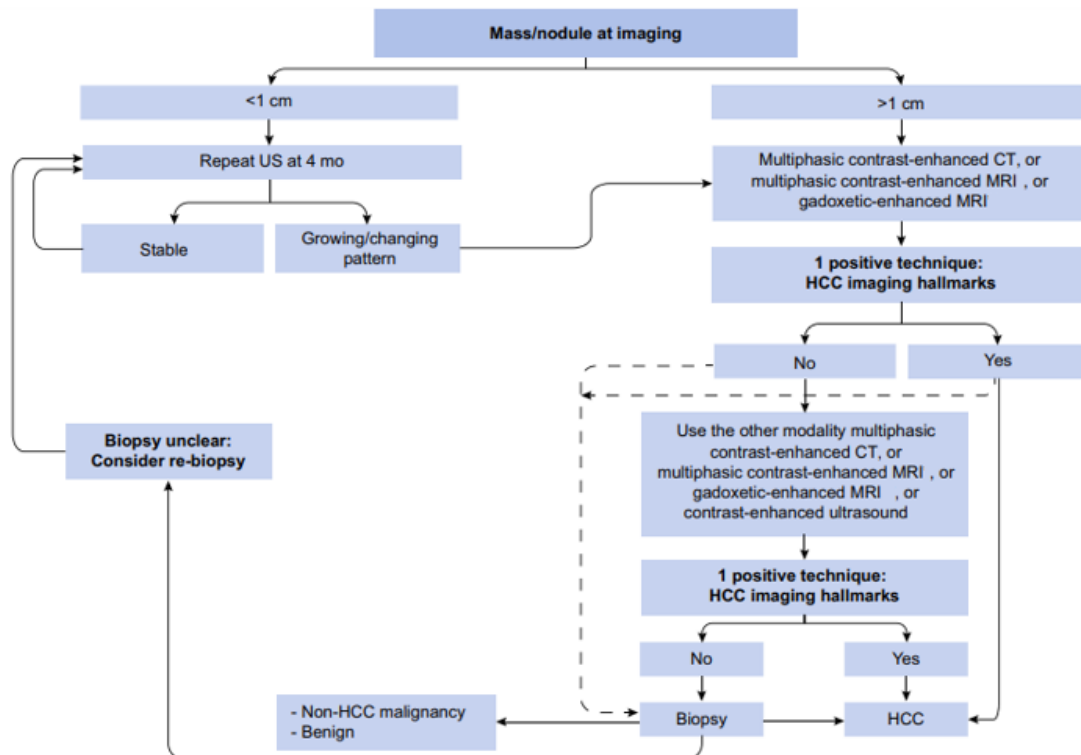


Figure 3 - Guidelines for HCC diagnosis and recall policy, provided by EASL. Abbreviations: CT - computed tomography; HCC - hepatocellular carcinoma; MRI - magnetic resonance imaging; US - ultrasonography. Adapted from Galle *et al.* 2018³⁶.

According to the National Cancer Institute, once HCC is diagnosed, it is important to investigate whether metastasis has occurred within the liver or to other organs. The search for metastatic lesions can be done using CT and MRI but also using positron emission tomography (PET) scan. At this point it is important to determine the size, location and possible metastasis of the tumor, called tumor staging, to select the most appropriate therapeutical strategy.

1.1.5. Disease Management

The Barcelona Clinic Liver Cancer (BCLC) staging system (**Figure 4**) is an algorithm used to classify patients in terms of prognosis and possible treatment. There

are five stages of HCC in this type of classification (0, A, B, C and D) according to three different factors: tumor burden, liver function and physical status (PS)^{37,38}.

The BCLC 0 subgroup corresponds to HCC in a very early stage. In this situation a single nodule with a diameter of ≤ 2 cm is observed, with no vascular invasion or extrahepatic metastasis. The liver function remains preserved, and no cancer-related symptoms are present, which means there is a good PS (PS 0). The early-stage HCC (BCLC A) presents the same characteristics as BCLC 0, differing in the number and size of the tumor. In this stage it is possible to have up to three nodules measuring ≤ 3 cm each. Patients who belong to one of these subgroups are the preferred candidates for surgical resection, liver transplantation and local ablation, treatment modalities that are potentially curative, and provide an expected average survival of more than 5 years^{30,37,38}.

Patients with large, multifocal tumors, but no vascular invasion or metastasis, belong to the BCLC B subgroup (intermediate stage). These patients still present a preserved liver function and a good PS. BCLC B is a very heterogeneous subgroup, which means that depending on the patients' characteristics and overall health status, different treatments may be indicated: liver transplantation, transarterial chemoembolization (TACE) or systemic therapies (e.g. sorafenib). The expected survival time for these patients is more than 2.5 years^{30,37,38}. TACE is the most used method to treat HCC patients and consists of the local delivery of high concentrations of chemotherapy through the artery that feeds the tumor, resulting in its blockage by embolizing particles and causing intratumoral ischemia. Importantly, the healthy liver tissue is spared as it continues to be supplied by blood from the portal vein, due to the liver's unique dual blood supply^{30,39}.

Patients included in the advanced stage HCC belong to the BCLC C subgroup. In addition to presenting multinodular disease, vascular invasion and extrahepatic metastasis are also observed. Although the liver function remains intact, the main HCC symptoms are already present (PS 1-2). For these patients, the treatment is mostly based on systemic therapies (e.g. sorafenib), with the expected median survival being more than 2 years^{30,37,38}.

The BCLC D subgroup refers to the terminal stage, in which the life expectancy is in average 3 months. These patients have impaired liver function and a poor PS presenting several cancer-related symptoms. Any of the previously mentioned treatments would be of no benefit, so it is mandatory that they receive the best supportive care^{30,37,38}.

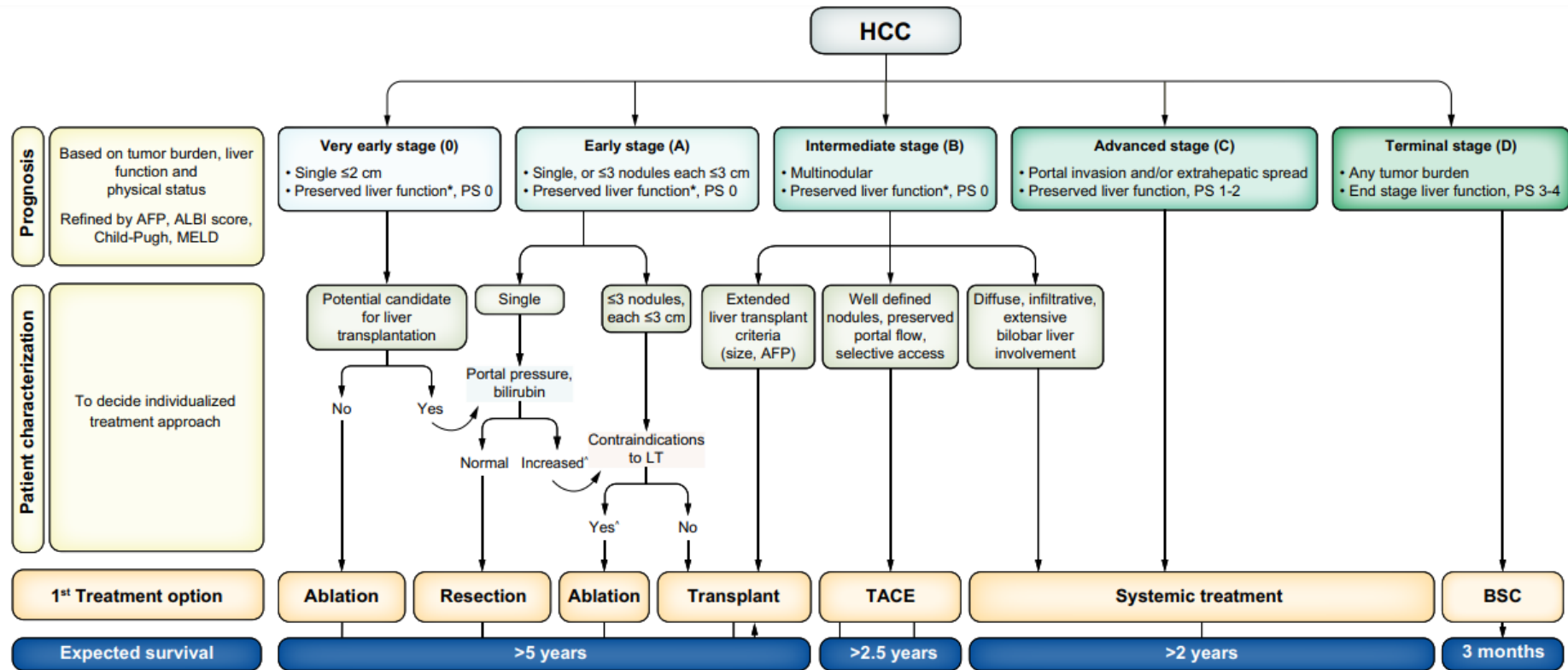


Figure 4 - BCLC staging system. BCLC algorithm to divide patients into five different groups (0, A, B, C and D), based on tumor burden, liver function and PS. The first-line treatment options for each situation and their expected survival times are also presented. Abbreviations: AFP – alpha-fetoprotein; ALBI – albumin-bilirubin; BSC – best supportive care; HCC – hepatocellular carcinoma; LT – liver transplantation; MELD – model of end-stage liver disease; PS – performance status; TACE - transarterial chemoembolization. Adapted from Reig *et al.* 2022³⁸.

1.2. Iron Metabolism and Cancer

1.2.1. Iron: Element and Physiology

Iron is one of the most abundant elements on Earth and an inorganic nutrient of utmost importance for human health, playing a major role in processes such as DNA synthesis and repair and cell respiration, signaling, growth and death^{40,41}. This element is able to interconvert between ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms, which makes it crucial in the composition of cytochromes, electron-transfer enzymes and oxygen transport proteins, such as hemoglobin and myoglobin⁴².

However, iron interconversion between two redox states induces cellular toxicity, as the cation Fe^{2+} reacts with hydrogen peroxide (H_2O_2) producing the highly reactive hydroxyl radical ($\text{HO}\cdot$), in the Fenton reaction⁴². The resulting hydroxyl radicals damage different biomolecules causing oxidative stress, which contributes to the development of several pathologies such as cancer. Specifically, $\text{HO}\cdot$ can react with DNA at the four nitrogen-containing bases and the deoxyribose backbone forming strand breaks, and thus causing genomic instability. Furthermore, $\text{HO}\cdot$ can oxidize proteins and alter their structure with consequent changes or even loss of function. Oxidative-reactions with lipids are also known to occur, creating peroxyl radicals that are responsible for the peroxidation of membrane lipids. Lipid peroxidation, in turn, is the initiating process of a form of iron-dependent regulated cell death called ferroptosis^{43–45}.

Maintaining a balance between cell death and survival is essential for homeostasis and prevention of disease development. Cell death can be programmed, with the best described type being apoptosis, in which apoptotic bodies are formed and quickly phagocytosed, without inflammation occurring. Lytic cell death is another form of programmed death that includes pyroptosis and necroptosis, both inflammatory processes. Necrosis is a form of non-programmed cell death, that involves destruction of cell membranes and release of intracellular contents⁴⁶. Ferroptosis, proposed by Stockwell *et al.*⁴⁷ in 2012, is defined as a form of regulated necrosis catalyzed by iron that occurs through unrestrained peroxidation of phospholipids containing polyunsaturated fatty acids (PUFAs) chains⁴⁸. The exact mechanism behind ferroptotic cell death is not yet well defined. However, this cell death process is characterized by oxidation of macromolecules through the action of reactive oxygen species (ROS), and consequent loss of cell membrane integrity due to disruption by lipid cross-linking⁴⁹. According to Hassania *et al.*⁴⁸, a cell undergoing ferroptosis presents the typical

morphology of necrotic cells, along with smaller dysmorphic mitochondria with a condensed membrane, as well as a disrupted outer membrane. The induction of ferroptosis has already been studied as a potential anticancer therapy to control tumor proliferation and progression ⁴⁹.

1.2.2. Molecular Basis of Iron Metabolism

The adult human body has around 2 to 4 g of iron, of which approximately 80% is allocated to the hemoglobin of red blood cells. The rest is stored in other cell types, such as macrophages and hepatocytes, or active in the form of other heme-groups or Fe-S clusters⁴¹.

Only a small portion of the ingested iron is actually absorbed by enterocytes in the duodenum and upper jejunum (1-2 mg/day). The diet can provide heme iron, from meat and poultry, and non-heme iron, from cereals and vegetables^{40,41}. To be absorbed, non-heme iron must be reduced from Fe^{3+} to Fe^{2+} for uptake through the apical membrane of the enterocytes via the divalent metal transporter 1 (DMT1). If the body needs iron, it will be exported through the basolateral membrane of enterocytes by ferroportin 1 (FPN1) and then oxidized back to Fe^{3+} by a copper-dependent serum ferroxidase, but if not required it will be stored in ferritin inside the cells, since free intracellular iron is toxic⁵⁰. Heme iron, on the other hand, is able to enter the cell directly, without the need for reduction to Fe^{2+} , and it is hypothesized that iron is released within the cell and exported into the bloodstream using FPN1, similarly to the non-heme form⁵¹.

Iron in its ferric form is loaded into transferrin (Tf) and transported through the blood to be used by cells, for example muscle cells to produce myoglobin. However, most iron in the organism is consumed by erythropoiesis in the bone marrow, which consists in the generation of around 200 billion red blood cells per day^{52,53}. In erythrocytes, ferrous iron is incorporated into hemoglobin which comprises four subunits, each containing one heme group and one polypeptide chain. Hemoglobin is responsible for transporting oxygen to all organs and tissues⁵⁴. Senescent erythrocytes are recognized by reticuloendothelial macrophages that act by phagocytosing the cell and recycling its iron for further use. Erythrophagocytosis is a highly efficient process, allowing only a small amount of iron to be absorbed through the intestine, important to compensate for the iron loss in events such as menstrual and accidental bleeding and pregnancy^{40,55}.

The liver plays a major role in iron metabolism, constituting the main iron storage site⁵⁵. Excess iron, not required for metabolic processes elsewhere in the

human body, is maintained mainly in hepatocytes, as these cells are able to synthesize high amounts of ferritin⁵⁶. When ferritin is saturated, iron storage in an insoluble complex called hemosiderin elevates, making it less readily available if required by the organism⁴⁰. The liver is also essential for mobilization of iron into circulation when it is required in other tissues⁵⁵. In this situation, ferritin is degraded in the hepatocytes and iron is exported by FPN1 into the bloodstream, where it is loaded into Tf and transported throughout the body⁵⁷ (**Figure 5**). Besides, the liver is the main responsible for the production of proteins important for the regulation of iron homeostasis⁵⁵. In addition to ferritin, the liver also produces Tf, with smaller amounts being produced in the brain and testis⁵⁰. Iron absorption, recycling and efflux from the liver are negatively regulated by hepcidin, a hormone also produced in hepatocytes that inhibits FPN1, and thus prevents the release of iron from enterocytes, reticuloendothelial macrophages and hepatocytes into the plasma^{58,59}. Hepcidin deficiency is considered the main cause of hereditary hemochromatosis⁵¹. Therefore, the liver's ability to maintain iron homeostasis is of the utmost importance and its disruption leads to iron-related disorders.

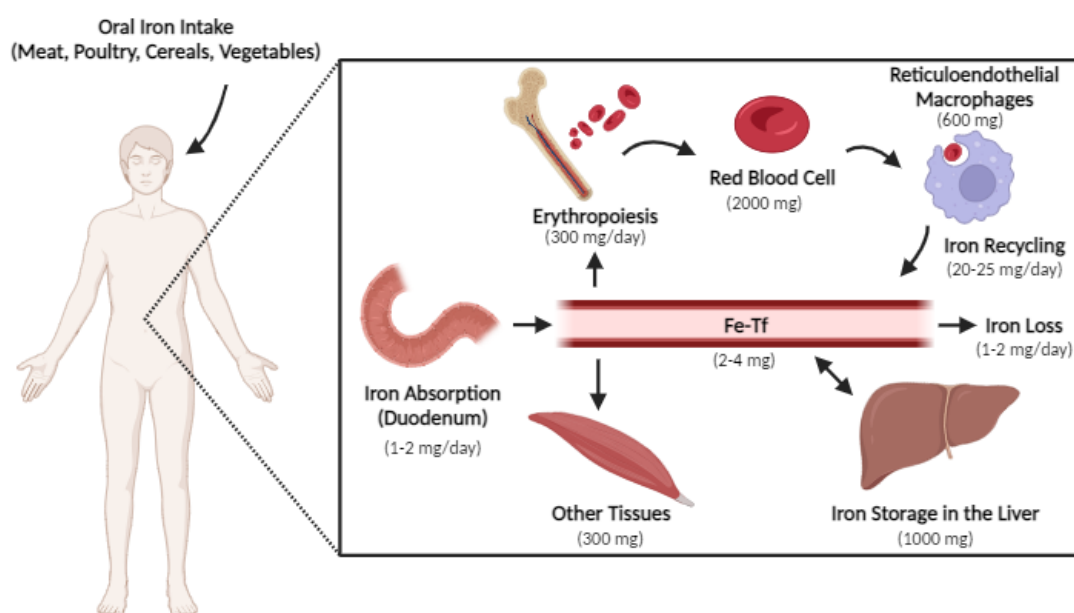


Figure 5 – Schematic representation of iron metabolism. Iron is acquired through the consumption of certain foods so that a small part can be absorbed from the duodenum into the bloodstream where it circulates in complex with Tf. Part of this iron is used to produce erythrocytes and another part for other tissues such as muscle. Excess iron is stored in the liver until required. Most of the iron that circulates in the body is obtained through recycling, carried out by reticuloendothelial macrophages. A small amount of iron is lost in events such as bleeding. These values are an average of the amount of iron stored or spent in each process. Image created with Biorender.com.

1.2.3. Dysregulation of Iron Homeostasis and Related Disorders

The human body has three main mechanisms to balance iron metabolism *in vivo*: (i) intracellular iron homeostasis is mainly controlled by iron regulatory proteins (IRPs) that bind to iron responsive elements (IREs) in specific mRNAs and, depending on the location of the IREs (5' vs 3'), either prevent their translation or stabilize the mRNA, (ii) serum iron levels depend on the negative action of hepcidin upon the cellular iron exporter FPN1, (iii) hypoxia inducible factors (HIFs) regulate the transcription of genes related to iron metabolism, for example, genes responsible for hepcidin suppression and increased FPN1 synthesis^{60–62}. Dysregulation at a systemic level, related to the hepcidin/ferroportin system, is responsible for the two major iron-related diseases: anemia and HH⁶⁰.

Anemia is a public health problem that affects up to a billion people worldwide, caused either by malnutrition, blood loss or genetic conditions. To be diagnosed with anemia, hemoglobin levels must be <120 g/L for women and <130 g/L for men over 15 years of age⁶⁰. Moreover, iron deficiency anemia requires ferritin concentrations <15 µg/L for women and <30 µg/L for men⁶³. In these situations, a greater production of hepcidin by the liver inhibits the action of FPN1, decreasing iron export into circulation⁶⁴. In the anemia of chronic disease (ACD), an inflammatory anemia, cytokines mediating inflammatory reactions include interleukin 6 (IL-6), which activate the STAT signaling pathway in liver cells, promoting hepcidin transcription. In iron-refractory iron deficient anemia (IRIDA), a rare genetic condition, iron deficiency is caused by a mutation in the *TMPRSS6* gene, which results in elevated hepcidin levels⁶⁵.

HH is an autosomal disease frequent in western populations, characterized by an accumulation of iron in vital organs, with the liver being the most affected. HH is caused by mutations in genes that limit iron export into the blood⁶⁶. The most common type of HH is due to a homozygous mutation (C282Y) in the *HFE* gene (~90% of cases) which encodes the HFE protein essential for the normal synthesis of hepcidin by the liver^{66,67}. As *HFE* is mutated in this disease, hepcidin levels are decreased with a consequent increase in serum iron levels, Tf saturation and ferritin levels⁶⁸. Iron accumulation in target organs leads to oxidative stress and organ damage, resulting in severe conditions, such as cirrhosis and cancer⁶⁰.

Diseases related to cellular iron overload include alcoholic liver disease, MAFLD, NASH, chronic HCV, which are all risk factors for HCC development, as previously addressed⁶⁰.

1.2.4. Importance of Iron Metabolism in Carcinogenesis

Systemic iron metabolism is often dysregulated in cancer patients, detected in the form of anemia in >40% of cases. This is a cancer-induced anemia, in which cytokines are produced, such as tumor necrosis factor- α (TNF- α) and IL-6, leading to iron restriction (through the hepcidin/ferroportin system) and reduced erythropoiesis⁶⁹.

At the cellular level, cancer cells tend to acquire and retain more iron. Therefore, proteins that mediate iron import are commonly upregulated in several types of cancer, such as the importer DMT1 and the transferrin receptor protein 1 (TfR1), which mediates the entry of iron into the cell through endocytosis. In turn, the exporter FPN1, tends to be downregulated since hepcidin is overexpressed, which is in line with anemia^{70,71}. Ferritin is upregulated in various types of cancer, such as acute myeloid leukemia, HCC and glioblastoma. In HCC, ferritin upregulation translates into an increased cell proliferation, since greater iron storage capacity will decrease the production of ROS and, consequently, cell damage and death by ferroptosis. In glioblastoma, ferritin upregulation fosters cell growth by deactivation of pro-apoptotic pathways⁷². These alterations are achieved through regulation by oncogenes and tumor suppressor genes^{70,71}.

Intracellular iron plays a crucial role in the initiation, growth and metastasis of tumor cells, which are considered to have a greater dependence on iron than non-malignant cells^{73,74}. Iron is carcinogenic, as it plays a role in tumor initiation through the production of free radicals and consequent genomic instability and mutagenicity⁷⁴. Elevated iron levels in cancer cells foster tumor growth because iron and iron-containing molecules are essential for DNA synthesis, energy production and cell division⁷¹.

Iron dysregulation in tumor cells has been explored for therapeutic benefit⁷¹. Iron chelators are small molecules that bind iron with high affinity, such as deferoxamine (DFO)⁷⁰. These molecules have traditionally been used to deplete tumor cells of iron to prevent proliferation, and more recently developed to increase the redox activity to generate intracellular ROS and induce cell death^{70,75}. The exploitation of TfR1 as a cancer therapy has also been investigated, either with the use of antibodies to prevent the entry of iron into cells or through the delivery of chemotherapeutic compounds through this membrane receptor^{71,76,77}.

1.3. Heme

1.3.1. The Physiology of Heme

Heme, a complex of iron and protoporphyrin IX, acts as a prosthetic group in several hemoproteins, with more than 95% of the heme in the human body being found in hemoglobin and myoglobin^{78–80}.

This iron-containing porphyrin carries out different cellular functions, depending on the hemoprotein in which it is incorporated: oxygen transport and storage in hemoglobin and myoglobin, respectively; H₂O₂ inactivation in catalases and peroxidases; electron transport, energy generation and chemical transformation in cytochromes; oxidation of tryptophan and others. Heme also influences the expression of several genes that code for other important proteins such as globin, heme biosynthetic enzymes and TfR1^{78,80}.

Heme-bound iron is involved in oxidation-reduction reactions that generate ROS, which oxidize cellular components, such as proteins, lipids and nucleic acids, causing oxidative damage. The intracellular free heme pool, comprised by heme that has been recently synthesized and not bound to a protein or that has been released, is responsible for these oxidative attacks, due to the lipophilic structure of heme that allows it to intercalate cell membranes^{80,81}. Excessive ROS have a pro-inflammatory effect and lead to erythrocyte hemolysis, and endothelial dysfunction⁸⁰.

Therefore, heme is a double-faced iron-containing molecule whose metabolism must be tightly controlled to maintain homeostasis⁸⁰.

1.3.2. Intracellular Heme Regulation

Heme synthesis, mainly performed by developing erythroid cells in the bone marrow and hepatocytes, is a highly conserved process that involves eight enzymes, four of which are cytoplasmic, whilst the remaining four are mitochondrial^{79,82}. The first and rate-limiting reaction, is the condensation of succinyl-coA and glycine to form 5-aminolevulinic acid (ALA), which is catalyzed by ALA synthase (ALAS) in the mitochondrial matrix. There are two different genes for ALAS: *ALAS1*, expressed ubiquitously, and *ALAS2*, expressed exclusively in erythroid precursors⁸³. It has been demonstrated that, through the inhibition of transcription of *ALAS1*, heme regulates its own synthesis^{80,83,84}.

Heme degradation is an important mechanism to protect cells from heme-induced oxidative injury and is catalyzed by heme oxygenase (HO). Most of the iron-containing porphyrin that is degraded comes from the hemoglobin present in

senescent erythrocytes that are phagocytosed by reticuloendothelial macrophages in the spleen and liver. The reaction releases iron, which is recycled, and the final products carbon monoxide (CO) and biliverdin, which is eventually reduced to bilirubin. Oxidative stress, hypoxia and high heme levels stimulate HO-1, the inducible isoform of HO, producing compounds with antioxidant properties, hence its protective capacity against cellular damage^{79,80,85}.

A tight regulation of heme import and export is also essential to control the amount of intracellular heme. Heme is imported into the cells by: two putative importers found in the plasma membrane, solute carrier family 46 member 1 (SLC46A1 or HCP1) and feline leukemia virus subgroup C receptor-related protein 2 (FLVCR2), and by one transmembrane transporter on endolysosomes, solute carrier family 48 member 1 (SLC48A1 or HRG1). In turn, heme is known to be exported from the cell by two transporters present in the plasma membrane, the feline leukemia virus subgroup C receptor-related protein 1a (FLVCR1a) and the ATP binding cassette subfamily G member 2 (ABCG2). The latter is known for extruding different molecules and xenobiotics^{80,86}. Once synthesized in the mitochondria, heme is exported to the cytoplasm through the feline leukemia virus subgroup C receptor-related protein 1b (FLVCR1b) found in the mitochondrial membrane⁸⁷.

1.3.3. Role of Heme in Cancer

The main enzymes involved in heme synthesis, such as ALAS, are often upregulated in cancer cells, which results in an accumulation of ALA and protoporphyrin IX. This leads to an increase in heme synthesis, which is supported by the observation of greater activity of heme-containing proteins and increased heme content in cancer cells^{86,88}. Higher heme levels may be important to maintain the activity of the mitochondrial respiratory chain for energy production, since it is part of the composition of cytochromes⁸⁸. This hypothesis contradicts the “Warburg effect”, firstly described by Otto Warburg, which stipulates that tumor cells undergo metabolic changes and rely primarily on glycolysis rather than oxidative, with a larger uptake of glucose and production of lactate, even in aerobic conditions⁸⁹. However, in the presence of oxygen and excess heme, tumor cells shift their energy production back to the oxidative phosphorylation pathway, and thus mitochondrial respiration becomes essential for the metabolism and bioenergetics of these cells^{88,90}.

Heme importers also tend to be overexpressed in different types of cancers, leading to a greater hemoprotein activity. Interestingly, heme export has also been shown to be upregulated in cancer. Increased heme efflux may occur to establish a

balance in intracellular heme homeostasis, but may also be the result of cellular compartmentalization, in a way that newly synthesized heme traffic may be handled differently than exogenous heme imported into cells⁸⁶. In lung cancer cells, researchers have shown that decreased heme synthesis and import leads to limited proliferation and migration, while supplying this iron-containing porphyrin has the opposite effect⁸⁸.

HO-1, the enzyme responsible for heme breakdown, has also been shown to be increased in several types of cancer such as brain tumors and melanoma⁸⁸. It has been suggested that this enzyme has a dual role, as moderate levels of HO-1 expression have a cytoprotective effect for tumor cells, while over-activation of HO-1 has anti-tumor properties since the reaction it catalyzes releases iron, so greater production of ROS is expected, resulting in cellular damage. Clinically, patients with higher HO-1 levels are associated with poorer outcomes⁹¹.

In addition to the effect at the tumor cell level, heme is also able to modulate the tumor microenvironment, composed of immune and stromal cells, blood vessels and extracellular matrix, for instance, by modulating the activity of tumor-associated macrophages and the angiogenic process^{86,92}.

1.4. The Heme Exporter FLVCR1

1.4.1. FLVCR1 Structure and Physiology

FLVCR1 was first identified as the receptor of the feline leukemia virus subgroup C (FeLV-C), a retrovirus that mainly infects cats and affects all hematopoietic cells by preventing the function of its receptor due to continuous binding, culminating in severe anemia and red blood cell aplasia^{93,94}. However, the *FLVCR1* gene has been conserved throughout evolution and has orthologs in several other living organisms, including humans⁹⁴.

FLVCR1 is a known heme exporter mainly expressed in the liver and small intestine, the organs that require greater heme trafficking⁹⁴. This protein belongs to the major facilitator superfamily (MFS) of transporters, recognized for the transport of small solutes across membranes⁹⁵. The *FLVCR1* gene, also known as *SLC49A1*, is a ubiquitously expressed gene positioned on the long arm of chromosome 1 (1q.32.3) that codes for two isoforms^{96–98}. The first isoform, FLVCR1a, is composed of 560 aminoacids and has a molecular mass of about 55-70 kDa⁹⁹. It presents 12 transmembrane α -helices connected by hydrophilic loops and is found in the plasma membrane (**Figure 6**)^{95,97}. According to the initial reports, its function is to export heme

out of the cell, being important for heme detoxification^{95,97,100}. This export was found by Yang *et al.* to be totally dependent on the presence of a heme-binding protein on the extracellular space, specifically hemopexin¹⁰¹. The second isoform, FLVCR1b, is composed by 6 transmembrane domains that require homodimerization or heterodimerization to be functional¹⁰². This protein is found in the mitochondrial membrane and its main function is to export newly synthesized heme from the mitochondria to the cytosol⁹⁵. The joint action of both isoforms is essential to control the intracellular free-heme pool in order to maintain homeostasis.

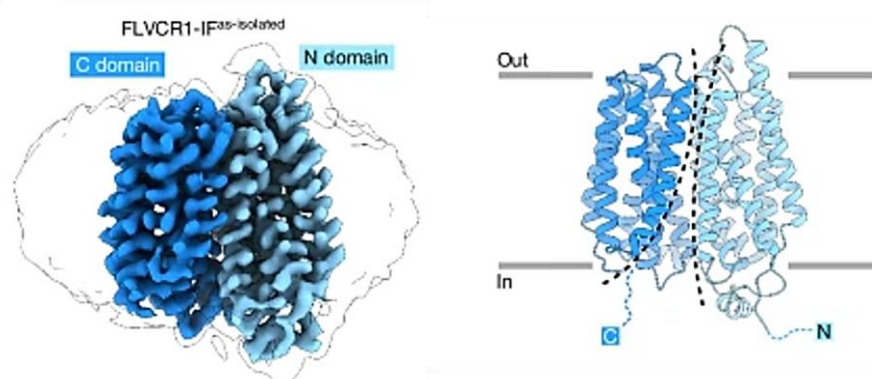


Figure 6 – FLVCR1a architecture. Inward-facing FLVCR1a protein structure obtained by cryogenic electron microscopy (left) and the respective atomic model (right). Adapted from Ri *et al.* 2024⁹⁸.

FLVCR1 has been reported to have an important role in erythropoiesis, since its isoforms control the size of the heme pool necessary for hemoglobinization^{94,102}. In the study carried out by Mercurio *et al.* it was concluded that FLVCR1a is required for the expansion of committed erythroid progenitors, as the heme pool must be restricted enough to allow cell proliferation but maintain the necessary levels for metabolic activity. In turn, only FLVCR1b is required for the terminal differentiation of these cells, as a greater amount of heme is needed to allow hemoglobinization^{102,103}. In another study, Petrillo *et al.* elucidated the role of FLVCR1a in the establishment of a functional vascular network, with the protein being preferentially expressed in angiogenic endothelial cells where it is essential for efficient developmental and adult angiogenesis¹⁰⁴. Erythropoiesis and angiogenesis are both hypoxia-stimulated processes and Fiorito *et al.* proved that the expression of this exporter is regulated by hypoxia through HIF2 α and ETS1, in colorectal adenocarcinoma cells⁹⁷. Additionally, Fiorito *et al.* concluded that FLVCR1 and ALAS1 are related to the transition between the glycolytic metabolism and oxidative phosphorylation that occurs in the shift from proliferation and differentiation, as well as to the reverse that is verified in the carcinogenesis process^{99,105}.

Importantly, FLVCR1 has recently been demonstrated to be involved in choline and ethanolamine import across the plasma membrane^{98,99,106}. Choline and ethanolamine are the precursors of the two most common phospholipids in mammalian cells, phosphatidylcholine and phosphatidylethanolamine, essential for cellular proliferation¹⁰⁷. Choline is also crucial for the synthesis of an important neurotransmitter, acetylcholine. Kenny *et al.* demonstrated that *FLVCR1* depletion together with choline deprivation led to extensive mitochondrial dysfunction¹⁰⁶. There is an ongoing debate about the impact of FLVCR1 as a heme exporter or a choline/ethanolamine importer on *FLVCR1*-related disorders that needs elucidation to determine the physiological role of the protein⁹⁹.

1.4.2. *FLVCR1*-related disorders

Over the past few years, *FLVCR1* has been associated with different diseases. Its involvement in diamond blackfan anemia (DBA), an inherited disorder characterized by a moderate to severe decrease in the production of erythroid progenitor cells, was described in 2008 by Rey *et al.* As previously discussed, FLVCR1 is crucial for the expansion and differentiation of erythroid progenitors. It was concluded that in DBA there is an increase in alternative splicing of *FLVCR1* transcripts, leading to disruption of its expression and function, which culminates in a reduced production of these cells¹⁰⁸. In 2010, Rajadhyaksha *et al.* described the role of homozygous missense mutations in the *FLVCR1* gene in posterior column ataxia and retinitis pigmentosa (PCARP), an autosomal-recessive neurodegenerative syndrome. These mutations interfere with the transmembrane export of heme, initiating a cascade of cell death that results in the degeneration of retinal photoreceptors and sensory neurons in the posterior spinal cord¹⁰⁹. However, FLVCR1 has also been reported to have a role in retinitis pigmentosa without posterior column ataxia, by Yusuf *et al.* in 2018. In this condition, splice-site variants were found in patients, which may result in sufficient functional *FLVCR1* translation for tissues where the protein is expressed at lower levels but not sufficient for tissues that express FLVCR1 at higher levels, such as the retina, in which the toxic effects of excess intracellular heme will be detected⁹⁶. The role of FLVCR1 in hereditary sensory and autonomic neuropathy (HSAN), a genetic condition that is characterized by the debilitation of sensory neurons that transmit information about sensations such as pain, was discovered by Chiabrando *et al.* in 2016. In HSAN, mutations in *FLVCR1* prevent the export of heme causing its accumulation in sensory neurons, which leads to cellular damage due to oxidative stress⁹⁵. Regarding malignant tumors, FLVCR1 was reported to be overexpressed in

synovial sarcoma, by Peng *et al.* in 2018, and esophageal squamous cell carcinoma, by Zhou *et al.* in 2021^{100,110}. In the former, FLVCR1 expression contributes to tumor proliferation and progression by inhibiting autophagy and apoptosis¹⁰⁰. In the latter, the proliferation, invasion and metastasis are promoted by the increased expression of FLVCR1 through upregulation of CSE1L, a homolog of the chromosome segregation protein¹¹⁰.

FLVCR1 has also been reported to be overexpressed in HCC and in bovine papillomavirus-associated urinary bladder cancer. In the former case, analyses of publicly available RNAseq data revealed that increased FLVCR1 expression was found to be associated with vascular invasion, inflammation in adjacent tissues, higher neoplasm disease staging and histologic grade, as well as poorer outcomes^{86,99,111}. However, the specific mechanism by which *FLVCR1* affects HCC remains unclear¹¹².

2. Aims

HCC is the most prevalent subtype of liver cancer, comprehending 75-85% of all cases, which in turn is the sixth most common cancer worldwide¹¹³. Its poor prognosis is due to the fact that the diagnosis is only formed at more advanced stages, therefore it is vital that new effective therapies are developed in order to attempt to improve patients' quality of life and expected lifespan²⁷. *FLVCR1* has been detected to be overexpressed in several tumors including HCC, as well as associated to a worst prognosis, which suggests that tumor cells have a greater export of heme than normal cells^{86,111}. Since excess heme has a known toxic effect due to the presence of iron in its structure and its role in the production of ROS, its exporter may act as a mechanism to reduce the presence of these molecules in cancer cells and consequently decrease cell damage and death by ferroptosis^{45,80}.

Our group has previously observed that liver-specific *FLVCR1* knockout (KO) decreases the incidence of HCC in mice treated with diethylnitrosamine (DEN) and carbon tetrachloride (CCl₄), a well-established mouse model of chemical-induced carcinogenesis. Furthermore, we induced the KO of *FLVCR1* in a human hepatoma cell line, Huh-7, using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. In our published work, we concluded, that *FLVCR1*-KO cells have a lower proliferative and migratory capacity, as well as more accumulation of heme, *in vitro*, compared to *FLVCR1*-WT cells, and less capable of invading the chick embryo chorioallantoic membrane model.

The main goal of this dissertation is to further explore the role of *FLVCR1* in hepatocellular carcinoma.

The specific objectives of the project are:

1. Evaluate the putative contribution of *FLVCR1* to the energy metabolism of HCC cells by measuring the metabolic phenotype of *FLVCR1*-WT and *FLVCR1*-KO cells *in vitro*.
2. Evaluate the impact of *FLVCR1* loss in human HCC cells *in vivo*, by generating *FLVCR1*-KO/WT human HCC cells (Huh-7) with stable luciferase expression and implanting them in the liver of immune-deficient mice (orthotopic xenograft model).
3. Perform a first assessment of the therapeutic potential of silencing *FLVCR1*, by evaluating the potential of small interfering RNAs (siRNA) targeted at *FLVCR1* to inhibit HCC cell (Huh-7) migration *in vitro*.

3. Methods

3.1. Cell Lines and Culture Conditions

The HEK293T cell line is derived from human embryonic kidney and carries the SV40 T-antigen, which makes it highly used for lentivirus production. This cell line was available at i3S, University of Porto. The Huh-7 hepatoma cell line, isolated from a 57-year-old Japanese male in 1982, was provided by Prof. Maria Carmo-Fonseca from the Institute of Molecular Medicine, University of Lisbon. The Huh-7 *FLVCR1*-KO clones (Clones 1 and 2) were previously generated by our group through the CRISPR/Cas9 gene editing technique, using single-guide RNAs targeting exon 1 to specifically knockout the expression of the *FLVCR1* isoform that is expressed at the cell membrane (FLVCR1a).

All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide (CO₂) atmosphere, using Dulbecco's modified eagle medium (DMEM; Gibco) containing 4.5 g/L D-Glucose, 110 mg/L Sodium Pyruvate and supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Cells were sub-cultured twice a week, at a split ratio of 1:12 for the HEK293T cell line and 1:6-1:9 for Huh7 *FLVCR1*-WT and *FLVCR1*-KO cell lines. The subculturing was performed by detaching cells through incubation with TrypLE™ Express (Gibco) for 5-8 minutes. All cell culture procedures were carried out in a laminar flow chamber (NUAIRE), under sterile conditions.

3.2. Seahorse Assay

Cellular oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and the rate of adenosine triphosphate (ATP) production in both mitochondrial respiration and glycolysis were measured in a Seahorse XFe24 Analyzer (Agilent), using the Seahorse ATP Real-Time rate assay (Agilent). Briefly, 5×10^4 Huh-7 *FLVCR1*-WT, Clone 1 or Clone 2 cells were plated in Seahorse XFe24 Cell Culture Microplates and incubated at 37 °C and 5% CO₂ for 24 hours. At the same time, the sensory cartridge was hydrated by adding XF Calibrant to the wells of the utility plate and left overnight in a non-CO₂ incubator at 37 °C. The Seahorse Analyzer was turned on the day before the assay to allow temperature to stabilize. On the next day, the cell culture growth medium was replaced by Seahorse XF DMEM supplemented with XF Glucose (1M), XF Pyruvate (100 mM) and XF L-Glutamine (200 mM) and incubated for 1 hour at 37 °C in a non-CO₂ incubator. Before loading the cell plate into the Seahorse XFe24 Analyzer, the medium was replaced once again by fresh Seahorse XF medium. OCR and ECAR were measured at baseline and following sequential

injections of Oligomycin (1.5 μ M) and Rotenone/antimycin A (0.5 μ M). To normalize the results for the number of cells, cells were fixed and stained with 0.1% crystal violet in 20% methanol for 15 minutes with agitation. The following day, the staining was removed by adding 10% acetic acid and the absorbance was read at 550 nm using μ Quant Universal Microplate Spectrophotometer (Biotek). Three independent experiments were performed.

3.3. Plasmid Recovery

The pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase plasmid encoding both firefly luciferase and EGFP, was sent as agar stab, as a gift from Ethan Abel (Addgene plasmid, #119816). The packaging vector (psPAX2) and the envelope vector (pVSVg) were available at the i3S, University of Porto, in the form of glycerol stock, so a series of procedures were necessary to recover them, following Addgene's instructions. LB agar (NZYtech) plates were prepared by pouring, containing 100 μ g/mL ampicillin, and stored at 4 °C. A sterile pipette tip was used to touch the bacteria growing on the stab and streak it onto a plate, which was left inside an incubator (VWR INCU-Line) *overnight* at 37 °C. Four single colonies were picked from the agar plate and left to grow in LB Broth (Sigma-Aldrich) supplemented with 100 μ g/mL ampicillin, as well as a control (without bacteria), at 37 °C and 200 rpm in a shaking incubator (Shel Lab). The following day, after confirming the turbidity of the cultures containing bacterial growth compared to the transparency of the control, DNA extraction was performed with NZYMiniprep kit (NZYtech) following the manufacturer's instructions. The cultures were centrifuged at 12,000 $\times$ g for 30 seconds to form a pellet and the supernatants were discarded. To lyse the cells, the pellets were resuspended in 250 μ L of Buffer A1 by vortexing, followed by the addition of 250 μ L of Buffer A2 and mixing gently by inverting the microcentrifuge tubes. The samples were left to incubate at room temperature for 4 minutes. Then, 300 μ L of Buffer A3 was added to the samples, which were mixed carefully by inversion. A centrifugation was performed for 10 minutes at 12,000 $\times$ g and room temperature. The supernatants obtained were transferred to NZYtech spin columns placed in collecting tubes along with 500 μ L of Buffer AY, followed by a centrifugation at 12,000 $\times$ g for 1 minute, after which the flow through was discarded. Following this, 600 μ L of Buffer A4 was added to the tubes prior to centrifugation at 12,000 $\times$ g for 1 minute, with disposal of the flow through. Another

centrifugation at 12,000 $\times$ g for 2 minutes was performed to dry the silica membrane. In the next step, 50 μ L of sterile Buffer AE was added to the columns followed by an incubation of 1 minute at room temperature and a centrifugation for 1 minute at 12,000 $\times$ g, which was then repeated one more time. The DNA concentration and purity were quantified using a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies).

3.4. Enzymatic Digestion

In order to confirm the plasmid identity and integrity, the restriction enzymes *EcoRI* (ER0271, Thermo Fisher Scientific) and *XhoI* (ER0692, Thermo Fisher Scientific) were used to digest the plasmid DNA. Both single and double digestions were performed by combining the following constituents in a 50 μ L reaction: 1 μ g of DNA, 5 U of both *EcoRI* and *XhoI*, 10 $\times$ Tango buffer and distilled water to make up the volume. The solution was mixed carefully by pipetting and incubated at 37°C for 1.5 hours.

3.5. Gel Electrophoresis

Immediately after enzymatic digestion, the samples were mixed with DNA Loading Dye (R0611, Thermo Fisher Scientific) and run in a 1% agarose gel prepared in Tris-Acetate-EDTA buffer (1 $\times$, TAE; NZYtech) with GelGreen (1:25; Biotium) working as the DNA stain. To verify the size of the bands, 0.5 μ g/ μ L GeneRuler 1 kb DNA Ladder (SM0311, Thermo Fisher Scientific) was added to the first well of the gel. The gel was run at 80 V for 1 hour and the DNA bands visualized in a GelDoc imaging system (Bio-Rad) using the Image Lab 5.2.1 software.

3.6. Plasmid Transfection

To obtain the lentiviral supernatant required for the subsequent procedures, the HEK293T cell line was transfected with the lentiviral transfer vector (pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase), the packaging vector (psPAX2) and the envelope vector (pVSVg). For this, 7 $\times$ 10⁵ cells were plated in a 6 cm tissue culture dish and incubated at 37 °C and 5% CO₂. After 24 hours to allow the establishment of a desired confluence of approximately 80%, the following constituents were added to OptiMEM (Thermo Fisher Scientific): 2 μ g of transfer vector, 1.25 μ g of

psPAX2 and 1.25 µg of pVSVg. In the meantime, FuGeneHD (E2311, Promega) was added to OptiMEM, in another tube, in a 3:1 transfection reagent:DNA ratio, and the mixture was left incubating for 5 minutes at room temperature. The content of the second tube was carefully transferred to the first tube, making up a final volume of 250 µL. The final solution was incubated at room temperature for 20 minutes before being added to the culture dish containing the previously plated cells. Cells were incubated in the lentivirus incubator *overnight* at 37 °C and 5% CO₂. Both 40 and 64 hours after transfection, the viral supernatant was collected and green fluorescent protein (GFP) expression was checked with ZOE Fluorescent Cell Imager (Bio-Rad). The total volume collected was passed through 0.45 µm cellulose acetate filter (FiltrateCH) and concentrated using an ultrafiltration device (Amicon Ultra-15 50K Filter; Merck Millipore) by centrifugation at 4,000 ×g for 20 minutes. Finally, the concentrated viral supernatant was gently resuspended, centrifuged at 1,000 ×g for 10 minutes to remove any debris and stored at -80 °C.

3.7. Transduction

3.7.1. Transduction Test

In order to estimate the volume of virus to be used in transduction, a transduction test was performed. Huh-7 *FLVCR1*-WT cells (7.5×10^4) were plated in a 24-well plate and incubated at 37 °C and 5% CO₂. After 24 hours, DMEM supplemented with 10% FBS, 5 µg/mL polybrene (10 mg/mL) was added to cells, as well as different volumes of viral concentrate (10 µL and 40 µL) in duplicate. The plate was incubated at 37 °C and 5% CO₂ for 72 hours, at which point cells were checked for GFP expression with ZOE Fluorescent Cell Imager and immediately detached and centrifuged at 1,000 rpm for 3 minutes. The supernatant was carefully removed, and the cells were washed with phosphate-buffered saline (1×, PBS; GRISP) supplemented with 2% FBS, prior to centrifugation at 1,000 rpm for 3 minutes. Cells were resuspended in an appropriate volume of PBS 2% FBS and passed through a 70 µm cell strainer (Corning). The samples were acquired by flow cytometry in a FACSCanto II (BD) and analyzed with the FlowJo software, using non-infected cells as the negative control.

3.7.2. *FLVCR1*-WT and *FLVCR1*-KO Transduction

Transduction of the *FLVCR1*-WT and *FLVCR1*-KO (Clone 2) Huh-7 cells was the final step towards the establishment of luciferase-expressing cells. To achieve this, 7.5×10^4 *FLVCR1*-WT or *FLVCR1*-KO Huh-7 cells were plated in 24-well plates and incubated at 37 °C and 5% CO₂ for 24 hours. After this time, the media was replaced by transduction medium, containing DMEM supplemented with 10% FBS, 5 µg/mL polybrene and 40 µL of concentrated virus, and incubated at 37 °C and 5% CO₂ for 72 hours. By the end of this period, 1 µg/mL of puromycin was added to each well, thus initiating the antibiotic selection, and cells were continuously incubated at 37 °C and 5% CO₂ in the presence of puromycin, until a stable population of cells that contained the antibiotic resistance gene was evident. Furthermore, these cells were sorted to select the GFP-positive (GFP⁺) cells with a brighter GFP expression, using flow cytometry (BD FACSAria™ Fusion). Cells were prepared for flow cytometry as previously described and were sorted into either a T25 cell culture flask, in the case of *FLVCR1*-WT Huh-7 cells, or into a well of a 48-well plate, in the case of *FLVCR1*-KO Huh-7 cells, in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (Thermo Fisher Scientific). GFP expression was checked with ZOE Fluorescent Cell Imager. Luciferase expression was confirmed after addition of D-Luciferin Potassium Salt (40 mg/mL; BioThema), using IVIS Spectrum In Vivo Imaging System (Perkin-Elmer).

3.8. Cytotoxicity Assay

To evaluate the cytotoxic effects of puromycin and determine its optimal concentration for the Huh-7 cell line, a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, 1.5×10^4 Huh-7 cells were plated in a 96-well plate and incubated at 37 °C and 5% CO₂ overnight, to obtain a desired confluence of ≥80%. Cells were treated with different concentrations of puromycin (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 µg/mL), in quadruplicate, for 48 hours. After this period, the treatment was removed and MTT solution (0.5 mg/mL; M5655, Sigma) was added to each well, including a blank quadruplicate, for 1.5 hours at 37 °C. Then, 100% DMSO was added for 5 minutes to dissolve the formazan product formed in the previous reaction. The optical density was measured at 570 nm using µQuant Universal Microplate Spectrophotometer (Biotek). The cell viability was estimated using the following equation: cell viability (%) = [OD (puromycin)

– OD (blank)]/[OD (control) – OD (blank)] × 100. Morphological changes were monitored using Leica DMI1 Inverted Microscope.

3.9. Animal Studies

The NSG-SGM3 mouse strain (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ) was acquired from The Jackson Laboratory. For the intrahepatic injections, 7-11-week-old males and females were used. Animals were housed at the i3S animal facility under specific pathogen-free conditions, in a temperature-controlled environment and subjected to 12h light - 12h dark cycles. Animals had free access to standard rodent chow and water. All animals received humane care according to the criteria outlined by the Federation of European Laboratory Animal Science Associations for the care and handling of laboratory animals (EU Directive 2010/63/EU). Experimental procedures were approved by the i3S Animal Ethics Committee (DD_2021_11) and Direção-Geral da Alimentação e Veterinária.

3.10. Echo-guided Intrahepatic Injections

Before anesthetizing the mice with 5% isoflurane and 1 L/min O₂, analgesia (0.08 mg/kg buprenorphine) was administered subcutaneously using a 26G needle. The anesthesia was maintained in the mask throughout the procedure at 3% isoflurane and 0.8 L/min O₂. Drops of ophthalmic gel (Siccafluid) were applied to each eye to prevent corneal damage and dehydration and a rectal probe was inserted to monitor the animal's temperature during the procedure. The abdomen was shaved using a shaving machine and tape was placed around that area of the animal in order to stretch the skin that was then disinfected by alternating between 70% alcohol and iodine. After placing ECG gel in the desired location, identification of the liver was possible using a Vevo F2 microultrasound (Fujifilm VisualSonics) with an UHF57X (57-25 MHz) transducer (Fujifilm VisualSonics), in brightness mode (B-Mode). Afterwards, 1×10^6 *FLVCR1*-WT or *FLVCR1*-KO Huh-7 cells, resuspended in 50 µL PBS, were injected into the liver using a 30G needle, whose syringe was held in a micromanipulator belonging to the Vevo injection mount device (Fujifilm VisualSonics), at an angle of approximately 30 degrees. Before injection in the right liver lobe, alignment between the needle and the probe was confirmed. Afterwards the animals' welfare (including body weight and behavior) was inspected twice a week.

3.11. Intrahepatic Injections After Laparotomy

Animals were anesthetized in 5% isoflurane and 1 L/min O₂ and maintained in the anesthesia mask throughout the procedure at 3% isoflurane and 0.8 L/min O₂. The procedure was performed on a heating pad to maintain body temperature and drops of ophthalmic gel were placed in each eye. Pre-operative analgesia (0.08 mg/kg buprenorphine) was administered subcutaneously using a 26G needle. The rodent's fur was removed from the surgical site using a shaving machine and the skin was disinfected by alternating between 70% alcohol and iodine. At this moment the animal was ready to be placed in the surgery area, in a supine position. The surgery was conducted in a disinfected, uncluttered area to promote asepsis during the procedure. Firstly, an incision was made in the abdomen midline, leading to the opening of both the cutaneous and muscular layers and the exposure of the left liver lobe, which was maintained using a retractor. Then, 1×10^6 *FLVCR1*-WT or *FLVCR1*-KO Huh-7 cells, resuspended in 50 μ L PBS, were injected into the liver using a 0.3 mL insulin syringe, making it possible to observe the formation of a transparent bleb of cells in this organ. After injection, a slight pressure was applied using a sterilized swab to prevent cell leakage and bleeding. The peritoneum layer was closed in a continuous line using PGA 5.0 absorbable suture, also used to close the skin layer intradermally, and a drop of surgical glue was placed on top of the incision. Post-operative analgesia (buprenorphine) was administered in the three following days, twice a day. Afterwards the animals' welfare (including body weight and behavior) was inspected twice a week.

3.12. *In vivo* Imaging

3.12.1. Bioluminescence Imaging

To monitor tumor formation and growth, luciferase activity was measured on days 11, 16, 44 and 49, for mice that received echo-guided intrahepatic injections, and on days 13, 27, 30, 34, 37 and 41, for mice in which intrahepatic injections were performed after laparotomy, after cell injection using a NEWTON 7.0 small animal optical imaging system for bioluminescence (Vilber). Animals were anesthetized in 5% isoflurane and 1 L/min O₂ and maintained in the anesthesia mask inside the imaging chamber throughout the acquisitions at 3% isoflurane and 0.8 L/min O₂. To measure luciferase activity, 60 μ L of the substrate D-Luciferin Potassium Salt (40 mg/mL; BioTherma) was administered subcutaneously. First, the kinetic curve of luciferin was established by acquiring images every 5 minutes for 35 minutes in order to determine

the peak signal time, with an exposure time of 4 minutes and 22 seconds. After that, all image acquisitions were performed approximately 10 minutes after injection with an exposure of 4 minutes and 22 seconds. After sacrifice, images of the organs were acquired with an exposure of 30 seconds. Tumors were measured with a caliper and tumor volume was calculated using the modified ellipsoidal formula: Tumor volume = $\frac{1}{2}$ (length $\times$ width²). Tumor bioluminescence was analyzed by Kuant software and expressed as photons/second/cm²/steradian (p/s/cm²/sr).

3.12.2. Ultrasound Imaging

Tumor growth was also monitored using ultrasound imaging before sacrificing the mice. After anesthetizing the mice with 5% isoflurane and 1 L/min O₂ and maintaining anesthesia using the mask at 3% isoflurane and 0.8 L/min O₂, a Vevo F2 microultrasound (Fujifilm VisualSonics) was used with an UHF57X (57-25 MHz) transducer (Fujifilm VisualSonics) that was manipulated on the animal's abdomen in supine position and imaged in B-mode.

3.13. Histology

Immediately after sacrificing the mice by cervical dislocation, the livers were harvested, weighed and measured, and immersed in 10% neutral buffered formalin (Bio-Optica). These samples were sent to the i3S Histology and Electron Microscopy platform, where the tissues were embedded in paraffin and cut in 3 μ m sections, which were stained with Haematoxylin and Eosin (H&E). The images were obtained with a Leica DM2000 LED brightfield microscope (Leica Microsystems).

3.14. Serum Biochemical Analysis

Blood was withdrawn by retroorbital bleeding under anesthesia, prior to euthanizing the mice by cervical displacement. Blood samples were incubated at room temperature for at least 30min, prior to centrifugation at 10,000 $\times$ g for 10 minutes, after which sera were collected and stored at -80 °C. Serum biochemistry was analyzed in a AU5800 Chemistry Analyzer (Beckman Coulter).

3.15. siRNAs Transfection

In order to obtain a transient knockdown of *FLVCR1*, *FLVCR1*-WT Huh-7 cells were transfected with siRNAs. For this, 1.25×10^5 cells were plated in 6-well plates, to reach a desired confluence of 30-50% on the day of transfection and incubated at 37 °C and 5% CO₂ for 24 hours. The following day, 10 nM of either three different Dicer-Substrate Short Interfering RNAs (DsiRNAs) that specifically target *FLVCR1* (si-*FLVCR1*), non-targeting siRNA (si-Scramble) that functions as a negative control or TYE™ 563 DS transfection control (included in the TriFECTa kit, Integrated DNA Technologies IDT) (**Table 1**) were diluted in 150 µL of Opti-MEM and added to a mix of 9 µL of Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) and 150 µL of Opti-MEM, per well, which was incubated at room temperature for 20 minutes. The mixture was added dropwise to the cells, whose medium had been replaced with Opti-MEM, and cells were incubated at 37 °C and 5% CO₂ for 6 hours. After this time, the medium was replaced with DMEM supplemented with 10% FBS.

Table 1 – siRNAs, respective sequence and mRNA and exon targets, used in the transfection of Huh-7 cells.

siRNA	Sequence	Target mRNA (location)	Target Exon
hs.Ri.FLVCR1.13.1	5'-CAUUGGACCUUAGAUAUAUUAUCAT-3'	<i>FLVCR1</i> (CDS)	6
hs.Ri.FLVCR1.13.2	5'-UUGAAAUACACUUACCCUGAAUCUGA-3'	<i>FLVCR1</i> (CDS)	7
hs.Ri.FLVCR1.13.3	5'-CCAAAAAAGCUAGUCAGGUAAUGAA-3'	<i>FLVCR1</i> (3' UTR)	10
Negative Control (DS NC1)	n/a	n/a	n/a
TYE™ 563 DS Transfection Control	n/a	n/a	n/a

3.16. Knockdown Validation

After transfecting Huh-7 cells as described in **3.15.**, cells were maintained in culture for 7 days, during which samples were collected daily to evaluate the variation in *FLVCR1* mRNA and protein levels.

3.16.1. RNA Extraction

Total RNA was obtained by harvesting and lysing cells by adding NZYol reagent (NZYtech), following the manufacturer's protocol. Subsequently, samples were incubated for 5 minutes at room temperature, followed by a phase separation achieved by adding 0.2 mL chloroform (per mL of NZYol reagent used) and vigorously

shaking for about 15 seconds to guarantee homogenization. Following an incubation at room temperature for 2-3 minutes, samples were centrifuged at 12,000 ×g and 4 °C for 15 minutes, and it was possible to distinguish three different phases, in which the upper aqueous phase contained the RNA. Therefore, this phase was carefully transferred to a new tube, to which 0.5 mL of isopropyl alcohol (per mL of NZYol reagent used) was added to promote RNA precipitation, and samples were incubated at -20 °C for 10 minutes. Following a centrifugation at 12,000 ×g and 4 °C for 10 minutes, the RNA pellet was washed with 1 mL of 75% ethanol (per mL of NZYol reagent used), centrifuged for 5 minutes at 12,000 ×g and 4 °C, and left to air dry for approximately 10 minutes. Finally, RNA was dissolved in RNase-free water and its concentration was quantified using a NanoDrop 1000 spectrophotometer.

3.16.2. Complementary DNA (cDNA) Synthesis

Contaminating genomic DNA was digested using the TURBO DNA-free kit (Thermo Fisher Scientific), following the manufacturer's protocol. First, RNA was combined with TURBO DNase Enzyme, 10× TURBO DNase Buffer and nuclease-free water. Different amounts of RNA were treated, always ensuring that time-matched samples contained the same quantity. Samples were incubated at 37 °C for 30 minutes. After addition of DNase Inactivation Reagent to the mixture and incubation at room temperature for 5 minutes, samples were centrifuged at 10,000 ×g for 1.5 minutes. cDNA was synthesized using the NZY First-strand cDNA synthesis kit (NZYtech), following the manufacturer's protocol. DNased RNA (6 µL, 0.24-1.2 µg) was mixed with 1 µL of Oligo (dT)18 Primer Mix and 1 µL of 10× Annealing Buffer in a final volume of 8 µL. Subsequently, RNA was incubated at 65 °C for 5 minutes, promptly placed on ice and spun briefly. In the next step, 10 µL of a NZYRT 2× master mix, containing MgCl₂ and dNTPs, and 2 µL of a NZYRT Enzyme Mix, containing NZY Reverse Transcriptase and NZY Ribonuclease Inhibitor, were added to each sample. The cDNA synthesis reaction occurred at 50 °C for 30 minutes and was inactivated at 85 °C for 5 minutes. Finally, after cooling on ice, 1 µL of NZY RNase H was added and the mix was incubated at 37 °C for 20 minutes to degrade traces of the template RNA.

3.16.3. RT-qPCR

In order to evaluate *FLVCR1* mRNA expression, an RT-qPCR was performed using *HPRT1* as a housekeeping gene or endogenous control. For each gene, a

reaction mixture was prepared, containing 5 μ L of iTaq Universal SYBR Green Supermix, 0.2 μ L of both the forward and reverse primers (10 μ M), whose sequences are present in **Table 2**, and 3.6 μ L of dH₂O. Each sample was amplified in duplicates in 384-well plates. To each well, 1 μ L of cDNA and 9 μ L of the previously prepared master mix were added. The reaction was carried out on a CFX384 Real-Time System (Bio-Rad), following these conditions: initial denaturation at 95 °C for 3.5 minutes, followed by 39 cycles of denaturation at 95 °C for 20 seconds and annealing at 59 °C for 30 seconds. At the end of the amplification reaction, a denaturing curve was obtained by setting the temperature to 65 °C for 5 seconds, and then increasing to 95 °C at 0.5 °C/cycle. The result was expressed in the form of fold change ($2^{-\Delta\Delta C_t}$) in the gene expression of the treated samples at a given time point compared to the time-matched control. ΔC_t refers to the threshold cycle (C_t) of *FLVCR1* minus the C_t of the housekeeping gene, whereas $\Delta\Delta C_t$ refers to the ΔC_t of samples treated with si-*FLVCR1* minus the ΔC_t of samples treated with si-Scramble.

Table 2 - Sequences of the human *FLVCR1* and *HPRT1* primers used in RT-qPCR.

Gene	Forward	Reverse
<i>FLVCR1</i>	5'-GTCCACAGACCAAGAAC-3'	5'-CCACCACATACAAACCCAG-3'
<i>HPRT1</i>	5'-GCAGACTTTGCTTTCCTTGTCAG-3'	5'-GTCTGGCTTATATCCAACACTTCGTG-3'

3.16.4. Protein Quantification

Protein extracts were obtained from harvested cells by lysis with RIPA buffer supplemented with 35% protease inhibitor cocktail (PIC, Roche), followed by an incubation at 4 °C for 30 minutes. Samples were clarified through a 10-minute centrifugation at 8,000 $\times$ g and 4 °C. Then, the protein content of the cell lysates was quantified using the DC Protein Assay kit (Bio-Rad). Absorbance was measured at 750 nm using μ Quant Universal Microplate Spectrophotometer (Biotek). A calibration curve was prepared using the kit's standards with a protein range of 0 - 1.5 mg/mL.

3.16.5. Western Blot

Proteins (5.5 μ g of the samples from day 1 and 11 μ g from the other samples) were denatured and reduced by mixing with a loading buffer containing 25% 2-mercaptoethanol (final concentration of 5% on the sample), followed by an incubation at 37 °C for 15 minutes. Proteins were resolved in a 10% polyacrylamide gel during an

electrophoresis that was conducted in a Mini-PROTEAN Tetra Cell System (Bio-Rad) for 1.5 hours at 120 V. Subsequently, proteins were transferred to nitrocellulose membranes (Bio-Rad) at 60 V for 1.5 hours. Once the transfer was completed, its efficiency was assessed through staining with Ponceau S. Membranes were blocked with 5% milk in Tris-Buffered Saline (TBS)-Tween 20 0.1% at room temperature for 1 hour, to prevent non-specific binding, and then probed with the primary antibodies, described in **Table 3**, overnight at 4 °C. The following day, the membranes were incubated with the secondary antibodies, listed in **Table 3**, for 2 hours at room temperature. In addition, before and after this incubation the membranes were washed three times (5 minutes each) with TBS-Tween 20 0.1%. Finally, membranes were incubated for 2 minutes at room temperature with Clarity Western ECL Substrate (Bio-Rad) and the chemiluminescence signal was detected using the ChemiDoc Imaging System (Bio-Rad).

Table 3 - Primary and secondary antibodies used in Western Blot assay.

Antigen (Origin)	Dilution	Provider	Catalog No.
FLVCR1 (mouse)	1:500	Santa Cruz Biotechnology	Sc-390100
Beta Actin (rabbit)	1:5000	Abcam	Ab8227
Secondary Antibody	Dilution	Provider	Catalog No.
Peroxidase AffiniPure™ Goat α-mouse IgG (H+L)	1:5000	Jackson ImmunoResearch Europe	AB_2338504
Peroxidase AffiniPure™ Goat Anti-Rabbit IgG (H+L)	1:10000	Jackson ImmunoResearch Europe	AB_2313567

3.17. Single-Cell Migration Assay

After transfecting Huh-7 cells as described in **3.15.**, Huh-7 *FLVCR1*-WT and knockdown cells were harvested and plated (2×10^4 cells) in duplicate in a μ -Plate 24 Well Black ID 14 mm ibiTreat (Ibidi) and incubated at 37 °C and 5% CO₂ for 5 hours, to allow cell adherence. Afterwards, the plate was placed in the Operetta CLS (Perkin-Elmer) imaging system, with controlled temperature and CO₂ concentration of 37 °C and 5%, respectively. Brightfield microscopic images of four fields per well were acquired every 10 minutes during 16 hours. Image analysis was performed using the Harmony High-Content Imaging and Analysis Software. Displacement graphs were plotted using Chemotaxis and Migration Tool (Ibidi) Three independent experiments were performed.

3.18. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9.0.0. Paired and unpaired *t* test and Mann-Whitney test were used to compare the means of two groups. Comparisons between two or more groups having more than one variable were performed by 2-way ANOVA, followed by Dunnett's multiple comparisons tests. Differences between groups were considered significant when $P < 0.05$.

4. Results

4.1. Contribution of *FLVCR1* to the Energy Metabolism of HCC Cells

4.1.1. FLVCR1-KO Shifts the Metabolic Phenotype in Huh-7 Human Hepatoma Cells

To determine whether *FLVCR1* expression modulates the energy metabolism of human HCC cells, we compared the metabolic phenotype of *FLVCR1*-WT Huh-7 cells and of two *FLVCR1*-KO Clones, in a Seahorse ATP Real Time rate assay. This assay allows observing the evolution of OCR and ECAR over time, measuring the basal rates, as well as the rates after the injection of oligomycin, which is an ATP synthase inhibitor (complex V), and after the injection of a mixture of rotenone and antimycin A, inhibitors of complexes I and III of the respiratory chain, respectively. Besides, this assay allows the quantification of the ATP production rate associated with the glycolytic pathway (glycoATP production rate) and with oxidative phosphorylation (mitoATP production rate).

As depicted in **Figure 7A**, *FLVCR1*-KO Huh-7 Clone 2 had a significantly higher basal OCR ($P < 0.0001$) than *FLVCR1*-WT Huh-7 cells. This was accompanied by an also significant decrease in ECAR ($P < 0.05$) (**Figure 7B**) compared to the *FLVCR1*-WT cells. There was an increase in ATP production derived from mitochondrial respiration and a decrease in ATP production derived from the conversion of glucose to lactate, in *FLVCR1*-KO Huh-7 Clone 2 relatively to *FLVCR1*-WT Huh-7 cells, both significant ($P < 0.05$) (**Figure 7C**). A significant decrease in ECAR at baseline was also observed in *FLVCR1*-KO Clone 1 ($P < 0.05$) (**Figure 7B**). However, this difference was not accompanied by large variations in the rate of mitochondrial and glycolytic ATP production (**Figure 7C**). After the injections of the respiratory chain inhibitors, which inhibit oxidative phosphorylation, we observed that the extracellular acidification values, derived from lactate production, of Clone 1 were closer to the *FLVCR1*-WT values, while ECAR of Clone 2 continued to depart from *FLVCR1*-WT Huh-7 cells significantly ($P < 0.001$) (**Figure 7B**).

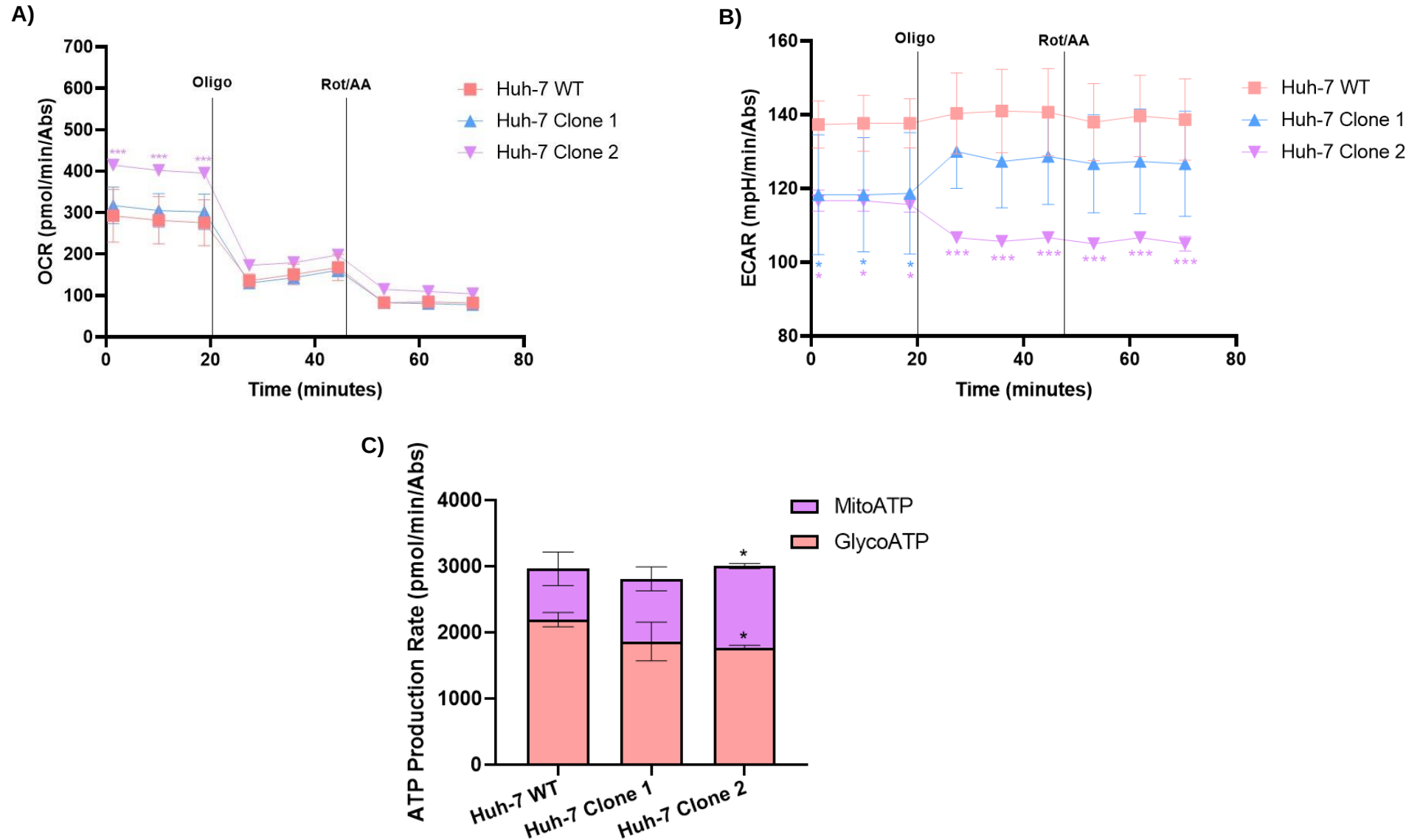


Figure 7 - *FLVCR1* deletion causes a shift in the metabolic phenotype of Huh-7 cells. OCR, ECAR and ATP production rate were analyzed by Seahorse XFe24 Analyzer. **A)** and **B)** OCR and ECAR values are presented as mean $\pm$ standard deviations of three independent experiments. The first three measurements represent basal mitochondrial respiration, whereas the next three time points correspond to measurements after the injection of oligomycin (1.5 μ M), and the last three time points follow the injection of a mixture of rotenone and antimycin A (0.5 μ M). **C)** Mitochondrial and glycolytic ATP production rates were assessed using Seahorse ATP Real-Time rate assay. Statistical significance was determined by 2way ANOVA followed by Dunnett's multiple comparisons tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: OCR – Oxygen Consumption Rate; ECAR – Extracellular Acidification Rate.

4.2. Impact of *FLVCR1* Loss in Human HCC Cells *in vivo*

4.2.1. Assessment of Plasmid DNA Integrity

In order to generate FLVCR1-WT/KO human HCC cells (Huh-7) with stable luciferase expression, a plasmid encoding both firefly luciferase and EGFP was ordered from a commercial source, as described in Methods. To confirm the identity and integrity of the pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase plasmid, an enzymatic digestion was performed, using the restriction enzymes *EcoRI* and *XhoI*, followed by an electrophoresis to evaluate the sizes of the bands corresponding to the number of base pairs of the resulting DNA (**Figure 8A**). These enzymes were chosen as they flank the insert (EGFP-Firefly Luciferase) and do not present restriction sites elsewhere in the plasmid, verified in **Supplementary Figure 1** and schematically represented in **Figure 8B**. The lane corresponding to the migration of undigested DNA (uncut) presented a band slightly above the 10,000 bp, which was in agreement with the sum of the sizes of the insert and the backbone vector (10,489 bp), thus corresponding to the linear conformation. Furthermore, a smear corresponding to DNA that migrated more slowly was visible, which should coincide with a different plasmid conformation such as open-circular. In the case of the lanes corresponding to the digestion exclusively with either *EcoRI* or *XhoI*, we observed a well-defined band with the same size as the undigested band corresponding to linear DNA, since digesting with just one of the enzymes only linearizes the plasmid DNA. Digestion with both restriction enzymes guaranteed the presence of the insert of interest since a band close to 2,500 bp was detectable, which corresponds to the size of the insert (2,373 bp), as well as a band around 8,000 bp coinciding with the backbone vector (8,116 bp).

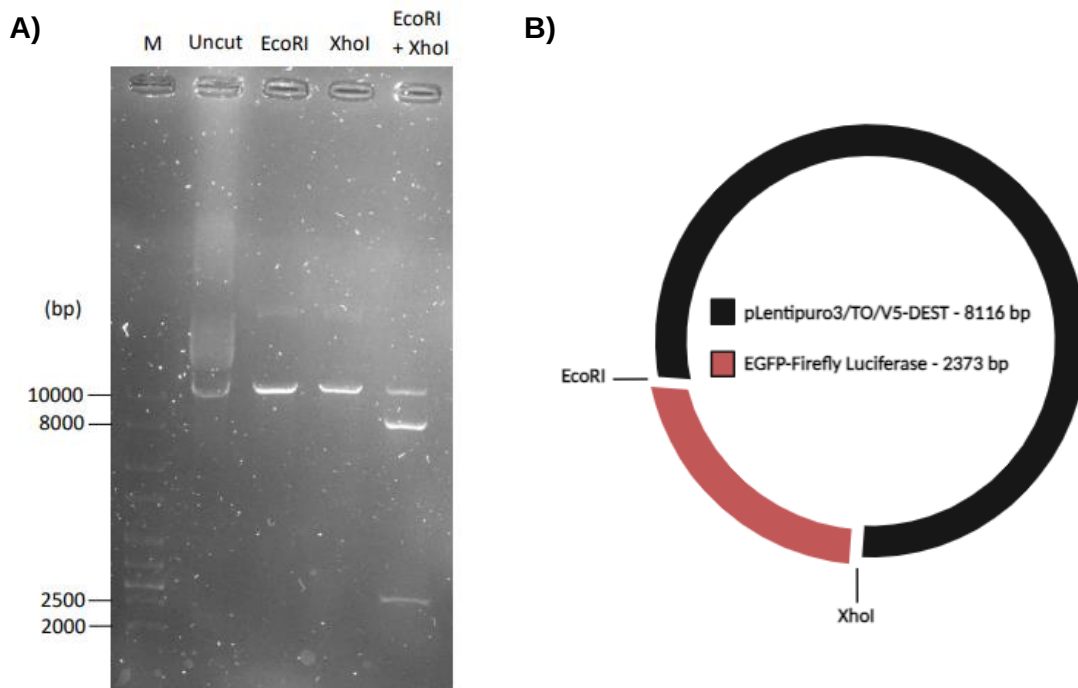


Figure 8 - Restriction pattern of pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase vector. **A)** M, GeneRuler™ 1kb DNA Ladder molecular weight marker; Uncut, undigested plasmid; EcoRI, plasmid digested using *EcoRI* restriction enzyme; XhoI, plasmid digested using *XhoI* restriction enzyme; EcoRI + XhoI, plasmid digested using both *EcoRI* and *XhoI* restriction enzymes. Electrophoresis conditions: 1% agarose, 1 hour, 80 V. **B)** Schematic illustration representing the transfer vector *EcoRI* and *XhoI* restriction sites and the size of its backbone (pLentipuro3/TO/V5-DEST) and insert (EGFP-Firefly Luciferase). Image created with Biorender.com.

4.2.2 Puromycin Selection Dose

The pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase plasmid encodes an antibiotic resistance gene (puromycin), which can be used as a selection marker for transduced cells. The administration of puromycin kills all cells without the antibiotic resistance gene, allowing the survival of cells that have been successfully transduced with the plasmid of interest. In order to determine the optimal puromycin dose for selecting Huh-7 cells after transduction, an MTT assay was performed, which allowed us to evaluate the cytotoxicity of different concentrations of puromycin compared to the untreated control. **Figure 9A**, shows the effect of the antibiotic on Huh-7 cells 48 hours after treatment. At a concentration of 1 $\mu\text{g/mL}$ we observed morphological changes that suggested the induction of cell death by apoptosis, such as shrinkage of the cells and membrane blebbing, which were not detected in untreated cells. **Figure 9B** depicts the results of the MTT assay, in which the percentage of viable cells with increasing concentrations of puromycin is represented. The control, which was not treated with puromycin, showed a cell viability of 100%. As expected, the percentage

of viable cells decreased with increasing antibiotic concentration. The optimal dose for selection corresponds to the lowest concentration that results in the death of all untransduced cells after 48 hours. This was not the case for concentrations of 0.25, 0.5 and 0.75 $\mu\text{g/mL}$ at which a viability of approximately 24.4%, 5.5% and 1.7%, respectively, was obtained. At an antibiotic concentration of 1 $\mu\text{g/mL}$ or higher (1.5, 2, 2.5, 3, 3.5, 4 and 5 $\mu\text{g/mL}$), there were no surviving cells after 48 hours, since no metabolically active cells capable of reducing MTT to formazan were detected through the assay. Therefore, the concentration of puromycin chosen for the selection of transduced cells was 1 $\mu\text{g/mL}$.

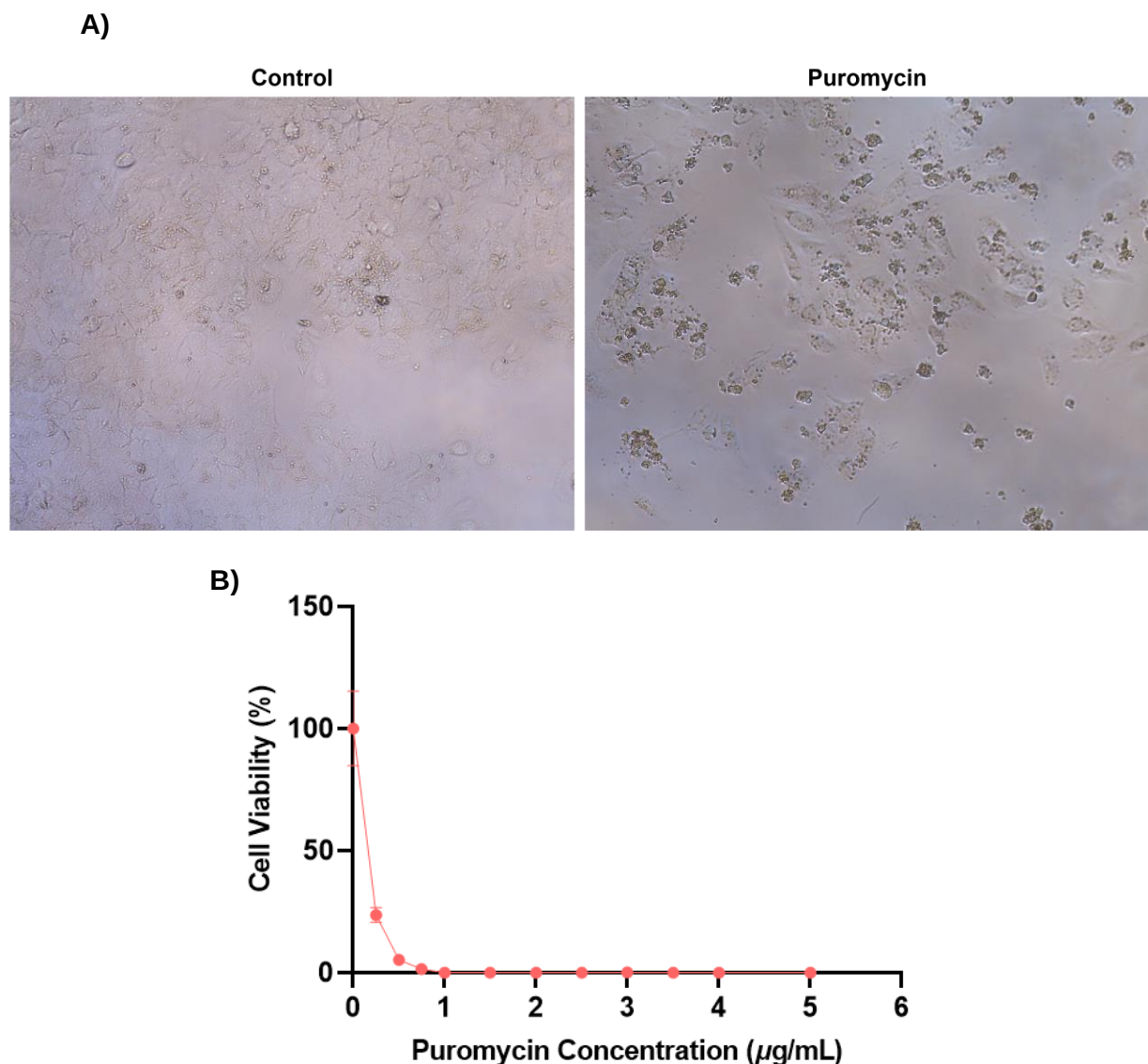


Figure 9 - Puromycin exerts a dose-dependent cytotoxic effect on Huh-7 cells. **A)** Brightfield microscopic images revealing morphologic alterations in Huh-7 cells when treated with 1 $\mu\text{g/mL}$ of puromycin for 48 hours compared to the untreated control. Cells show signs of typical hallmarks of apoptosis, such as shrinkage of the cells and membrane blebbing. **B)** Dose-response plot of the viability of Huh-7 cells when treated with increasing puromycin concentrations for 48 hours, determined by the MTT assay. Data represented as mean $\pm$ SD of quadruplicates cultures.

4.2.3 Transfection Efficiency for Lentiviral Production

HEK293T cells were used due to their efficient lentivirus production capacity when transfected with the transfer, packaging and envelope vectors. The cells were co-transfected with the enhanced green fluorescent protein (EGFP) and firefly luciferase-expressing plasmid, the psPAX2 plasmid, responsible for the expression of enzymatic and structural proteins of the virus, and the pVSVg plasmid, coding for the envelope protein. The expression of GFP, resulting from the integration of the transfer plasmid into the cell genome, was evaluated using fluorescence microscopy at approximately 64 hours after transfection (**Figure 10**). The expression of the reporter protein was confirmed by a large percentage of GFP⁺ cells in transfected versus control cells. Some degree of cell death resulting from transfection was also noticeable through brightfield microscopy, which was not observed in the control.

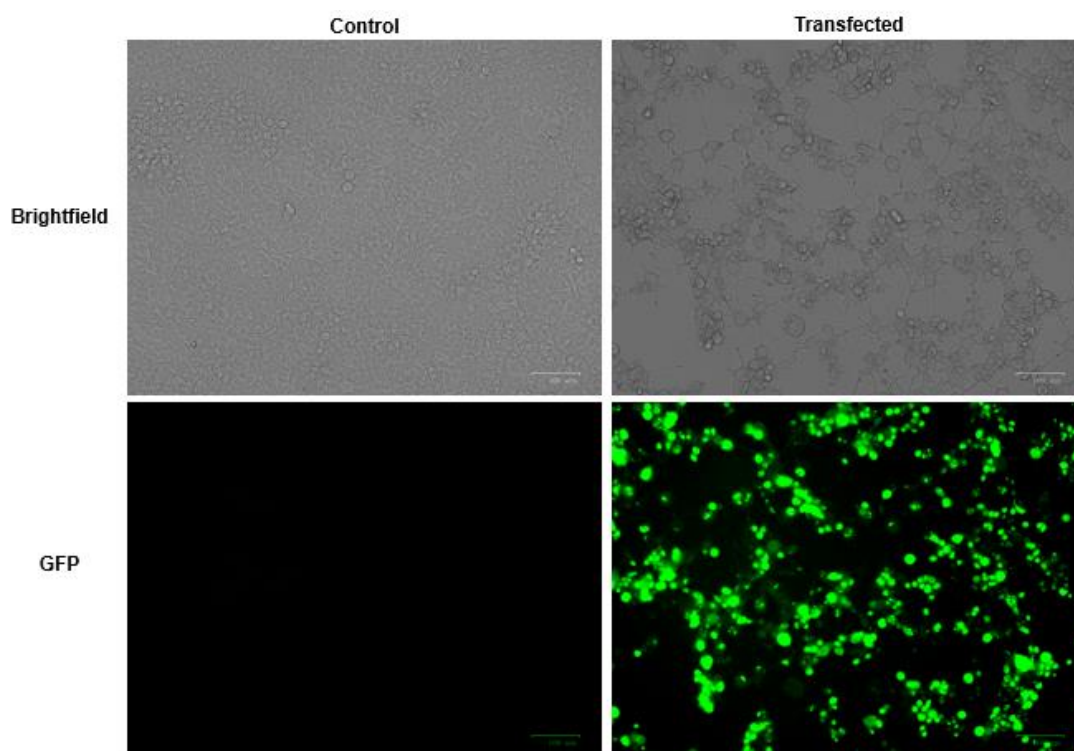


Figure 10 - HEK293T cells expressing GFP after transfection. Brightfield and fluorescence images of control and transfected HEK293T cells 64 hours post transfection resorting to ZOE Fluorescent Cell Imager (scale bar = 100 μ m). Transfected cells received 2 μ g of the transfer vector (pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase), 1.25 μ g of the packaging vector (psPAX2) and 1.25 μ g of the envelope vector (pVSVg), using FuGeneHD in a 3:1 transfection reagent:DNA ratio.

4.2.4. Establishment of Luciferase-expressing Huh-7 Cells

In order to determine the viral concentrate volume that should be used to obtain the greatest transduction efficiency of Huh-7 cells, volumes of 10 μ L and 40 μ L of concentrated virus were tested. The addition of 5 μ g/mL of polybrene, a cationic polymer, aimed to further enhance the infection efficiency. **Figures 11A** and **11B** represent the expression of GFP 72 hours post transduction (acquired through fluorescence microscopy) and the respective percentage of GFP⁺ cells (determined using flow cytometry), for 10 μ L and 40 μ L of concentrated viral particles, respectively. The highest percentage of GFP⁺ cells (29.7%) was achieved with the addition of 40 μ L of concentrated viral particles, compared to the 6.87% obtained when adding 10 μ L. The representative gating strategy applied in flow cytometry for GFP⁺ Huh-7 cells is shown in **Supplementary Figure 2**. These results were in agreement with what was observed in the fluorescence microscopy images, in which the greater positivity, and therefore higher transduction efficiency, was evident when a greater volume of virus was used.

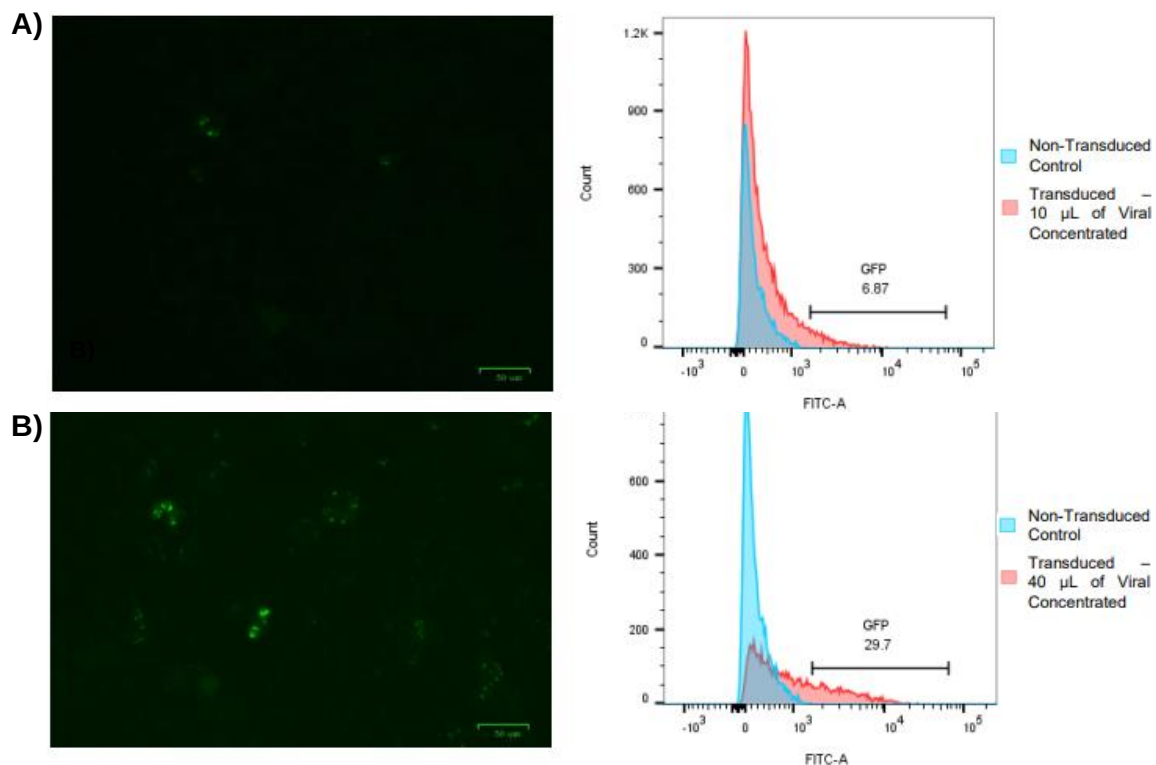


Figure 11 - Huh-7 cells expressing GFP after lentiviral transduction. Huh-7 cells in DMEM 10% FBS containing 5 μ g/mL of polybrene were transduced either with 10 μ L (**A**) or 40 μ L (**B**) of viral concentrated. 72 hours after transduction, GFP expression was evaluated by ZOE Fluorescent Cell Imager (scale bar = 50 μ m) and by flow cytometry

After transduction with 40 μ L of concentrated virus and 5 μ g/mL of polybrene, *FLVCR1*-WT and *FLVCR1*-KO Huh-7 cells were positively selected with 1 μ g/mL puromycin. To further ensure that the cells injected into the mice would be those with the brightest GFP signal, *FLVCR1*-WT and *FLVCR1*-KO cells were FACS-sorted, using the gating strategy shown in **Figure 12**. The percentage of sorted cells was 38.3% for *FLVCR1*-WT cells (yielding $\sim 1.5 \times 10^6$ cells (**Figure 12A**) and 1.4% for *FLVCR1*-KO (yielding $\sim 4 \times 10^4$ cells) (**Figure 12B**).

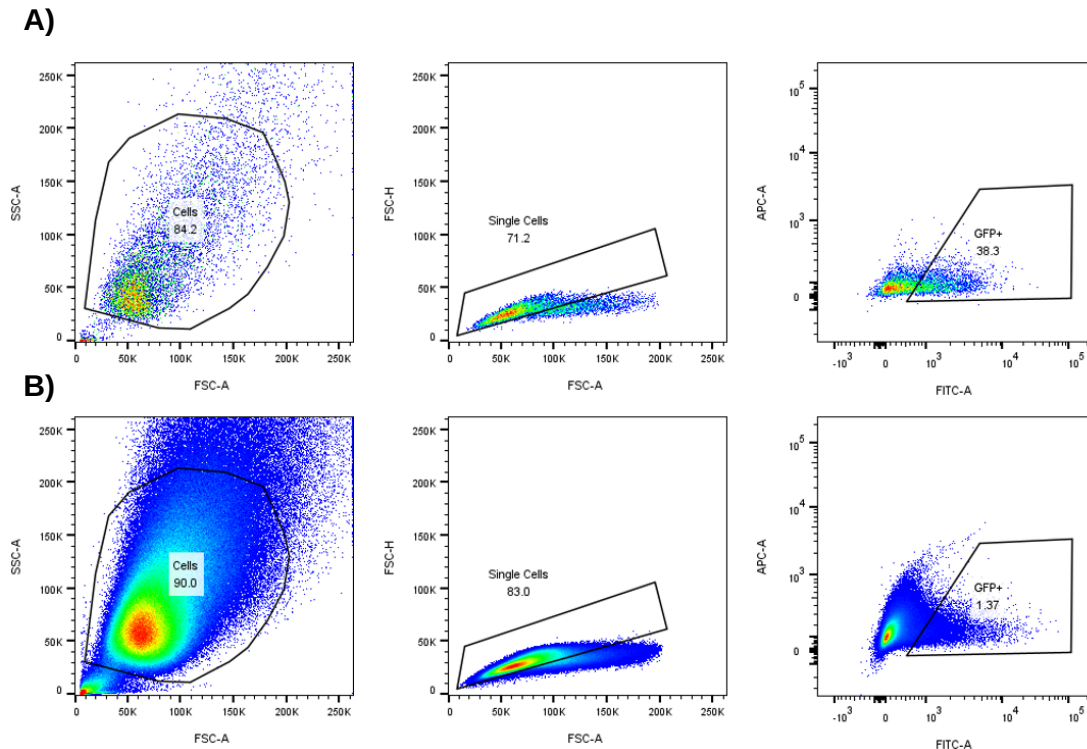


Figure 12 - Gating strategy for cell sorting of transduced Huh-7 cells. Gating strategy for the sorting of transduced *FLVCR1*-WT (**A**) and *FLVCR1*-KO (**B**) Huh-7 cells, to obtain the cells with the brightest GFP signal, using flow cytometry. FSC-A vs SSC-A plot to gate Huh-7 cells. FSC-A vs FSC-H plot to exclude doublets between cells. FITC-A vs APC-A plot to obtain the percentage of cells expressing GFP with more intensity.

4.2.5 Xenotransplantation of Modified Huh-7 Cells

After having the cell model established, the goal was to evaluate tumor growth and metastases formation after the injection of human HCC cells into the liver of severely immunodeficient mice (NSG-SGM3), generating an orthotopic model.

In order to obtain bioluminescence images whose intensity is directly proportional to the amount of tumor cells expressing luciferase, we generated a luciferin kinetic curve. Simply put, we evaluated the evolution of the signal emitted by the luciferase catalyzed reaction over time after the injection of its substrate luciferin. After luciferin subcutaneous injection, images were acquired every 5 minutes starting

5 minutes after injection, with an exposure time of 4 minutes and 22 seconds. The bioluminescence images of the mouse obtained at different time points (**Figure 13A**) and the resulting luciferin kinetic curve (**Figure 13B**) showed that from 10 minutes until at least 35 minutes after injection, there was not a large variation in the signal, since a plateau was reached. We concluded that to obtain a reliable bioluminescent signal in this model, images should not be acquired within the first 10 minutes after the luciferin injection.

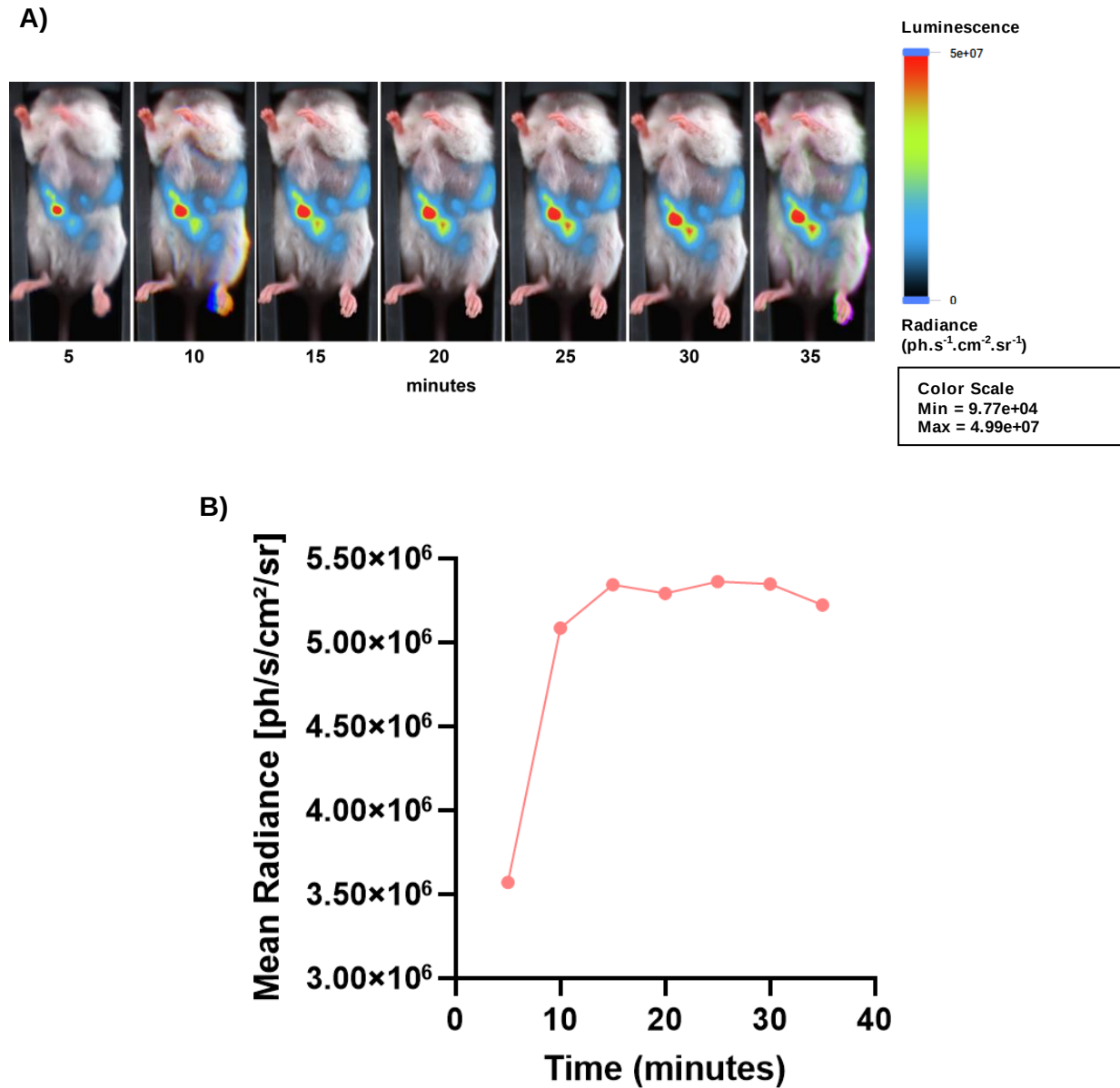


Figure 13 - Kinetic curve of luciferase bioluminescence after luciferin injection. (A) Evolution of the bioluminescent signal in the mouse over time. Images were taken every 5 minutes after luciferin injection, for 35 minutes, with an exposure time of 4 minutes and 22 seconds. (B) The kinetic curve represents the radiance from the luciferase catalyzed reaction when luciferin is injected, per minutes. The average radiance, corresponding to the signal intensity, reaches a peak at around 15 minutes, after which it stabilizes.

To establish the orthotopic model, 18 mice were initially injected intrahepatically, using ultrasound as a guide, with either *FLVCR1*-WT ($n = 9$) or *FLVCR1*-KO ($n = 9$) Huh-7 luciferase-expressing cells (1×10^6 cells per mouse). Tumor development was monitored by ultrasound and bioluminescence imaging. From the 18 injected mice, cell engraftment only failed in one mouse, which was injected with *FLVCR1*-KO luciferase-expressing cells. However, in most animals we observed a bioluminescent signal, derived from the expression of luciferase from cells that deposited in tissues other than the liver, as evident in **Figure 14**. Animal E was the most notorious example of signal appearance elsewhere before appearing in the liver. Hence, we conclude that the echo-guided intrahepatic injections failed to deliver human HCC cells specifically to the mouse liver. It is worth noting, however, that *FLVCR1*-WT cells appeared to be more capable of engraftment and tumor growth than *FLVCR1*-KO cells.

Figure 15 depicts two mice whose tumors were found exclusively in the liver, one of which was injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells and the other with *FLVCR1*-KO Huh-7 luciferase-expressing cells. After sacrifice, livers were harvested and their bioluminescence was immediately evaluated, revealing a larger tumor area in the *FLVCR1*-WT recipient liver compared to the *FLVCR1*-KO counterpart (**Figure 15B**), which was in agreement with the higher radiance value showed in the *FLVCR1*-WT recipient mouse compared to the *FLVCR1*-KO recipient (**Figure 15A**). The *FLVCR1*-WT tumor had a volume of 226 mm^3 , while the volume of the *FLVCR1*-KO tumor was 0.1 mm^3 . Tumor development was also confirmed by histologic analysis of liver sections of both animals, stained with H&E and visualized by brightfield microscopy. The tumors were readily identified by the cellular morphology, distinct from host cells, and by a more basophilic appearance when compared to the eosinophilic non-tumorous liver (**Figure 16**). **Figure 16A** represents a small, well-defined tumoral region in the liver of the *FLVCR1*-WT mouse recipient, whereas **Figure 16B** depicts a large tumor region in the same liver with an invasive front and an abundance of erythrocytes suggestive of intra-tumor hemorrhage. The tumor is surrounded by several fibrotic areas, cell death and inflammatory cells infiltrates. **Figure 16C** depicts a liver section of the *FLVCR1*-KO recipient mouse. The tumor is surrounded by several necrotic areas, which are heavily infiltrated by inflammatory cells. The presence of erythrocytes within the tumor are suggestive of small vessels irrigating the tumor (**Figure 16C**).

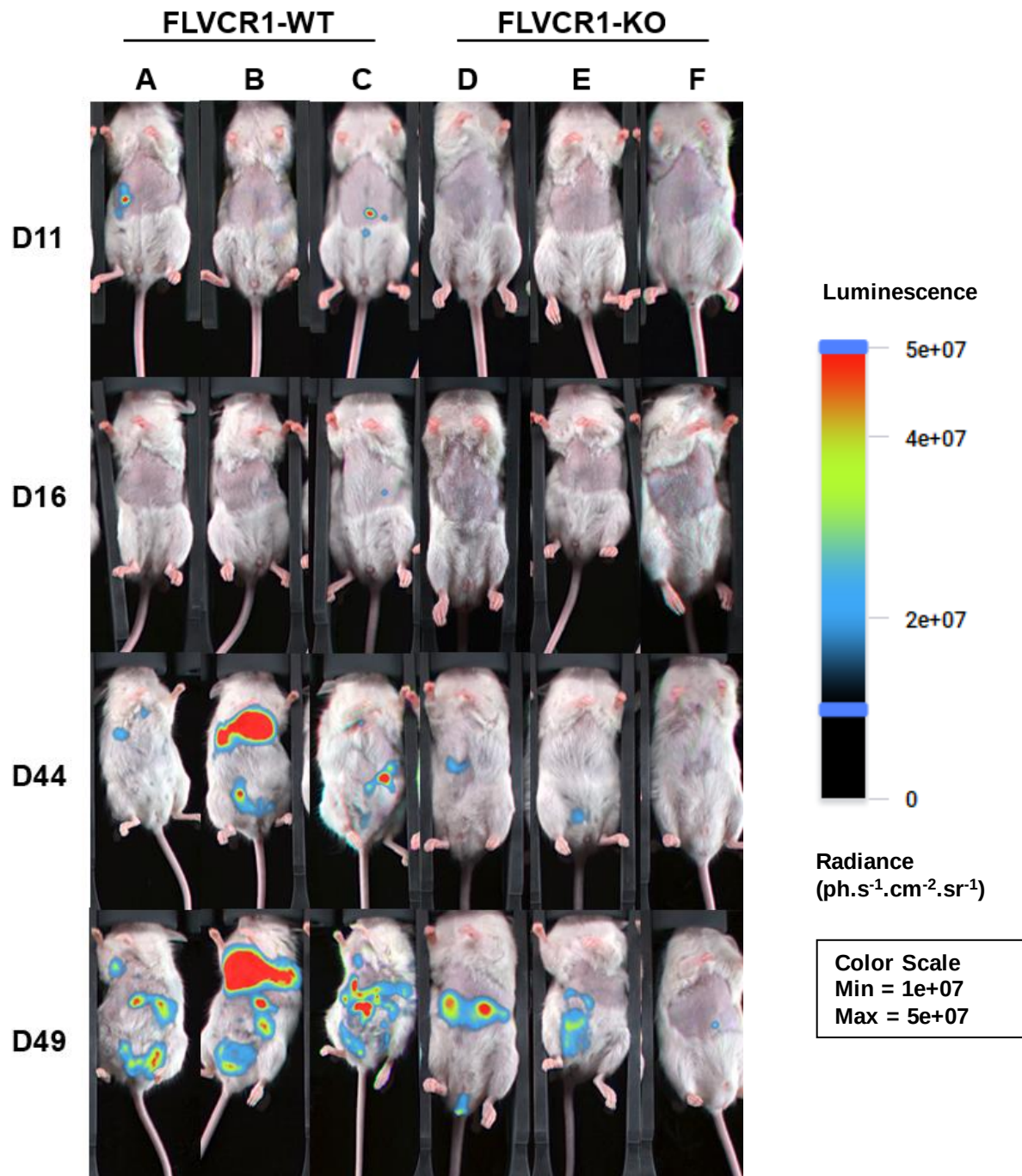


Figure 14 - Bioluminescence imaging, on days 11, 16, 44 and 49, of mice that received echo-guided intrahepatic injections. Animals A, B and C were injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells and animals D, E and F were injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells. These animals are representative of the 18 animals injected. The scale bar indicates radiance and the images are all normalized to the same scale. All the mice revealed tumors in sites other than the liver, such as intestine and spleen.

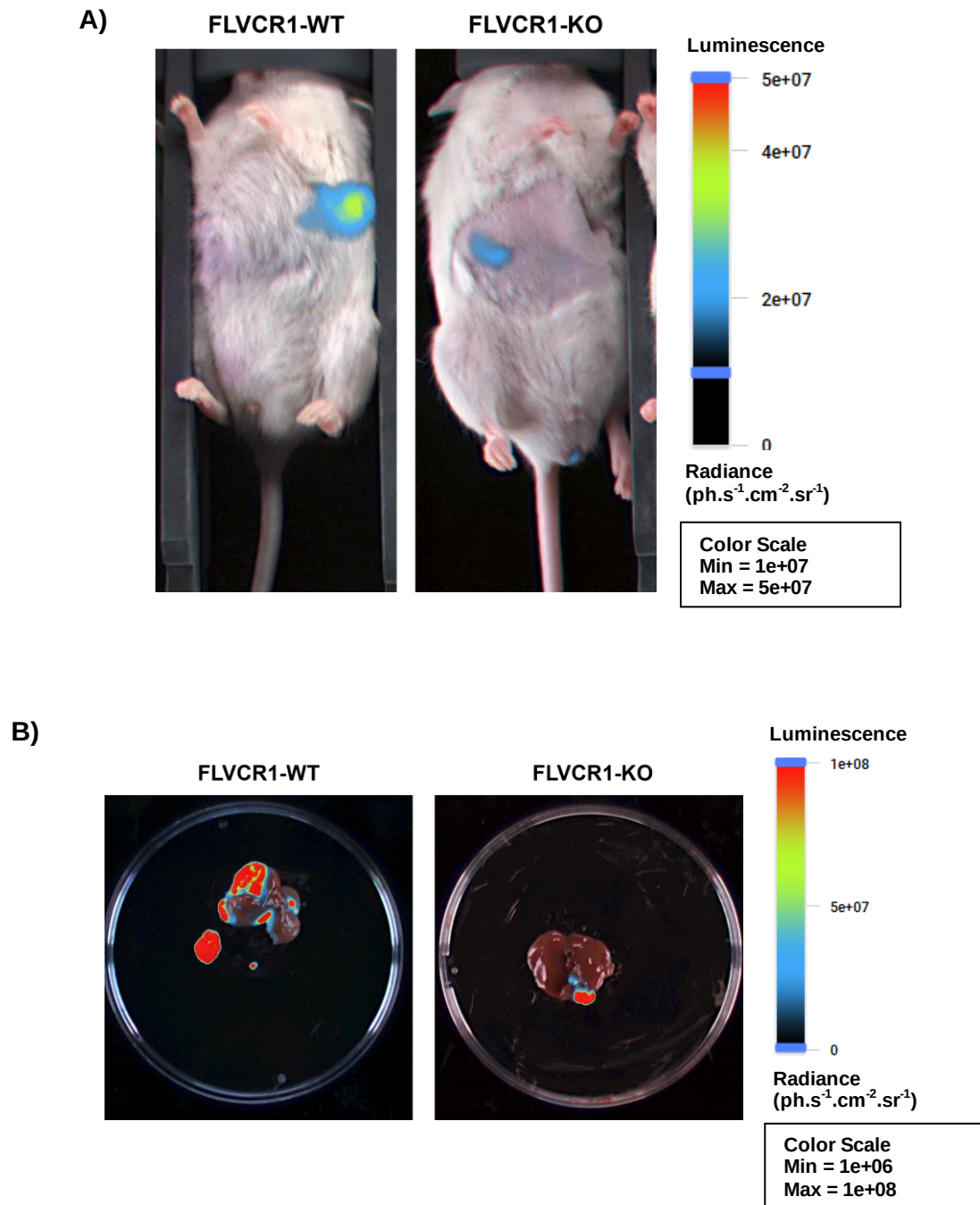


Figure 15 - Mice bioluminescence imaging and respective liver tumors. (A) Bioluminescence images from a mouse injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells sacrificed on day 44 due to low body condition, and a mouse injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells sacrificed on day 49. **(B)** Respective liver tumors collected immediately after cervical dislocation, WT tumor measuring 226 mm³ and KO tumor measuring 0.1 mm³.

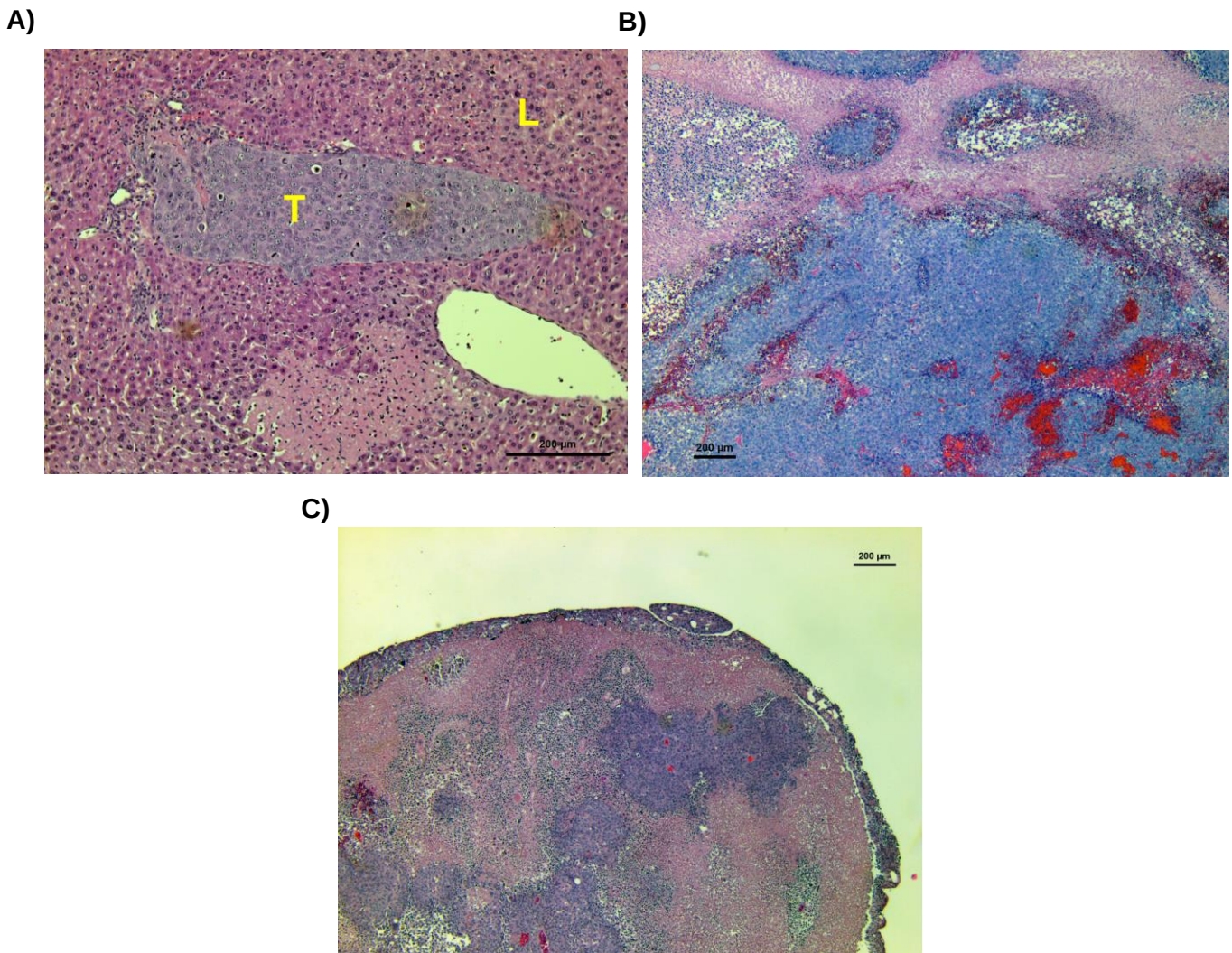


Figure 16 - Histology of tumor-bearing mouse livers following echo-guided intrahepatic injections of Huh-7 cells. H&E staining of tumors (T) or normal liver (L) derived from animals injected intrahepatically with either *FLVCR1*-WT Huh-7 luciferase-expressing cells (**A**, **B**) or *FLVCR1*-KO Huh-7 luciferase-expressing cells (**C**). (**A**) is in a 10× magnification, (**B**) and (**C**) are in a 4× magnification. Scale bar = 200 μm.

We then tested intrahepatic injections after performing laparotomy. In this experiment, 12 mice were injected with either 1×10^6 *FLVCR1*-WT (n = 6) or *FLVCR1*-KO (n = 6) Huh-7 luciferase-expressing cells. The death of an *FLVCR1*-KO recipient mouse was registered during surgery. A, B, C, D, E and F mice were injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells and G, H, I, J and K were injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells. Injections after laparotomy resulted in bioluminescence signals originating exclusively from the liver (**Figure 17**). No bioluminescence signal was detected in F (*FLVCR1*-WT) and I, J and K (*FLVCR1*-KO) mouse recipients. The recipient mouse K died, 34 days after injection, of unknown causes, without the presence of tumors. The bioluminescence signal in *FLVCR1*-WT mouse recipients was more intense and with a superior area, when compared to a

more contained signal in *FLVCR1*-KO counterparts. In animal A, bioluminescence was even detected in extrahepatic sites.

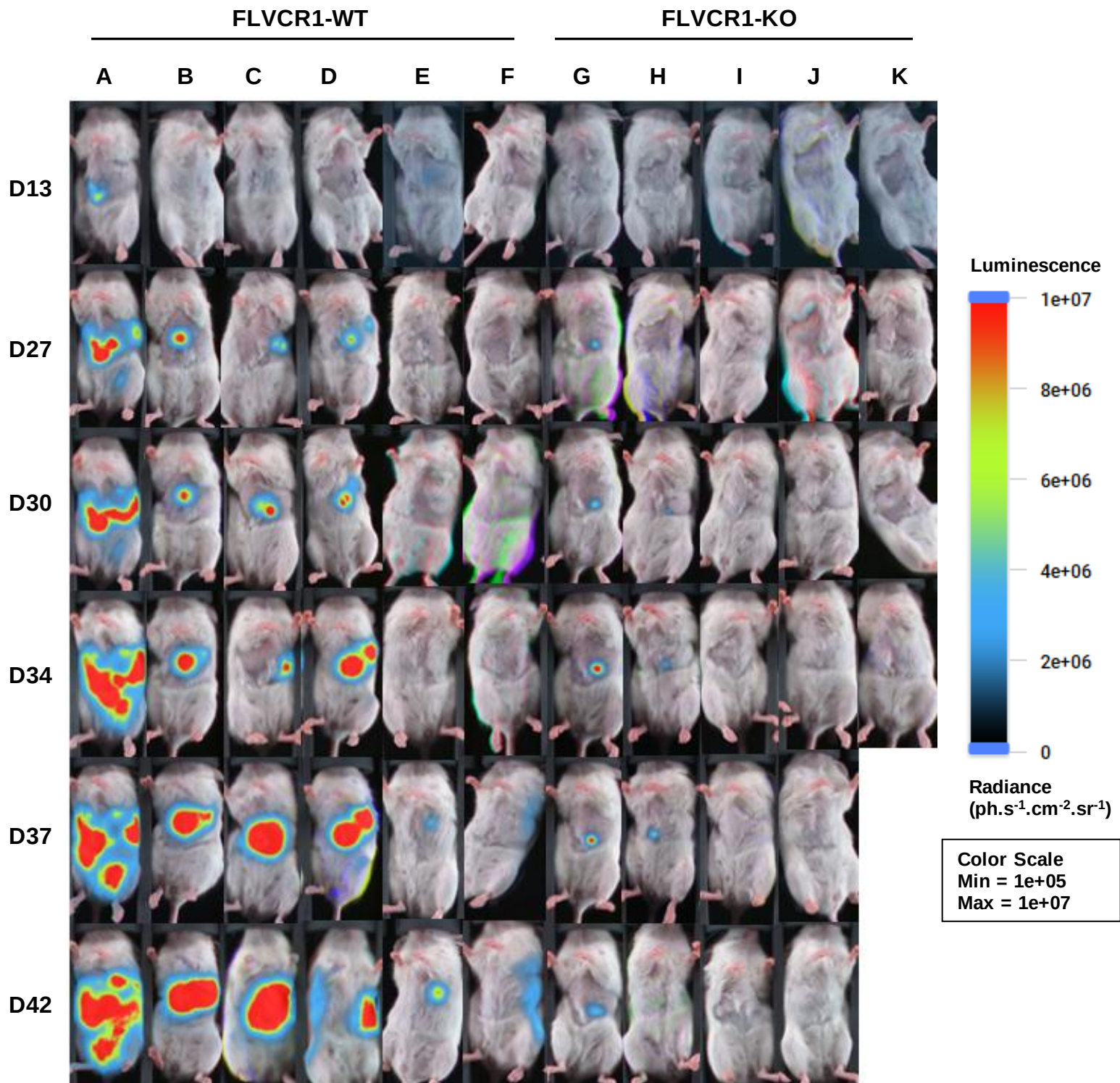


Figure 17 - Bioluminescence imaging of mice that received intrahepatic injections of human HCC cells after laparotomy. Animals A, B, C, D, E and F were injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells and animals G, H, I, J and K were injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells. Bioluminescence was measured at days 13, 27, 30, 34, 37 and 42 after injection. From the 11 injected mice, no bioluminescence signal was detected in one mouse recipient of *FLVCR1*-WT Huh-7 luciferase-expressing and in three recipients of *FLVCR1*-KO Huh-7 luciferase-expressing cells. Animal K died of unknown causes. The scale bar indicates radiance and the images are all normalized to the same scale.

Ultrasound was used to monitor tumor size to ensure it did not exceed the maximum allowed of 1 cm in any direction (**Figure 18**). Mice were sacrificed 42 days after injection and organs were immediately harvested to evaluate liver bioluminescence and the presence of metastases in lungs and inguinal lymph nodes (**Figure 19A**). The livers of mice A, B, C and D presented bioluminescence signals corresponding to macroscopically visible tumors with a total tumor volume of 919.5, 153.6, 266.0 and 65.5 mm³, respectively. Mouse A also presented inguinal lymph node metastases, which corresponds to the extrahepatic signal obtained before sacrifice. Animal E depicted bioluminescence signal in the liver region before sacrifice, although it had no macroscopically visible tumors or luciferase signal in this organ after sacrifice. In turn, animal F had no bioluminescence before sacrifice nor a macroscopically visible tumor but showed a foci of bioluminescence signal in the liver. *FLVCR1*-KO mouse recipients (G, H, I and J) had no visible tumors or bioluminescence signal in the respective livers even though luciferase expression was detected in mice G and H before sacrifice. The radiance values showed to be significantly superior ($P < 0.05$) in the bioluminescence signal emitted in the livers of the *FLVCR1*-WT mouse recipients compared to *FLVCR1*-KO counterparts (**Figure 19B**). *FLVCR1*-WT Huh-7 luciferase-expressing cells were able to develop larger tumors and, in the case of animal A, had the ability to form metastases in the inguinal lymph nodes.

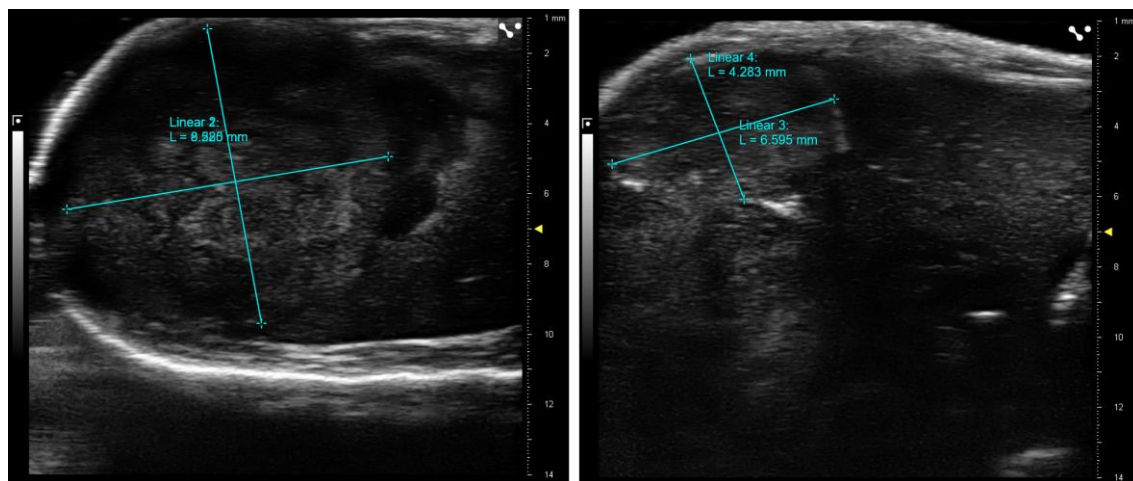


Figure 18 – Tumor ultrasound imaging. Representative ultrasound image of tumors in mice injected intrahepatically with human Huh-7 cells after laparotomy. Ultrasound was performed to ensure that mice were humanely sacrificed before the tumor size reached a maximum volume of 1 cm in any direction. The animals are oriented with the ventral side up and dorsal side down.

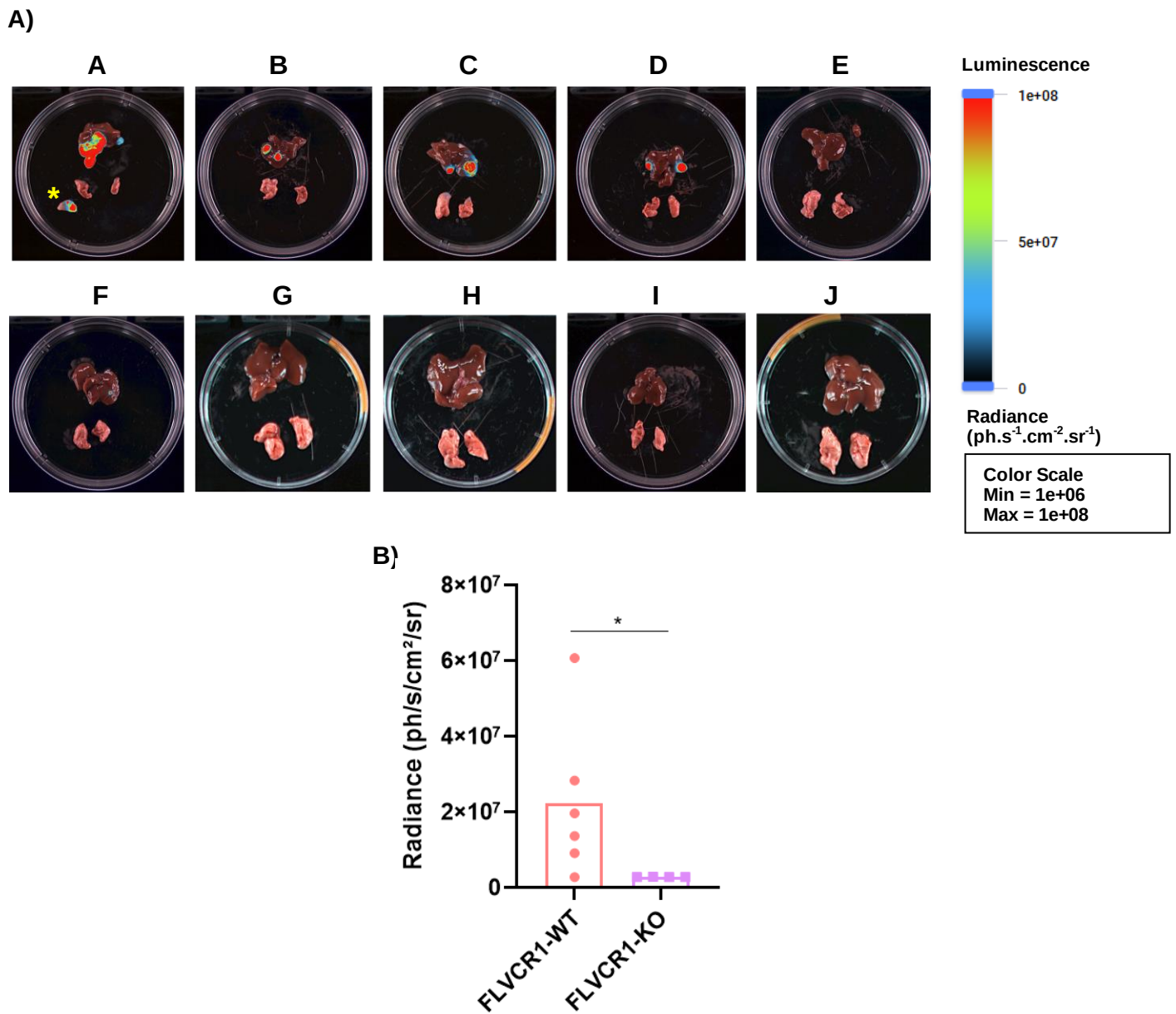


Figure 19 – Tumor development in mice injected intrahepatically with Huh-7 luciferase-expressing cells after laparotomy. (A) Bioluminescence in liver, lungs and inguinal lymph nodes. A-F mice were injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells. Mouse E did not show bioluminescence signal. The yellow asterisk next to the organs of the first animal reveals metastases in the inguinal lymph nodes. G-J mice were injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells and did not show luciferase expression in the liver or lungs. **(B)** Radiance comparison between the livers of *FLVCR1*-WT and *FLVCR1*-KO mouse recipients. Statistical significance was determined with the Mann-Whitney test $*P < 0.05$.

Tumor development in mice that received *FLVCR1*-WT Huh-7 luciferase-expressing cells was confirmed by histologic analysis of liver sections. **Figure 20A** depicts a large tumor, distinguished by a more basophilic appearance when compared to the eosinophilic non-tumorous liver. As expected, the tumors showed loss of normal hepatic tissue architecture, with absence of portal triads, as well as presence of necrosis and hemorrhage. A higher magnification of a tumor shows abundant mitotic figures (**Figure 20B**). In mouse recipients of *FLVCR1*-KO Huh-7 luciferase-expressing cells, we observed no liver tumors. Instead, histologic analysis of sections from the liver lobule in which the injection of Huh-7 cells took place revealed the presence of necroinflammatory foci comprising histiocytes, siderophages, cell debris and apoptotic bodies (**Figures 20C,D**).

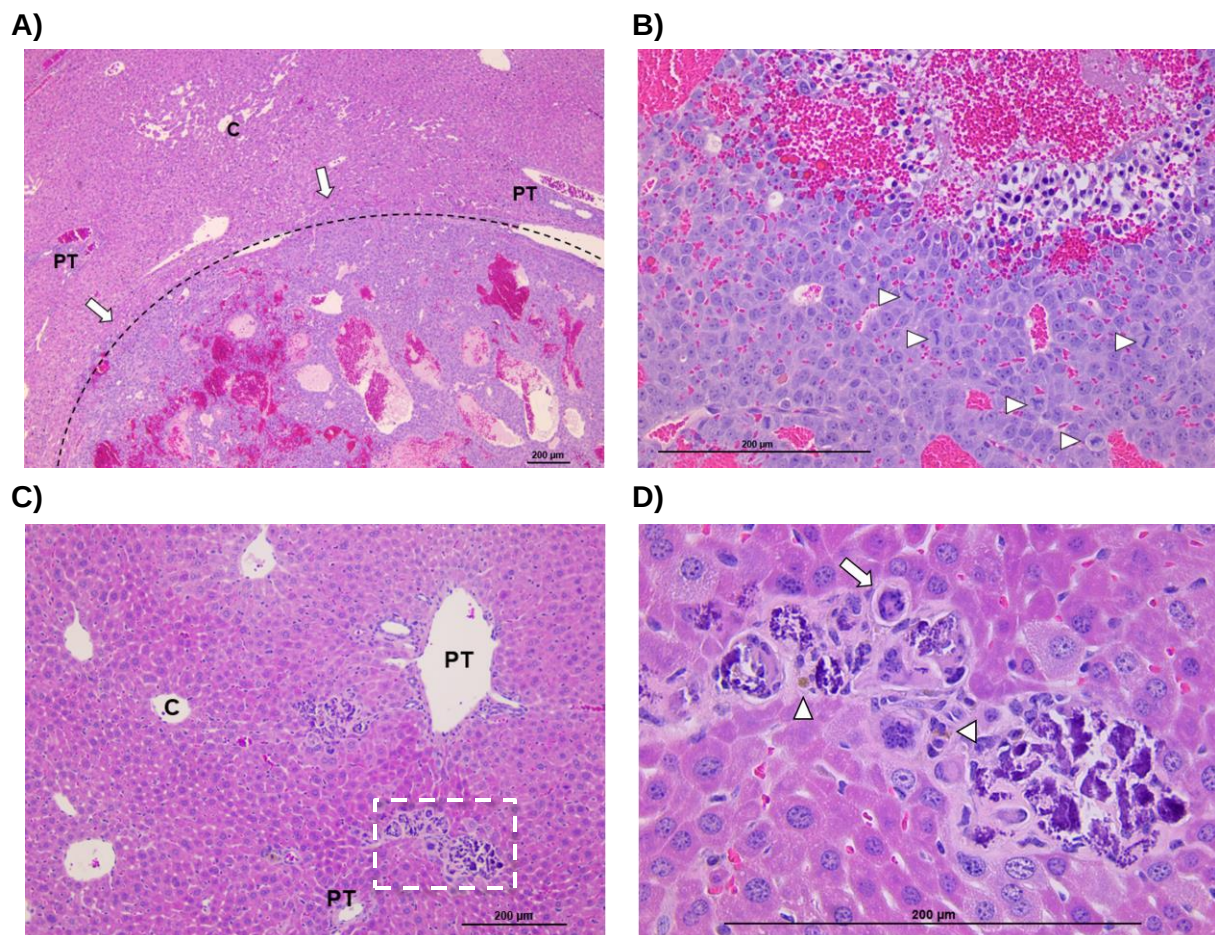


Figure 20 - Histology of tumor-bearing mouse livers following intrahepatic injections of Huh-7 cells after laparotomy. H&E staining of livers derived from animals injected intrahepatically with either *FLVCR1*-WT Huh-7 luciferase-expressing cells (**A, B**) or *FLVCR1*-KO Huh-7 luciferase-expressing cells (**C, D**). (**A**) The dotted line marks the border of a large tumor. Arrows point to areas of tumor invasion. Original magnification: 4× (**B**) Arrow heads point to mitotic features. Original magnification: 20× (**C**) Typical morphology of the liver of a mouse recipient of *FLVCR1*-KO Huh-7 luciferase-expressing cells, denoting normal liver architecture. Original magnification: 10× (**D**) Higher magnification of the area contained within the dotted rectangle, depicting a necroinflammatory focus with cell debris and immune cells. Arrow points to an apoptotic body and arrow heads point to siderophages. Original magnification: 40×. Scale bar = 200 μm. Abbreviations: C – Centrilobular; PT – Portal triad.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) enzyme activity levels, as well as total, direct and indirect bilirubin, were measured to assess HCC-associated liver damage through serum biochemical analysis (**Figure 21**). The enzymatic levels of AST and ALT, which are two liver injury markers, showed a tendency toward increased AST ($P = 0.147$) and a borderline increase in ALT activity ($P = 0.059$) in *FLVCR1*-WT mouse recipients compared to *FLVCR1*-KO counterparts. There were no significant differences between the two groups regarding ALP activity and total, direct and indirect bilirubin levels, which are also markers of liver dysfunction.

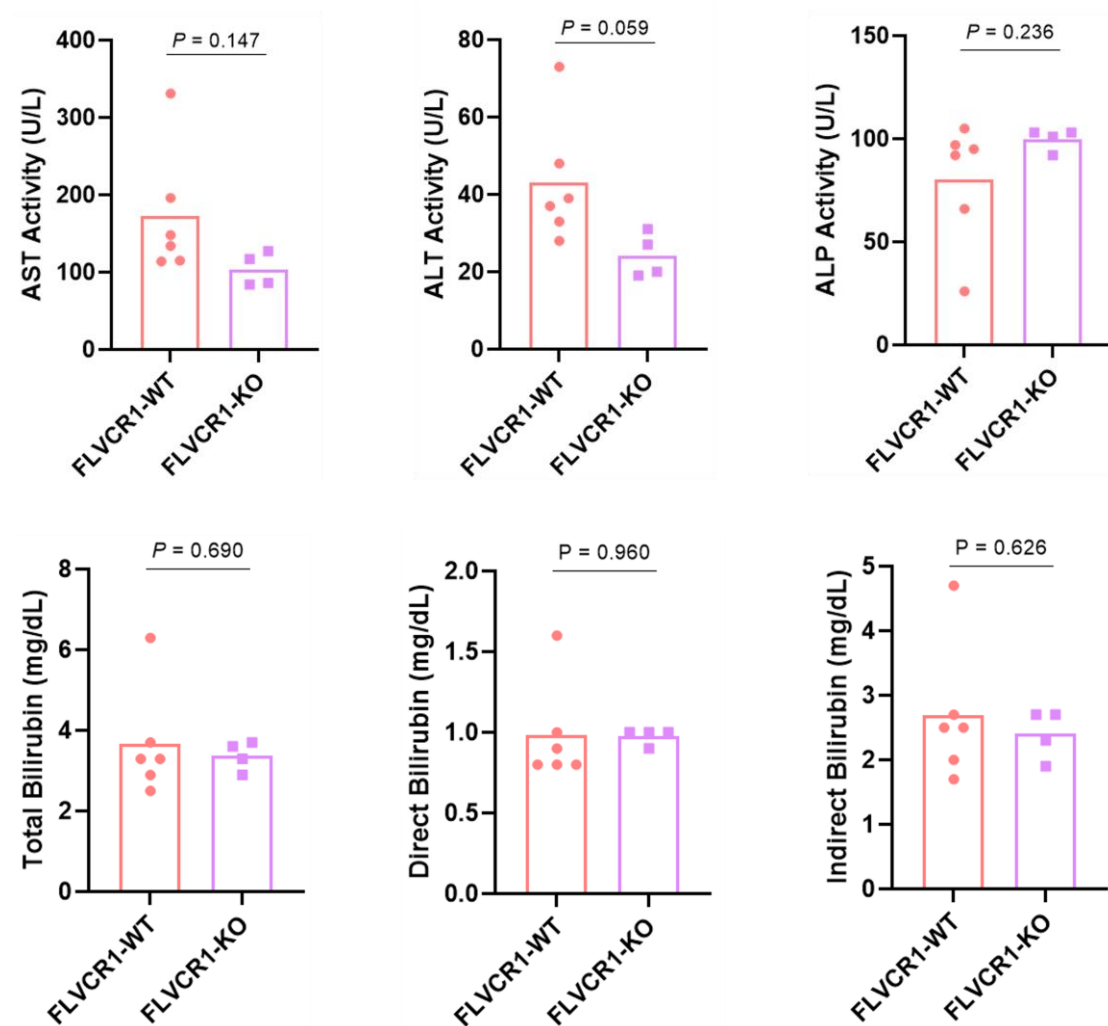


Figure 21 – Liver function comparison between *FLVCR1*-WT and *FLVCR1*-KO mice. AST, ALT and ALP activity and total, direct and indirect bilirubin in *FLVCR1*-WT (n=6) and *FLVCR1*-KO (n=4) mice. Statistical significance was determined with unpaired t test. Abbreviations: AST – Aspartate Aminotransferase; ALT – Alanine Aminotransferase; ALP – Alkaline Phosphatase.

4.3. Therapeutic Potential of *FLVCR1* Silencing

4.3.1. Time Course of *FLVCR1* Knockdown

As transfection with siRNAs results in a transient knockdown, it was crucial to know how long FLVCR1 protein levels remained reduced in order to carry out functional assays. Briefly, after cells were transfected with a scrambled siRNA (si-Scramble) or with a set of three *FLVCR1*-specific siRNAs (si-FLVCR1), samples were collected every 24 hours for 7 days. RNA and protein expression were evaluated using RT-qPCR and Western Blot, respectively.

As shown in **Figure 22A**, the effect of the siRNA-mediated knockdown on the *FLVCR1* mRNA levels was evident as early as 24 hours after transfection, when the expression was barely detectable. The knockdown persisted until day 4, except for day 3 in which mRNA expression appeared to show no differences compared to the si-Scramble control. From day 5 onwards, *FLVCR1* mRNA expression increased gradually, even exceeding control expression levels on day 7. *FLVCR1* and *HPRT1* amplification curves and melt peaks are shown in **Supplementary Figures 3 and 4**, confirming the specificity of the amplification.

Regarding FLVCR1 protein expression (**Figure 22B**), the knockdown effect was evident at 24 hours after transfection and became more pronounced at 2 days after transfection. In agreement with mRNA expression, protein expression levels appeared to increase from day 5 after transfection, approaching the respective si-Scramble control.

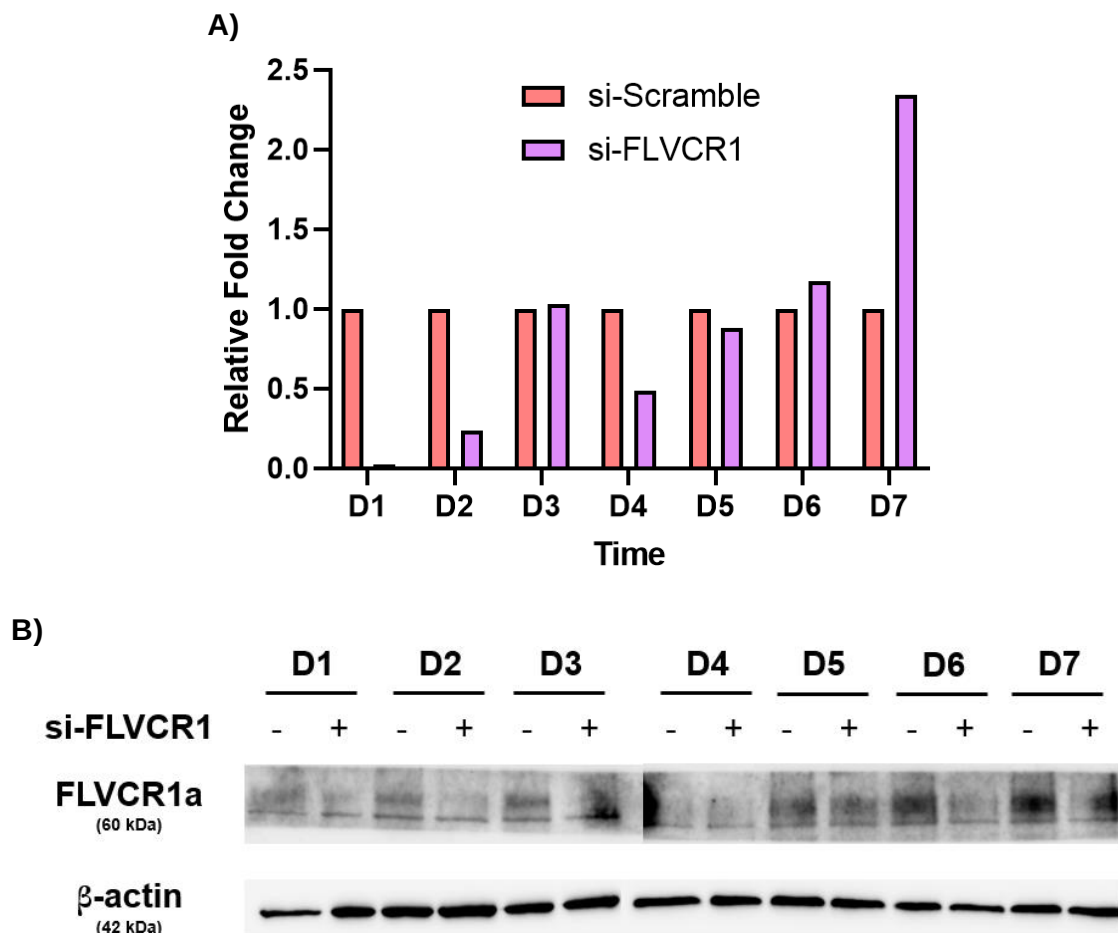


Figure 22 - Time course of siRNA knockdown of *FLVCR1* in Huh-7 cells. Huh-7 cells were transfected with 10 nM of either a scrambled siRNA (si-Scramble) or a mix of three different siRNAs specific to *FLVCR1* (si-*FLVCR1*). Total RNA and protein were collected from day 1 to day 7 post transfection. **A)** *FLVCR1* mRNA expression was measured by RT-qPCR using HPRT1 as an endogenous control gene. **B)** *FLVCR1* protein expression was evaluated by Western Blot, using β -actin as a loading control to normalize protein levels.

4.3.2. siRNA-mediated *FLVCR1* Knockdown Compromises Huh-7 Cell Migration

A Single-Cell Migration assay was performed to assess the effect of *FLVCR1* knockdown in the migration capacity of Huh-7 cells. Cells were seeded at low-density and images were acquired every 10 minutes in a live cell imaging system during 16 hours.

The mean total distance traveled by the tracked cells, which is the sum of all the individual steps taken by each cell, proved to be significantly lower in cells transfected with si-*FLVCR1* compared to si-Scramble ($P < 0.05$) (**Figure 23A**). Likewise, the mean speed of the monitored cells was lower in *FLVCR1* knockdown

cells ($P < 0.01$) (**Figure 23B**). The graph depicting displacement along the X and Y axes shows the change in position from the cells' initial to final point. Some difference was noticeable between the two conditions, with the si-FLVCR1 cells appearing to have migrated less over time compared to the si-Scramble control (**Figure 23C**). Finally, the distance between two consecutive positions (i.e. steps) and the speed, over time, were also significantly lower in the si-FLVCR1 condition compared to control cells ($P < 0.001$) (**Figure 23D and Figure 23E**).

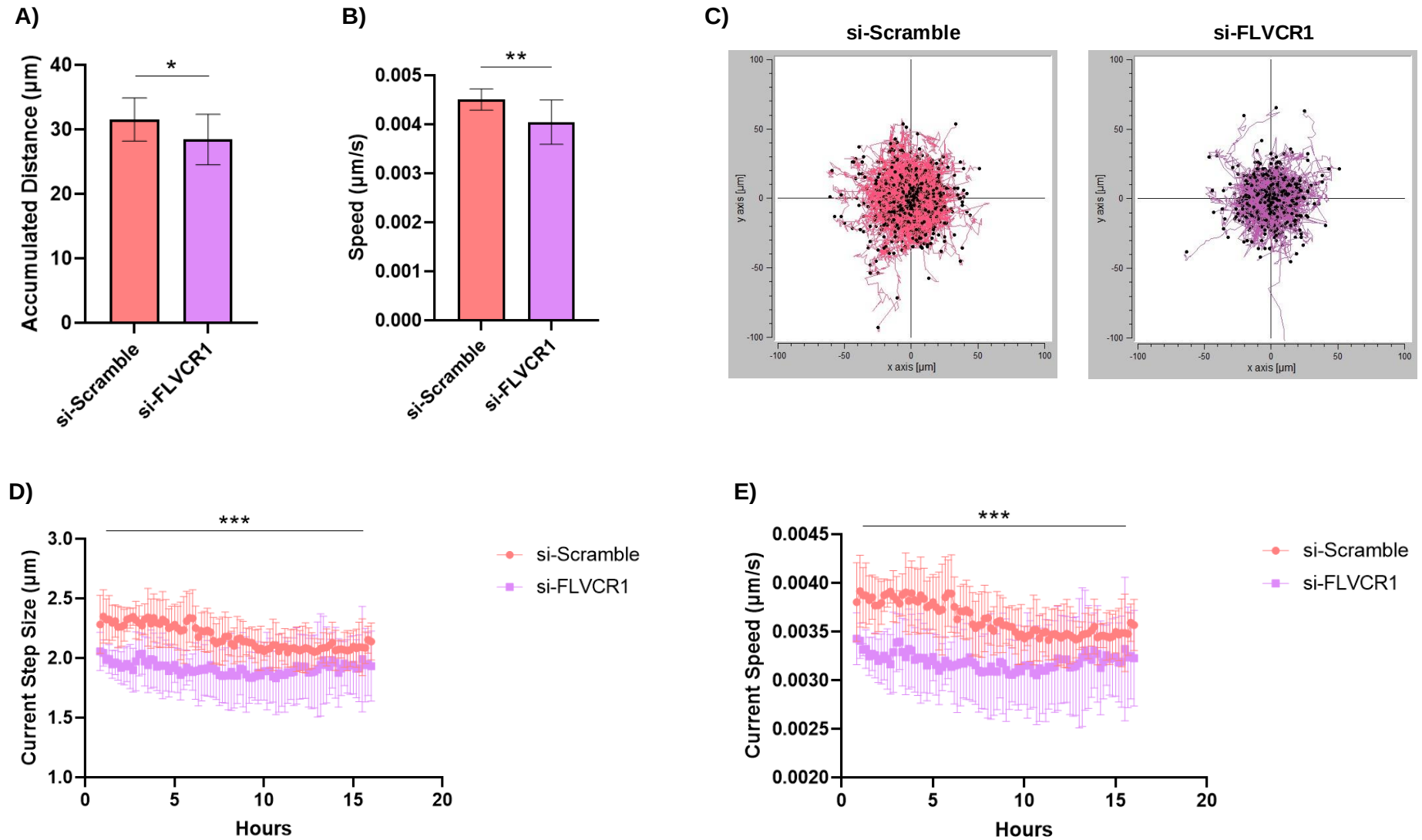


Figure 23 – siRNA-mediated *FLVCR1* knockdown reduces the migratory capacity of Huh-7 cells. A single-cell migration assay was performed for 16 hours with image acquisitions every 10 minutes. **A)** Accumulated distance of control and knockdown single cells and **B)** speed of migrating control and knockdown single cells for 16 hours, the results are the average and SD of the pooled replicates. **C)** Representative trajectory of each cell for 16 hours. **D)** Single cells' step size and **E)** speed over time. Results are the average and SD of 3 independent experiments. Statistical significance was determined with paired t-test and 2way ANOVA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5. Discussion

The overexpression of *FLVCR1* in HCC and its correlation with worse prognoses, reported in 2018 by Shen, *et al.*, makes it imperative to understand the mechanism by which *FLVCR1* affects this disease, with the aim of directing more efficient treatments for these patients. Therefore, we aimed to further understand the impact of the *FLVCR1* KO in HCC.

Preliminary results previously obtained by our group showed that Huh-7 Clones 1 and 2, in which *FLVCR1* KO was induced by CRISPR/Cas9, have lower proliferative, migratory and invasive capacity, compared to *FLVCR1*-WT counterparts. Furthermore, the results showed an accumulation of heme in Huh-7 *FLVCR1*-KO cells. Since *FLVCR1*-mediated regulation of heme metabolism was previously shown to modulate cell energetic metabolism¹¹⁴, it became a priority to assess whether there was a shift in metabolism between *FLVCR1*-WT and *FLVCR1*-KO human HCC cells.

As previously mentioned, Warburg demonstrated that tumor cells alter their metabolism to one in which there is a greater conversion of glucose into lactate, even in the presence of oxygen, this being one of the hallmarks of cancer^{90,115}. This occurs because, although oxidative phosphorylation produces more ATP per glucose molecule, glycolysis can produce energy faster, in addition to providing metabolites required for cell growth^{115,116}. However, an excess of heme in tumor cells forces them to increase mitochondrial respiration⁸⁸. Therefore, it would be expected a shift from glycolytic to oxidative metabolism from *FLVCR1*-WT Huh-7 cells to the respective *FLVCR1*-KO clones, since there is an accumulation of heme derived from the deletion of its main exporter.

Basal OCR levels revealed a preference of *FLVCR1*-KO cells, specially Clone 2, for oxidative phosphorylation compared to *FLVCR1*-WT Huh-7 cells. During oxidative phosphorylation, mitochondria consume oxygen to generate an electrochemical gradient that is necessary for energy production. Thus, OCR is a measure of oxidative phosphorylation. On the other hand, ECAR is a measure of glycolysis, since the extracellular acidification assessed herein is caused by the final product of glycolysis, lactate. Therefore, a higher ECAR value for *FLVCR1*-WT Huh-7 cells relatively to their *FLVCR1*-KO clones would be expected. The fact that *FLVCR1*-WT Huh-7 cells have a more glycolytic metabolism than Clone 2 of *FLVCR1*-KO Huh-7 cells, in which there is a significant increase in oxidative phosphorylation, is confirmed by the ATP production rate results. The glycolysis ATP production rate shows the inverse trend of the mitochondrial ATP production rate, revealing a shift towards a more oxidative metabolism in Clone 2 of *FLVCR1*-KO Huh-7 cells compared to *FLVCR1*-WT Huh-7 cells. After injection of oligomycin, which blocks ATP synthesis, and a mix of rotenone and antimycin A, which completely block mitochondrial

respiration, OCR levels in all conditions decrease, as expected. However, in this situation the ECAR levels of Clone 1 of *FLVCR1*-KO Huh-7 cells are similar to the levels of *FLVCR1*-WT Huh-7 cells, while Clone 2 of *FLVCR1*-KO Huh-7 cells do not increase their glycolytic metabolism despite the blockage in oxidative phosphorylation. These results are in agreement with data from Fiorito *et al.*, who showed that the silencing of *FLVCR1*, in colorectal adenocarcinoma cells (Caco-2), achieved through transfection with *FLVCR1*-specific short hairpin RNAs (shRNAs) resulted in an increase in the activity of all complexes that compose the respiratory chain, indicating an increase in oxidative phosphorylation that was also supported by increased mitochondrial membrane potential.

The metabolic shift of the *FLVCR1*-KO Clone 2 compared to *FLVCR1*-WT cells was more pronounced than that of Clone 1, which is in agreement with greater decreases in cell proliferation, migration and invasion, previously observed by our group. This may be explained by the higher levels of intracellular heme in Clone 2. For this reason, Clone 2 was chosen to be transduced with lentivirus containing the pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase plasmid, which expresses GFP and luciferase as a fusion protein, hereafter referred to as *FLVCR1*-KO Huh-7 cells. After transduction, *FLVCR1*-WT and *FLVCR1*-KO Huh-7 cells were selected with puromycin, so that only the cells that incorporated the plasmid would survive. However, even after selection, not all cells had GFP expression as determined by fluorescence microscopy. This could be due to: greater difficulty for Huh-7 cells to be transduced with this specific plasmid, delivery method, DNA quality, low stability of GFP mRNA and protein, cellular stress derived from transduction or the existence of variation between cells due to stochastic processes. To overcome this problem, cells with the highest GFP signal intensity were sorted and *FLVCR1*-WT Huh-7 cells showed a much higher percentage of GFP⁺ cells than *FLVCR1*-KO Huh-7 cells (38.3% and 1.4%, respectively). This difference may indicate that *FLVCR1* KO indirectly influences GFP expression, for example due to heme accumulation and consequent oxidative stress that can impact GFP stability. We should have carried out a single cell sorting in order to guarantee the homogeneity of the GFP signal and consequently the one emitted in the luciferase reaction.

NSG-SGM3 was the chosen mouse model to inject Huh-7 luciferase-expressing cells due to its lack of T, B and Natural Killer cells and their deficient innate immunity and cytokine signaling pathways. This severe immunodeficiency allows the engraftment of human cells and the establishment of orthotopic models. Initially, echo-guided intrahepatic injections were chosen as the method to generate the orthotopic model, as it is a less invasive method, which does not require surgery and its

associated complications. Echo-guided injections were already performed in other organs, such as the heart and in embryos, and applied in clinics¹¹⁷. Moreover, orthotopic bladder and pancreatic cancer models using echo-guided injection have been successfully established¹¹⁸. However, our ultrasound-guided intrahepatic injections were not successful since in 8 out of 9 *FLVCR1*-WT recipient mice, we detected a bioluminescence signal, derived from luciferase expression of tumor cells, in organs other than the liver. In the case of *FLVCR1*-KO recipient mice, a bioluminescence signal was found outside the liver in 7 out of 9 mice and engraftment did not occur in 1 mouse. This shows that in many cases there was cell leakage out of the liver during the injection, whereas in other cases cells were not injected in the liver at all. However, in two mice, tumors were found exclusively in the liver, one in a *FLVCR1*-WT recipient and one in a *FLVCR1*-KO recipient. *FLVCR1*-KO Huh-7 luciferase-expressing cells showed lower proliferative capacity compared with *FLVCR1*-WT Huh-7 luciferase-expressing cells, as indicated by both the emitted bioluminescence signal and the tumor volume. Bou About *et al.*, who were the first to standardize the method for echo-guided intrahepatic injections, did not observe tumor engraftment in the liver but in other organs of a small proportion of mice (< 2%), suggesting that the injection was outside the liver lobe¹¹⁷. McVeigh *et al.*, used echo-guided intrahepatic injections to generate an orthotopic model of colorectal cancer liver metastases. In this study, authors present a panel demonstrating tumor development over time with bioluminescence imaging, in which the appearance of bioluminescence signal outside the liver is visible and reveals the occurrence of extrahepatic leakage of tumor cells during injections¹¹⁸. For a successful injection, the needle tip must not be too deep in the liver lobe but also not too superficial so as not to obtain tumors in the peritoneum, and the use of a solid matrix, such as Matrigel, could have been useful to prevent some leakage^{117,118}. Although ultrasound-guided intrahepatic injections are less invasive and a measure of refinement in animal research, the method turned out to be too unpredictable, even after extensive training. This made us move on with intrahepatic injections after laparotomy.

Unlike the echo-guided intrahepatic injections, in intrahepatic injections after laparotomy the bioluminescence signal was observed firstly in the liver, which shows that no cell leakage occurred. Therefore, despite the fact that intrahepatic injections are more invasive, these showed to be more reliable. In mice injected with *FLVCR1*-WT Huh-7 luciferase-expressing cells in which the engraftment occurred (5/6), cells have given rise to larger bioluminescence signals, which is proportional to tumor size, and also seemed to appear in other tissues over time. On the contrary, the signal obtained in *FLVCR1*-KO mouse recipients remained contained in the liver region

without appearing in other organs. In the case of mice injected with *FLVCR1*-KO Huh-7 luciferase-expressing cells, engraftment occurred in 2/5 animals. However, the bioluminescence signal obtained in the *FLVCR1*-KO mouse recipients before sacrifice did not correspond to the signal observed in the recipient livers after sacrifice, and no macroscopically visible tumors were found. **Figure 17** shows that the bioluminescence signals of G and H mice (*FLVCR1*-KO) decreased in intensity between days 37 and 42 after injection. This may indicate that cells in which engraftment occurred ended up dying, probably because they were insufficient in number given that part of the injected cells may not have engrafted. This is supported by the histology of the livers of *FLVCR1*-KO mouse recipients, which shows necroinflammatory foci.

The quantification of AST, ALT and ALP enzymes levels, as well as total, direct and indirect bilirubin levels, are commonly used for clinical assessment of liver function. ALT and AST are metabolic enzymes found in the liver and elevated serum levels indicate hepatocyte necrosis and inflammation¹¹⁹. Mice presenting larger tumors presented higher levels of AST and ALT, indicating more severe hepatocellular injury at more advanced HCC stages, as expected. Results showed that in *FLVCR1*-WT mouse recipients there is a tendency toward increased hepatocellular injury compared to *FLVCR1*-KO counterparts. ALP is an enzyme associated with dephosphorylation and elevated levels are indicative of cholestasis or bile duct obstruction. Indirect or unconjugated bilirubin results from hemoglobin breakdown during erythrocyte phagocytosis, whereas direct bilirubin has been conjugated in the liver and is excreted in the bile. Elevated levels of total, direct and indirect bilirubin indicate liver dysfunction. Only the mouse with higher tumor volume (an *FLVCR1*-WT recipient) presented high bilirubin levels, indicating hepatocellular dysfunction at more advanced HCC stages. The levels of bilirubin and AST, ALT and ALP enzymes show that mice were sacrificed prior to reaching a state of tumor development in which liver function and animal health had been severely compromised.

Treatments developed for HCC, such as Sorafenib, improve patient prognosis but with a consequent substantial loss of quality of life. Thus, there is a need to develop new therapeutic approaches with fewer adverse effects and greater specificity^{120,121}. siRNAs are designed to cleave and degrade the mRNA of specific genes before translation causing gene knockdown¹²¹. siRNAs are able to decrease the expression of proteins with aberrant activity in cancer, which makes them a promising tool in the therapy of HCC¹²⁰. When siRNAs are administered intravenously, they accumulate in the liver with high affinity, through the reticuloendothelial system, which can be a problematic factor for therapies targeting other organs, but beneficial for therapies that target the liver¹²². For this reason, and because it is a method used in the treatment of

other liver diseases, we decided to transfect Huh-7 cells with *FLVCR1*-specific siRNAs to obtain gene knockdown.

To perform functional assays aimed at assessing the impact of *FLVCR1* knockdown on HCC, it was crucial to measure *FLVCR1* mRNA and protein levels over several days to evaluate the duration of the knockdown. *FLVCR1* mRNA expression in Huh-7 cells transfected with *FLVCR1*-specific siRNAs decreased substantially at 24 hours after transfection and gradually increased over the following days. The increasing trend did not appear to occur on day 3 after transfection, which may indicate an error during RT-qPCR, as expression was still low at day 4. From day 5 after transfection, mRNA expression in cells with knocked down *FLVCR1* approached the control mRNA expression, even exceeding these levels by day 7. The elevated mRNA expression in day 7 compared to the control may be the result of a negative feedback response from the cell to compensate *FLVCR1* levels. In the case of *FLVCR1* protein expression, *FLVCR1* knockdown was particularly evident at day 2 after transfection, which would be expected since siRNAs act at the mRNA level, i.e. protein reduction is a consequence of RNA degradation. Protein knockdown was maintained until day 5, after which any functional assay performed would no longer be reliable. It is worth noting another protein band that remained visible in the Western Blot throughout the days in both conditions (si-*FLVCR1* and si-Scramble), with a slightly lower molecular weight than that of *FLVCR1* (**Figure 21B**). It appears to be a non-specific band resulting from non-specific binding of primary and secondary antibodies, possibly due to an incomplete blocking. The hypothesis that the band corresponds to the non-glycosylated form of *FLVCR1* was ruled out since it did not present the same pattern of decreased protein expression visible in the band with higher molecular weight. In this Western Blot, the amount of protein from cells harvested on day 1 after transfection was not sufficient to match the remaining samples, so a smaller amount of protein was used, which explains the lower intensity of the bands in these samples.

The results of the Single-Cell Migration assay indicated that *FLVCR1* knockdown cells have slower and less extensive movements, with smaller steps, compared to the control (si-Scramble). We can conclude that cells with *FLVCR1* knockdown have compromised migration, which supports our previous evidence that *FLVCR1* has an important role in regulating the migration of Huh-7 cells. This result, in addition to agreeing with what was previously obtained in the group using Huh-7 cells whose *FLVCR1* was KO by CRISPR/Cas9, is also corroborated by the data from Zhou *et al.* In the Zhou *et al.* study, transducing esophageal squamous cell carcinoma cells (TE-1 and KYSE-150) with an sh*FLVCR1* recombinant lentivirus resulted in the knockdown of *FLVCR1*, which negatively affected cell migration and proliferation¹¹⁰.

Importantly, the recent discovery that FLVCR1 besides being an heme exporter is an importer of choline and ethanolamine, leaves doubt whether the phenotypes here observed are due to heme metabolism, choline and ethanolamine transport, or both. Further assays are needed to understand whether modulation of heme and/or choline/ethanolamine levels explain the impact of FLVCR1 on migration, proliferation, invasion and metabolism of HCC cells. Examples include the treatment of *FLVCR1*-WT Huh-7 cells with ALA, which would mimic the heme increase observed in *FLVCR1*-KO Huh-7 cells and the treatment of *FLVCR1*-KO Huh-7 cells with choline, to increase its import by the cell through other transporters.

6. Conclusion and Future Perspectives

Altogether, our orthotopic xenotransplant model revealed while *FLVCR1*-WT Huh-7 luciferase-expressing cells gave rise to large hepatic tumors in mice and metastasis to the inguinal lymph nodes, *FLVCR1*-KO Huh-7 luciferase-expressing cells engrafted but ended up dying, showing no capacity for tumor formation and growth. Based on our *in vitro* work, this may be due to the fact that *FLVCR1* KO causes a metabolic shift in human HCC cells, from a glycolytic metabolism to a more oxidative profile, thereby impairing cell growth and cell proliferation. Furthermore, our results suggest that siRNA-induced *FLVCR1* silencing decreases HCC cells' migration capacity.

These findings highlight the relevance of *FLVCR1* expression to HCC progression. However, further studies are needed to understand whether the resulting phenotype is caused by heme metabolism, choline/ethanolamine transport, or both. To achieve a complete mechanistic study of the phenotype observed with *FLVCR1* loss, changes in these products should also be linked to particular cellular processes important for HCC, including DNA replication, cell death and metabolism. Furthermore, it would be important to understand the role of the immune system in HCC progression after deletion or depletion of *FLVCR1*. After realizing the impact of *FLVCR1* silencing on HCC cell migration, it will also be important to study the effect on HCC cell proliferation. In addition, to bring this study closer to the clinic, it would be relevant to test the delivery of *FLVCR1*-specific siRNAs to HCC mice. The knowledge obtained from this study will be essential for the development of new therapies against HCC that will lead to an improved quality of life of patients.

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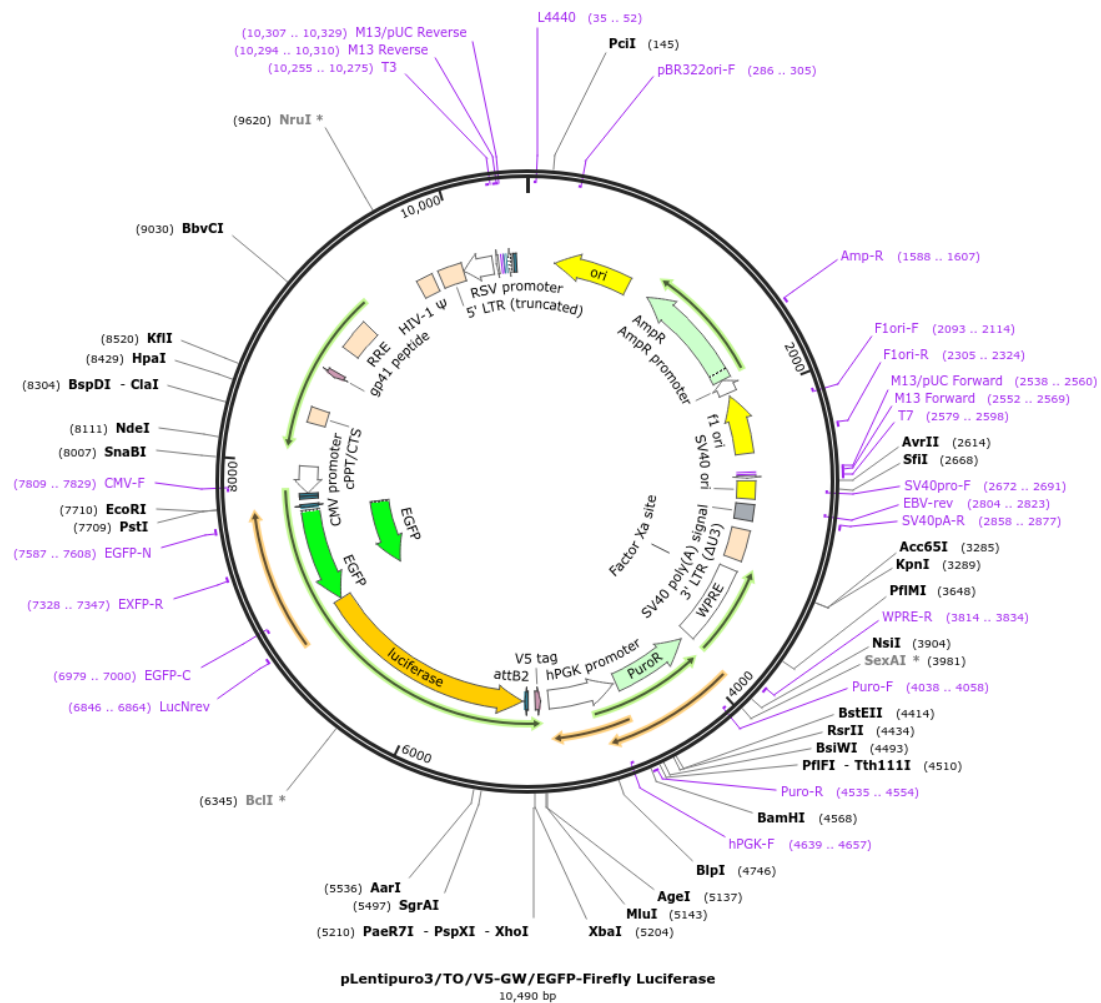
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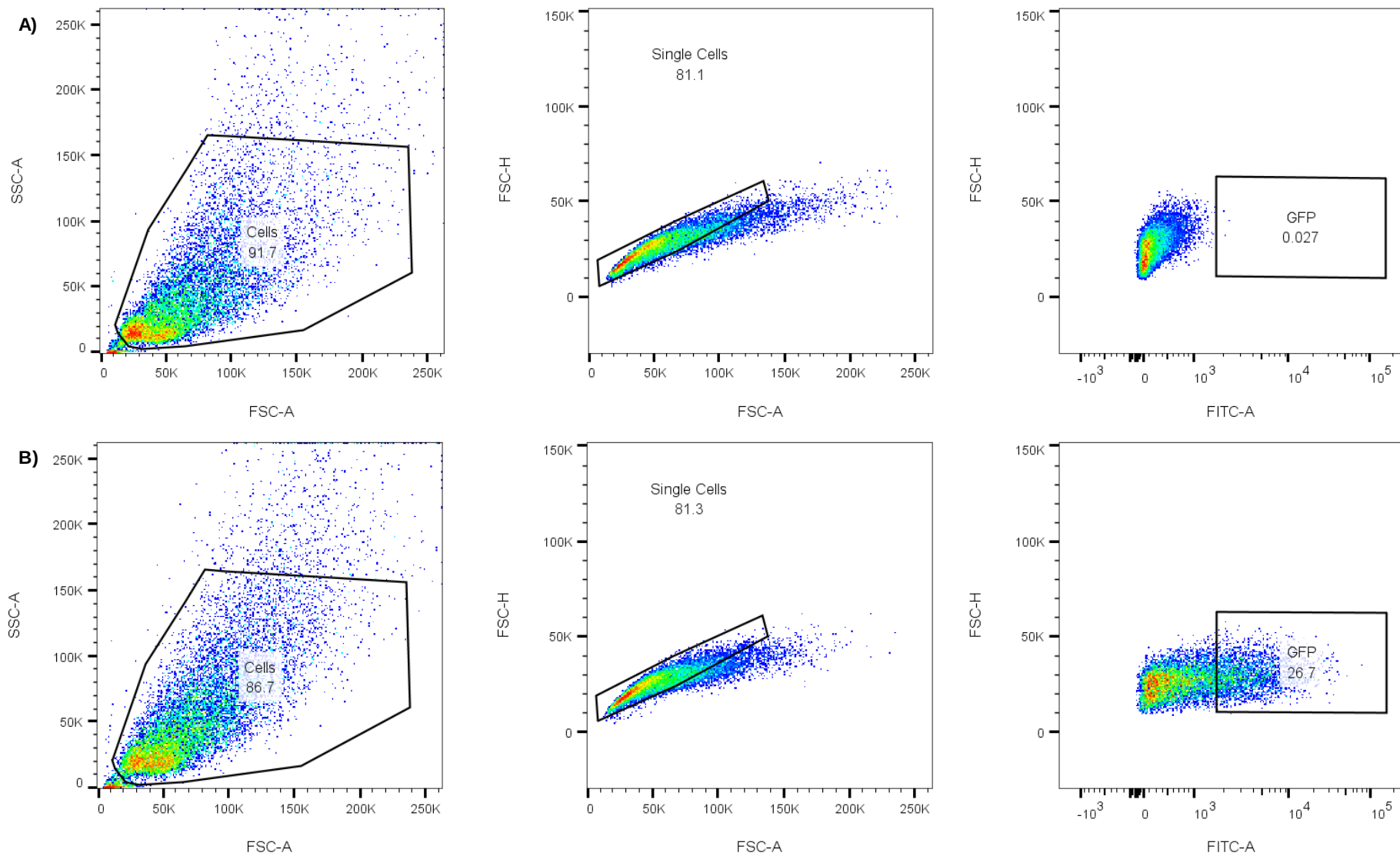
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8. Appendix

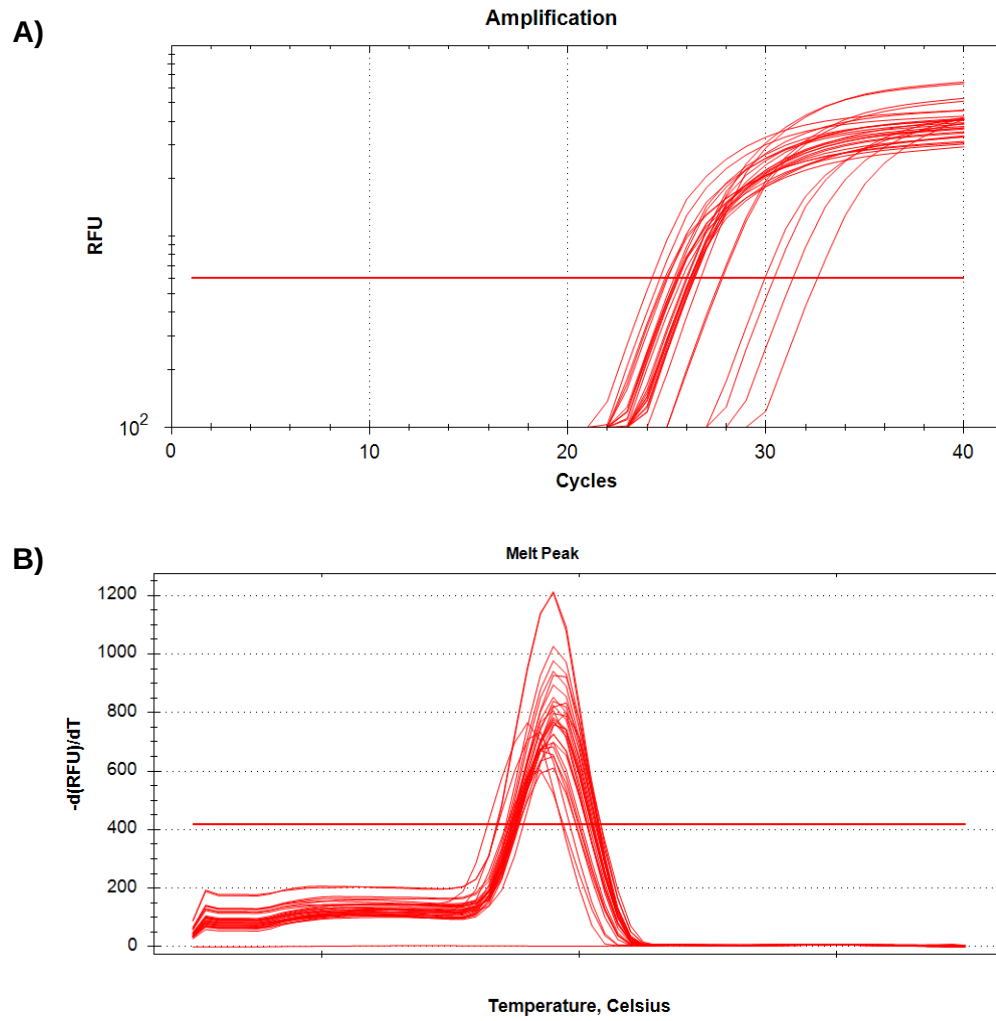
8.1. Appendix I: Supplementary Figures



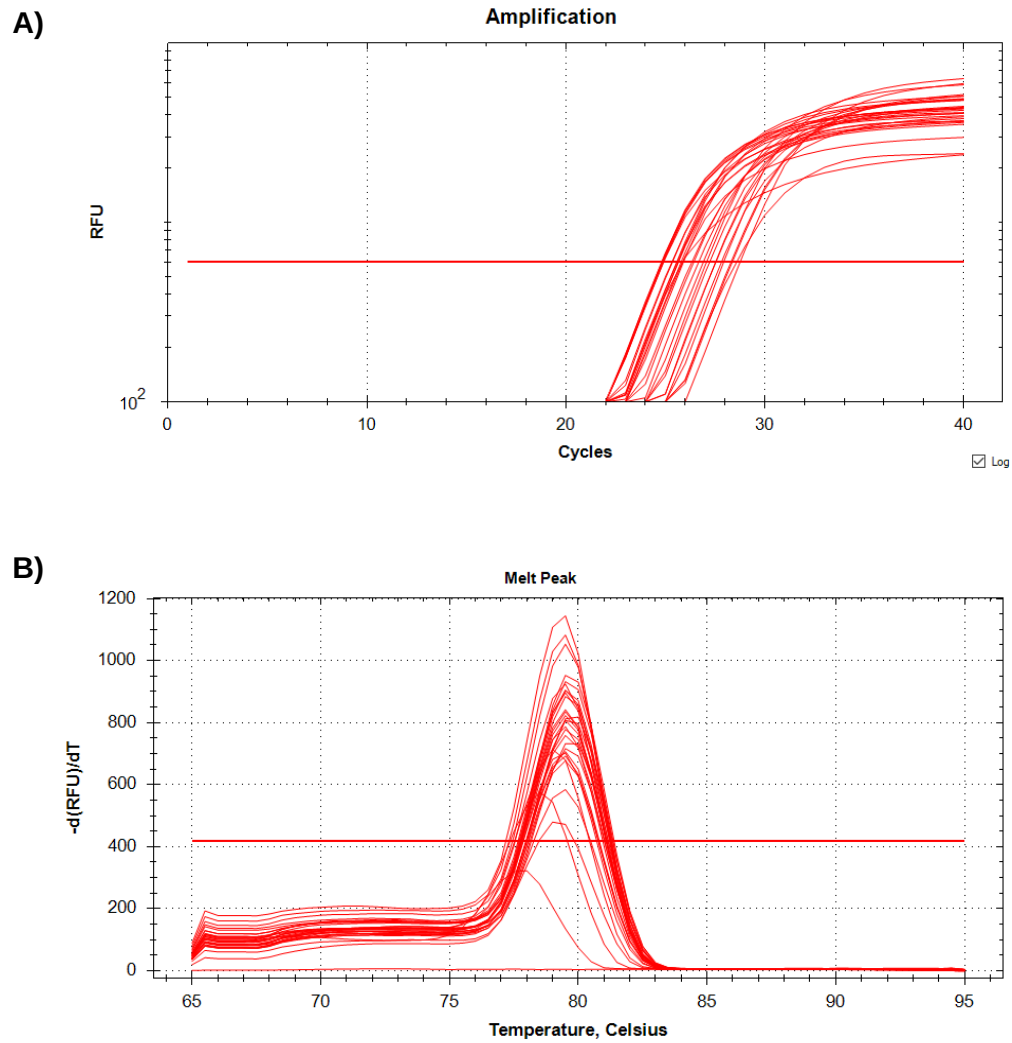
Supplementary Figure 1 – Plasmid restriction map. Map showing the restriction sites and genes included in the pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase plasmid. Adapted from Addgene.



Supplementary Figure 2 – Gating strategy of Huh-7 cells. Representative gating strategy for Huh-7 cells untransduced **(A)** or transduced with 40 µL of viral concentrated **(B)** and analyzed by flow cytometry 72 hours post transduction. FSC-A vs SSC-A plot to gate Huh-7 cells. FSC-A vs FSC-H plot to exclude doublets between cells. FITC-A vs FSC-H plot to obtain the percentage of cells expressing GFP.



Supplementary Figure 3 – RT-qPCR *FLVCR1* amplification and melting curves. *FLVCR1* amplification (A) and melting curves (B) performed to validate the siRNA knockdown.



Supplementary Figure 4 – RT-qPCR *HPRT1* amplification and melting curves. *HPRT1* amplification **(A)** and melting curves **(B)** performed to validate the siRNA knockdown. *HPRT1* was used as the housekeeping gene or endogenous control.

8.2. Appendix II: Abstract submitted to IJUP 2024

Development of an Orthotopic Xenograft Model to Determine the Impact of FLVCR1 in Tumor Growth, Metastasis and Resistance to

Chemotherapy *in vivo*

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Background & Aim: Primary liver cancer is the sixth most common neoplasm worldwide and the third cause of cancer-related deaths¹. Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer, comprising 75-85% of all cases, so it is vital that new effective therapies are developed to improve its patients' quality of life and expected lifespan^{1,2}. The liver represents the major storage site for iron, an element that has a dual role in cancer progression. Iron is essential for growth and proliferation of tumor cells, but excessive iron can generate reactive oxygen species, which damage cells and promote cell death by ferroptosis³. Around 95% of functional iron is in the form of heme, which requires that its intracellular concentration be tightly controlled⁴. Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) is a heme exporter that has been shown to be overexpressed in HCC, as well as associated to a worse prognosis⁵, although its role in this disease remains unexplored. We have evidence that liver-specific FLVCR1 KO decreases the incidence of HCC in mice treated with diethylnitrosamine and CCl₄. In parallel, we generated FLVCR1 KO in human HCC cells (Huh-7) by CRISPR/Cas9. The loss of FLVCR1 function decreased cell migration *in vitro* and the ability to invade the chorioallantoic membrane model. We hypothesized that FLVCR1 plays a pro-tumorigenic role in HCC. Our aim is to evaluate the impact of FLVCR1 in tumor growth, response to chemotherapy and metastasis in an HCC orthotopic xenograft model. **Methods & Results:** For this, we are establishing luciferase-expressing FLVCR1 WT and KO human hepatoma cell lines, by resorting to lentivirus as the plasmid delivery method. The cells will then be injected intrahepatically under ultrasound guidance in severe immune-deficient mice (NSG). Finally, tumor growth, chemotherapy response and metastasis will be

evaluated through bioluminescence imaging. **Conclusion:** We expect to disclose FLVCR1 as a key player in the pathophysiology of HCC.

Keywords: Hepatocellular Carcinoma, Feline Leukemia Virus Subgroup C receptor-related protein 1, Heme, Orthotopic Xenograft Model

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8.3. Appendix III: Published Mini Review



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Heme (dys)homeostasis and liver disease

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Heme is essential for a variety of proteins involved in vital physiological functions in the body, such as oxygen transport, drug metabolism, biosynthesis of steroids, signal transduction, antioxidant defense and mitochondrial respiration. However, free heme is potentially cytotoxic due to the capacity of heme iron to promote the oxidation of cellular molecules. The liver plays a central role in heme metabolism by significantly contributing to heme synthesis, heme detoxification, and recycling of heme iron. Conversely, enzymatic defects in the heme biosynthetic pathway originate multisystemic diseases (porphyrias) that are highly associated with liver damage. In addition, there is growing evidence that heme contributes to the outcomes of inflammatory, metabolic and malignant liver diseases. In this review, we summarize the contribution of the liver to heme metabolism and the association of heme dyshomeostasis with liver disease.

KEYWORDS

heme, iron metabolism, liver disease, porphyria, ferroptosis, liver cancer, hemolysis, immune response

1 Introduction

Heme, a complex of iron with protoporphyrin IX, serves as a prosthetic group in several hemoproteins involved in oxygen transport and storage (hemoglobin and myoglobin), peroxide inactivation (catalase, peroxidases), electron transport, energy generation and chemical transformation (cytochromes), oxidation of tryptophan (tryptophan dioxygenases), among others (Ponka, 1999). In humans and other higher animals, both heme synthesis and degradation are highly regulated processes. Heme biosynthesis, mainly performed by developing erythroid cells and hepatocytes (which are responsible for 15% of the daily heme production) is a highly conserved process that involves eight enzymes, four of which are cytoplasmic, whereas the remaining four are mitochondrial (Ajioka et al., 2006). In both erythroid and nonerythroid tissues, heme biosynthesis is mainly regulated at the level of the first and rate-controlling enzyme, Aminolevulinic acid synthase (ALAS), albeit by different mechanisms. Tissue-specific regulation is ensured by the existence of 2 different genes for ALAS, one expressed ubiquitously (ALAS1) and the other expressed only in erythroid precursors (ALAS2), which are differentially regulated. ALAS2 is regulated by erythroid specific factors (Surinya et al., 1997) and by the interaction of IRE binding protein (IRP) with an iron regulatory element (IRE) in the 5'-untranslated region of ALAS2 mRNA. The IRE-IRP complex prevents translation of the ALAS2 mRNA, whereas addition of an iron-sulfur cluster (Fe/S) abrogates the ability of IRPs to bind to the

IRE and allows translation to occur. This ensures that the rate-limiting step of erythroid heme production is controlled by iron availability (Wingert et al., 2005; Ajioka et al., 2006). Likewise, expression of ferroportin (FPN1), the unique cellular iron exporter, is mostly regulated by erythroid-specific factors, as erythroid precursors make use of an alternative upstream promoter to express FPN1 transcript that lacks the IRE and is thus not repressed in iron-deficient conditions (Zhang et al., 2009). As a result, in iron-depleted conditions, iron export from FPN1 increases, which further contributes to repress heme synthesis and erythropoiesis (Zhang et al., 2011). On the other hand, ALAS1 is regulated by the Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) (Handschin et al., 2005) and the hepatic heme synthesis is regulated by heme-mediated feedback inhibition, through inhibition of transcription of ALAS1 gene and translation of ALAS1 mRNA, destabilization of ALAS1 transcript, and inhibition of translocation of the ALAS1 protein precursor into the mitochondrial matrix (Ponka, 1999).

Heme breakdown is also a highly controlled process, regulated by Heme oxygenase (HMOX). Most of the iron-containing porphyrin that is degraded comes from the hemoglobin present in senescent erythrocytes that are phagocytosed by reticuloendothelial macrophages in the spleen and liver. During erythrophagocytosis, heme transport from the phagolysosome to the cytoplasm relies on SLC48A1 (also known as Heme-responsive gene-1, HRG1), a heme transporter that is highly expressed in reticuloendothelial macrophages (White et al., 2013). HRG1 deficiency in mice induces hemozoin formation due to heme accumulation into lysosomes, thus preventing heme recycling (Pek et al., 2019). The reaction catalyzed by HMOX leads to the release of iron, which is recycled, and the final products carbon monoxide (CO) and biliverdin, which is eventually reduced to the antioxidant bilirubin (Kikuchi et al., 2005). HMOX1, the inducible isoform of HMOX, is highly expressed in splenic macrophages and Kupffer cells (Bauer et al., 1998). Increased intracellular heme levels induce HMOX1 expression via transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2), upon release of the heme-sensitive transcriptional repressor BTB domain and CNC homology 1 (BACH1) from stress response elements located in an enhancer region of the gene (Ogawa et al., 2001).

In addition, the liver plays an important role in recycling the heme iron of damaged erythrocytes. In mice, stress erythrophagocytosis is performed by a population of bone marrow-derived Ly-6C⁺ monocytes that home to the liver, where they differentiate into Ferroportin 1 (FPN1)-expressing macrophages, which deliver iron to hepatocytes (Theurl et al., 2016). The differentiation depends on growth factor Colony Stimulating Factor 1 (CSF1) and on NRF2 (Theurl et al., 2016), which is known to mediate the transcriptional activation of the FPN1 gene in macrophages (Marro et al., 2010).

In addition to regulating genes involved in its own biosynthesis or breakdown, heme regulates genes coding for globins, cytochromes, myeloperoxidase, and iron import/export proteins (Transferrin receptor, FPN1) (Chiabrando et al., 2014).

Whilst heme proteins are essential for a variety of vital physiological functions in the body, free heme is potentially

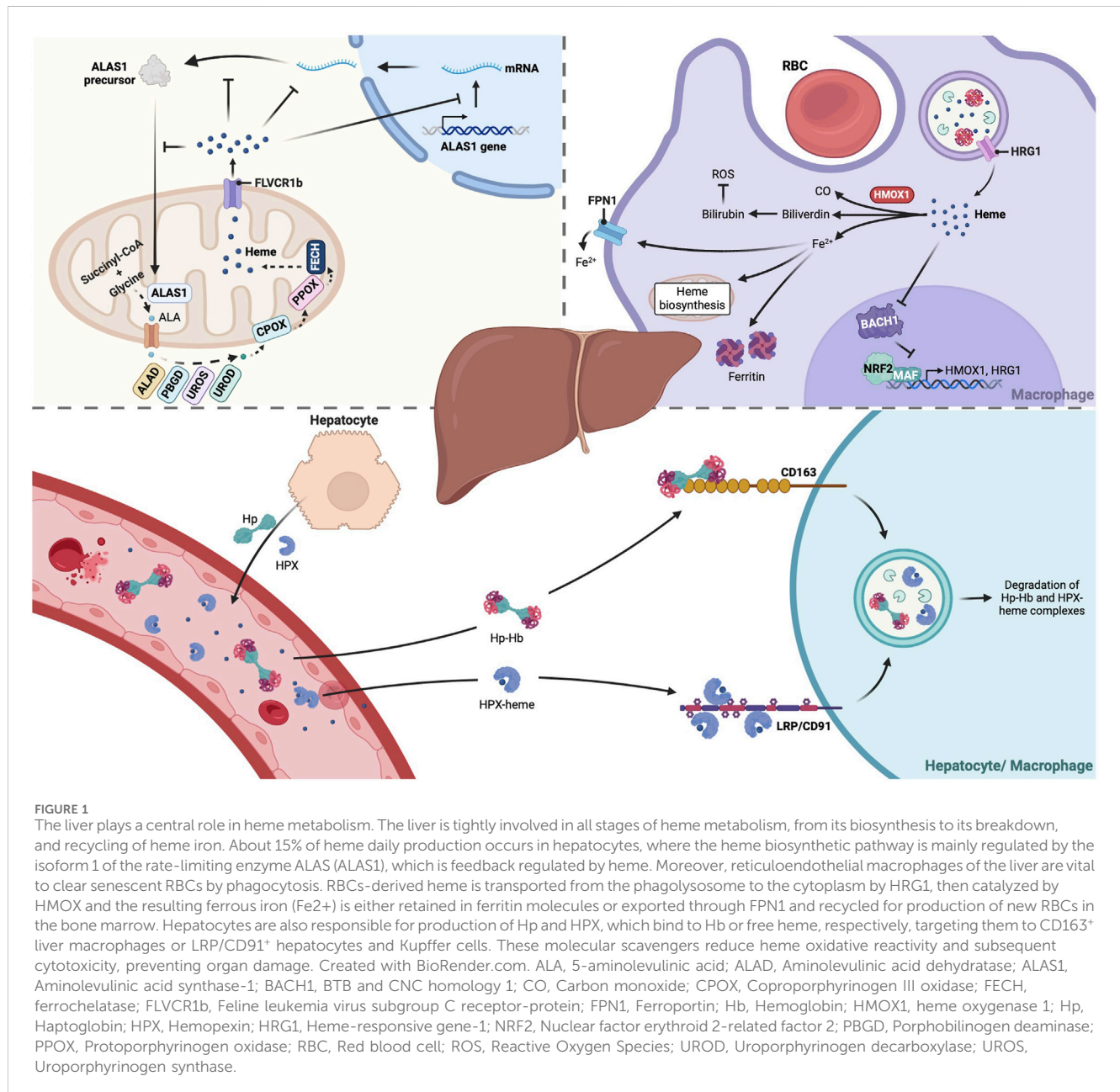
cytotoxic due to the capacity of heme iron to promote the oxidation of cellular proteins, lipids and DNA (Chiabrando et al., 2014). The liver plays a major role in the regulation of circulating heme levels, by producing the two soluble scavengers of free hemoglobin and heme, haptoglobin (Schaer and Buehler, 2013; Schaer et al., 2013) and hemopexin, respectively. Haptoglobin binds to circulating hemoglobin, preventing its extravascular translocation, as well as its reaction with nitric oxide (NO) and peroxides, and the release of hemin (Schaer et al., 2013). Haptoglobin-hemoglobin complexes are endocytosed in liver macrophages through the hemoglobin scavenger receptor CD163 (Kristiansen et al., 2001). Hemopexin binds free heme forming hemopexin-heme complexes that are internalized by Low-density lipoprotein receptor-related protein (LRP)/CD91-expressing hepatocytes and the Kupffer cells (Hvidberg et al., 2005). These hepatic cell types catabolize heme, thus preventing heme-mediated oxidative stress and heme-bound iron loss, particularly in pathologic conditions associated with intravascular hemolysis (Tolosano et al., 2010). Besides hemopexin and haptoglobin, other circulating proteins produced by the liver are known to bind heme, thus preventing heme-mediated oxidative stress: albumin, high/low-density lipoprotein (LDL/HDL), and α 1-microglobulin (Larsen et al., 2012).

In summary, the liver contributes significantly to heme synthesis and recycling, and the detoxification of circulating heme relies on proteins produced in the liver (Figure 1).

2 Heme biosynthesis gone wrong: hepatic and erythropoietic porphyrias

The porphyrias are a group of eight genetic diseases that result from defects in the different enzymatic steps of the heme biosynthetic pathway (Dickey et al., 2024): X-linked protoporphyria (XLP), δ -aminolevulinic acid dehydratase (ALAD) deficiency porphyria (ADP), acute intermittent porphyria (AIP), congenital erythropoietic porphyria (CEP), porphyria cutanea tarda (PCT), hereditary coproporphyria (HCP), variegate porphyria (VP), and erythropoietic protoporphyria (EPP). The accumulation of specific enzyme substrates explains the clinical symptoms, which include either acute neurovisceral attacks or photosensitivity or both. Neurovisceral symptoms are due to neurotoxic effects of porphyrin precursors, whereas the photosensitivity is due to the fluorescent properties of porphyrins. Some authors have classified porphyrias according to their clinical symptoms as acute hepatic porphyrias (AHP) (AIP, VP, HCP, and ADP) and cutaneous, with the latter category comprising both blistering (PCT, CEP, VP, HCP) and nonblistering (EPP, XLP). Porphyrias are also traditionally divided into two categories, depending on the primary site of heme precursor overproduction: hepatic (ADP, AIP, PCT, HCP and VP) and erythropoietic porphyrias (XLP, CEP and EPP). For an up-to-date classification of the porphyrias and for insights on their clinical management, readers are advised to consult the excellent review of Dickey and colleagues (Dickey et al., 2024).

Regardless of whether they are classified as hepatic or erythropoietic, nearly all porphyrias relate in some way to the



liver. Firstly, because some porphyrias are caused by liver damage. PCT, the most common type of porphyria, is in most cases (type I) an acquired deficiency of Uroporphyrinogen decarboxylase (UROD), the fifth enzyme in heme biosynthesis, caused by underlying liver diseases triggered by alcohol, iron overload, chlorinated hydrocarbons, oestrogens or viral hepatitis (Frank and Poblete-Gutierrez, 2010). Secondly, some porphyrias are associated with increased risk of primary liver cancer. This is the case of both PCT and AHP, as discussed below (Section 4). Thirdly, even erythropoietic porphyrias are known to damage the liver. EPP is caused by an inherited loss-of-function mutation in the gene for the final enzyme of heme biosynthesis, Ferrochelatase (FECH), whereas XLP patients carry gain-of-function pathogenic

variants in erythroid-specific ALAS2, which encodes the first enzyme of heme synthesis. Erythropoietic protoporphyrias (EPP and XLP) are characterized by tissue accumulation of hydrophobic protoporphyrin, which can absorb energy from light and damage the endothelium and subcutaneous tissues through a process that is mediated by the production of reactive oxygen species (ROS). Patients are mostly affected by severe painful cutaneous phototoxicity after light exposure (Hussain et al., 2023). However, since protoporphyrin is excreted in the bile, some patients accumulate porphyrin-containing bile plugs, leading to severe cholestatic liver disease (Anstey and Hift, 2007). A recent study proposed the silencing of the hepatic bile acid-related nuclear farnesoid x receptor (FXR), which induces the expression of genes involved in heme

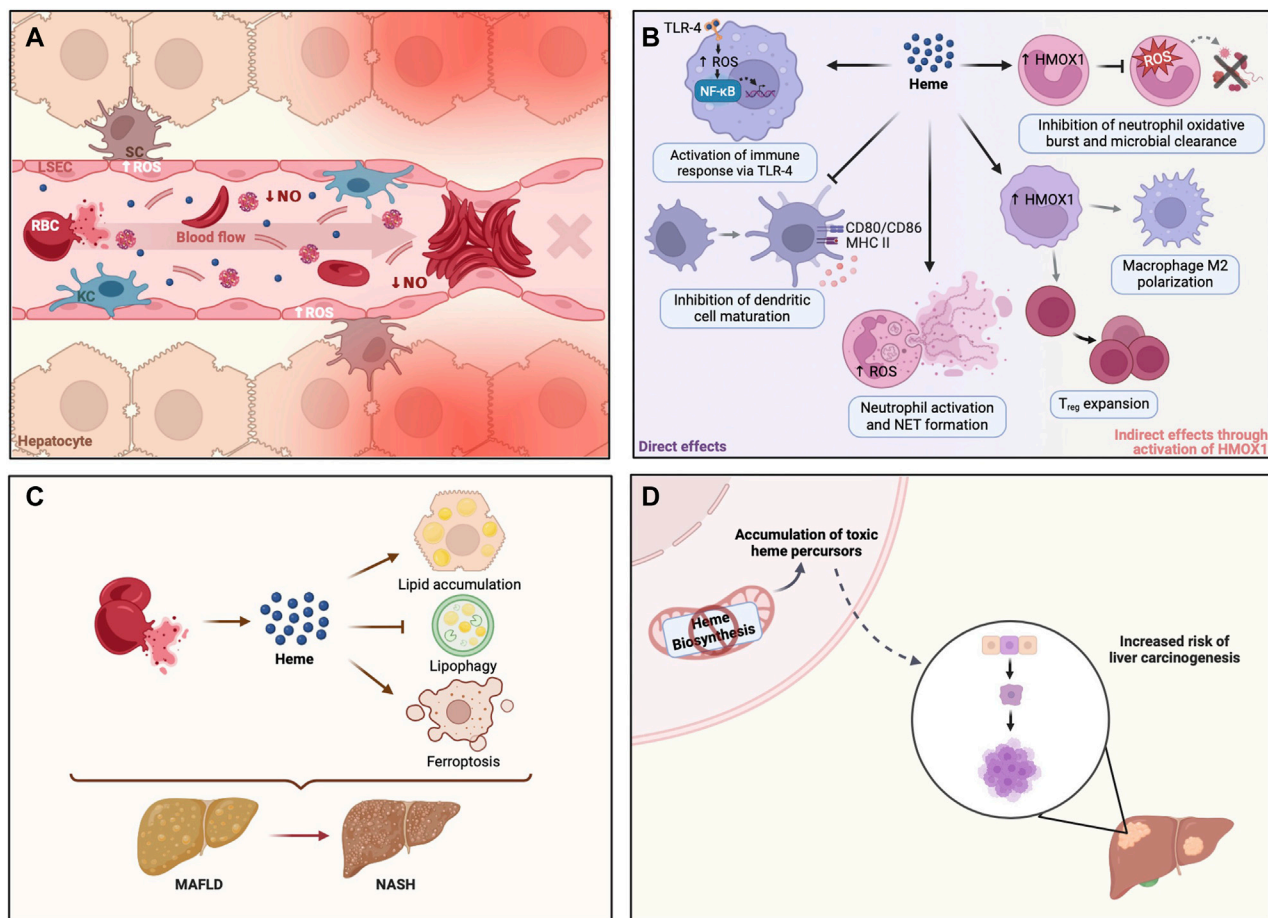


FIGURE 2

Heme dyshomeostasis is associated with liver disease. **(A)** In sickle cell disease, RBCs are more fragile and break apart during circulation, releasing significant amounts of heme and heme-enriched membrane microparticles. Such amounts of cell-free heme in circulation activate endothelial cells via ROS production and promote abnormal RBC adhesion, originating clumps that can block blood flow. This vascular occlusion may occur in liver sinusoids, preventing liver oxygenation and thus causing severe tissue damage. **(B)** Heme is a danger-associated molecular pattern (DAMP) that can modulate different cells in the immune compartment. Heme can activate innate immune receptors (e.g., TLR-4) and neutrophil NET formation, activating and amplifying inflammation. In turn, heme may inhibit dendritic cell maturation or modulate HMOX1 expression in monocytes, favoring T_{reg} expansion and macrophage M2 polarization. These anti-inflammatory responses are of the utmost importance in sterile liver inflammation or liver transplantation. Heme can also increase the expression of heme-inducible HMOX1 in immature neutrophils, inhibiting a proper oxidative burst in resulting mature neutrophils, thereby preventing host defense against pathogens targeting different organs, including the liver. **(C)** Cell-free heme, released during hemolysis, may promote metabolic liver disease by disruption of hepatic lipid metabolism. Hemolysis has been shown to promote lipid accumulation and block intracellular breakdown of lipid droplets by lipophagy, resulting in liver steatosis, a well-known trait of MAFLD. Heme-iron may also initiate lipid peroxidation, mediating the death of hepatocytes by ferroptosis, which potentially plays a role in the progression of MAFLD to NASH. **(D)** Enzymatic defects in the heme biosynthetic pathway result in metabolic disorders known as porphyrias, due to accumulation of different heme precursors (i.e., porphyrins), some of which are genotoxic. Their accumulation is associated with an increased risk of carcinogenesis. Created with BioRender.com. HMOX1, heme oxygenase 1; KC, Kupffer cell; LSEC, Liver sinusoidal endothelial cell; MAFLD, Metabolic dysfunction-associated fatty liver disease; NASH, Non-alcoholic steatohepatitis; NET, Neutrophil extracellular trap; NF- κ B, Nuclear factor kappa B; NO, nitric oxide; RBC, Red blood cell; ROS, Reactive Oxygen Species; SC, Stellate cell; TLR-4, Toll-like receptor 4; T_{reg}, Regulatory T cell.

biosynthesis, as a potential new therapeutic approach against EPP-associated cholestasis (Dean et al., 2023). Lastly, the treatment of some porphyrias may require liver transplantation (Lissing et al., 2024), either to treat the porphyria or its symptoms, as is the case of AIP (Lissing et al., 2021) or EPP (Anstey and Hift, 2007). Likewise, increasing the hepatic production of specific enzymes of the heme biosynthetic pathway via systemic messenger RNA therapy (e.g., hPBGD mRNA) is emerging as a potential treatment for porphyria patients (Jiang et al., 2018).

3 Heme, vascular occlusion, immune response and metabolic alterations in liver disease

Liver damage is a common finding in patients with sickle cell disease (SCD), mostly due to vascular occlusion from intrahepatic sickling of erythrocytes with concomitant acute ischemia (Theocharidou and Suddle, 2019). In SCD, hemolysis results in the release of large quantities of free heme and heme-laden erythrocyte membrane microparticles into the circulation (Figure 2A). This

triggers endothelial cell activation, via production of ROS, and abnormal red blood cell adhesion (Camus et al., 2015; An et al., 2023). In addition, free plasma hemoglobin depletes circulating levels of NO, which causes smooth muscle contractions and vasoconstriction (Piccin et al., 2019). Another consequence of chronic hemolysis in SCD is the development of pigment gallstones, whereas viral hepatitis and iron overload may develop secondary to multiple blood transfusions (Theocharidou and Suddle, 2019). Additionally, some SCD patients present autoimmune hepatitis (Lynch et al., 2023).

Free heme is a well-established danger-associated molecular pattern (DAMP), which can initiate immune responses upon binding to Toll-like receptor 4 (TLR4) (Canesin et al., 2020). However, there is accumulating evidence that hemolysis can modulate immune cell differentiation and function in different ways (reviewed by Zhong and Yazdanbakhsh, 2018). Via the production of ROS, free heme can induce the formation of neutrophil extracellular traps (NETs), which contributes to the vaso-occlusive crises in SCD patients. Heme can also induce HMOX1 in immature neutrophils, which inhibits the oxidative burst and impairs the capacity of leukocytes to destroy pathogenic agents (Evans et al., 2015). Hemolysis can also favor anti-inflammatory immune cell polarization by inhibiting dendritic cell maturation required for effector T-cell responses (Zhong and Yazdanbakhsh, 2018), and induce the differentiation of liver macrophages into anti-inflammatory erythrophagocytes (Pfefferlé et al., 2020), which was shown to provide protection against sterile liver inflammation (Pfefferlé et al., 2021). Likewise, hemolysis may promote regulatory T-cell (T_{reg}) expansion through modulation of HMOX1 expression in nonclassical monocytes (Zhong and Yazdanbakhsh, 2018; Figure 2B).

The heme-inducible enzyme HMOX1 was reported to play a protective role in hepatic ischemia–reperfusion injury (IRI) following orthotopic liver transplantation. Low HMOX1 expression by liver macrophages correlates with hepatocellular death and worse patient survival (Nakamura et al., 2017). Mechanistically, HMOX1 prevents macrophage M1 polarization (Zhang et al., 2018) and TLR4-driven inflammatory responses (Rao et al., 2015). Another study reported the ability of HMOX1 to modulate T_{reg} expansion and to inhibit infiltration of $CD4^+$ and $CD8^+$ cells in transplanted livers (Sun et al., 2011).

Hemolysis may enhance liver damage by promoting metabolic dysfunction-associated fatty liver disease (MAFLD) (Figure 2C). Using a mouse model of acute intravascular hemolysis, Rayego-Mateos et al. (2023), demonstrated that heme disturbs lipid metabolism and promotes liver steatosis. Specifically, hemolysis exacerbates lipid accumulation and blocks the lipophagy pathway. On the other hand, heme-inducible enzymes and products of heme breakdown with antioxidant properties seem to be protective. The catabolism of heme mediated by HMOX produces biliverdin, which is reduced to bilirubin by Biliverdin reductase (BVR) (O'Brien et al., 2015). HMOX1 expression is increased in non-alcoholic steatohepatitis (NASH) patients (Malaguarnera et al., 2005) and an *in vitro* study suggests that HMOX1 may have a protective role by suppressing endoplasmic reticulum stress in hepatocytes (Li X. et al., 2020). Several studies have shown that serum bilirubin levels are inversely correlated with the prevalence of MAFLD in the

general population (Kwak et al., 2012; Han et al., 2024), and with less severe liver disease among MAFLD patients (Kumar et al., 2012; Puri et al., 2013; Salomone et al., 2013; Han et al., 2024), which was repeatedly hypothesized to be due to the antioxidant effect of bilirubin. However, this was never demonstrated experimentally and remains a speculation. In fact, a study by Stec et al. (2016) demonstrated that bilirubin inhibits lipid accumulation in mice through direct binding to Peroxisome proliferator-activated receptor α (PPAR α). Moreover, Biliverdin reductase A (BVRA), the enzyme that reduces biliverdin IX α to bilirubin IX α , was also shown to prevent hepatic lipid accumulation in a study using mice with liver-specific BVRA KO fed with high-fat diet, through inhibition of Glycogen synthase kinase (GSK) β and activation of PPAR α (Hinds et al., 2016).

In addition to the effects on hepatic steatosis, hepatocellular free heme may also promote the progression of MAFLD to NASH by catalyzing Fenton-like reactions, lipid peroxidation and ultimately hepatocellular death by ferroptosis (Zhao et al., 2023). Ferroptosis is an iron-dependent and lipid peroxidation-mediated nonapoptotic cell death that was shown to be the initiator of inflammation in the methionine-choline deficient diet mouse model of NASH (Tsurusaki et al., 2019; Li D. et al., 2020; Qi et al., 2020). Taking all the evidence above into consideration, reducing free heme may constitute a therapeutic approach in the treatment of metabolic liver disease (Zhao et al., 2023).

4 Heme and liver cancer

The AHP, especially AIP, are associated with a marked increased risk of primary liver cancer, mainly hepatocellular carcinoma (HCC) (Baravelli et al., 2017; Saberi et al., 2021; Lissing et al., 2022; Figure 2D). Notably, HCC in AHP occurs in the absence of cirrhosis, unlike other chronic liver diseases (Saberi et al., 2021). The pathogenesis of hepatocarcinogenesis in AHP remains unknown but it may be related to the intrahepatic accumulation of 5-Aminolevulinic acid, which is pro-oxidant and genotoxic (Onuki et al., 2002). Alternative hypotheses such as a loss of antioxidant effects due to heme deficiency, or direct or indirect effects of mutations in heme metabolism genes have also been proposed (Peoc'h et al., 2019). This is in contrast with PCT patients, who also show increased risk of primary liver cancer (Baravelli et al., 2019), but whose porphyria is concomitant to an underlying liver disease that increases the risk of cirrhosis and primary liver cancer. Likewise, chronic exposure of laboratory rodents to some chemicals, drugs and pesticides that cause hepatic porphyria is associated with liver carcinogenesis (reviewed by Smith and Foster, 2018).

Heme per se is also believed to be carcinogenic, at least in colorectal cancer. There is wide evidence that heme iron is the critical component of red meat that promotes colorectal carcinogenesis (Seiwert et al., 2020). Excess heme iron may promote carcinogenesis by: favoring ROS production and the oxidation of DNA, lipids and proteins; suppressing TP53 activity; modulating immune cell function, inflammation, and gut dysbiosis (Gamage et al., 2021).

Cancer cells are reported to have greater activity of heme-containing proteins and increased heme content, which may be partly explained by the upregulation of the main enzymes in heme synthesis (e.g., ALAS) (Fiorito et al., 2020; Gamage et al., 2021). In other cases, such as in acute myeloid leukemia, heme biosynthesis is downregulated (Lin et al., 2019). The heme-iron exporter Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) is also over-expressed in several tumors (Peng et al., 2018; Russo et al., 2019), possibly to establish a balance in intracellular heme homeostasis. In fact, both increased heme synthesis and increased heme export were shown to control the energetic metabolism of cells with high-energy demand, such as tumor cells (Fiorito et al., 2021). Notably, in the particular case of HCC, analyses of publicly available RNAseq data at the “The Cancer Genome Atlas” (TCGA) showed that: a) FLVCR1 mRNA expression is significantly increased in HCC when compared with normal liver tissue; b) FLVCR1 amplification or mRNA upregulation are observed in 21% of HCC cases; c) FLVCR1 mRNA expression is significantly associated with HCC disease status, histological grade, and vascular invasion; and d) higher expression of FLVCR1 is associated with poor overall survival in HCC (Shen et al., 2018; Tang et al., 2020; Wei et al., 2020). Likewise, analyses of tissue samples obtained from the Human Protein Atlas showed that FLVCR1 protein is strongly detected in HCC tissue, but not in normal liver tissue (Shen et al., 2018; Wei et al., 2020). While FLVCR1 is emerging as a new significant predictor of prognosis and a useful diagnosis marker in HCC, its role in the pathophysiology of HCC remains unknown.

Increased expression of proteins involved in heme uptake has also been implicated in cancer (Hooda et al., 2013). HRG1 may contribute to cancer cell invasiveness (Fogarty et al., 2014) by regulating the activity of vacuolar-(H(+)) ATPase (V-ATPase), which is essential for endosomal acidification and receptor trafficking in mammalian cells (O’Callaghan et al., 2010). Although this has not been addressed specifically in the context of liver cancer, HRG-1 may also represent a target for disrupting V-ATPase activity and decrease the metastatic potential of cancer cells (Fogarty et al., 2014).

Finally, excess iron may promote cell death by ferroptosis, which is a mechanism of tumor suppression that has been implicated in the action of clinical agents used to treat HCC (e.g., sorafenib). There is increasing evidence that activating ferroptosis may potentially inhibit the growth of HCC cells (Chen et al., 2022; Jiang et al., 2024). The fact that cancer cells exhibit higher levels of heme could potentially be explored in the search for a novel therapeutic strategy for HCC, which is among the leading causes of cancer-related

mortality worldwide. However, the contribution of heme-bound iron to ferroptosis remains unclear.

5 Conclusion

Heme is a key component of cellular respiration and function. Heme biosynthesis is therefore tightly regulated and changes in enzymatic activity of the pathway are associated with disease. Heme also plays an essential role in oxygen transport and its uncontrolled release (e.g., hemolysis) is associated with vascular damage and malfunction and tissue oxidation. An emerging topic of research is how cellular heme impacts on cancer cell proliferation and death. This is particularly relevant in tissues enriched for heme proteins, such as the liver. Its study may reveal cancer-specific cell dependencies related with heme production and heme handling that are still unknown.

Author contributions

TD: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing. NV: Visualization, Writing–review and editing, Writing–original draft. CG: Writing–review and editing, Writing–original draft. DD: Funding acquisition, Writing–review and editing, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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