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Rewiring lipid metabolism by targeting PCSK9 as a new therapeutic approach in liver cancers

Malak Alannan

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

SPÉCIALITÉ BIOLOGIE CELLULAIRE ET PHYSIOPATHOLOGIE

Par Malak ALANNAN

**Remodelage du métabolisme lipidique en ciblant PCSK9 comme
nouvelle approche thérapeutique dans les cancers du foie**

Sous la direction de Aksam MERCHED
(co-direction: Hussein FAYYAD-KAZAN)

Soutenue le 19 Novembre 2021

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« I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. »

Mary Curie

*To my parents, **Iman and Ghassan**, for raising me to believe that anything was possible*

*To my amazing husband, **Mohammad Nour**, for constantly being by my side*

You are my backbone and the reason of what I become today

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I am grateful for every single person who has passed along the way during this journey, you have been either a lesson or a friend.

Abstract

Rewiring lipid metabolism by targeting PCSK9 as a new therapeutic approach in liver cancers

Deregulation of energetic metabolism, and specifically enhanced lipid biosynthesis, is an emerging hallmark of many cancers including the adult and pediatric forms of liver cancer, hepatocellular carcinoma (HCC) and hepatoblastoma (HBL), respectively. HCC is a primary malignant liver disease that ranks third in cancer-related deaths worldwide. HBL, on the other hand, is a rare malignant embryonic tumor whose incidence has been increasing drastically over the past decade. The analysis of our transcriptomic data as well as other available ones revealed deregulation of the expression of genes involved in the lipid uptake and metabolism in both forms of liver cancers. For instance, we found that PCSK9, a pro-protein convertase that negatively regulates LDLR, was overexpressed in both tumors. Moreover, the enzymes that catalyze the *de novo* synthesis of cholesterol, such as HMGCR, were also upregulated. The role of PCSK9 in liver cancer, especially HBL, is not very well understood. Therefore, the aim of my project is to rewire this lipid metabolic hub controlled by PCSK9 using available drugs and by overexpressing a PCSK9 maturation enzyme e.g., meprin alpha (Mep1a), which is overexpressed in liver cancers. My results show that targeting PCSK9, whether pharmacologically or genetically, in hepatic cancer cells significantly decreased cellular growth and migration, whilst the mitochondrial oxygen consumption rate (OCR) increases. Moreover, combination of these treatments with statins, famous HMGCR inhibitors, showed synergetic anti-proliferative effects. Silencing of PCSK9 using small interfering RNA led to a strong increase in the intracellular levels of neutral and phospho-lipids, as well as a massive accumulation of lipid droplets. Molecular signaling of cell death involved the disruption of the p62/KEAP1/Nrf2 anti-oxidative axis leading to ferroptosis. The anti-tumoral effects of PCSK9 deficiency were validated using two *in vivo* xenograft models, zebrafish and CAM models. As for Mep1a, I created cell lines with higher expression to mimic the profile observed in liver tumors. Proteomic analysis of the secretome derived from meprin-overexpressing HepG2 cells revealed upregulation in the expression of many PCSK9 interacting proteins. Interestingly, my *in vitro* and *in vivo* analyses revealed many changes in lipid metabolism depending on Mep1a, probably in connection to PCSK9 and its lipid-related activities.

Collectively, my data reveal a critical role of PCSK9 in lipid metabolism and novel functional involvement of this enzyme in maintaining cell redox homeostasis and as a new player of ferroptosis. They also provide strong evidence supporting the potential therapeutic benefit and drug repositioning of anti-PCSK9 approaches to treat liver cancers. And for the first time, we revealed a novel role for meprin α in lipid metabolism which needs further investigations.

Keywords: PCSK9, ferroptosis, meprin, hepatocellular carcinoma, hepatoblastoma, lipid metabolism

Résumé

Remodelage du métabolisme lipidique en ciblant PCSK9 comme nouvelle approche thérapeutique dans les cancers du foie

La dérégulation du métabolisme énergétique, et plus particulièrement l'augmentation de la biosynthèse des lipides, est une caractéristique émergente de nombreux cancers, y compris les formes adultes et pédiatriques du cancer du foie, le carcinome hépatocellulaire (CHC) et l'hépatoblastome (HBL), respectivement. Le CHC est une tumeur maligne primaire du foie qui se classe au troisième rang mondial des décès liés au cancer. L'hépatoblastome, quant à lui, est une tumeur maligne embryonnaire rare dont l'incidence a considérablement augmenté au cours de la dernière décennie. L'analyse de nos données transcriptomiques ainsi que d'autres données disponibles a révélé une dérégulation de l'expression des gènes impliqués dans l'absorption et le métabolisme des lipides dans les deux formes de cancer du foie. Ainsi, nous avons constaté que PCSK9, une pro-protéine convertase qui régule négativement le LDLR, était surexprimée dans les deux tumeurs. De plus, les enzymes qui catalysent la synthèse *de novo* du cholestérol, telle que HMGCR, étaient également régulées à la hausse. Le rôle de PCSK9 dans le cancer du foie, en particulier le HBL, n'est pas très bien compris. Par conséquent, l'objectif de mon projet est de remodeler cette plaque tournante du métabolisme des lipides autour de PCSK9 en utilisant des médicaments disponibles et en surexprimant une enzyme impliquée dans la maturation de PCSK9, la méprine alpha (Mep1a) dont l'expression augmente fortement dans les cancers du foie. Mes résultats montrent que le ciblage de PCSK9, que ce soit pharmacologiquement ou génétiquement, dans les cellules cancéreuses hépatiques diminue significativement la croissance et la migration cellulaires, tandis que le taux de consommation mitochondriale d'oxygène augmente. De plus, la combinaison de ces traitements avec des statines, des inhibiteurs connus de HMGCR, a induit des effets antiprolifératifs synergiques. L'inhibition de PCSK9 à l'aide de petits ARN interférents entraîne une forte augmentation des niveaux intracellulaires de lipides neutres et de phospholipides, ainsi qu'une accumulation massive de gouttelettes lipidiques. La signalisation moléculaire de la mort cellulaire implique la perturbation de l'axe antioxydant p62/KEAP1/Nrf2 qui conduit à la ferroptose. Les effets anti-tumoraux de la déficience en PCSK9 ont été validés à l'aide de deux modèles de xéno greffe *in vivo*, le poisson zèbre et le modèle CAM. Concernant la

méprine, j'ai créé des lignées cellulaires qui surexpriment cette enzyme afin de mimer le profil des tumeurs. L'analyse protéomique du surnageant des cellules HepG2 surexprimant la méprine montre une augmentation significative de nombreuses protéines connues pour interagir avec PCSK9. Les analyses fonctionnelles et lipidomiques montrent un changement au niveau du métabolisme lipidique *in vitro* et *in vivo*, probablement en relation avec PCSK9 et son rôle dans le métabolisme lipidique.

L'ensemble de mes données met en évidence un rôle essentiel de PCSK9 dans le métabolisme lipidique ainsi qu'une nouvelle implication fonctionnelle de cette enzyme dans le maintien de l'homéostasie redox cellulaire et en tant que nouvel acteur de la ferroptose. Il apporte également de solides arguments en faveur du repositionnement de médicaments anti-PCSK9 pour traiter les cancers du foie avec un bénéfice thérapeutique réel. Et pour la première fois, nous avons identifié une nouvelle implication de la méprine α dans le métabolisme lipidique qu'il nous faudra mieux comprendre par la suite.

Mots-clés : PCSK9, ferroptose, méprine, carcinome hépatocellulaire, hépatoblastome, métabolisme lipidique

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List of Abbreviations

A

ABCA1: ATP-binding cassette transporter
ACBD: adenylyl cyclase-binding domain
ACLY: ATP citrate lyase
Ac-SDKP: *N*-acetyl-seryl-aspartyl-lysyl-proline
ADAM: a disintegrin and metalloprotease domain
AD: Alzheimer's disease
ADH: alcohol dehydrogenase
AFB1: aflatoxin-B1
AFP: α -fetoprotein
AKT: or called PKB, protein kinase B
ALL: acute lymphoblastic leukemia
Alox15: arachidonate 15-lipoxygenase
AML: acute myeloid leukemia
AnxA2: Annexin A2
APC: adenomatous polyposis coli
ApoB: apolipoprotein B
ApoER2: apolipoprotein E receptor 2
Arg1: arginase 1
ARH: autosomal recessive hypercholesterolemia
ASCVD: atherosclerotic cardiovascular disease
ASO: antisense oligonucleotides
ATG5: autophagy related 5
ATG7: autophagy related 7
ATP: adenosine triphosphate

B

BAK: Bcl-2 antagonist killer
Bax: Bcl-2 associated X protein
BBR: berberine
Bcl-2: B-cell lymphoma 2
BCLC: Barcelona Clinic Liver Cancer
 β -TrCP E3: β -transducin repeat-containing protein, an E3 ubiquitin ligase
BWS: Beckwith-Wiedemann syndrome

C

CAM: chick chorioallantoic membrane

CAP1: cyclase-associated protein 1
Cas9: CRISPR-Associated Protein 9
CCL2: chemokine CC motif ligand 2
CDDP: cis-diamminedichloroplatinum (II)
CDH1: cadherin type 1
CDKN1C: Cyclin dependent kinase inhibitor 1C
CDX2: *Caudal*-related homeobox TF 2
CD36: cluster of differentiation 36
CD81: cluster of differentiation 81
CHD: coronary heart disease
CHIC: Children's Hepatic tumors International Cooperation
CHO-K1: Chinese hamster ovarian cells
CK1 α : casein kinase 1 α
CLL: chronic lymphocytic leukemia
CML: chronic myeloid leukemia
COG: Children's Oncology Group
CoQ₁₀: coenzyme Q₁₀
CRD: cysteine-rich domain
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat
CTL: cytotoxic T-cells
CTNNB: β -catenin

D

DCP: Des-gamma-carboxy-prothrombin
DNA: deoxyribonucleic acid
DN: dysplastic nodules
DSS: dextran sulfate sodium
Dvl: Dishevelled

E

EASL: European Association for the Study of Liver
ECM: extracellular matrix
EGFA: Epidermal growth factor-like repeat A
EGFR: epidermal growth factor receptor
eIF2 α : eukaryotic initiation factor 2 α
EMT: epithelial-mesenchymal transition
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase

ERS: ER stress

F

FA: fatty acid

FAP: Familial Adenomatous Polyposis

FB1: fumonisin B1

FDA: Food and Drug Administration

FDB: familial defective Apo-B100

FGFR: fibroblast growth factor receptor

FH: familial hypercholesterolemia

Flt-3: Fms-like tyrosine kinase 3

FN: fibronectin

FSP1: ferroptosis suppression protein 1

FXR: farnesoid X receptor

Fzd: frizzled

G

GOF: Gain-of-function

GPC3: glypican-3

GPOH: German Society for Pediatric Oncology and Hematology

GRP: glucose-related proteins

GPX4: glutathione peroxidase 4

GS: glutamine synthetase

GSK3 β : glycogen synthase kinase 3- β

H

HBcAg: hepatitis B core antigen

HBL: hepatoblastoma

HBV: hepatitis B virus

HBx: HBV protein x

HCC: hepatocellular carcinoma

HCV: hepatitis C virus

HDAC: histone deacetylase

HDGF: hepatoma-derived growth factor

HDL: high-density lipoprotein

HDR: homology-directed repair

HEK293: human embryonic kidney cells

HER2: human epidermal growth factor receptor 2

HFD: high fat diet

HGF: hepatocyte growth factor

HMGCR: 3-hydroxy 3-methylglutaryl Coenzyme A reductase

HNF: hepatocyte nuclear factor

HSD17B6: hydroxysteroid 17-beta dehydrogenase 6

HSPB1: heat shock protein beta 1

HSP70: heat shock protein 70

HuR: Hu antigen R

I

IBD: inflammatory bowel disease

IC₅₀: half-maximal inhibitory concentration

IFN α : interferon- α

IGF: insulin-like growth factor

IHC: immunohistochemistry

IL: interleukin

IR: insulin resistance

ITGA6: integrin alpha 6

J

JAK: Janus kinase

JNK: c-Jun N-terminal kinase

JPLT: Japanese Pediatric Liver Tumours study group

K

Keap1: kelch-like ECH-associated protein 1

KLK: kallikrein-related peptidase

KO: Knock-out

L

LDL-C: low density lipoprotein cholesterol

LDLR: low density lipoprotein receptor

LEF: lymphoid enhancer factor

L-IFPTA+: Liposomal Immunogenic Fused PCSK9-Tetanus plus Alum adjuvant

LOF: loss-of-function

LOX-1: lectin-like oxidized LDL receptor 1

LPS: lipopolysaccharide

LRP1/5/6: LDLR related protein 1/5/6

LXR: liver X-activated receptor

M

mAbs: monoclonal antibodies

MAPK1: mitogen activated protein kinase 1
Mcl-1: myeloid cell leukemia 1
MCP: monocyte chemotactic protein 1
MDCT: multidetector computed tomography
MEK: Mitogen-activated protein kinase
Mep1a: meprin 1 alpha
Mep1b: meprin 1 beta
MHC1: major histocompatibility complex 1
miRNA: mirco ribonucleic acid
MMP: matrix metalloproteinases
MRI: magnetic resonance imaging
mRNA: messenger ribonucleic acid
mTOR: mammalian target of rapamycin
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MS: metabolic syndrome
MVA: mevalonate pathway
MYC: Myelocytomatosis

N

NAD(P)H: nicotinamide adenine dinucleotide (phosphate) + hydrogen
NAFL(D): non-alcoholic fatty liver (disease)
NARC-1: neural apoptosis-regulated convertase 1
NASH: non-alcoholic steatohepatitis
NCOA4: nuclear receptor coactivator 4
NFKB: nuclear factor kappa-light chain-enhancer of activated B cells
NHEJ: non-homologous end-joining
NK: natural killer
NNGH: N-isobutylN-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid
NPC1L1: Niemann-Pick C1-like 1
Nrf2: nuclear factor erythroid 2-related factor 2
NT: non-tumoral

O

OCR: oxygen consumption rate
OS: overall survival

oxLDL: oxidized LDL

P

PC : proprotein convertase
PCSK9: proprotein convertase subtilisin/kexin type 9
PD-1: programmed cell death protein 1
PDGF(R) β : platelet-derived-growth-factor (receptor) β
PD-L1/2: programmed cell death protein-ligand 1/2
PE: phosphatidylethanolamine
PERK: protein kinase R-like ER kinase
PG: proteoglycans
PI3K: phosphatidylinositol 3-kinase
PKC: protein kinase C
PMA: propidium monoazide
POP: prolyl oligopeptidase
PPAR: peroxisome proliferator-activated receptor
PRETXT: PRETreatment EXTent of tumor
PS: Pseurotin A
PUFA: polyunsaturated fatty acid
P53: tumor protein p53
P62: also called sequestosome 1

Q

q-RT PCR: quantitative real time polymerase chain reaction

R

RAF-1: Rapidly Accelerated Fibrosarcoma
RAS: Rat sarcoma
RBP: RNA-binding protein
R-IMPP: (R)-N-(isoquinolin-1-yl)-3-(4-methoxyphenyl)-N-(piperidin-3-yl)propanamide
ROS: reactive oxygen species

S

SAT1: spermidine/spermine N1-acetyltransferase 1
SCAP: SREBP cleavage-activating protein

sgRNA: single guide RNA
SHARP: Sorafenib HCC Assessment
Randomized Protocol
shRNA: short hairpin ribonucleic acid
SH3BD: Src homology 3 binding domain
SIL: silibinin A
SIOPEL: European Société Internationale
d'Oncologie Pédiatrique
siRNA: small interfering ribonucleic acid
SIRT: sirtuins
SLC7A11: solute carrier family 7 member
11
SOCS3: suppressor of cytokine signaling 3
SRE: sterol regulatory element
SREBP: sterol regulatory element binding
protein
STAT: signal transducer and activator of
transcription
STS: sodium thiosulphate

T

TACE: Transcatheter arterial
chemoembolization
TAM: tumor associated macrophages
T β 4: thymosin- β 4
TCF: T-cell factor
TCGA: The Cancer Genome Atlas
TERC: telomerase RNA component
TERT: tolemrase reverse transcriptase
TF: transcription factor
TFR1: transferrin receptor 1
TGF α : transforming growth factor α
TGF- β : transforming growth factor- β
TLE: transducing-like enhancer protein
TLR4: toll-like receptor 4
TNF α : tumor-necrosis factor α
TOP2A: topoisomerase 2-alpha
TTP: tristetraprolin

U

UK: United Kingdom
UKCCS: United Kingdom Childhood Cancer
Study
UTR: untranslated region

V

VEGFA: vascular endothelial growth factor
A
VEGFR: vascular endothelial growth factor
receptor
VIM: vimentin
VLDLR: very low density lipoprotein
receptor

W

WHO: World Health Organization
WNT: wingless-type MMTV integration site
family member
WT: wild type

X

Xc: System Xc⁻ cysteine/glutamate
antiporter
xCT: alternative name of SLC7A1

XIAP: X-linked inhibitor of apoptosis
protein

Z

ZEB1: zinc-finger E-box binding homeobox
1

Introduction

I- Cancer

Cancer cannot be considered a new disease as in fact it goes back thousands of years, when the very first cancer was found in an ancient Egyptian mummy who was suffering from bone cancer. This word originates from the time of Hippocrates, the Father of Medicine, who came up with a Greek word “**karkinos**” to describe carcinoma tumors. Today, cancer comes in the second rank for lethal diseases. Unfortunately, no treatments have been registered along the history for this disease, but luckily for us, with all the technological and research advances, nowadays millions of people suffering from cancer have had their life line extended due to early detection and available treatments [1].

The way by which normal cells grow out of control leading to cancer development is nowadays less mysterious and in general can be caused by several factors, whether environmental (i.e. tobacco, diet, radiation & infectious organisms) or internal (e.g., inherited mutations, hormones & immune conditions) [2]. Thus, the interaction between the human body itself (as referred to the genes) and the acquired environmental factors can be considered as drivers for cancer pathogenesis. Craig Venter, a pioneer genomic researcher who sequenced his own genome, talked about the complexity of human biology that doesn't rely entirely and exclusively on the genes we receive from our parents. He mentioned that genes can give us information about the increased risk of a disease, but they will not determine the actual cause of it, thus I quote “Genes are absolutely not our fate”. Because of that, he came up with a conclusion, which says that biology is the result of the complex interaction of proteins and cells that work under environmental factors rather than genetic code [2]. His conclusion is quite significant to dig more into the multiple gene-environment interactions that would increase the risk of driving tumor development.

Generally, cancer can be divided into 5 types depending of the cell type it comes from [3]:

- **Carcinoma:** it is the most common type of cancer and 80% of UK cancer cases are related to this type. It begins in the epithelial tissues, such as liver, lung, prostate, etc.
- **Sarcoma:** it is much less common than the carcinoma (1%) and appears in the connective tissues like the bone, cartilage, fat, muscle or blood vessels.
- **Leukemia:** it accounts for 3% of cancers and originates from the blood forming tissues, like the bone marrow, leading to the production of abnormal white blood cells that

circulate in the blood (e.g., acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML)).

- **Lymphoma and myeloma:** they make about 5% and 1% of cancers, respectively. They develop in the cells of the immune system like lymphocytes, which tend to stay in the lymph nodes, causing for example Hodgkin lymphoma (known by the presence of B Reed-Sternberg cells) and non-Hodgkin lymphoma derived from B cells, NK cells and T cells.
- **Brain and spinal cord cancers:** 3% of cancer cases are registered as being of this type. It starts in cells of the brain or spine, the most famous example being glioma.

Some cancers can be metastatic by gaining certain properties that allow them to travel from one part of the body to the other, and thus start to grow there. In addition, most of the identified cancers form solid tumors except for leukemia, which involves blood and blood forming organs. Generally, tumors can be either **malignant**- and hence called cancerous- or they could be **benign**, and thus called non-cancerous. The difference between both is the fact that the latter tumors do not threaten the life of patients [1].

It took centuries for scientists to solve the mysterious way by which cancer develops. It was believed that cancer was contagious and could spread through infections. It was not until the 20th century that the real problem began to be solved. In year 2000, the six hallmarks of cancer were introduced by Hanahan and Weinberg [4], which included the ability of tumor cells to be self-sufficient from the surrounding growth signals along with being resistant to any anti-growth ones, having uncontrolled proliferation going on and on, escaping apoptosis, inducing angiogenesis as well as being capable of invasion and metastasis. These hallmarks were then defined as acquired novel capabilities necessary for the tumor growth and proliferation.

Eleven years later, Hanahan and Weinberg published a new update to these hallmarks of cancer, which they termed “the next generation”, and among which two were emerging: energetic metabolism reprogramming and escaping immune destruction (Figure 1) [5]. A growing body of research suggests that these two hallmarks are implicated in the pathogenesis of most, if not all, cancers. Two consequential characteristics of neoplasia, genome instability and mutation, in addition to tumor-promoting inflammation facilitate the acquisition of all the hallmarks of cancer [5].

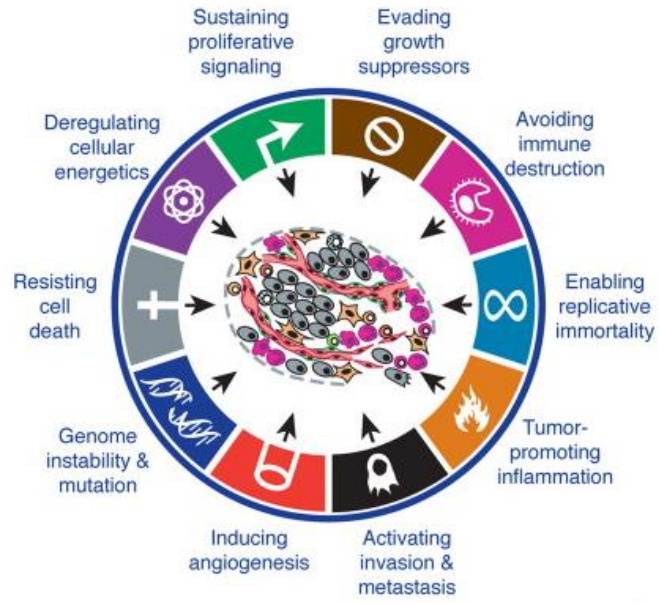


Figure 1: The Next Generation Hallmarks of Cancer [5]

II- Liver Cancers

Liver is the largest solid organ of all, located on the right side of our body consisting mainly of cells called the hepatocytes. Liver has numerous vital functions essential for human survival, notably its role in toxins' removal by blood filtering, drug metabolizing, bile synthesis and excretion into the duodenum, and blood sugar level regulation. Due to the high vascular nature of this organ, it is a very common site for cancer development, especially metastatic ones.

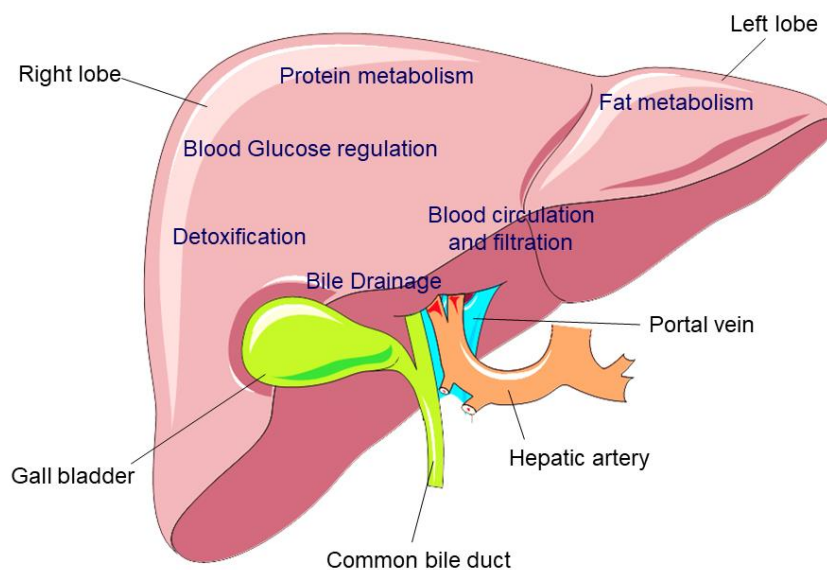


Figure 2: Liver anatomy and function

According to the GLOBOCAN 2020 estimates of cancer incidence and mortality, liver cancer ranks the sixth among the most commonly diagnosed cancers and the third leading cause of cancer-related deaths worldwide. Every year about 906,000 new cases are diagnosed and 830,000 deaths are recorded [6]. In 2019, the American Society of Cancer has made an estimation of the number of new liver cancer cases and deaths in males and females in the United States, which are summarized in Table 1 [7].

Table 1: Estimated New cancer Cases and Deaths by Sex in the United States in 2019

Cancer Type	Estimated New Cases			Estimated Deaths		
	Both sexes	Males	Females	Both sexes	Males	Females
Liver & intrahepatic bile duct	42,030	29,480	12,550	31,780	21,600	10,180

The cancer that starts in the liver is called primary liver cancer and can be distinguished into four kinds according to the American Cancer Society [8].

- 1- **Hepatocellular Carcinoma (HCC):** most common form of adult liver cancer.
- 2- **Intrahepatic cholangiocarcinoma:** bile duct cancer.
- 3- **Angiosarcoma and hemangiosarcoma:** rare cancer that starts in the cells lining the blood vessels of the liver.
- 4- **Hepatoblastoma (HBL):** pediatric form of liver cancer affecting children usually younger than 4 to 5 years old.

In this manuscript, I will be talking in details about two primary liver cancers, the hepatocellular carcinoma and the hepatoblastoma affecting adults and children, respectively.

III- Hepatocellular Carcinoma (HCC)

1. Generalities:

The majority of primary liver cancers are hepatocellular carcinoma (HCC). HCC is associated with poor prognosis with an overall survival rate of 3-5% [9]. HCC is the fifth most common cancer in males and the ninth in females [6], [10], [11]. In females, the incidence rate and the mortality of this disease are significantly lower than in males. Interestingly, it has been shown that women are at higher risk of developing HCC after menopause, highlighting the protective role of estrogen on HCC progression [12], [13]. Therefore, the gender difference seems to have a great role on the development of HCC.

It is of no surprise that the incidence rate of HCC is increasing worldwide (Figure 3) as it is accompanied with many different risk factors. According to GLOBOCAN 2020, for men, the high incidence regions are Eastern and South-Eastern Asia, intermediate ones are Southern Europe and Northern America whilst the lowest regions are in Northern Europe and South-Central Asia. On the contrary, the women's rates are basically much lower where they record the highest rates in Eastern Asia and Northern Africa. The same goes for the mortality rate, which is also 2 to 3 times higher in males, making it the second most common cancer-related death worldwide [6], [10], [11].

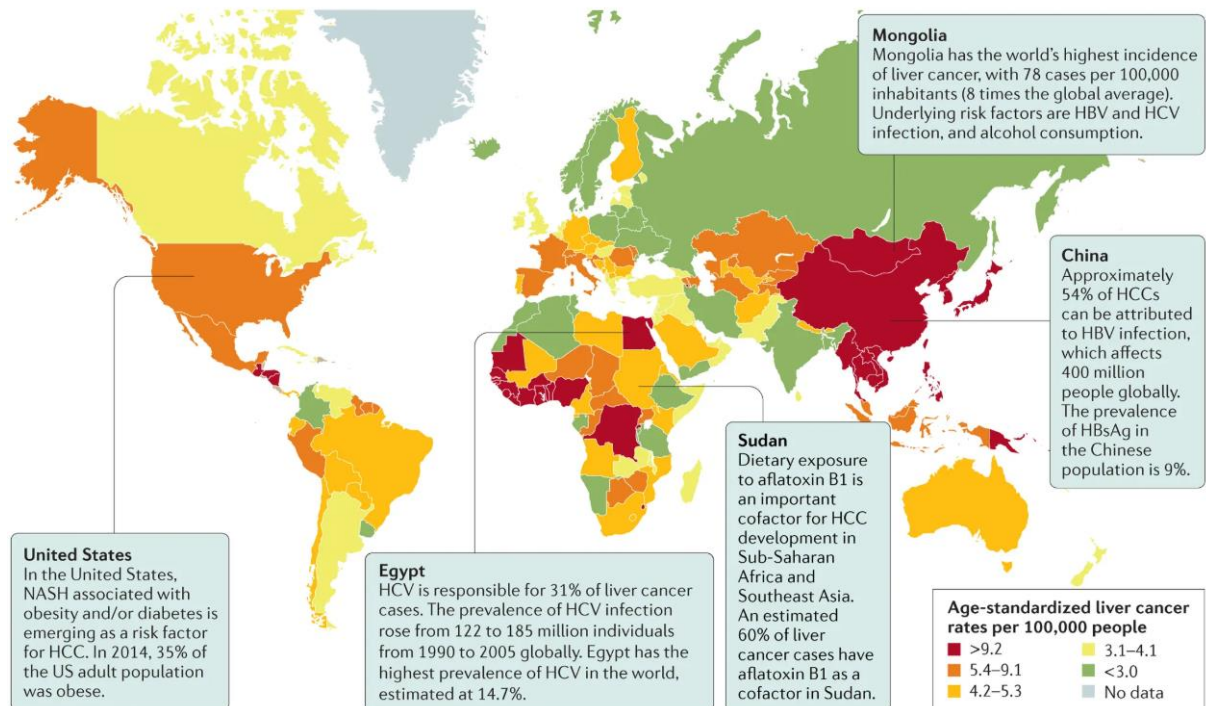


Figure 3: The incidence rate of HCC associated with the risk factors in different countries worldwide [14].

The European Association for the Study of Liver (EASL) was the first to publish clinical management guidelines for HCC in 2001 [15]. Ten years later, it was followed by several updated guidelines of which 8 current guidelines for HCC around the world are summarized in Table 2.

Table 2: Current Guidelines for HCC management over the world (Adapted from [16])

Publishing Year	Guidelines	Abbreviations	Region
2010	Asian Pacific Association for the Study of the Liver	APASL	
2014	Korean Liver Cancer Study Group & the National Cancer Center	KLCSG-NCC	Asia
2015	Japan Society of Hepatology	JSH	
2012	European Association for the Study of the Liver & the European Organization for Research and Treatment of Cancer	EASL-EORTC	
2012	European Society for Medical Oncology-European Society of Digestive Oncology	ESMO-ESDO	Europe
2011	American Association for the Study of Liver Disease	AASLD	
2014	American College of Gastroenterology	ACG	USA
2016	National Comprehensive Cancer Network	NCCN	

Guidelines were then defined as “systematically developed statements to help medical practitioners and patient decisions about the suitable healthcare for certain medical conditions”. The use of these guidelines makes it possible to achieve these goals: (a) serving as a guide for proper medical decision-making by doctors, (b) improving the quality of healthcare and patients’ outcomes and (c) supporting and influencing local as well as national

experts who allocate resources [17]. It is important to note that HCC is very heterogeneous among patients of different geographic origins, making it unfeasible to have general universally accepted guidelines, as compared to other malignancies [16].

2. Risk factors:

HCC can affect population all over the world despite the significant difference in its incidence, which is related to many etiological factors. The most common of these factors are chronic hepatitis B and C viral (HBV/HCV) infection, alcohol abuse, exposure to aflatoxin B1 and all cirrhosis-causing conditions such as non-alcoholic fatty liver diseases (NAFLD) [18] (Figure 4).

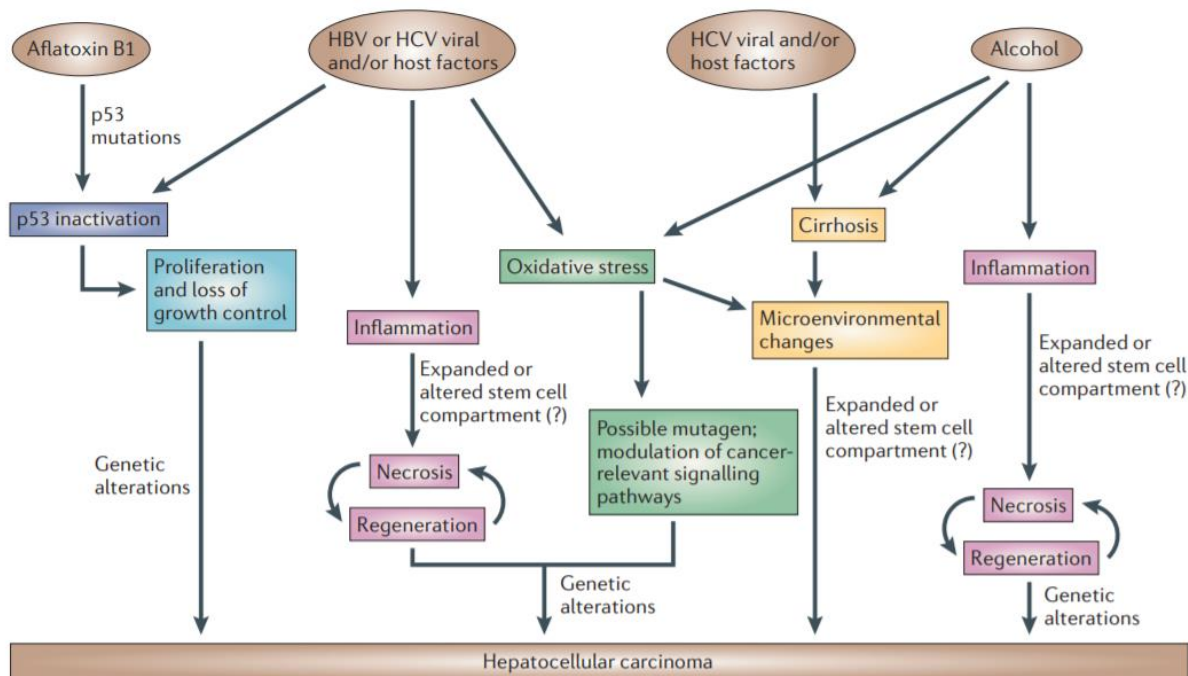


Figure 4: Mechanisms of hepatocarcinogenesis associated with multiple risk factors [18]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; p53: tumor suppressor protein 53

The exposure of the liver to such risk factors enrolls it into vicious cycles of continuous necrosis and regeneration. If left untreated, this will cause a severe irreversible hepatic damage that eventually results in cirrhosis, the very first step to develop HCC. Cirrhosis is simply defined as abnormal nodules all over the liver tissue surrounded by collagen and scarring. Cirrhotic livers unveil abnormal, immature hepatocytes called dysplastic foci (<1 mm) or dysplastic nodules (DN) (≥1 mm), which can be considered as precancerous lesions [19]. We can distinguish two

types of nodules: low-grade DNAs (LGDNs) and high-grade DNAs (HGDNs), both of which can potentially develop HCC, the latter being at greater risk.

- **HBV:**

This virus infects around 2 billion people per year and around 50% of HBV-related deaths are due to HCC [20]. HBV belongs to the family of *Hepadnaviridae*, and is a non-cytopathic, partially double-stranded hepatotropic DNA virus. Just like many other viruses, HBV genome encodes different viral proteins that are necessary for its life cycle, such as a DNA polymerase with reverse transcriptase activity (pol), the capsid protein named hepatitis B core antigen (HBcAg), and the L, M & S envelope proteins necessary for the association with endoplasmic reticulum (ER) membrane during the replication process and finally the protein x (HBx) whose function needs to be further studied [21]. The mechanism by which HBV infection would lead to HCC development is related to the genome integration of this virus into the host DNA, resulting in DNA microdeletions of cancer-related genes like the telomerase reverse transcriptase (TERT), platelet-derived-growth-factor receptor β (PDGFR β) and its ligand (PDGF β) and mitogen activated protein kinase 1 (MAPK1) [22], [23].

- **HCV:**

The rate of infection of HCV is less compared to HBV, as more than 170 million persons are currently infected and 2.5% of these people are capable of developing HCC [18]. HCV belongs to the *Flaviviridae* family of non-cytopathic viruses. It consists of a positive-stranded RNA genome that encodes structural (core, E1, E2 & p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A & NS5B) proteins [24]. Unlike HBV, HCV's RNA genome lacks the ability to integrate into the host genome, thus cannot form DNA intermediates [25]. For instance, the core protein gives rise to the viral nucleocapsid, which surrounds and protects the genomic RNA, while E1 & E2 envelope glycoproteins make up the structural components of the virion. Moreover, HCV is capable of evading the immune system and immune-killing machinery thanks to its core protein and NS5A non-structural protein, which, for example, can interact with tumor-necrosis factor- α (TNF α) receptor, interferon- α (IFN α) and others [26]–[28].

- **Excessive Alcohol Consumption:**

As defined by Ramadori *et al.* [29], alcohol is a small polar organic molecule that can diffuse through cell membranes and *via* bloodstreams to be distributed into all tissues. It is mainly oxidized in the liver by the action of an enzyme called alcohol dehydrogenase (ADH) in order to produce acetaldehyde. The latter can then diffuse into the bloodstream and exerts its mutagenic effect directly by causing DNA damage, thus “adding fuel to the flame” to quote Ramadori. Alcohol abuse is the third leading risk factor for disease and mortality in Europe. It is associated with liver cirrhosis and cancer, where in both genders, it is responsible for 18-33% of liver cancer cases [30].

- **Aflatoxin-B1 (AFB1):**

AFB1 is a mycotoxin produced by the commonly widespread fungal species; *Aspergillus flavus* and *Aspergillus parasiticus*. It can be found in certain foods like corn, rice, oil seeds, peanuts and dried fruits, which have been stored in inappropriate conditions of humidity and unsanitary conditions [31]. AFB1 is considered to be the most potent hepatotoxic and hepatocarcinogenic agent. AFB1 is a genotoxic mutagen that causes DNA damage in cells. For example, it can cause a mutation in *TP53* gene of codon 249 (R249S substitution) resulting in inhibition of apoptosis, p53 mediated transcription as well as stimulation of liver cell growth *in vitro* [32]. It is noteworthy to mention that there is a synergistic effect between HBV infection and AFB1 exposure, where HBx protein produced by the virus is correlated with 2-fold increase in the incidence of R249S mutation in *TP53* gene in the presence of AFB1 [33].

- **NAFLD:**

NAFLD is a benign form of liver disease where fat starts to accumulate in hepatocytes. It is expected to become a dominant cause of HCC in the upcoming 10 years. NAFLD mainly occurs in patients with the following underlying risk factors: metabolic syndrome (MS), insulin resistance (IR), persistent inflammation and altered gut flora. However, the exact molecular mechanism by which NAFLD progresses from simple steatosis, to steatohepatitis and finally to HCC is not very well understood [34], [35]. Two subgroups can be distinguished: NAFL (non-alcoholic fatty liver) and NASH (non-alcoholic steatohepatitis). The first is known to have low risk of progression into advanced fibrosis, while the latter is considered as the active form of NAFLD [36] and is characterized by inflammation, hepatocyte ballooning and faster fibrosis

progression [37]. The mechanism by which NAFLD becomes HCC is diverse. For instance, IR leads to fat accumulation in hepatocytes and genetic mutations resulting from oxidative stress and reactive oxygen species (ROS) induce liver cancer [34].

3. Genetic & Epigenetic Alterations in HCC:

Significant progress has recently been made to reveal the genetic and epigenetic alterations occurring in HCC, including potentially targetable aberrations. These alterations make HCC heterogeneous at the phenotypic as well as genomic levels, where they result in the deregulation of key oncogenes and tumor-suppressor genes that will be discussed hereafter.

- **Mutation in *TP53*:**

TP53 gene encodes for p53 transcription factor which functions as a key tumor suppressor and main regulator of many important signaling pathways like cell cycle arrest, DNA repair and apoptosis. Mutations in *TP53* occur in almost every type of cancer, and result in the loss of the ability of p53 to bind to the DNA and activate the transcription of p53 target genes [38]. The contribution of p53 mutations to cancer initiation and progression is not very well understood, but it is known to participate in the development of HCC. It was shown in HBx transgenic mice that the sequestration of p53 by HBx in the cytoplasm, and thus its inactivation, was observed in HCCs implicating a potential role of p53 mutation in HCC progression [18]. As mentioned earlier, *TP53* mutation drives the initiation of HCC in the case of aflatoxin B1 poisoning.

- **Mutation in β -catenin:**

β -catenin is the famous most important downstream component of the Wnt signaling pathway. The absence of Wnt ligand renders the Wnt signaling inactive (Figure 5, left), and this results in the phosphorylation of β -catenin by the action of the “destruction complex”. This complex is composed of the scaffold protein Axin, the adenomatous polyposis coli (APC) and two kinases: casein kinase 1 α (CK1 α), and glycogen synthase kinase 3- β (GSK3- β). The phosphorylation of β -catenin by GSK3- β and CK1 α will lead to its ubiquitination by β -TrCP E3 linker (β -transducin repeat-containing protein, an E3 ubiquitin ligase) and further proteasomal degradation. Therefore, in the absence of nuclear β -catenin, a repressive complex containing

TCF/LEF (T-cell factor/lymphoid enhancer factor) and transducing-like enhancer protein (TLE/Groucho) recruits HDACs to repress target genes [39].

The activation of the Wnt signaling pathway is made possible by Wnt ligands (e.g., Wnt3a & Wnt1), which will bind to the frizzled (Fzd) receptor and its co-receptors, the low-density lipoprotein receptor related protein 5/6 (LRP5/6) (Figure 5, right). LRP5/6 are then phosphorylated by GSK3- β and CK1 α , which recruits Dishevelled (Dvl) proteins to the plasma membrane where they will undergo polymerization and become activated. In turn, the latter will inactivate and destroy the destruction complex resulting in the stabilization and accumulation of β -catenin, which is now capable of translocating into the nucleus. Once inside, β -catenin will associate with TCF/LEF proteins by displacing TLE/Groucho, thus forming an active complex that will recruit histone modifying co-activators in order to switch on the transcription of several genes involved in proliferation and growth [39].

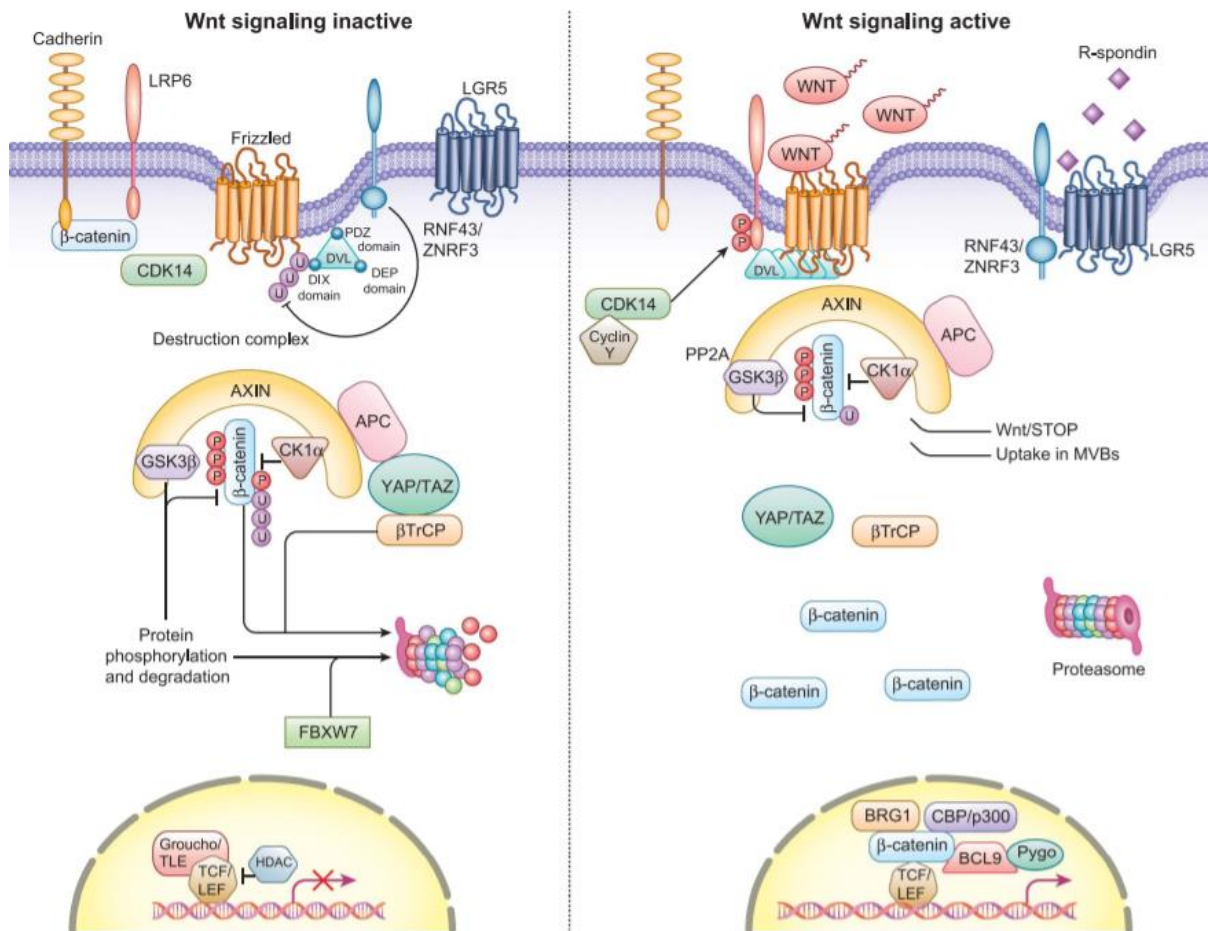


Figure 5: The canonical Wnt/ β -catenin signaling pathway (Adapted from [39])

(Left) Wnt signaling inactive: degradation of β -catenin by destruction complex and inhibition of gene transcription. (Right) Wnt signaling active: degradation of destruction complex, stabilization and translocation of β -catenin into nucleus where it leads to activation of gene transcription

In HCC, mutations in β -catenin and other Wnt signaling pathway partners have been detected. For instance, in 11-37% of HCC cases, Wnt/ β -catenin pathway is frequently activated due to a mutation in *CTNNB1* that activates β -catenin. Moreover, it has also been reported to have inactivating mutations or deletions in Axin 1 (*AXIN1* in 10% of HCC cases) or more rarely in APC (*APC* in 1-2% of HCC cases) [14]. All of these mentioned mutations lead to the constitutive activation of Wnt/ β -catenin pathway.

- **The family of ErbB receptors:**

This family of tyrosine kinase receptors consists of four members, ERBB1 to 4, which showed a role in the development of many types of tumors. ERBB1 is better known as the EGFR

(epidermal growth factor receptor) whose expression was detected to be higher in 68% of HCC cases. This overexpression correlates with more aggressiveness of the tumors, i.e. higher proliferation, advanced disease stage, de-differentiation and intrahepatic metastasis [40]. ERBB2, also known as HER2 (human epidermal growth factor receptor) was reported to be overexpressed in 21% of HCC cases unrelated to the clinicopathological parameters [40]. Moreover, ERBB3 was the highest among its family members in terms of overexpression in HCC (84% of cases). It is correlated with more aggressiveness, differentiation and staging. Finally, ERBB4 is upregulated in 61% of cases with no significant correlation with the clinicopathological features of HCC. However, it was later discovered to act as a suppressor gene in the development of HCC [41]. It was also shown that ErbB4 acts as a suppressor in colitis-associated cancer by negatively regulating cholesterol metabolism [42].

- **Telomeres and telomerase:**

Telomeres are ribonucleoprotein complexes located at the end of each chromosome arm in order to maintain genome stability. They are highly conserved in mammals and consist of hexameric (TTAGG) tandem repeat DNA sequence. Telomerase is made up of telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) [43]. In HCC, there is a robust activation of telomerase in around 90% of cases [44]. The shortening of telomeres is a characteristic feature of chronic liver diseases where it triggers DNA damage thus engaging the cells into irreversible cellular senescence or even apoptosis. This will eventually result in cirrhosis. Nonetheless, telomerase activity is absent in normal hepatocytes, so in order to promote liver carcinogenesis and uncontrolled hepatic cell proliferation, it needs to be reactivated. Therefore, during the early stages of hepatocarcinogenesis, telomerase is reactivated to promote the disease progression by the occurrence of point mutations at two hotspots in the *TERT* promoter in 60% of HCC cases [14]. These mutations create a new binding site for the transcription factor responsible for inducing the expression of *TERT* mRNA.

- **Genomic alterations in HCC:**

Genomic alterations, such as copy number variation and allelic imbalances that lead to genomic gain or loss, are responsible for the instability of HCC genome. Chromosomal comparative genome hybridization (CGH) has identified recurrent regions of copy number changes and imbalances in terms of genomic gains and losses. These include gains in 1q

(57.1%), 8q (46.6%), 6p (23.3%), and 17q (22.2%); and losses of 8p (38%), 16q (35.9%), 4q (34.3%), 17p (32.1%) and 13q (26.2%) [18], [45]. For some loci, the resident cancer gene has been detected and confirmed: retinoblastoma (*RB*, 13q), *TP53* (17p), hepatoma-derived growth factor (*HDGF*, 1q) and cadherin type 1 (*CDH1*, 8q) [18].

It is noteworthy to mention that many evidences have revealed the important roles played by the genetic and genomic alterations in the development and progression of HCC. However, more effort and research are needed to better understand the role of these alterations in the pathogenesis of HCC.

4. Molecular classes of HCC:

The classification of HCC is important for categorizing patients according to their biological backgrounds into homogeneous groups in order to help better target the disease with the best proposed treatments. Genomic studies have identified two main molecular subclasses of HCC: proliferative and non-proliferative HCC [46] (Figure 6). The proliferative class is characterized by the activation of signaling pathways involved in cell proliferation and survival, such as, PI3K-AKT-mTOR (class 1 phosphatidylinositol 3-kinase; serine/threonine kinases; mammalian target of rapamycin) pathway, RAS (Rat sarcoma)-MAPK and IGF (insulin-like growth factor) signaling pathways. It is also related to chromosomal instability (*TP53* inactivation) and HBV-infections, resulting overall in higher aggressiveness and poor clinical outcomes. On the other hand, the non-proliferative class is more heterogeneous and involves the constitutive activation of Wnt signaling pathway due to *CTNNB1* mutations, and is also related to higher *TERT* promoter mutations. This subclass is associated with alcohol- and HCV-related etiologies and proposed to have better outcomes [14], [46].

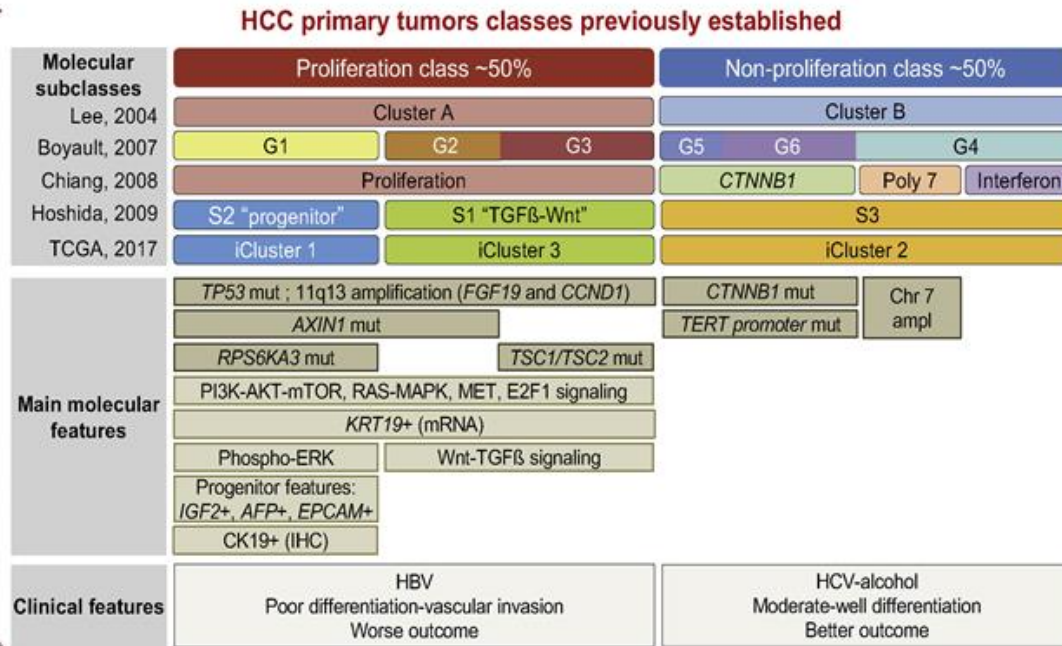


Figure 6: Molecular classification of HCC (Adapted from [47])

This figure shows the different molecular subclasses of HCC as depicted by different research teams, as well as their molecular and clinical features. CTNNB1: β-catenin; TGFβ: transforming growth factor-β; TP53: p53 transcription factor; Chr: chromosome; mut: mutation; amp: amplification; AXIN1: axin 1; RPS6KA3: ribosomal protein S6 kinase A3; TSC1/2: TSC complex subunit 1/2 also known as hamartin; PI3K-AKT-mTOR: class 1 phosphatidylinositol 3-kinase- serine/threonine kinases- mammalian target of rapamycin; RAS-MAPK: rat sarcoma-mitogen activated protein kinase; MET: MET proto-Oncogene, receptor tyrosine kinase; E2F1: E2F transcription factor 1; KRT19 and CK19: keratin 19; ERK: extracellular signal-regulated kinase; IGF2: insulin-like growth factor 2; AFP: alpha-fetoprotein; EPCAM: epithelial cell adhesion molecule; HBV/HCV: hepatitis B/C virus

In 2007, HCC was classified based on unsupervised transcriptomic analyses of 120 tumors into six subgroups named G1 to G6 [48]. High level of association with genetic alterations and clinical factors were observed, where G1-G3 groups were characterized by high chromosomal instability as compared to the G4-G6 groups. Furthermore, G1-subgroup had low viral DNA copies of HBV while G2-subgroup showed higher viral DNA copies along with *TP53* and *PIK3CA* (PI3-Kinase Subunit Alpha) mutations. However, only the G3-subgroup had *TP53* mutation. G4-subgroup was associated with rare mutations in *TCF1* and *HNF1A* (*hepatocyte nuclear factor-1A*). Finally, the G5- and G6-subgroups were highly related to *CTNNB1* mutation that results in Wnt pathway activation, and hypermethylation of E-cadherin 1 (*CDH1*) resulting in its downregulation [48].

Two years later, Hoshida *et al.* analyzed samples from 603 patients representing HBV- and HCV-etiologies of HCC [49]. In their integrative transcriptomic analyses, they showed that HCC

could be divided into 3 subclasses, which they termed: S1, S2 & S3 (Figure 6). S1 is characterized by the activation of Wnt signaling pathway, not because of *CTNNB1* mutation, but rather because of the activation of transforming growth factor- β (TGF- β). S2 was characterized by higher proliferation, α -fetoprotein (AFP) levels, as well as MYC and AKT activation. Finally, the S3 subclass showed very well differentiated hepatocytes with smaller tumors. It is important to note that S1 & S2 subclasses are associated with poor survival, whilst S3 is associated with good survival [49].

All of these proposed classes have been confirmed and further characterized by The Cancer Genome Atlas (TCGA) Research Network, harboring the largest cohort of HCC data, based on different platforms: DNA copy number changes, DNA methylation profiling, expression of mRNA and microRNA (miRNA), and reverse phase protein array (RPPA) assays [50]. They analyzed 196 tumors, which yielded 3 subtypes (Figure 6). The iClust1 subtype associated with poor prognosis and similar gene expression profile to the progenitor cell subclass tumors. iClust2, a lower grade subtype, was also identified with *CTNNB1* mutations similar to the non-proliferative class. And finally, iClust3 that has *TP53* mutations resulted in chromosomal instability and poor prognosis.

5. Management of HCC

Over the last decade, the incidence rate of HCC is rising in many countries, but the overall survival of patients can be prolonged if earlier screening and diagnosis are made. HCC is one of the cancers whose prevention is made easy and possible simply by using vaccination against HBV infection. In 2009, the World Health Organization (WHO) recommended that all newborns and high-risk-groups be vaccinated against hepatitis B [51]. For instance, HBV vaccination reduces the incidence of HCC in high prevalence regions like Taiwan [52]. Multiple lines of evidence indicate that the prevention strategies reduce HCC development.

The management of HCC not only includes its early diagnosis, but also the staging of the disease and the suitable treatments of patients and those who are at risk of developing the disease. That's why the surveillance of HCC is very important, where subjects at risk are periodically diagnosed for HCC development. Cancer surveillance allows the early detection of tumors in patients at high risk, thus increasing the opportunity for curative treatments and

improving survival by decreasing the disease-related mortality (European Association for the Study of the Liver and European Organization for Research and Treatment of Cancer, 2012). The AASLD and EASL guidelines suggest surveillance programs of high-risk patients, including all cirrhotic patients (HBV/HCV infection, AFLD, NAFLD/NASH), HBV carriers from regions of high HBV incidence like Asia and Africa, as well as HCV patients for whom infection is not associated with cirrhosis [14], [53].

- **Diagnosis of HCC:**

The diagnosis of HCC can be done using these steps: serologic testing, diagnostic imaging and histology. AFP is the most famous serological marker for early HCC detection. This protein is produced during the fetal development by the liver and the yolk sac. After birth, its levels drop off remarkably and only traces of it can be detected in the serum. The diagnosis of HCC requires AFP serum levels to be higher than 500 ng/mL, but negative values usually do not exclude HCC. Elevated AFP levels have been found not only in HCC but also in many other diseases including chronic liver disease with no cancer, pregnancy and gastrointestinal tumor [54]. Due to limited AFP sensitivity and specificity, three different variants (AFP-L1, AFP-L2, AFP-L3) have been studied in order to improve the diagnostic performance. Among these variants, AFP-L3 was more specific for HCC diagnosis than AFP [55]. Moreover, another biomarker for HCC has been studied called Des-gamma-carboxy-prothrombin (DCP), but it was not accepted for clinical use by itself in the United States, although it's being routinely used in Asia as part of both the screening and surveillance after curative therapy [56].

Diagnostic imaging can be done using ultrasound in case the hepatic nodules are <1 cm in a cirrhotic liver. Once the nodule becomes >1 cm in a cirrhotic liver, the imaging should switch to either multidetector computed tomography (MDCT) or magnetic resonance imaging (MRI). If the lesion is found to be intermediate in cirrhotic and non-cirrhotic patients, a biopsy should be taken from the lesion [53], [54]. It is important to agree on the fact that biopsies should only be reviewed and examined by an expert "hepatopathologist" since the distinction between a dysplastic nodule and HCC can be tricky. The sensitivity and specificity of biopsies have been increased by the use of immunostaining for glypican-3 (GPC3), heat shock protein 70 (HSP70), and glutamine synthetase (GS) [57]. If at least two of these markers were positively stained, the patient is diagnosed with HCC.

- **Staging of HCC:**

In 1999, the Barcelona Clinic Liver Cancer (BCLC) group proposed for the first time the what so called BCLC staging [58]. So far, BCLC is the best staging system proposed, as it is the most commonly used one for HCC. The algorithm used by this system links the tumor stages to their corresponding treatments [59]. Treatments are the fundamental therapies that aim to either cure HCC using radical therapies or find palliative ones that help to improve overall survival. Radical therapies include ablation, resection and transplantation, whilst palliative therapies involve chemoembolization and systemic therapies [59] (Figure 7).

The choice of therapy depends on the tumor stage (BCLC 0, A, B, C & D) and this will be discussed in the next section. BCLC system relies on the number and size of the tumors as well as the liver function and health status of the patient (Eastern Cooperative Oncology Group [ECOG] Performance Status [PS]) [14].

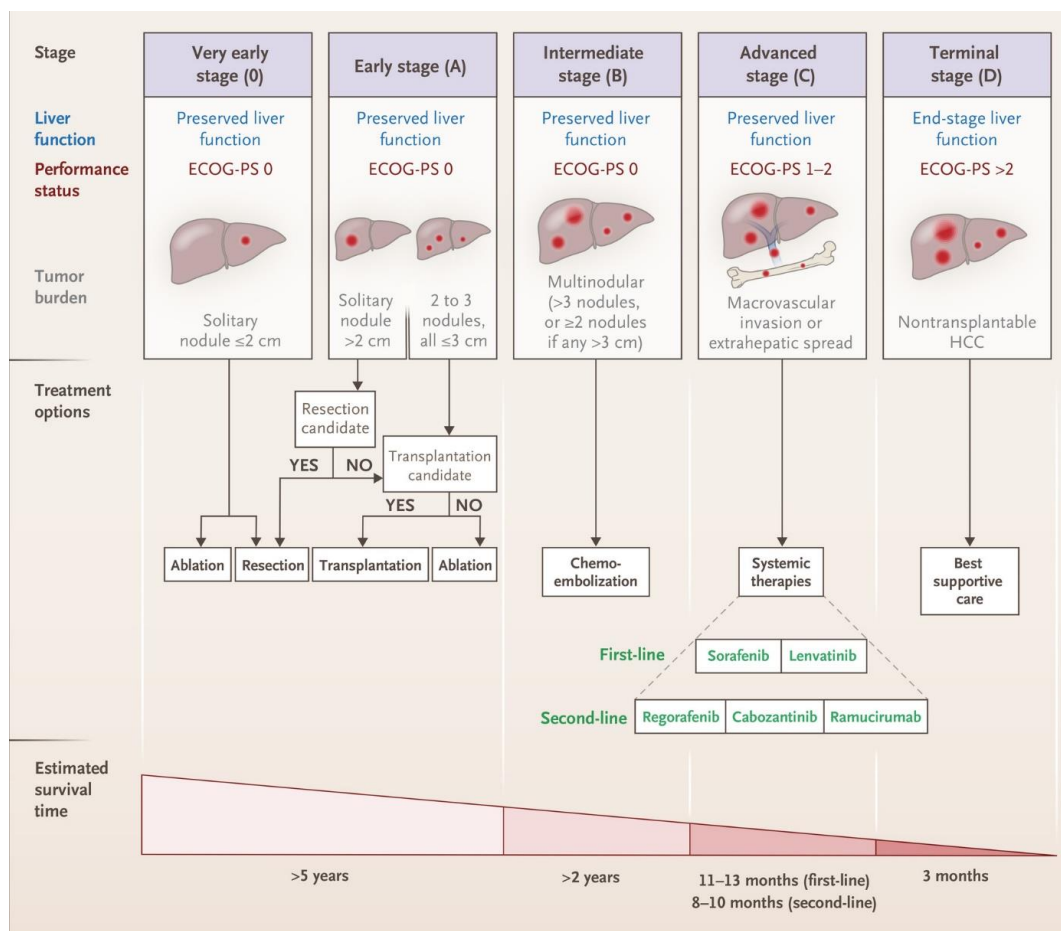


Figure 7: The clinical algorithm for the management of HCC: BCLC staging system [60]

➤ **Early stage disease:**

Very early HCC (BCLC 0) is defined by the presence of a single tumor ≤ 2 cm in diameter while early HCC (BCLC A) is characterized by a solitary tumor ≤ 3 cm or by the presence of three nodules of diameter ≤ 3 cm. In both cases, the liver has a well-preserved function without macrovascular invasion or extrahepatic spread, and the patient feels well (ECOG PS 0). These patients can eventually benefit from radical therapies like ablation, resection, or liver transplantation.

➤ **Intermediate stage disease:**

BCLC B or intermediate stage HCC patients are asymptomatic with preserved liver function. They have large, multifocal tumors without vascular invasion and extrahepatic spread (ECOG PS 0). Transcatheter arterial chemoembolization (TACE) is an effective measure at this stage, in which this minimally invasive technique is based on combining intra-arterial injection of chemotherapy followed by the embolization of the blood vessels using beads in order to restrict blood supply to the tumor.

➤ **Advanced stage disease:**

Patients with BCLC C have at least one or more of the following symptoms: extrahepatic spread, vascular invasion and mild-cancer related symptoms (ECOG PS 1-2). Systematic therapies are available for these patients. The Sorafenib HCC Assessment Randomized Protocol (SHARP) trial showed that the oral administration of the multikinase tyrosine inhibitor sorafenib, is capable of increasing the overall survival of BCLC C patients [61]. If patients showed any HCC progression after sorafenib treatment, they are then subjected to the second-line treatment, regorafenib, which is a systematic multikinase inhibitor, shown to prolong patients' survival [62].

➤ **Terminal stage disease:**

Patients with end stage disease (BCLC D) have poor liver function, marked cancer-related symptoms (ECOG PS >2). Such patients require the best supportive care from nutritionists and psychologists but unfortunately are not candidates for clinical trials.

6. Treatment of HCC

The BCLC staging classification is the major determinant used to arrange patients and propose suitable treatment for each. Early stages HCC patients (BCLC 0-A) can benefit from resection, liver transplantation or even local ablation. Such treatments usually lead to a median overall survival duration for up to 5 years. BCLC 0 patients with normal liver function, normal bilirubin and without portal hypertension are usually selected for tumor resection [63]. If this is not possible, they can benefit from liver transplantation or percutaneous ablative therapies (BCLC 0 and A) by thermal ablations (e.g., cryotherapy, microwave or radiofrequency ablations) or by using chemicals such as ethanol.

BCLC B patients with intact liver function are selected for TACE. As mentioned earlier, TACE involves the use of embolizing agents, like gelatin sponge particles, known to block or slow down the blood supply to tissues, followed by the injection of chemotherapeutic agents. Sometimes, antineoplastic agents can also be used, such as mitomycin, cisplatin and doxorubicin alone or in combination to lipiodol emulsion [64]. By this method, the embolic agents trigger ischemia and local hypoxia while the lipiodol prolongs the lifespan of the drug in the tumoral tissues. It is noteworthy to mention that the portal vein supplies blood to three quarters of the liver parenchyma while the rest comes from the hepatic artery. And tumor cells are known to have their supply from the latter, hence making TACE the best treatment strategy in this case because it will only block the hepatic artery without affecting the rest of the liver [63]. The median overall survival is 2-2.5 years [46]. Unluckily, patients who still suffer from disease progression after TACE will eventually receive systemic therapy.

On the contrary, BCLC C patients are usually registered for systemic therapies. In 2007, a breakthrough in the management of advanced HCC was done where sorafenib demonstrated survival benefits versus the placebo [61]. Sorafenib (Nexavar, BAY 43-9006) is an oral bi-aryl urea drug approved by the Food and Drug Administration (FDA). Its chemical name is N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl) oxyphenyl) urea. The first discovery of this drug by Bayer HealthCare and Onyx Pharmaceuticals goes back to 1995. Only in 2001 was it confirmed to be a RAF1 (serine/threonine kinase) inhibitor, whose role is to stimulate the transcription of genes that promote tumor growth [65]. Nowadays, sorafenib is identified as a multikinase inhibitor where it can target around 40 kinases such as vascular endothelial growth factor receptor (VEGFR)-1/2/3, platelet-derived growth factor receptor- β

(PDGFR- β), fibroblast growth factor receptor 1 (FGFR1), B-RAF, and Fms-like tyrosine kinase 3 (Flt-3) [66], [67].

Sorafenib treatment is usually accompanied with some adverse events, yet uncomplicated ones, like hypertension, fatigue, hand-foot skin reaction and diarrhea. In spite of these events, sorafenib succeeded in increasing the median overall survival of advanced-stage HCC patients from 8 to 11 months [46].

- **Mode of action of sorafenib:**

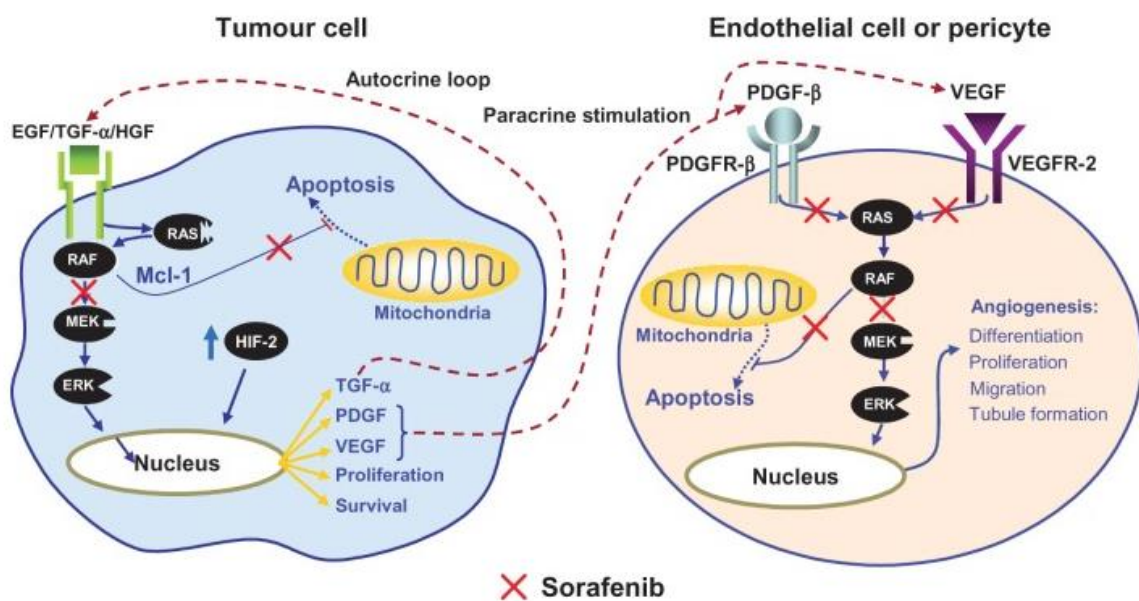


Figure 8: Mode of action of sorafenib [68]

As shown in figure 8, sorafenib exerts its action by directly inhibiting RAF and its isoforms in the liver tumors. When RAF is inhibited, the phosphorylation of MEK/ERK (Mitogen-activated protein kinase/ extracellular signal-regulated kinase) is suppressed leading eventually to a decrease in the cellular proliferation. Moreover, it will induce apoptosis by inhibiting the translation of anti-apoptotic proteins such as, myeloid cell leukemia 1 (Mcl-1) and B-cell lymphoma 2 (Bcl-2). As a result, a pro-apoptotic protein called BAK (Bcl-2 antagonist killer) will be released and thus inducing apoptosis. In parallel, sorafenib can inhibit angiogenesis by targeting important receptors expressed on endothelial cells like VEGFRs and PDGFR- β . These receptors are known for activating RAS/MAPK pathway and finally the transcription of genes promoting angiogenesis [67], [69].

- **Resistance to sorafenib:**

Despite the promising effects of sorafenib in extending the survival of patients, in the end, it's still a chemotherapeutic agent against which some patients will develop resistance. It has been shown that resistance could be due to increased expression of EGFR. That's why, a combination treatment of sorafenib with EGFR inhibitors (erlotinib or gefitinib) enhanced the efficacy of sorafenib by ensuring a significant blockade of RAF kinases, hence illustrating the important role played by these kinases in HCC [70]. Another possible resistance mechanism can be through the activation of PI3K/AKT pathway as a compensation for the inhibition of RAF/RAS/MAPK signaling. This will induce the cellular proliferation and decrease apoptosis [71].

In order to overcome sorafenib resistance, other drugs have been tested. For instance, regorafenib, a second-line treatment, has been developed in 2017 [62]. It is FDA-approved and very similar to sorafenib in terms of chemical structure and mode of action. However, regorafenib showed more tendencies towards the inhibition of VEGFR kinase. The RESORCE trial showed that treatment with regorafenib improved the overall survival of patients with sorafenib resistance from 7.8 to 10.6 months when compared to placebo. Also, the adverse events were similar to that observed with sorafenib [62]. Further work is needed to further explore and understand the exact mechanism of action of regorafenib.

- **Immunotherapy:**

The failure of many chemical drugs tested for HCC treatment urges the development of novel approaches as therapeutic strategies. Immunotherapy has opened a new door in the axis of therapy with many promising results. At the beginning, immune therapies have focused on vaccination strategies, cytokines or even non-specific T-cell activation in HCC, all of which were unfortunately not fruitful. Later on, other strategies have emerged like the inhibition of immune checkpoints, which are defined as membrane bound proteins regulating the immune response. These checkpoints are expressed on immune cells like B- and T-lymphocytes, tumor associated macrophages (TAM) and natural killer (NK) cells. They display an immunosuppressive activity to block the uncontrolled long term T-cell activation in order to avoid tissue damage [72]. For instance, programmed cell death protein 1 (PD-1) is one of the most studied checkpoints in human cancer. It is expressed by activated CD8⁺ and CD4⁺ T-cells.

During cancer pathogenesis, PD-1 can recognize its ligands PD-L1 and PD-L2, which are expressed by tumor cells, and thereby blocks T-cell and immune response activation [46]. Monoclonal antibodies have been developed against PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab, avelumab and durvalumab) and are approved for treatment in many diseases [73]. Nivolumab is now considered a second-line treatment for patients with advanced HCC. Results obtained using this treatment showed a good response, enhanced overall survival of patients and reduced toxicity [74].

IV- Hepatoblastoma (HBL)

1. Generalities:

Hepatoblastoma (HBL) is a rare malignant embryonic tumor accounting for only 1% of all pediatric neoplasms, yet it is the most common primary hepatic tumor in babies [75]. Approximately one in million children are affected by HBL, which usually develops between six months and three years after birth in around 95% of cases, with a median age of 18 months [76]. The remaining 5% are diagnosed in children above 4 years old and exceptionally in some adult cases [76], [77]. HBL is often detected by palpation of an asymptomatic hepatomegaly, in which the liver is enlarged beyond its normal size due to pathological conditions, and the physician can detect this enlargement using medical examination by touch. Other clinical features can be helpful such as jaundice, weight loss and sometimes pain and fatigue depending on the tumor progress [75]. This disease is also characterized by elevated level of AFP, which is not only helpful for diagnosis, but also in assessing the efficacy of a given treatment [78]. The overall survival rate of HBL recorded so far is around 80%, thanks to the modern imaging techniques used to detect it [79] as well as the adapted chemotherapeutic regimes being used [80].

Despite the relatively low number of HBL cases, the Children's Hepatic tumors International Cooperation (CHIC) group developed an international clinical database by collecting 1,605 HB cases that have been treated in prospective multicenter trial, including: the Société Internationale d'Oncologie Pédiatrique — Epithelial Liver Tumor Study Group (SIOPEL) located in Europe; the Children's Oncology Group (COG) located in North America; the German Society for Pediatric Oncology and Hematology (GPOH); and the Japanese Study Group for Pediatric Liver Tumors (JPLT) [81].

The etiology of HBL is still not fully understood; however, tumorigenesis is believed to arise from immature hepatocytes that go off track and differentiate into other cell types, such as hepatocytes, epithelial, biliary and mesenchymal cells. Therefore, this explains the heterogeneity observed at the cellular and histological levels among patients: 56% epithelial or 44% mixed forms (epithelial and mesenchymal). The epithelial form is further divided into subgroups: 31% pure fetal, 19% embryonic, 3% macrotrabecular and 3% undifferentiated small-cells, which are associated with poor prognosis [75].

2. Risk Factors

Most of the HBL tumors develop sporadically. It has been assumed that HBL starts *in utero* due to many lines of evidence. Since the incidence of HBL development is the highest at birth, it is most likely to start during the gestation period, where tumor cells resemble the embryonic liver cells. Therefore, many researches done on HBL focus on events happening around gestation [82].

- **Inherited Syndromes:**

The incidence of HBL in patients with Familial Adenomatous Polyposis (FAP) and Beckwith-Wiedemann syndrome (BWS) is increased. FAP is associated with inactivating mutations in the tumor suppressor gene *APC* resulting in the constitutive activation of Wnt/ β -catenin signaling pathway, a major player in many cancers including HBL and HCC. The risk of developing HBL is around 750 times higher in patients with FAP than in the general population [83]. BWS is characterized by excessive growth and congenital malformations such as gigantism, macroglossia and neonatal hypoglycemia. It is attributed to anomalies that deregulate the expression of *IGF2* (Insulin-like growth factor), *H19* (Tumor suppressor gene) and *CDKN1C* (Cyclin dependent kinase inhibitor 1C) located on chromosome 11p15 and are known to be involved in cell cycle [82], [84]. The result of such anomaly is increased expression of IGF2, and decreased expression of H19 and CDKN1C. The rate of incidence of HBL is 2,280 times higher in patients with BWS (0-4 years old) [82].

Occasionally, some HBL cases are associated with trisomy 18, also known as Edwards Syndrome. It is characterized by multiple congenital anomalies like heart defects, skeletal anomalies, short stature and mental retardation and the survival beyond 1 year is rare in such a case. Because of the high mortality during infancy, it is very difficult to estimate and know the incidence of HBL among these children [82].

Some connection of HBL with other inherited syndromes such as Simpson-Golabi-Behmel syndrome, Prader-Willi syndrome, Sotos syndrome, Kabuki syndrome, Neurofibromatosis type 1, Fanconi Anemia, Tyrosinemia type 1, Noonan syndrome, DiGeorge syndrome and *FGFR3* (Fibroblast growth factor receptor 3) mutation were also described. However, such syndromes have been reported in one or two cases of HBL, only indicating their insignificance as to be the causes of HBL [82].

- **Gestational risk factors:**

HBL is also strongly associated with pre-mature birth and very low birth weight (VLBW; < 1.5 kg) in diverse countries like Japan, United Kingdom, US, and China. Different explanations were proposed. First, HBL may be launched by iatrogenic hazards encountered in Neonatal Intensive Care Units (NICUs) in combination to the declined xenobiotic and antioxidant defense system of these premature infants [85]. HBL could also be the consequence of pre-eclampsia, high maternal pre-pregnancy weight and the treatment for infertility [82].

- **Environmental risk factors:**

Tobacco smoking by both parents, pre- or post-conception, has been identified as a potential risk factor for HBL. This strong and significant association between tobacco use and HBL was first noted by the United Kingdom Childhood Cancer Study (UKCCS) based on a study done in 28 cases [86].

HBL was confirmed not to be associated with maternal exposure to alcohol or estrogen use, but it was significantly related to maternal and paternal exposure to metals [87].

3. PRETEXT Staging System

The PRETEXT (PRETreatment EXTent of tumor) system has been first introduced in 1992 by the SIOPEL as an approach to regularize the imaging evaluation and stratify patients of pediatric liver tumors prior to the administration of neoadjuvant chemotherapy [88]. This system is made of two components: the PRETEXT group and the annotation factors. The first describes the extent of the tumor in the liver whilst the annotation factors describe the associated features [89].

Based on the medical imaging examinations, the liver can be divided into four sections: I, II, III and IV in order to perform the PRETEXT classification. The latter is defined as the number of hepatic sections that have been affected by the tumor at the time of diagnosis (Figure 9) [90]:

- I: tumor is present in one section of the liver; it is typically small; the other three ones are tumor free
- II: tumor is found in one or two sections; two adjacent sections are tumor free

- III: three sections of the liver are infected by the tumor; one section is tumor free
- IV: tumor is present in all four sections of the liver

Then, in order to refine the classification, PRETEXT annotation factors were introduced:

- V: Hepatic venous/inferior vena cava involvement
- P: Portal venous involvement; participation of the main portal vein and its 2 branches
- E: Extrahepatic disease contiguous with the main liver tumor
- F: Multifocality
- R: Tumor rupture or intraperitoneal hemorrhage
- C: Caudate lobe involvement
- N: Lymph node metastasis
- M: Distant metastasis

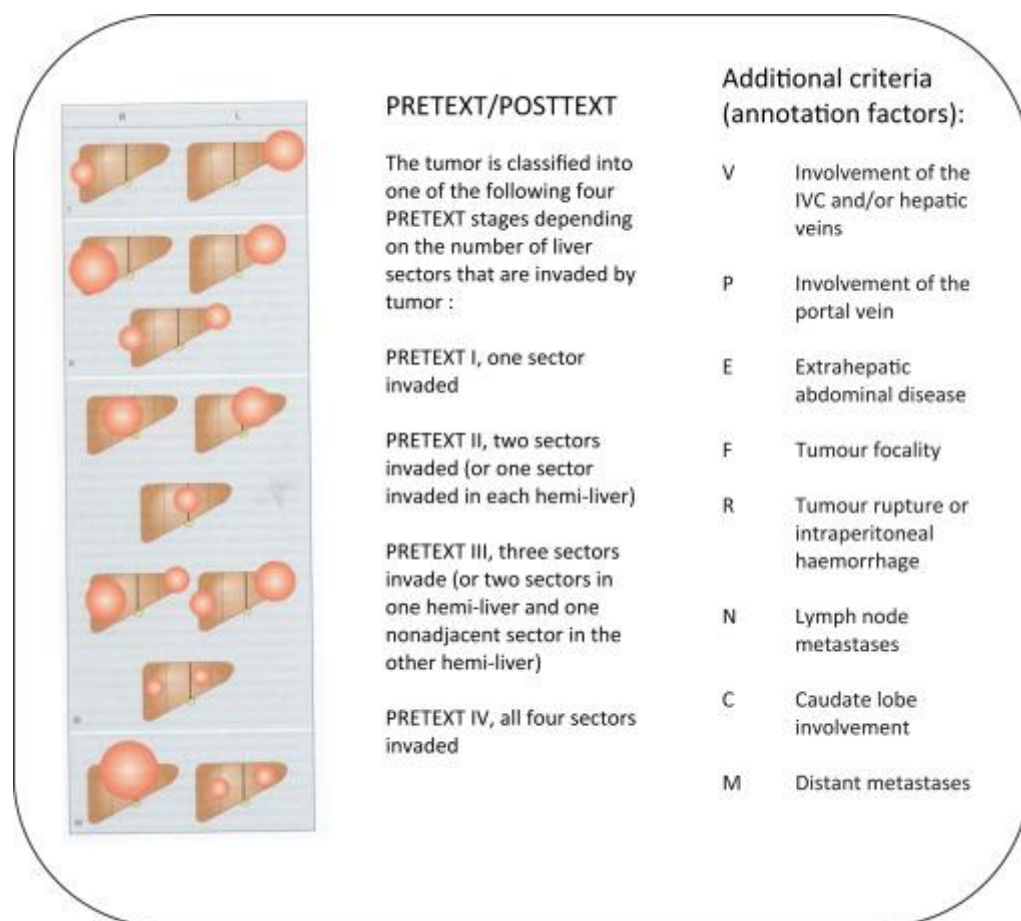


Figure 9: The PRETEXT and POSTTEXT classification systems [91]

The importance of PRETEXT classification resides in facilitating the determination of therapeutic approaches in HBL patients. It is used to predict whether tumors can be resectable or not, and to reclassify patients after being subjected to chemotherapy, and in this case, it is called POSTTEXT.

4. Molecular Classification of HBL

In 2008, a microarray analysis was done in order to identify the molecular signatures characteristic of the tumor subclasses of HBL [92]. 76% of the examined cases had a mutation in β -catenin gene (*CTNNB1*), and this was further confirmed in many other studies [93]–[95]. Mutations in *APC* and *AXIN1* genes have been also detected. Therefore, the total of 82% of analyzed tumors carried mutations in genes linked to Wnt/ β -catenin signaling pathway. This shows the importance of Wnt signaling pathway in the tumorigenesis of HBL.

Using transcriptional profiling, Cairo *et al.* [92] were able to classify HBL tumors into two distinct subgroups termed C1 and C2 according to the differential expression of 16 gene signatures. Both groups were characterized by similar, high mutation rates of *CTNNB1* as well as strong expression of β -catenin in the cytosol and nucleus of tumor cells. However, C1 tumors show predominant localization of β -catenin on the membrane and cytoplasm, whilst C2 tumors show strong accumulation of β -catenin in the nucleus. Histologically speaking, C1 tumors are of fetal type for the most part whereas C2 tumors are more immature, mostly embryonic, macrotrabecular, or fetal type that is associated with high proliferation. Generally, C2 HBL tumors are mainly associated with a more advanced stage and the presence of vascular invasion and extrahepatic metastasis. This renders the tumors of this group more aggressive with usually poor prognosis and an estimated survival at 2 years of age of 44% of patients, unlike 92% of patients with the C1 tumors.

In 2018, another group of researchers came to better refine this classification of HBL tumors using RNA-seq data collected from HBL tumor samples matched with the respective normal liver (NT). Hooks *et al.* [96] distinguished three groups of HBL named C1, C2A and C2B using the following four-gene signature: hydroxysteroid 17-beta dehydrogenase 6 (*HSD17B6*), integrin alpha 6 (*ITGA6*), topoisomerase 2-alpha (*TOP2A*) and vimentin (*VIM*). Each gene was a signature for a certain group; *HSD17B6*, *ITGA6*, *TOP2A* and *VIM* are signatures of NT liver,

C1 group, C2A group and C2B group, respectively. This newly identified classification brought into light new prognostic markers that help in bringing molecular precisions with respect to histological classification and PRETEXT, and propose the use to bortezomib (FDA-approved proteasome inhibitor) as a possible treatment for patients presenting a C2A tumor phenotype characterized by the highest proliferation among other groups and the activation of Fanconi-Anemia (FA) pathway.

5. HBL Treatment

According to the CHIC consortium, a standardization of risk criteria and patient stratification has been put in place, evolving continuously with the results from various studies. To date, CHIC recommends using the PRETEXT classification. Only patients classified as PRETEXT I and II, with well-differentiated tumors and without major involvement of the blood system, are operated without any preoperative chemotherapy. Other standard risk patients (PRETEXT I, II and III) benefit from surgery associated with pre- and post-operative chemotherapy. PRETEXT IV, or high-risk patients, are recommended to get pre-operative chemotherapy combining intense doses of several compounds, followed by resection of the tumor or liver transplantation, and finally followed by post-operative chemotherapy [80], [97].

- **Chemotherapy:**

Cisplatin, or cis-diamminedichloroplatinum (II) (CDDP), is a well-known chemotherapeutic drug, the use of which has been adapted by all protocols and proven to be the most effective chemotherapeutic treatment for HBL [98]. Cisplatin is a platinum based molecule that disrupts DNA replication and transcription, and interferes with its repair machinery, causing DNA damage and hence activation of apoptosis [99]. In order to reduce drugs' cytotoxicity, cisplatin can be used in neo-adjuvant chemotherapies in combination with doxorubicin, carboplatin, etoposide or even ifosfamide, as part of the treatment protocols [100].

As previously mentioned, the main disadvantage of cisplatin, like many other chemotherapeutic agents, is its ototoxicity. In fact, it induces more or less severe irreversible hearing loss in around 50% of HBL cases [101]. Recently, randomized phase 3 clinical trial, SIOPEL 6, was done to investigate the effect of delayed administration of sodium thiosulphate (STS) with cisplatin on the hearing loss caused by the latter [102]. The addition of STS to

cisplatin led to 48% lower risk of hearing loss. The ACCL0431 trial also showed similar protective effects of delayed STS administration by reducing the incidence and severity of cisplatin-induced ototoxicity [103].

- **Liver Transplantation:**

Not all HBL patients are candidates for liver transplantation. Usually, for high-risk HBL patients, complete surgical resection is required and accompanied with chemotherapy prior and post resection. In most severe cases, such as multifocal PRETEXT IV, PRETEXT III + V, PRETEXT III + P and intrahepatic relapse patients, tumor resection is not possible and the relapse rate of such patients is high, leaving them with only one more possible approach, which is undergoing liver transplantation [104]. The cure rate of high-risk patients has been increased using liver transplantation and they showed a long-term survival of 80% compared to 30% after incomplete resection [105].

Liver cancer and PCSK9

As it was mentioned earlier, liver cancer ranks the sixth among the most commonly diagnosed cancers and the fourth leading cause of cancer-related deaths worldwide. An emerging characteristic that promotes the tumor aggressiveness and progression is the altered energetic metabolism, especially lipid metabolism. Whenever we speak about lipid metabolism, we directly think of many closely connected pathways that lead to the anabolism and catabolism of lipid molecules. But to make the picture simpler, I will focus on two inversely related pathways, the lipoprotein uptake mediated by lipoprotein receptors expressed on the surface of hepatocytes and the *de novo* synthesis of cholesterol catalyzed by many rate-limiting enzymes.

The uptake of lipoprotein molecules that circulate in the blood stream, such as low-density lipoprotein cholesterol (LDL-C), is an essential process to maintain blood homeostasis and to avoid their accumulation in blood and hence, to prevent cardiovascular diseases. This uptake is made possible by the action of recyclable low-density lipoprotein receptors (e.g., LDLR) that are expressed on the cell surface. Once the level of cholesterol is back to normal in the blood, this will trigger a negative feedback regulation of LDLRs to prevent them from taking more cholesterol. This negative regulation is mediated by a very important convertase named proprotein convertase subtilisin/kexin type 9 (PCSK9), which we are interested in. In simple words, PCSK9 binds to LDLR and guides it toward lysosomal degradation instead of recycling, thereby decreasing the level of receptors on cell surface. Interestingly, many available transcriptomic databases that were done on HCC and HBL show an increase in the level of PCSK9 in the tumors when compared to the non-tumoral tissues, indicating the presence of the negative regulation of LDLR, and thus a decrease in the uptake of LDL-C in such tumors.

Inversely, the alternative pathway by which the cells can have a supply of cholesterol is through the mevalonate pathway, also known as *de novo* cholesterol biosynthesis pathway. During this process, the cells are capable of producing their own cholesterol, along with non-sterol molecules. This pathway is catalyzed by a rate-limiting enzyme named HMGCR, whose expression is significantly increased in the tumors compared to normal tissues in the different available databases of HCC and HBL.

All these observations led us to hypothesize that the tumor cells have a preference towards the *de novo* cholesterol synthesis rather than the uptake. Hence, digging deeper into this altered lipidic profile, the role of PCSK9 and the interplay between PCSK9 and HMGCR in liver cancer was our main focus.

V- Pro-Protein Convertase Subtilisin/Kexin type 9 (PCSK9/NARC-1)

1. Generalities

Amongst the 30,000 genes encoded by the mammalian genome, ~1.7% are proteolytic enzymes that can belong to one of these six classes of proteases: serine, threonine, metallo, cysteinyl, glutamyl and aspartyl [106]. Generally, proteases are capable of irreversibly cleaving a wide number of substrates that include hormones, growth factors, other proteases and cytokines. This cleavage can result in either activation or inhibition of the target molecule.

In 2003, Seidah *et al.* [107] identified a putative convertase named neural apoptosis-regulated convertase 1 (NARC-1) through cloning of cDNAs that were upregulated in primary cerebellar neurons after inducing apoptosis by serum deprivation. However, at that time, little was known about the activity, cleavage specificity, cellular/tissue expression and function of this protein. All what was known was its gene localization in the human chromosome 1p33-p34.3 and the more abundant expression of NARC-1 mRNA in liver than in testis and kidney. With further investigations, Seidah and his group identified NARC-1 as a mammalian protein convertase member of the proteinase K subfamily of subtilases that is composed of multiple domains: signal peptide, pro-domain, catalytic domain, putative P-domain and finally the C-terminal domain [107]. This zymogen undergoes an autocatalytic prosegment cleavage in the endoplasmic reticulum (ER) in order to become mature, and hence exits the ER. It is important to note that the cleaved prosegment of NARC-1 remains associated with it even after being secreted. Moreover, a very high expression of NARC-1 was recorded in neuroepithelioma, hepatic and colon carcinoma cell lines. This expression seemed to be related to cells that can proliferate and differentiate like hepatocytes [107].

The discovery of NARC-1 by Seidah was the basis for revealing that gain-of-function mutations in this gene, leading to higher production, were the cause of familial hypercholesterolaemia (i.e. a genetic form of extremely high levels of LDL cholesterol) [108], being an important risk factor for coronary heart disease.

Journal editors later changed NARC-1 nomenclature to PCSK9 as they considered this name as being more adapted for standard nomenclature. PCSK9 stands for proprotein convertase subtilisin/kexin type 9 and it is a serine protease that belongs to the family of proprotein convertases (PCs). This family comprises other members namely: PC1, PC2, Furin, PC4, PC5,

PACE4, PC7 and isoenzyme 1 subtilisin kexin. All of these proteins function as enzyme modulators due to their catalytic centers that can cleave many substrates including, precursor proteins, growth factors, receptors, hormones and even transmembrane proteins [109]. However, PCSK9 stands in here as an exception for all of these. This protein exerts its action only through self-activation, making it the one and only substrate for itself [110]. As mentioned above, the autocatalytically cleaved prosegment of PCSK9 zymogen remains associated with the protein even after secretion making it the only PC that is secreted as catalytically inactive [107], [111].

2. Structure of PCSK9

PCSK9 gene encodes a 74.3-kDa zymogen composed of 692 amino acids. This protein has multiple domains as revealed by Lambert *et al.* [112]: signal peptide (aa 1-30), prodomain (aa 31-152), catalytic domain (aa 153-451) and C-terminal domain (aa 452-692), which is rich in cysteine and histidine (Figure 10). The only domain lacking in this protein as compared to its family members is the P-domain [113], which is known to be involved in protein folding and regulation of the protease activity [114]. Unlike its respective mates in the family, which cleave after basic residues in the presence of calcium, PCSK9 undergoes an autocatalytic intramolecular cleavage after a non-basic residue, without any requirement for calcium [111], [115] in order to form a ~ 14-kDa prodomain that remains tightly non-covalently associated with a ~60-kDa domain (catalytic and C-terminal domain) in the secretory pathway [111], [112], [116], [117]. The self-inhibition action of the prodomain allosterically blocks the action of the other two domains. However, the total removal of PCSK9 prodomain results in almost ten times higher affinity between this protein and LDLR and a four times higher degradation rate of LDL receptor [110], [118], [119]. Moreover, PCSK9 prodomain and its mature form both undergo a post-translational modification termed as sulphation of tyrosyl residues. This step occurs in the late Golgi complex before secretion, but the exact role of it is not yet defined [111].

On another note, multiple mutations of PCSK9 can be found, which either induce higher activity -thus associated with hypercholesterolemia- or lower activity -thereby associated with hypocholesterolemia (Figure 10). These mutations will be discussed later in the next section.

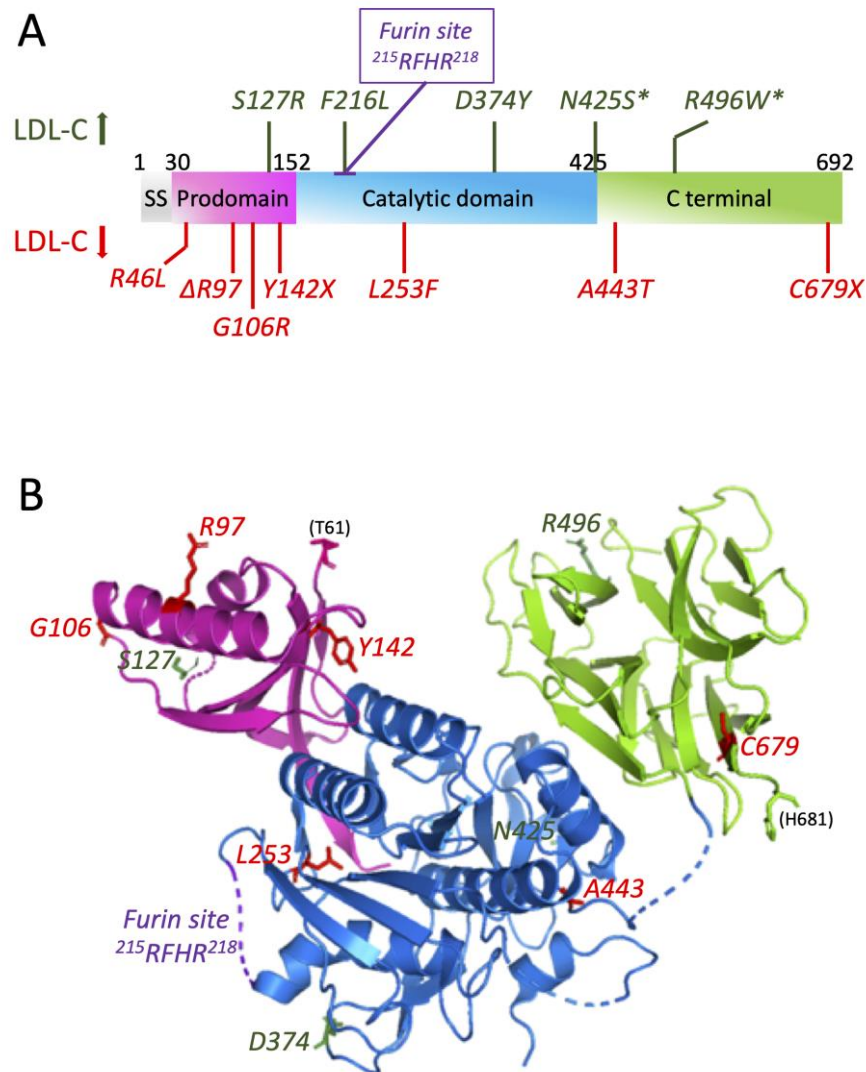


Figure 10: PCSK9 domains, mutations and 3D structure.

A) Different domains of PCSK9 along with the naturally occurring mutations (Adapted from [115]). The mutations increasing LDL-C are in green; those decreasing LDL-C are in red. B) 3D structure representation of PCSK9 from amino acid 61 to 681 (PDB 2PMW) generated with Pymol 2.4.2.

3. Function of PCSK9

The main role of PCSK9 is to promote the degradation of the hepatic low-density lipoprotein receptor (LDLR) present on hepatocytes' surface, thereby blocking the primary pathway of LDL-cholesterol (LDL-C) uptake from the circulation, thus leading to the increase of blood cholesterol levels. Lagace *et al.* [119] were the first to show the molecular mechanism by which PCSK9 represses LDLR. PCSK9 is secreted from cells and is subsequently internalized with LDLR to promote its degradation. This internalization process is dependent on the presence of an adaptor protein called ARH (autosomal recessive hypercholesterolemia) that

binds to the cytosolic tail of LDLR [119], [120]. However, another study has showed that PCSK9 can still degrade LDLR even in ARH knockout mice [121] and this is possibly done either *en route* from the synthesis of LDLR in the ER to the cell surface or on the cell surface before the internalization of LDLR. This proposed mode of action of PCSK9 is supported by the presence of two naturally occurring mutants –S127R and D129G- whose secretion is impaired, yet their function is intact [122].

So based on that, we can distinguish two pathways by which PCSK9 regulates LDLR: intracellular and extracellular pathways (Figure 11). Active PCSK9 binds through its catalytic subunit to the LDLR on its EGF-A (Epidermal growth factor-like repeat A) domain. In the intracellular pathway, this binding will facilitate the transport of LDLR from the *trans*-Golgi network to the lysosomes, instead of going to the cell surface, with the help of clathrin light chains, thus mediating its degradation. This is confirmed by using brefeldin A –a fungal toxin that induces that dissociation of Golgi complex. Brefeldin A prevented LDLR degradation by PCSK9, thus suggesting that PCSK9 can cause LDLR degradation as it migrates from ER to the cell membrane [123]. On the contrary, in the extracellular pathway, mature PCSK9 will be released from the Golgi apparatus, it will bind to LDLR expressed on the cell surface, and together, they will be internalized in clathrin-coated endosomes which will fuse later on with lysosomes to enter in the degradation process. It is important to remind that this internalization process necessitates the presence of ARH (autosomal recessive hypercholesterolemia) adaptor protein [110], [124]. Moreover, a third pathway by which PCSK9 induces LDLR degradation was recently discovered to be mediated by caveolin in a cyclase-associated protein 1 (CAP1) dependent-manner [125]. In this pathway, CAP1 is capable of binding to the C-terminal cysteine-rich domain (CRD) of PCSK9 as well as to caveolin-1 through two different domains, SH3BD (Src homology 3 binding domain) and ACBD (adenylyl cyclase-binding domain) respectively. This interaction will guide the PCSK9/LDLR complex towards caveolae-dependent endocytosis and further lysosomal degradation [125].

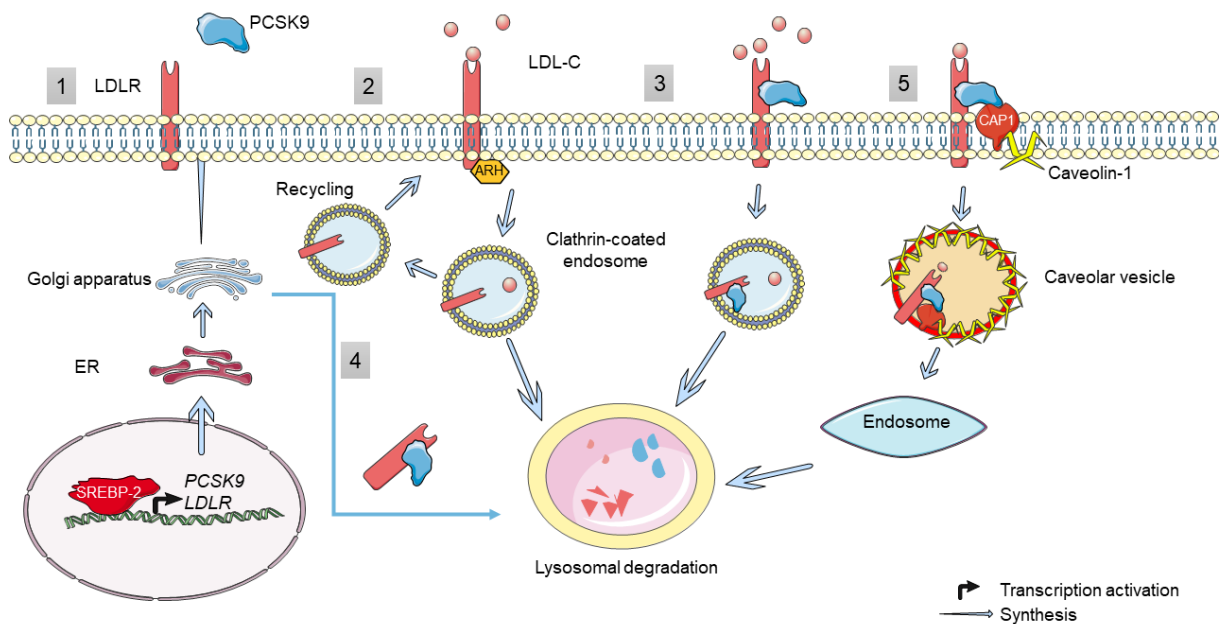


Figure 11: PCSK9, genetic regulation and function.

- 1) The synthesis of PCSK9 and LDLR is regulated transcriptionally by SREBP-2. Once translated, they will travel through ER and Golgi apparatus for maturation before being secreted.
- 2) LDLR expressed on the cell surface will bind to LDL-C and will be internalized in an AHR-dependent manner *via* clathrin coated endosomes. Inside the endosomes, LDL-C will dissociate from the receptor and be directed toward lysosomal degradation, whilst LDLR will be recycled back to the cell surface.
- 3) Extracellular and 4) intracellular pathway regulation of LDLR by PCSK9: in both pathways, PCSK9 binds to the EGF-like domain of LDLR and targets it to lysosomal degradation instead of recycling.
- 5) A novel extracellular pathway by which PCSK9 induces LDLR degradation by interacting with CAP-1, which in turn binds to caveolin-1 and induces caveolin dependent endocytosis followed by lysosomal degradation of LDLR/PCSK9/CAP1 complex

The secreted mature form of PCSK9 (~60 kDa) can be cleaved by the action of two convertases of the same family (furin and PC5/6A) after the recognition sequence R215-F216-H217-R218, giving rise to a truncated PCSK9 protein (50 kDa) with ten times less efficiency with respect to LDLR degradation. In humans, up to 40% of the circulating PCSK9 are found in this truncated form [126], [127].

As mentioned earlier, PCSK9 naturally occurring mutations can be present rendering the protein either more or less active. This is termed as gain-of-function mutations or loss-of-function mutations, respectively. These mutations and their impact have been studied in diseases such as Familial Hypercholesterolemia (FH), hypocholesterolemia and coronary heart disease (CHD).

- **Gain-of-function (GOF) mutations of PCSK9:**

There exist many missense mutations in *PCSK9* gene, which can cause dominant hypercholesterolemia in patients, either by increasing the activity of PCSK9 or by giving it a new activity. Three of these mutations: S127R, F216L and D374Y, have been detected in patients with symptoms of FH and FDB. Some other mutations have been detected only in patients who also have mutations in *LDLR* gene: N425S and R496W (Figure 10) [115]. In these hypercholesterolemia patients, the plasma levels of LDL-C were around 50% higher than in those having either mutation alone [128].

Both S127R and F216L mutant forms of PCSK9 have been tested *in vivo* in comparison to the wild type (WT) PCSK9. All of these forms were highly expressed in mice livers, and they were capable of remarkably decreasing the protein levels of LDLR without any change at the mRNA levels. This indicates a post-transcriptional mechanism of action of the highly expressed PCSK9 (WT or mutant) on reducing LDLR [111], [121], [129]. On the contrary, *in vitro* experiments showed variable effects of PCSK9 on LDLRs. In some cells such as human hepatoma cells (Huh7 & HepG2) or human embryonic kidney cells (HEK293), PCSK9 significantly decreases the levels of LDLRs. However, this was not the case in other cells like fibroblasts, Chinese hamster ovarian (CHO-K1) or rat liver cells. This difference in terms of action between cell lines can be attributed to some missing factor essential for PCSK9 function [115].

In addition, the D374Y mutant of PCSK9 has been shown to increase the affinity of PCSK9 to the EGF-A domain of LDLR by 25-fold compared to WT PCSK9. This mutant form is 10-fold more active than the WT in decreasing the levels of LDLR [116].

- **Loss-of-function (LOF) mutations of PCSK9:**

Nonsense, missense and even in-frame deletion mutations have been found in *PCSK9* gene to cause hypocholesterolemia by increased clearance of LDL-C (Figure 10). Three LOF mutations in African-Americans (Y142X and C679X) and Caucasians (R46L) have been shown to afford a great protection against CHD. A 15-year prospective study done in these populations has shown that nonsense mutations of *PCSK9* not only reduced the LDL-C levels by 28%, but also decreased the frequency of CHD by 88%. In case of R46L allele mutation, the frequency of CHD was decreased by 50% as the average reduction of LDL-C levels was only 15% [130]. These observations indicate that LOF mutations in *PCSK9* reduce the risk of CHD better than using

statins in a short-term clinical trial [131]. In addition, Y142X mutation results in undetectable protein expression, whilst the C679X mutant is not secreted into the medium of human liver cells because of the misfolding and retaining in the ER. L253F mutation is known to impair the autocatalytic cleavage and thus the secretion of PCSK9. Mutations in the prodomain (Δ 97, G106R) and catalytic domain (L253F) also interfere with the autocatalytic cleavage of the protein [115], [116]. Finally, A443T mutant form was shown to be more susceptible to furin cleavage leading to LOF [132].

Interestingly, a novel LOF mutation, Q152H, was discovered in a white French-Canadian family that results in very low plasma PCSK9 concentration and subsequently, lower concentration of circulating LDL-C. The amino acid substitution at position 152 results in the impairment of autocatalytic cleavage of proPCSK9, hence limiting its processing and secretion from the ER [133]. Not only is this variant capable of protecting against cardiovascular diseases, but it can also protect against liver injuries caused by ER stress. This was very recently demonstrated by Lebeau *et al.* [134], who identified a cochaperone function for the Q152H variant retained in ER, which increased the abundance of ER chaperones, glucose-regulated proteins of 78 and 94 kDa (GRP78 and GRP94), hence resulting in protection against ER stress-induced liver injury.

4. Transcription Regulation of PCSK9

PCSK9 gene was identified as the third locus to be associated with autosomal dominant hypercholesterolemia (ADH). Together with *LDLR* and *ApoB*, this gene triad plays an essential role in cholesterol metabolism [108]. The promoters of *PCSK9* and *LDLR* genes contain a functional sterol regulatory element (SRE) that is targeted by transcription factors called sterol-responsive element binding proteins (SREBPs) in response to any change in the intracellular levels of cholesterol [135]. Three isoforms have been distinguished to be encoded by 2 genes: SREBP-1a and SREBP-1c are encoded by a single gene on chromosome 17p11.2 and are the result of alternative transcription start sites; and SREBP-2 is encoded by a gene on chromosome 22q13 [136]. SREBP-1a is a more potent activator than the rest of the family members especially for genes important for cholesterol synthesis, fatty acids and triglycerides. However, SREBP-1c preferably induces the transcription of genes related to fatty acids

synthesis but not cholesterol synthesis, unlike SREBP-2, which preferentially induces cholesterologenesis [136].

SREBP-2 is known for its important role in regulating the synthesis and absorption of cholesterol by targeting the gene expression of HMG-CoA (3-hydroxy 3-methylglutaryl-Coenzyme A) reductase or HMGCR, HMG-CoA synthase, farnesyl diphosphate synthase and squalene synthase [137]. On the other hand, SREBP-1c induces the expression of ATP citrate lyase (ACLY), which leads to the production of acetyl-CoA. Both *PCSK9* and *LDLR* are regulated by the second member of this family, SREBP-2, which is capable of increasing the expression of PCSK9 and LDLR when the intracellular levels of cholesterol are minimal. In this case, the cholesterol sensor and the SREBP cleavage-activating protein (SCAP) will enable the transport of SREBP-2 from the ER to the Golgi apparatus to become completely mature. It then enters into the nucleus where it interacts with SRE-1 in the promoter region of *PCSK9* and *LDLR* thus increasing their expression [137]. In humans and animals, during fasting, the expression of SREBP-2 is suppressed in response to low dietary cholesterol concentrations, resulting in decreased levels of PCSK9 and LDLR proteins which can be increased again directly after feeding [138], [139].

SREBP-1c expression in hepatic cells can be regulated by insulin, liver X-activated receptors (LXRs) and glucagon (Figure 12). For instance, the presence of insulin in rodent primary hepatocytes increases the mRNA expression level of PCSK9 by 4-5 folds due to the increase in SREBP-1c expression [140]. Insulin also activates the mammalian target of rapamycin complex 1 (mTORC1)/protein kinase δ pathway, which in turn inhibits hepatocyte nuclear factor 1 α (HNF1 α) – another transcription factor that regulates the transcription of *PCSK9*– thus decreasing PCSK9 expression in cells [141]. This is confirmed both *in vitro* and *in vivo* under hyperinsulinemia condition in HepG2 cells and non-diabetic post-menopausal obese women [142]. Surprisingly, these observations were not the same in healthy men exposed to 24-hour hyperinsulinemia or even in type 2-diabetic patients; the expression and plasma PCSK9 levels were not altered [143].

As stated above, HNF1 α is a transcription factor that regulates the basal expression of *PCSK9*. It binds to HNF1 response site, an essential regulatory element for transcription, found in the promoter region of *PCSK9*. Hai Li *et al.* [144] showed that HNF1 α is involved in SREBP-2 induced maximal expression of *PCSK9* in conditions of cholesterol depletion in HepG2 cells. A

cooperative activity between HNF1 regulatory site and SRE was also discovered based on site-directed mutagenesis studies, where mutations in HNF1 site decreased the promoter activity of *PCSK9* by >90% as well as attenuated the activity of SREBP2 to transactivate *PCSK9* promoter. The abundant expression of PCSK9 in the liver is possibly because of the richness of HNF1 α in the liver tissue.

Another transcription factor that regulates the expression of *PCSK9* is the peroxisome proliferator-activated receptor α (PPAR α). It reduces the promoter activity thus decreasing the expression of *PCSK9*. At the same time, PPAR α enhances the expression of furin/PC5/6 which in turn leads to higher cleavage of PCSK9 [145]. However, phosphorylated PPAR γ , another member of the same family, increases the expression of PCSK9 in hepatic cells by a mechanism involving the inactivation of MAP kinase, thus increasing the level of processing of SREBP-2 [146].

Alternatively, other factors or transcription factors can also modulate the expression of PCSK9, such as LXR increases the expression of PCSK9 [140] while FXR (farnesoid X receptor) reduces its expression [147]. Moreover, histone deacetylases, sirtuins 1 and 6 (SIRT1/6), suppress the gene expression of PCSK9 leading to less secretion, higher LDLR expression and hence regulating LDL-C homeostasis [135] (Figure 12).

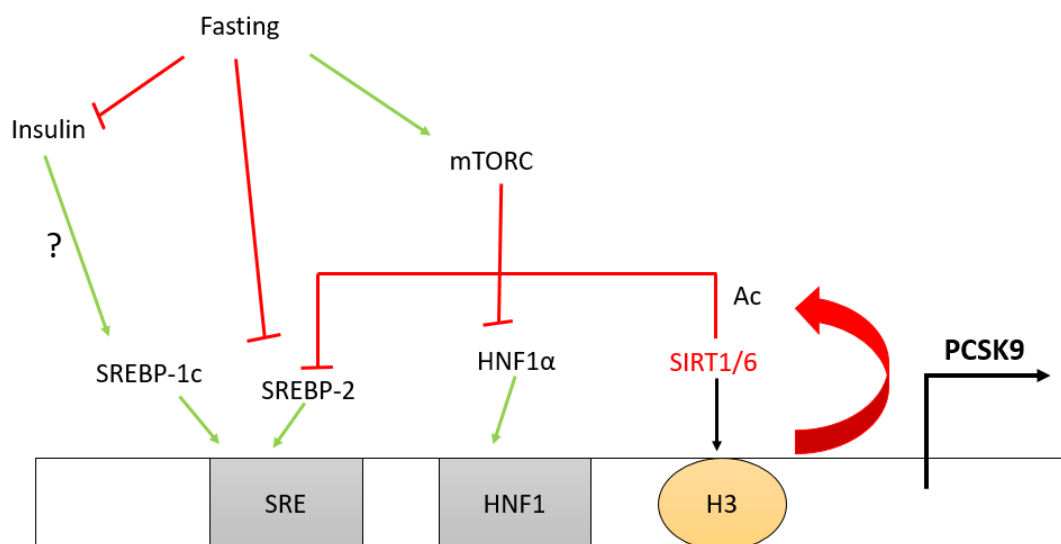


Figure 12: Multifactorial regulation of PCSK9 transcription.

Fasting has different consequences on activation and inhibition of factors and cofactors that modulate PCSK9 expression.

Pathways that increase PCSK9 expression are indicated in green and those that repress it are indicated in red. SRE is activated by SREBP-1c and SREBP-2, which in turn coactivate HNF1. The histone H3 will be deacetylated by SIRT1-6 thus blocking the expression. Ac: acetyl; H3: histone 3

5. Effect of Drugs on PCSK9 Expression

Because *PCSK9* gene expression can be regulated by many factors and transcription factors, this makes it easier to target it with many drugs that can affect its expression directly or indirectly.

- **Statins**

Statins, the famous HMGCR inhibitors, are the most commonly prescribed drugs for patients having high levels of LDL-C in blood. However, some limitations have been addressed to the use of statins such as the absence of linear dose-dependent decrease of LDL-C levels and most importantly resistance to statins due to long periods of therapy. Basically, statins tend to decrease the intracellular levels of cholesterol by inhibiting the rate limiting enzyme in the cholesterol biosynthesis pathway, HMGCR. This results in depletion of intracellular cholesterol and increase in translocation of SREBP-2 from the ER to the nucleus, which in turn binds to the SRE region and activates the expression of *LDLR* and *PCSK9* genes. So, both LDLR and circulating PCSK9 are elevated. The latter can bind to LDLR and directs it towards lysosomal degradation instead of recycling, thereby limiting the efficacy of statins to reduce the LDL-C levels in blood [148]. Recently, statins were shown to increase the levels of HNF1 α in hepatocytes resulting in increased expression of PCSK9 to a greater extent than LDLR [149]. Many statins can be found in the market such as atorvastatin, simvastatin, rosuvastatin and others. Several studies were done to check their effects on the expression of *PCSK9*. Atorvastatin was reported to increase the plasma levels of PCSK9 by various percentages and at different doses and times. A treatment of 10 mg atorvastatin for 6 weeks results in 7.4-14% increase in PCSK9 [150], [151]. A 35% increase in PCSK9 was observed with 20 mg atorvastatin administered for 8 weeks [152], while the highest percentage recorded was 47% increase with 80 mg of atorvastatin [153]. These results clearly suggest a dose-dependent effect of the drug on PCSK9 levels, where at higher doses of atorvastatin, there were higher concentrations of circulating PCSK9. Other statins have also been evaluated like simvastatin and rosuvastatin. 10 mg of simvastatin for 6 weeks didn't affect the levels of PCSK9 [154] while 40 mg for 14 weeks increased the concentration by 68% [155]. Moreover, 20 mg of rosuvastatin taken for 1 year by both sexes resulted in an increase in PCSK9 levels by 35% in females and 28% in males [156]. Taken together, all of these data reveal that each statin induces an increase in

the plasma levels of PCSK9 that can be dependent on the dose, duration and characteristic of the statin itself (lipophilic or hydrophilic nature) [148].

- **Ezetimibe:**

Ezetimibe, a cholesterol absorption inhibitor, is another LDL-C lowering drug. It can bind to the cholesterol transport protein Niemann-Pick C1-like 1 (NPC1L1) in order to inhibit the absorption of cholesterol by the liver, leading to an increase in the expression of LDLR and thus a higher uptake of LDL-C from blood. Some studies have shown that ezetimibe in combination with statins significantly induces higher plasma levels of PCSK9 as compared to the statins alone [157], [158]. On the contrary, other studies have shown no effect of ezetimibe on the plasma levels of PCSK9, whether taken alone or in combination with statins [154], [155], [159].

- **Fibrates:**

Fibrates, such as fenofibrate, are activators of PPAR α used in clinical practice due to their hypotriglyceridemic effects. They can reduce the triglyceride levels by around 50% and increase the high-density lipoproteins (HDLs) by around 15% [148]. They were shown to exert a dual mechanism of action on PCSK9 expression at the mRNA and proteins levels by activation of PPAR α and thus diminishing the mRNA levels and increasing the synthesis of furin and PC5/6 that can cleave the mature PCSK9 protein into a less active form [145]. Another study showed 13% decrease with fenofibrate in the serum levels of PCSK9 in type 2 diabetes patients who were taking statins [160]. However, there are some controversies about fibrates, which were shown in a short-term study to increase PCSK9 expression when compared to a placebo treatment [161]. Therefore, the effect of fibrates on the levels of PCSK9 is not yet clearly understood. Additional studies are required to elucidate the different effects induced by fibrates.

- **Nicotinic acid (Niacin):**

Niacin has been used in clinics for more than 50 years as an LDL-C lowering drug. A study done by Khera *et al.* revealed that the administration of niacin with simvastatin (20 mg) decreases the PCSK9 levels by 13%. Similar reduction was observed with combination treatments with atorvastatin and fenofibrate [162].

- **Glitazones**

Glitazones are a class of drugs that target insulin resistance, a characteristic feature of type 2 diabetes mellitus. They can activate extracellular-regulated kinases (ERK1/2) resulting in the phosphorylation of PPAR γ and thus reducing its activity. As it is known, PPAR γ induces the PCSK9 mRNA and protein productions, therefore, glitazones block the secretion of PCSK9 from hepatocytes [146].

- **Rapamycin:**

It is an antifungal metabolite that possesses immunosuppressive and anti-proliferative properties. It is an allosteric inhibitor of mTORC1 that blocks its activation resulting in increased activity of HNF1 α and eventually expression of PCSK9 [141].

- **Berberine:**

Berberine (BBR) is a natural cholesterol lowering drug that upregulates the expression of LDLR by a post-transcriptional mechanism requiring mRNA stabilization. It can transcriptionally inhibit PCSK9 expression in hepatocytes by inhibiting HNF1 α [144]. Moreover, BBR inhibited dyslipidemia in mice with lipopolysaccharide (LPS)-induced inflammation by decreasing the expression of PCSK9 on the one hand, and increasing LDLR expression on the other [163].

- **Fumonisin B1:**

Fumonisin B1 (FB1) is a toxic fungal metabolite commonly detected in agricultural commodities that are intended for human and animal consumption. The metabolic toxicity of FB1 results in the inhibition of ceramide synthase thus leading to the accumulation of sphingosine and its derivatives and reducing ceramide levels. The exposure to FB1 results in hypercholesterolemic effect as a result of a change in the influx and efflux of cholesterol in HepG2 cells. In these cells, the exposure to FB1 reduced the cell viability and metabolic activity without any change in the membrane integrity. Moreover, FB1 directly manipulated the influx/efflux of cholesterol by decreasing LDLR levels, indicating an inhibition in lipid uptake, whilst increasing the expression of ATP-binding cassette transporter (ABCA1), thus promoting cholesterol efflux. This observed change in cholesterol influx/efflux profile was due to the increased expression of PCSK9 and its transcriptional activator, liver X receptor (LXR) [164].

6. Effects of PCSK9 Distinct from Hepatic LDLR Regulation

The main effect exerted by PCSK9 is on the expression of LDLR in hepatocytes, thus regulating directly LDL-C concentrations in plasma and indirectly that of oxidized LDL (oxLDL) [165]. However, PCSK9 regulatory mechanism can have consequences on extra-hepatic tissues in which it is expressed, like kidney and heart, which usually respond to LDL-C and oxLDL. In such tissues, the cholesterol concentration is dependent on secretion rather than uptake by LDLR, LDLR-related protein 1 (LRP-1=CD91) and lectin-like oxidized LDL receptor 1 (LOX-1). The endogenous expression level of these receptors is controlled by PCSK9 along with their ligands [166]. Moreover, PCSK9 can target other members of LDLRs, like very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) independently of the presence of LDLR [167]. PCSK9 can also interact with cluster of differentiation 36 (CD36) to reduce the uptake of free fatty acids and triglycerides; it can also affect the degradation of cholesterol transporter NPC1L. In addition, CD81 (HCV receptor) levels are also regulated by PCSK9 [110].

- **PCSK9 in diabetes and inflammation:**

PCSK9 is necessary for the normal function of the pancreas because it is expressed by insulin-producing pancreatic islets β -cells along with LDLR. Mice lacking PCSK9 were hypoinsulinemic, hyperglycemic and glucose-intolerant compared to the control. The islets β -cells in these mice showed signs of apoptosis and inflammation due to cholesterol accumulation [168]. Moreover, another study has revealed the role played by insulin in regulating PCSK9 *in vitro* and *in vivo* [169]. Insulin increased the expression of PCSK9 and in turn the degradation of LDLR. PCSK9 levels were decreased markedly (up to 80%) in mice knockout for insulin receptor, or treated with streptozotocin or antisense oligonucleotides to inactivate their insulin receptors. These observations can be explained by an additional mechanism involving glucagon to influence PCSK9 levels. Glucagon decreased the mRNA and protein levels of PCSK9 by 50% in primary rat hepatocytes, along with decreasing the transcription of SREBP-1c and SREBP-2 by 20-50%. In addition, LDLR levels were also diminished by 20% at the mRNA levels, whilst their protein level was increased by 2-folds.

Another pathway that regulates PCSK9 levels was identified by Ai *et al.* [141]. During early stages of diabetes and type 2 diabetes, high LDL turnover is present and is dependent on hyperinsulinemia. In this study, the authors observed that obese mice suffering from

hyperinsulinemia had 60% lower mRNA levels of PCSK9 and increased LDLR. This was due to the fact that insulin activates mTORC1/PKC δ , which then inactivates HNF1 α and blocks PCSK9 transcription. Hence, the mTORC1 pathway herein links diabetes to lipid metabolism.

PCSK9 is expressed in smaller amounts in the kidney, adrenals, small intestine, brain and atherosclerotic plaque foam cells. Atherosclerosis is an inflammatory disease that involves many cytokines. The stimulation of THP-1 cells (human leukemic cell line 1) by oxLDL to macrophages leads to the synthesis of high amounts of IL-1 α , IL-6, TNF- α and PCSK9. The process is regulated by an important transcription factor: NF κ B (nuclear factor kappa-light chain-enhancer of activated B cells). OxLDL stimulation leads to the degradation of I κ B α , an inhibitor of NF κ B, resulting in the translocation of NF κ B to the nucleus and transcription activation. The inhibition of PCSK9 by small interfering RNA (siRNA) resulted in anti-inflammatory effects and blocked NF κ B [170]. Another study investigated the action of TNF- α on JAK/STAT (Janus kinase/ signal transducer and activator of transcription proteins) pathway. The high expression of the negative regulator of this pathway, called suppressor of cytokine signaling 3 (SOCS3), inhibits STAT3 phosphorylation and hence increased PCSK9 expression [171]. Moreover, Giunzoni *et al.* [172] showed that PCSK9 mediated inflammation in atherosclerosis was due to high recruitment of inflammatory monocytes and their differentiation to macrophages. This pro-inflammatory action of PCSK9 was dependent on the presence of LDLR. LPS-induced differentiation of monocytes increased the expression of pro-inflammatory cytokines (IL-1 β and TNF- α) and inhibited the inflammatory markers (Arg1 & IL-10).

- **PCSK9 in apoptosis:**

Apoptosis is a genetically regulated form of cell death that involves many molecular mechanisms. The ability of a cell to undergo mitochondrial apoptosis is governed by the ratio of pro-apoptotic proteins to anti-apoptotic ones: Bax/Bcl-2. Bax proteins are known for their pro-apoptotic effect by perforating the outer mitochondrial membrane and increasing its permeability, while Bcl-2 is a family of anti-apoptotic proteins that inhibit apoptosis [173]. A strong correlation between PCSK9 overexpression and increased oxLDL induced-apoptosis in vascular endothelial cells was observed [110]. This was further confirmed by inhibiting PCSK9 using siRNA which led to the reduction of Bcl-2/Bax ratio and inhibition of caspase 9 and 3, hence decreased apoptosis [174].

In neurons, PCSK9 was also found to regulate the neuronal apoptosis through JNK (c-Jun N-terminal kinase) pathway by adjusting ApoER2 levels and signaling [175]. The inhibition of PCSK9 showed anti-apoptotic effect on cerebellar granule cells (CGN) due to the reduction in nuclear phosphorylated, active, c-Jun and activated caspase 3. This was also accompanied with higher ApoER2 protein levels. The knockdown of ApoER2 reversed the action of PCSK9 inhibition, suggesting that PCSK9-mediated degradation of ApoER2 serves as a factor in JNK pathway and PI3K and ERK1/2 activity.

In another study done on brain glioma cells U251, the opposite action of PCSK9 was found. siRNA inhibition of PCSK9 in U251 cells promoted apoptosis through caspase 3 activation, decrease of anti-apoptotic proteins: XIAP (X-linked inhibitor of apoptosis protein) and pAKT, and increase in the ratio of Bax/Bcl-2 and cytochrome c distribution to the cytosol, whilst the overexpression of PCSK9 inhibited the apoptosis and decreased Bax/Bcl-2 ratio [176].

In cases of liver metastasis of melanoma cells (B16F1), mice lacking *PCSK9* gene showed lower levels of metastasis when on chow diet. This was directly reversed when the diet was switched to high-fat one. The lack of PCSK9 is associated with higher apoptosis in hepatocytes due to increased expression of TNF- α and its receptor (TNFR1) and decrease in Bcl-2 [177].

In HCC, the high expression of PCSK9 not only correlates with poor prognosis of patients, but it also promotes the proliferation of cells *in vitro* as well as HCC progression *in vivo*. This was due to the inhibition of apoptosis of HCC cells exerted by PCSK9 *via* the FASN/Bax/Bcl-2/Caspase9/Caspase3 pathway [178].

7. PCSK9 in cancer

The expression of PCSK9 is deregulated between the different types of cancers. In HCC tissue samples, immunohistochemical staining revealed that PCSK9 expression was downregulated along with an upregulation of the expression of LDLR as compared to adjacent cirrhotic tissues. This observation was confirmed using flash-frozen HCC samples where the mRNA level of PCSK9 was lower while that of LDLR was higher when compared to adjacent liver tissue as well as normal control tissue. However, the serum levels of PCSK9 in HCC patients were higher than in patients with chronic liver disease without HCC. Taken together, these data indicate that the microenvironment in HCC is modulated so as to have a constant energy supply in order to

fuel tumor growth [179]. In another study using different HCC cell lines (HepG2 & LM3), PCSK9 expression was enhanced using acRoots (*Actinidia chinensis* Planch root extract) resulting in decreased LDLR expression, inhibition of LDL uptake by LM3 cells, decrease in the intracellular cholesterol levels and thus diminished proliferation. These effects were confirmed using two approaches: PCSK9 overexpression and PCSK9 knockdown [180].

In prostate cancer (PCa), the inhibition of PCSK9 using siRNAs protected the PCa cells from ionizing radiation (IR)-induced cell damage. The IR-exposed PCa cells showed a decrease in cell viability along with an increase in apoptosis, which can be reflected by the increase in cytochrome c, caspase-3 and Bax/Bcl-2 ratio. All of these were reversed when the PCa cells were treated with siRNA against PCSK9 before IR, indicating that PCSK9 is capable of affecting the mitochondrial membrane stability and that PCSK9 siRNA can induce radio-resistance through mitochondrial signaling pathways [181].

In human lung adenocarcinoma cells (A549), PCSK9 was shown to regulate apoptosis through ER stress (ERS) and mitochondrial signaling pathways [182]. The use of PCSK9 siRNA resulted in an anti-tumor activity by the induction of apoptosis. This was possibly due to the activation of caspase-3 and downregulation of the anti-apoptotic proteins survivin and XIAP. In addition, an increase in Bax/Bcl-2 ratio was also recorded, leading to the release of cytochrome c after PCSK9 siRNA transfection. The ERS was also increased as a result of an increase in the levels of glucose related-proteins (GRP78 & GRP94), p-PERK (phosphorylated protein kinase R-like ER kinase) and p-eIF2 α (phosphorylated eukaryotic initiation factor 2 α), leading to cell death in A549 cells.

The inhibition of PCSK9 in melanoma, colon and breast murine cancer cells synergistically boosted the tumor response to murine anti-PD1 immune checkpoint inhibitor resulting in suppression of tumor growth. Moreover, evolocumab treatment, alone or in combination with anti-PD1 treatment, also inhibited the tumor growth of cells resistant to immune checkpoint therapy. The depletion of PCSK9 in tumors increased the intratumoral infiltration of lymphocytes, such as CD8⁺ cytotoxic T-cells (CTLs), CD4⁺ T helper (T_h) cells, $\gamma\delta$ T cells and natural killer (NK) cells, thus rendering the tumor responsive to immune checkpoint therapy. The molecular mechanism by which CTL killed PCSK9-deficient tumor cells involved an increase in the tumor cell surface MHC I (Major Histocompatibility Complex I) expression in PCSK9-deficient tumors as compared to control, indicating that PCSK9 downregulates MHC I

surface levels in a manner very similar to the downregulation of LDLR by lysosomal degradation [183].

Similar role of PCSK9 has also been observed in other types of cancers like human neuroglioma, breast cancer, colorectal cancer and others. And this poses a major question about the therapeutic strategies that could be adopted to target PCSK9 in these cancers.

8. Targeting PCSK9 using multiple approaches

The important role of PCSK9 in lipid homeostasis brings to the fore an attractive target for many diseases such as FH, cardiovascular disease and especially cancer. Several approaches can be used to target and inhibit PCSK9 including: (a) blocking the binding of PCSK9 to LDLR using monoclonal antibodies (mAbs), anti-PCSK9 vaccines, adnectins, mimetic peptides or novel molecules (Figure 13); (b) inhibiting the expression of PCSK9 using CRISPR/Cas9 genome-editing tool, antisense oligonucleotides (ASOs), siRNA or recently discovered pharmaceutical drugs (R-IMPP or PF-06446846, etc.); (c) interfering with the secretion of PCSK9 from the ER by sortilin or Sec24a (Figure 14).

a) Blocking PCSK9 binding to LDLR

This is the first approach used to inhibit PCSK9 (Figure 13). Within it lay multiple strategies:

- **PCSK9 monoclonal antibodies (mAbs)**

This strategy uses humanized mAbs that can recognize and bind the catalytic domain of PCSK9, thereby blocking its interaction with the EGF-A domain of LDLR. Due to that, PCSK9 fails to bind LDLR and the latter can escape the degradation [184]. This approach has been extensively studied in many clinical trials (Phase I to III), alone or in combination with other lipid lowering drugs like statins [185]. Two fully humanized anti-PCSK9 mAbs are currently approved for the treatment of hypercholesterolemia: (i) evolocumab (AMG145, trade name Rephata) developed by Amgen (Amgen, Thousand Oaks, CA, USA) was approved by the European Commission in July 2015 and FDA one month later; and (ii) alirocumab (SAR236553/REGN727, trade name Praluent) developed by Regeneron and Sanofi Pharmaceuticals (Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA; Sanofi, Paris, France) was also approved by Endocrinology and Metabolic Drugs Advisory Committee (EMDAC) of the US FDA in July 2015.

These drugs have shown a marked reduction of LDL-C levels in dyslipidemic patients. Several trials and meta-analyses showed around 50-60% reduction in LDL-C levels using these mAbs in patients with a diet-based therapy, whether used alone or in combination with different doses of statins, without any serious side effect [185]–[187].

Evolocumab was clinically evaluated in combination with statin treatment in 27,564 high-risk patients with atherosclerotic cardiovascular disease (FOURIER study; ClinicalTrials.gov number: NCY01764633). In this clinical trial, the levels of LDL-C were decreased by 59% using the combination treatment along with 15% decrease in the cardiovascular events (cardiovascular death, strokes, myocardial infarction, hospitalization for unstable angina or coronary revascularization) after a 2.2-year follow-up [188]. On the other hand, alirocumab was also evaluated in clinical trials in 18,924 patients who had acute coronary syndrome and high LDL-C levels despite of receiving high doses of statins (ODYSSEY OUTCOMES; ClinicalTrials.gov number: NCT01663402). LDL-C levels were decreased by 61% compared to placebo after one year although around 90% of patients were taking high doses of statin. Moreover, the adverse cardiovascular events were also reduced by 15%, including CHD death, non-fatal myocardial infarction, non-fatal ischemic stroke and hospitalization for unstable angina [189]. Taken together, both of these studies show the effectiveness of PCSK9 mAbs in high-risk patients with LDL-C > 70 mg/dL despite the use of high doses of statin.

Aside from these promising results, there are still some limitations for the use of mAbs. First of all, they need to be taken through a subcutaneous injection 1-2 times per month. Their price is quite high estimating that they cost 5000-7000\$ per person per year in Europe. It can be noted that the dose frequency of these injections is low compared to other types of injections like insulin. Nonetheless, improving the cost effectiveness of these mAbs is necessary to provide better treatment options for a wide range of population [124].

- **Anti-PCSK9 vaccine:**

Due to the many limitations associated with mAbs, active vaccination comes in hand to provide better efficiency and lower cost therapeutic approach. Nanoliposomal anti-PCSK9 (L-vaccine (L-IFPTA⁺: Liposomal Immunogenic Fused PCSK9-Tetanus plus Alum adjuvant) has been recently developed and resulted in an efficient and safe induction of long-lasting PCSK9-specific antibodies in vaccinated BALB/c mice. The vaccine stimulated Abs disrupted the

function of PCSK9 by blocking its interaction with LDLR [190], [191]. It also protected against hypercholesterolemia and atherosclerosis in C57BL/6 mice [192], [193]. Moreover, L-IFPTA⁺ vaccine was shown to be immunogenic and safe in healthy non-human primates in a pre-clinical study suggesting that it could be eligible for a phase I clinical trial in humans [194]. With respect to cancer, mice bearing breast cancer that were vaccinated with L-IFPTA⁺ vaccine showed a moderate, but not significant, decrease in tumor growth (21.2%) and a prolonged lifespan by 4.2%. However, this reveals that inhibition of PCSK9 by vaccination had no side effects on breast tumor endpoint and that it could improve breast cancer behavior [195]. On the other hand, vaccination with L-IFPTA⁺ vaccine in mice bearing melanoma did not show any effect on tumor growth nor survival of tumor-bearing mice [196]. Taken altogether, vaccination seems to be an interesting strategy to inhibit PCSK9, however, more studies and clinical research need to be done in order to investigate the efficacy of the vaccine in humans and to evaluate whether it is indeed effective in inhibiting tumor growth in other cancers.

- **Adnectin:**

Adnectins are small (~12 kDa) synthetic proteins based on the 10th type III domain of human fibronectin, whose variable loops can be designed to bind therapeutic targets with high affinity and specificity. They possess a β -sheet fold structure with diversified loops similar to Ab variable domains, however they differ from Abs in primary sequence and have a single domain structure lacking disulfide bonds [197]. Adnectin, BMS-962476 by Bristol-Myers Squibb/Adnexus, is a PCSK9-binding polypeptide engineered with polyethylene glycol to enhance pharmacokinetics and bind with a subnanomolar affinity to human PCSK9 [198]. Adnectin impedes the interaction between PCSK9 and EGF-A domain of LDLR thus preventing its degradation, similarly to mAbs. The production of adnectin is less expensive and easier than mAbs, since it can be produced using bacterial expression systems thereby serving as a potential alternative to mAbs. In a preclinical trial done in cynomolgus monkey, BMS-962476 (5 mg/kg) decreased the free PCSK9 levels by >99% and LDL-C by ~55% within 48 hours, followed by a 6-fold increase in total PCSK9 [198]. In humans, the single ascending subcutaneous (SC) or intravenous (IV) doses of BMS-962476 resulted in >90% decrease of free PCSK9 along with around 48% decrease in LDL-C levels at maximal dose [199], without any serious adverse event.

Taken together, these observations suggest that BMS-962476 is a promising substituent drug for mAbs that can target circulating PCSK9 and significantly decreases LDL-C levels. Nevertheless, its effects should be followed on a larger scale and over long periods of time to make sure of its efficacy and safety.

- **Mimetic peptides:**

They are small amino acid (aa) sequences designed to biologically look like the peptidic structure of a target protein. Peptides mimicking the EGF-A or EGF-AB binding domains of LDLR were designed as competitive inhibitors of PCSK9 that bind to the catalytic domain of PCSK9 thus preventing its interaction with LDLR. They can serve as therapeutic alternatives of small molecules and large Abs [200]. For instance, a truncated 26-aa EGF-A analog was recently synthesized serving as an inhibitor for PCSK9-LDLR binding. This peptide exhibited a high binding affinity for PCSK9 leading to increased recycling of LDLR and thus lowering LDL-C levels [201]. Moreover, another small peptide that mimics the secondary structural elements of EGF-A domain called Pep2-8 was discovered to attenuate the interaction between PCSK9 and LDLR, leading to the full restoration of LDLR surface levels and uptake of LDL-C by HepG2 cells treated with PCSK9 [202].

Annexin A2 (AnxA2) is a natural extrahepatic inhibitor that binds to the C-terminal domain of PCSK9 preventing its interaction with LDLR and hence PCSK9-mediated degradation. It is abundant in lung, pancreas, colon and adrenal glands, while lower levels are found in liver, kidney and spleen. AnxA2 knockout mice show ~2-fold increase in the circulating PCSK9 and ~1.4-fold increase in LDL-C levels, along with ~50% decrease in LDLR protein levels in extrahepatic tissues. However, overexpression of AnxA2 in liver leads to an increase of LDLR proteins [203]. Thus, the inhibitory role of AnxA2 in PCSK9 modulates the degradation of LDLR, and, for that reason, creating small molecules that mimic AnxA2 can be a good approach for PCSK9 inhibition.

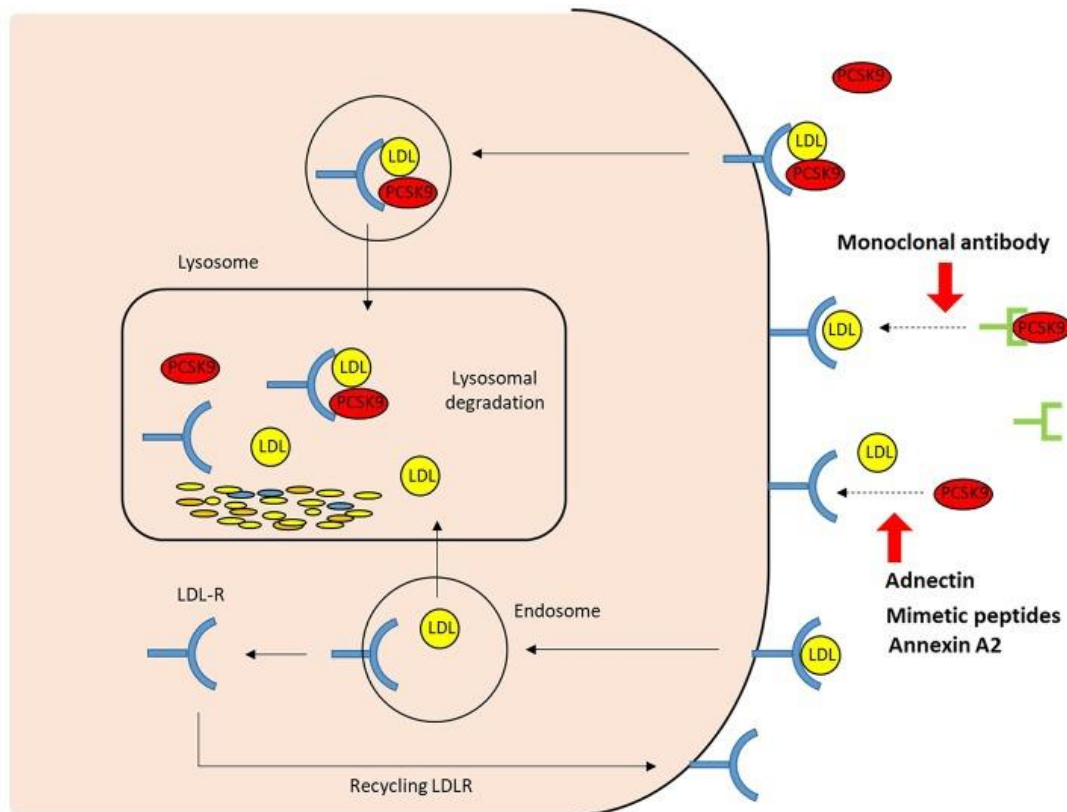


Figure 13: The inhibition of PCSK9 binding to LDLR using mAbs or mimicking peptides [124]

- **Pseurotin A**

Pseurotin A (PS) is a unique spiro-heterocyclic γ -lactam alkaloid isolated from the fungus *Aspergillus fumigatus*. It has an anti-inflammatory role, anti-seizure activity, and was shown to be a potential treatment and preventive for osteoporosis. Interestingly, in a very recent study, PS was shown to inhibit the expression and secretion of PCSK9 in hepatic cancer cells, HepG2 and Huh7, respectively. The mechanism by which PS exerts its function is through binding to the catalytic domain of PCSK9 (which usually accommodates the EGF-A domain of LDLR) thus inhibiting its interaction with LDLR. Moreover, PS also showed an anti-proliferative effect in breast cancer cell lines (BT-474 and T47D) and resulted in reducing PCSK9 levels while increasing LDLR levels in a dose-dependent manner. Similarly, *in vivo* experiments confirmed such observations, where PS treatment in orthotopic nude mice bearing BT-474 tumor cells xenograft model fed a high fat diet (HFD) resulted in more than 59% reduction in tumor growth, reduced the expression level of PCSK9, and decreased the level of circulating cholesterol as compared to vehicle control treatments. Finally, PS inhibited the locoregional

tumor recurrence by decreasing PCSK9 levels [204]. This interesting study has brought PS, a fungal metabolite, to the spotlight as a novel small molecule that targets PCSK9 and prevents tumor growth *in vitro* and *in vivo*. However, more research is needed to better understand the mode of action of PS in many cancer models.

- **Silibinin A**

Due to the high cost of FDA approved monoclonal antibodies targeting PCSK9, the development of low cost alternative is a necessity. Traditional Chinese Medicine (TCM) is the answer to low-cost effective treatments that has been in clinical practice for so long. Silibinin (SIL) is a polyphenolic compound that belongs to the flavonoids and represents the main bioactive ingredient of silymarin (silybin), a standard extract of milk thistle seeds [205]. It has been clinically used to treat liver diseases such as acute and chronic hepatitis, early cirrhosis and poisonous liver injury [206]. SIL was discovered to effectively decrease the promoter activity of *PCSK9* as well as the mRNA and protein levels in a dose/time-dependent manner in HepG2 cells. Moreover, statin-induced increase of PCSK9 expression was attenuated when SIL was co-incubated with atorvastatin in HepG2 cells. This effect was mediated by suppressing p38 mitogen-activated protein kinases (MAPK) pathway, thus enhancing the lipid-lowering activity of statin [207]. However, further studies are needed to confirm this additive effect of SIL along with statin especially *in vivo*.

- b) Inhibition of PCSK9 expression**

Different approaches can be used in this strategy including genomic and pharmaceutical ones (Figure 14):

- **CRISPR/Cas9 genome-editing tool**

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Protein 9 (Cas9) is a very useful genome-editing tool that is used to destroy or edit a target gene. It is an accurate, fast and cheap editing system. It consists of Cas9 endonuclease and a single guide RNA (sgRNA) that has, at its 5' end, a 20-nucleotide sequence capable of recognizing and binding with a complementary sequence on the target DNA. Cas9 will induce a DNA cleavage generating double strand break and triggering DNA repair machinery by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ is error-prone resulting in disruptive insertions and /or deletions at the target site, while HDR can use an

exogenous DNA repair template to induce a knock-in of the desired alteration in the genome [208]. Several studies have been successfully able to abrogate the *PCSK9* gene using this tool. As a result, a significant reduction in plasma PCSK9 levels was observed with an increase in hepatic LDLR levels. The plasma LDL-C levels were decreased by 35-40% [209]–[211].

This means that a tool like CRISPR/Cas9 can be used to permanently alter the human genome in order to prevent for instance CHD in hypercholesterolemic patients. However, many ethical, legal and safety issues need first to be resolved before integrating it as a therapeutic approach in humans [124] since it's associated with several limitations like the off-target mutations.

- **Antisense oligonucleotides (ASOs)**

These are short, single-stranded oligonucleotides 12-25 base pair long that bind directly to a target mRNA by Watson-Crick base pairing, in order to block its translation in cytosol and nucleus. The degradation of mRNA by these ASOs depends on the activity of the RNase H enzyme, which recognizes RNA-DNA heteroduplexes [212]. 2'-O-methoxyethyl RNA (ISIS 394814/BMS 844421 by Bristol Myers Squibb/ISIS Pharmaceutical) is a second-generation ASO that targets PCSK9 mRNA evaluated in preclinical studies. High doses of this ASO lead to a decrease in PCSK9 mRNA levels by 92% and an increase in LDLR protein levels by 2-fold, resulting in 38% reduction of LDL-C levels *in vivo* [213]. However, the development of this family of ASOs was stopped due to their low binding affinity. Later, a new generation of shorter ASOs (SPC4061/SPC5001 by Santaris Pharma A/S) was developed having better stability, stronger affinity and specificity to PCSK9 mRNA. These ASOs have proven to be effective 24 hours post-injection with 60% reduction in PCSK9 levels, persisting up to 16 days after treatment [214]. In another study, the subcutaneous injection of SPC5001 decreased PCSK9 and LDL-C levels by 50% and 25% respectively and dose-dependently, while decreasing also ApoB and increasing ApoA1 levels [215]. As promising as they seem, they caused acute kidney injury in one patient which led to termination of clinical development [216].

It is important not to neglect the potential therapeutic benefit of ASOs, which on the other hand need a lot of development to reduce their adverse effects, by making them accurately match the target mRNA and be delivered specifically to the desired cells to minimize off-target toxicity.

- **siRNA:**

Small interfering RNA (siRNA) is a synthetic RNA duplex that targets mRNA for degradation or translation silencing, thereby inducing a gene knockdown. Frank-Kamenetsky *et al.* [217] have developed siRNAs in lipidoid nanoparticles that can target PCSK9 from several species (murine, rat, nonhuman primate (NHP) and human), and checked their efficacy *in vivo*. They found 50-70% decrease in PCSK9 mRNA levels in mice and rats. This was accompanied by a 60% decrease in LDL-C levels. Furthermore, transgenic mice expressing human PCSK9 also showed >70% decrease in PCSK9 transcript and plasma levels upon siRNA administration. In NHP, similar observations were seen and the effects lasted for 3 weeks after a single intravenous injection.

Inclisiran (Leqivo®; Novartis) is the first-in-class chemically synthesized siRNA conjugated to N-acetylgalactosamine carbohydrate (GalNAc) designed to decrease cholesterol levels [218]. In December 2020, Europe gave Inclisiran its first approval for the use in adults suffering from heterozygous FH or non-familial, and mixed dyslipidaemia [219], [220]. Inclisiran targets PCSK9 mRNA and because it is conjugated to GalNAc, this will allow the precise and targeted uptake of the drug by hepatocytes. It is used in a combination treatment with statin or other lipid-lowering drugs in the case of statin-intolerant patients. The mode of administration of inclisiran is *via* a subcutaneous injection, which delivers an equivalent dose of 284 mg in 1.5 mL solution. It is recommended to get a single shot on day 1, day 90 and every 6 months [219]. The efficacy in a long-term reduction in the plasma PCSK9 levels, and hence reduction in LDL-C, is confirmed by all three phases III ORION studies (ORION-9, ORION-10 and ORION-11) in patients with heterozygous FH [221].

It is worth mentioning that a New Drug Application for inclisiran in patients with FH and atherosclerotic cardiovascular disease (ASCVD) was presented in December 2019 to the US FAD, however, a delay has occurred due to coronavirus disease-2019 (COVID-19)-related travel restrictions [222], [223].

- **Pharmaceutical drugs:**

Some studies have checked the capacity of small pharmaceutical molecules in inhibiting PCSK9 expression through a mechanism involving the stalling of human 80S ribosomal subunit during translation. Two molecules have been discovered, R-IMPP and PF-06446846, which suggest the therapeutic potential behind the use of selective inhibitors of mRNA translation.

R-IMPP ((R)-*N*-(isoquinolin-1-yl)-3-(4-methoxyphenyl)-*N*-(piperidin-3-yl) propanamide) is a small-molecule compound identified using phenotypic screening to inhibit PCSK9 secretion [224]. It is an anti-secretagogue of PCSK9 by Huh7 cells that endogenously express PCSK9, leading to higher LDLR surface levels and increased uptake of LDL. Since it didn't affect the secretion of transferrin by Huh7 cells, this suggests that it doesn't exert its action *via* secretion inhibition. However, the specificity by which R-IMPP blocks PCSK9 protein synthesis is by binding to the human 80S ribosomal subunit and a sequence at the N-terminus (signal peptide and a portion of prodomain) of PCSK9 mRNA, resulting in translation inhibition of PCSK9 mRNA. Yet, R-IMPP can also have some off-target effects on other enzymes, which may or may not play a role in modulating PCSK9 levels. The discovery of this molecule provides a new approach to target the synthesis of PCSK9 in the context of translating ribosomes.

PF-06446846 (by Pfizer) is the first example of an orally-active small molecule that is capable of reducing the synthesis of PCSK9, hence "inhibiting its function" [225]. It was capable of reducing plasma PCSK9 and total cholesterol levels *in vivo* without any sign of toxicity in the liver of rats. But this doesn't exclude the fact that the translation of very small number of transcripts was affected by PF-06446846 leading to some adverse effects outside the liver at elevated doses. The mechanism of action of PF-06446846 is based on ribosome stalling on PCSK9 transcript a few codons beyond to the end of signal peptide, leading to a direct and selective inhibition of PCSK9 translation during the elongation phase [226].

However, more experiments need to be done to further optimize both of these molecules in order to improve their selectivity for PCSK9 only with no off-target effect on other essential proteins.

c) Interfering with PCSK9 secretion

PCSK9 is synthesized and transported from ER to Golgi complex and finally secreted into circulation. Its secretion can be inhibited by some LOF mutations in the prodomain or even targeting molecules like sortilin or Sec24a (Figure 14).

- **Sortilin:**

SORT1 gene encodes sortilin that is a high affinity sorting receptor of PCSK9. It co-localizes with PCSK9 in the trans-Golgi network and facilitates its secretion to the plasma membrane. This type of interaction is pH-dependent and at pH 6.5 (in trans-Golgi), there is a strong

interaction between PCSK9 and sortilin, while at pH 5.5 (secretory vesicles), PCSK9 is released. Any deficiency in sortilin expression leads to increased intracellular levels of PCSK9 and decreased that of circulating PCSK9. Sortilin overexpression increases circulating PCSK9, reduces LDLR and increases LDL-C levels. All of these indicate a positive correlation between sortilin and PCSK9 [227]. Thus, targeting sortilin could be a useful tool to deregulate the levels of PCSK9

- **Sec24a:**

Sec24a is another protein required for the transport of PCSK9 from ER to Golgi apparatus. Observations similar to those for sortilin have been made regarding Sec24a deficiency or overexpression [124]. That's why it could serve as a potential therapeutic target as well.

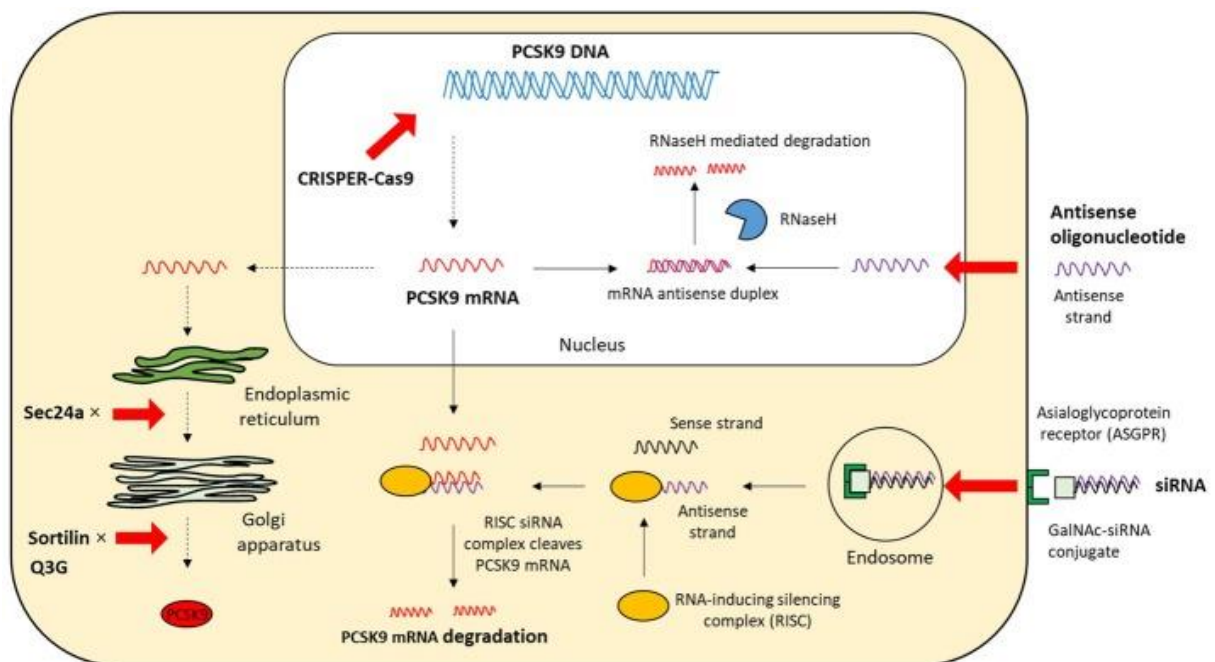


Figure 14: Strategies to inhibit PCSK9 expression and secretion [124]

Liver Cancer and Meprin α

Few are the studies that investigate the role of meprin α in liver cancer, especially HCC, whereas no research has been done so far to examine its role in HBL. In the next section, I will be digging deeper into the topic of the meprin α metalloprotease, its various roles and its relationship to HCC. Briefly, proteases are known to control many diseases since their functions are diverse. They can activate or inhibit essential proteins, hormones, receptors, or even other proteases, therefore contributing towards the progression of diseases. We were interested in this secreted protein and were willing to check its roles in HBL.

VI- Meprin 1 alpha (Mep1 α) Metalloprotease

1. Generalities

Over the last decade, many of the pathophysiological conditions were shown to be controlled by proteolytic enzymes that are necessary for the post-translational modification of different proteins. These proteases play a crucial role in many processes such as immune system regulation, development and degeneration of neurons, apoptosis and cancers [228]. In the early 1980s, an unexpected proteolytic activity was discovered in mouse kidney as well as in the intestine of patients after pancreatic surgery [229], [230]; this led to the identification of meprin (previously called PABA peptide hydrolase/PPH) as a novel metalloprotease from renal tissue. Later on, two meprin genes were identified, *MEP1A* on chromosome 6 and *MEP1B* on chromosome 18, coding for meprin α and meprin β proteases in humans, respectively [231], [232]. These meprins belong to the astacin family of zinc endopeptidases and the metzincin superfamily of metalloproteinases [231], hence being somehow genetically related to MMPs (matrix metalloproteinases) and ADAMs (a disintegrin and metalloprotease domain), which are famously known to be involved in different biological processes associated with cancer, metastasis and inflammation [233], [234]. Unlike MMPs and ADAMs, which have been extensively studied for their important biological roles, meprin α and meprin β were mysterious. This was due to the fact that most of the studies were focusing on the organ-specific activity of these proteases, and not their global action. However, the identification of the important roles of meprins were only possible after the generation of meprin-knockout mice, which exhibited clear immunological phenotypes [235], [236]. For instance, meprins were identified as susceptibility genes in inflammatory bowel disease (IBD) [237]; they had different functions in epidermal homeostasis in human skin, where meprin α promotes cellular proliferation in basal epidermis, whereas meprin β induces cell death in terminally differentiated keratinocytes [238]. Also, meprin α has an important role in promoting angiogenesis and meprin β is essential for organogenesis in zebrafish [239], [240]. Moreover, thanks to the proteomics approaches, many substrates have been identified for meprin α and meprin β . This has led to further focus on their role in regulating fibrosis, collagen assembly and neurodegenerative processes like Alzheimer's disease (AD). As a result, a wider role of meprins has been highlighted, prompting scientists to try to better understand their function and the multiple implications of their activity.

2. Structure of meprins

Meprins are complex multi-domain membrane bound enzymes that are highly glycosylated and require post-translational modifications in order to become fully active. Indeed, the activation of the inactive zymogens requires the removal of the N-terminal propeptide by different proteases [241], [242]. For instance, many serine proteases have been discovered to activate meprins such as pancreatic trypsin and human kallikrein-related peptidases (KLKs) [243]. Both human meprins α and β are composed of the domains depicted in Figure 15: an N-terminal signal peptide whose importance relies on targeting the polypeptide chain to the ER; an N-terminal propeptide (PRO), which needs to be cleaved for full activation; an astacin-like protease domain (CAT) that contains a conserved zinc-binding site motif (HExxHxxGxxHxxxRxDR) and a conserved methionine containing β -hairpin (Met-turn); a MAM (meprin A5 protein tyrosine phosphatase μ) domain and a TRAF (tumor-necrosis-factor-receptor-associated factor) domain, which are both necessary for protein-protein interaction; an EGF (epidermal growth factor)-like domain, a transmembrane domain (TM); and a cytosolic C-terminal tail (C) [228], [244].

What differentiates meprin α from meprin β is the presence of an additional domain called “inserted” domain (I), which can be cleaved by the action of furin so that meprin α is secreted to the extracellular environment after the loss of the EGF-like transmembrane and cytosolic domains. Once released, meprin α is capable of forming non-covalently linked oligomers [231], [245], making it the largest protease ever secreted (Figure 15). On the other hand, meprin β is predominantly restrained to the cell membrane as a dimeric type I integral protein, but it can be shed from the surface by the action of ADAM 10/17, resulting in a soluble form of meprin β [246], [247] (Figure 15).

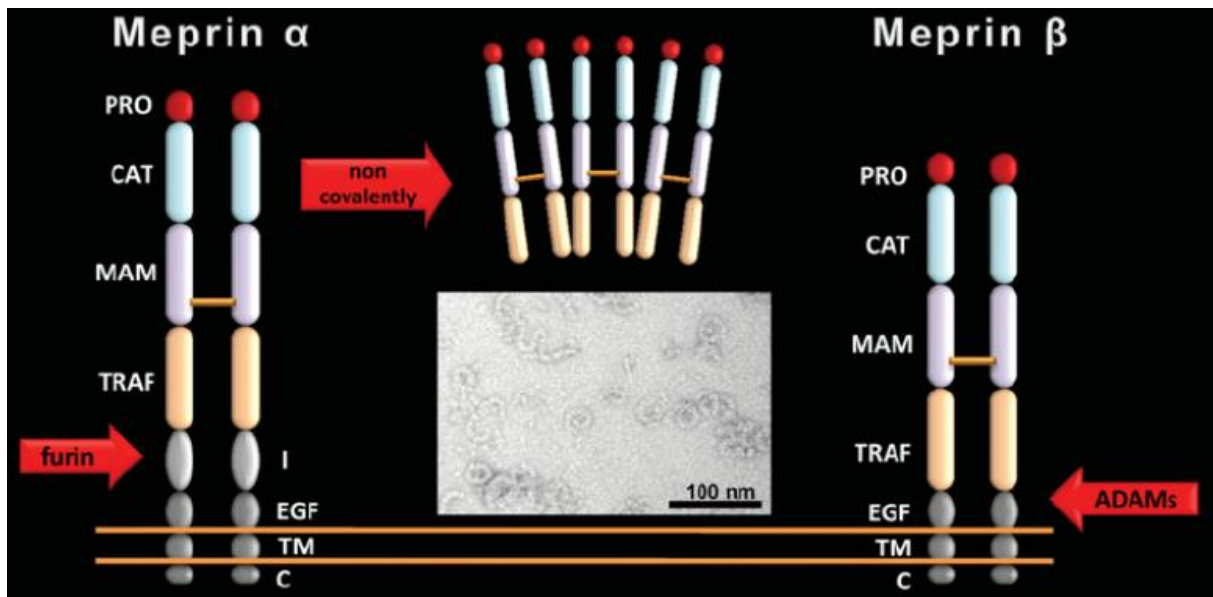


Figure 15: Schematic representation of human meprin α and meprin β (adapted from [228])

Meprins are expressed as zymogens with a propeptide (PRO) N-terminal linked to the protease domain (CAT) that must be cleaved off proteolytically to gain full activity. CAT: astacin-like protease domain; MAM: meprin A5 protein tyrosine phosphatase μ ; TRAF: tumor-necrosis-factor-receptor-associated factor; EGF: epidermal growth factor; TM: transmembrane domain; C: C-terminal cytosolic tail; I: "inserted" domain. Purified recombinant meprin as visualized by electron microscopy

Due to this difference between the two meprins, each one of them has distinct roles and can alter different biological functions. For instance, meprin α and meprin β are found to be differentially expressed in small and large intestine, leukocytes and several cancers [231]. In normal dermis, higher expression is observed for meprin α than meprin β , but in keloid tissues, both of them are upregulated [248]. On a different note, meprin α and meprin β are able to form hetero-oligomers when co-expressed in rodents, but not in humans. That's why, in rodents, we can distinguish: meprin A, composed of meprin α oligomers or meprin α/β hetero-oligomers; and meprin B, alternative name for meprin β dimers [249]. The hetero-oligomers formed by meprin α and β are membrane-associated proteases anchored to the brush-border membranes *via* meprin β subunit. However, in humans we stick with the nomenclature of meprin α and meprin β due to the absence of meprin α/β hetero-oligomers. Moreover, both meprins can form homodimers using a disulfide bridge linkage, where only one disulfide bridge between MAM domains of the human meprin β dimers is needed to do the job [250]. The main role for this S-S bridge is to stabilize the structure.

In this manuscript, meprin α (Mep1 α) will be the main focus in the upcoming sections.

3. Function of Mep1α

The function of Mep1α is better understood when looking at its targets. A wide variety of substrates can be hydrolyzed by Mep1α once it's activated and secreted. The substrates include: active peptides, cytokines (IL-6), receptors (IL-6R), extracellular matrix (ECM) proteins (fibronectin, pro-collagen I), and other proteases (MMPs, ADAMs) [231]. That's why we can find multiple implications of Mep1α in inflammation, fibrosis, diseases (e.g., IBD), cancer progression, migration and invasion. Some examples are given in Table 3 and more are discussed below.

Table 3: Some meprin substrates in relation to various pathophysiological conditions

Condition	Mep1α	Of note	Substrate	Cells/Organism	Drug
Inflammation	Mep1α		IL-6 and its cognate receptor (IL-6R)	Murine B-cell expressing gp130	Tocilizumab blocks proliferation
IBD	Mep1α	Decreased expression	Increased expression of IL-6	Colon cells	
IBD model	Mep1α	<i>Mep1α</i> ^{-/-} mouse becomes more sensitive to induced colitis	Increased expression of IL-6	Knock out mouse	
Acute and chronic inflammatory diseases	Mep1α		Pro-IL-1β	Monocytes, macrophages, neutrophils and hepatocytes	Actinonin inhibits Mep1α
Inflammation and fibrosis	Mep1α		Tβ4 (with prolyl oligopeptidase [POP] to generate the active peptide Ac-SDKP)	Mouse and rat	POP inhibitors or actinonin prevent release of Ac-SDKP from Tβ4
Inflammation: recruitment and traffic of mononuclear immune cells	Mep1α	Recombinant rat Mep1α	CCL2/MCP-1 (inactivation)	HEK-2933	

Cystic fibrosis	Mep1 α	EGF and TGF α (released from cell surface to transactivate EGFR/TLR4)	Caco-2 (colon carcinoma)	cells
Skin and fibrosis	Mep1 α & β	Pro-collagen I & III	ECM of the human epidermis	
Cell migration	Mep1 α & β	Collagen IV, fibronectin (FN), laminins and nidogen- 1	Recombinant human meprins	Hydroxamic acid derivatives (batimastat, galardin and Pro- Leu-Gly- hydroxamate), TAPI-0 (tumor necrosis factor α protease inhibitor- 0), TAPI-2, thiol-based compounds (captopril)
Angiogenesis	Mep1 α		VEGF-A	

a) Role in inflammation:

Inflammation is a process by which the body responds to tissue damage that can be caused by physical injuries, ischemic injuries, infection, exposure to toxins or other types of traumas. The response by inflammation to such harms can cause cellular changes and immune activation in order to repair the damage. However, when the inflammation persists or the control systems fail to resolve it, it will become chronic resulting in leukocyte infiltration, mutations, increased proliferation and often creating an environment suitable for cancer development or disease advancement. That's why, it is of great importance to treat the causes of inflammation [251]. Chronic IBD is a famous example of chronic gastrointestinal tract inflammation that is genetically associated with *MEP1A* gene single nucleotide polymorphisms (SNPs) [237]. The mRNA expression level of *MEP1A* is downregulated in the epithelium during intestinal inflammation. Evidence has accumulated regarding the action of meprins in modulating the immune microenvironment by processing and activating pro-inflammatory cytokines like interleukins (ILs) that are strongly associated with IBD.

Some important players in the initiation and propagation of inflammation are IL-6 and its cognate receptor (IL-6R), which are both found to be substrates for Mep1 α . What is

interesting about IL-6R signaling is that it can be done in two different manners: mainly a pro-inflammatory trans-signaling *via* soluble IL-6R (sIL-6R) or a classical signaling *via* membrane bound IL-6R. Soluble Mep1 α can cleave IL-6R between glutamine (Q357) and aspartate (D358) to release it from the cell surface as a bioactive sIL-6R. This has been proven by the significant increase in the proliferation of murine B-cell line (Ba/F3) expressing signal transducing receptor gp130 dependent on IL-6. The dependency on IL-6 was further confirmed by the use of tocilizumab, a drug that blocks IL-6 binding site of the α receptor, resulting in complete suppression of proliferation [252]. On the other hand, IBD is associated with decreased expression of Mep1 α in the colon of patients. Furthermore, *MEP1A* knockout mice were also more susceptible to dextran sulfate sodium (DSS)-induced colitis [237]. Taken together, this indicates that Mep1 α plays an important anti-inflammatory role in chronic intestinal diseases in humans and mice. IL-6 is known to be involved in the pathogenesis of IBD by promoting inflammation and neutrophil influx. The levels of IL-6 are highly elevated in the absence of Mep1 α in mice, whilst in its presence, Mep1 α can cleave IL-6 leading to a decrease in its biological activity, and hence preventing the stimulation of IL-6 dependent murine B-cell hybridoma cell line (B9 cells) growth [253], [254]. Furthermore, a key pro-inflammatory cytokine called IL-1 β is also regulated by Mep1 α . IL-1 β is produced by many cells like monocytes, macrophages, neutrophils and hepatocytes. It has a great role in many acute and chronic inflammatory diseases, whether we are talking about infections or even tumor invasiveness and angiogenesis [255]. In order to exert its function, inactive pro-IL-1 β needs to be activated by a proteolytic process to produce the biologically active IL-1 β . This can be made possible by caspase-1 cleavage, but caspase-1-independent processing may also occur. For instance, Mep1 α is capable of proteolytically cleaving pro-IL-1 β at the Histidine¹¹⁵-Aspartate¹¹⁶ bond, creating a 17-kDa IL-1 β fragment that is slightly larger than the one created by caspase-1. The biological activity of this IL-1 β produced by Mep1 α was higher than the one produced by caspase-1 or even meprin β . In addition, the inhibition of Mep1 α by actinonin prevented sepsis-induced generation of serum IL-1 β , thus highlighting the critical role of Mep1 α in processing pro-IL-1 β during tissue injury and inflammation [256].

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a natural tetrapeptide with anti-inflammatory and antifibrotic properties. It is released from the NH₂-terminal part of its precursor thymosin- β 4 (T β 4) by peptidases, which can be blocked by inhibitors of prolyl

oligopeptidase (POP). The inability of POP to hydrolyze peptides of more than 30 aa suggests that T β 4 needs to undergo hydrolysis before Ac-SDKP is released *via* POP cleavage. This can be made possible by Mep1 α , which was shown to be necessarily present with POP in order to release Ac-SDKP *in vitro*, whilst neither Mep1 α nor POP alone were able to do the job. Also, the incubation of T β 4 with rat kidney homogenates (KH) led to a significant release of Ac-SDKP, which was due to Mep1 α whose inhibition by actinonin blocked the release. Moreover, Ac-SDKP release was abrogated when T β 4 was incubated with KH coming from Mep1 α -knock out mice compared to WT. Taken altogether, these observations indicate the important hydrolyzing role of Mep1 α , along with POP, to release Ac-SDKP from T β 4 [257].

In addition, Mep1 α can also cleave small chemokines such as CCL2/MCP-1 (chemokine CC motif ligand 2/monocyte chemotactic protein 1) that is involved in recruiting and trafficking of mononuclear immune cells (e.g., monocytes) to the site of inflammation. Mep1 α cleaves the N-terminal domain of CCL2/MCP-1 leading to a significant loss of its biological activity [258].

Several studies have shown that Mep1 α can release ligands like EGF and TGF α (transforming growth factor α) from the cell surface in order to induce inflammation by transactivating EGFR [259]. Activated Mep1 α in colon carcinoma cells (Caco-2) was responsible for the shedding of EGF and TGF α from plasma membrane, leading to the activation of EGFR and extracellular regulated kinase 1/2 (ERK1/2) signaling cascade in a ligand-dependent manner. The overall result is enhancement of cell proliferation and migration, indicating that Mep1 α can be involved in the progression of colorectal cancer. Similar activation was also identified in human bronchial epithelial cells [260], where soluble TGF α binds to and activates EGFR/TLR4 (Toll-like receptor 4) leading to the increased release of IL-8, which serves as a neutrophil chemoattractant implicated in cystic fibrosis.

b) Role in skin and fibrosis:

Skin is the largest organ protecting the body against external physical, chemical and biological influences since it is composed of many distinct layers. As mentioned earlier, Mep1 α is expressed in the human epidermis, especially the *stratum basale*, and plays a role in enhancing the proliferation of human keratinocytes [238], [243]. Such cells, and like many others, are found in a well-organized network called the ECM (extracellular matrix) that is composed of a mixture of cells and non-cellular components in a given tissue or organ. ECM

provides physical scaffolds into which cells are incorporated, in addition to its important role in regulating cellular processes like growth, migration, differentiation and survival. A wide variety of macromolecules combine to form the ECM, where its major constituents are fibrous-forming proteins like collagens, elastin, fibronectin (FN), laminins, glycoproteins and proteoglycans (PGs). Basement membrane is a type of pericellular ECM mainly composed of type IV collagen (stabilizing overall structure), laminins (providing epithelial cell adhesion sites), nidogen 1 & 2, and PGs. The ECM interacts with cells embedded in it *via* their surface receptors, which in turn secrete matrix macromolecules aiding in the formation of ECM. Any variation in the composition and structure of ECM might disrupt the normal homeostasis, leading to many syndromes and pathological conditions -sometimes lethal. One important example is during tumorigenesis, where marked alterations in ECM are observed, favoring cell growth, migration and angiogenesis [261]. Another example is the fibrosis, a condition linked to excessive deposition of ECM proteins like large portions of collagen I [262].

Many studies have shown that the *in vitro* expression of *MEP1A* can induce cell migration mainly by processing components of the basal lamina like collagen IV, fibronectin (FN), laminins and nidogen-1, thereby leading to cell detachment from the basement membrane and hence migration [241], [246], [263]. In addition, *MEP1A* was found to be overexpressed in human dermal fibroblasts of keloids when compared to normal skin [248]. Therefore, any modulation in the *MEP1A* expression might affect the ECM and its components, which are substrate of meprin α .

For instance, fibril-forming collagens like pro-collagen I & III are secreted as precursor molecules into ECM and require the removal of both the C- and N-terminal propeptides in order to induce collagen fibril assembly. *In vitro*, it was shown that Mep1 α was capable of cleaving propeptides of both pro-collagen I & III triggering self-assembly of mature collagen into fibrils. The role of *MEP1A* was confirmed *in vivo* in mice deficient for *MEP1A*, which presented higher levels of full-length procollagen I as compared to WT mice. This led to reduced thickness of the fibrous layer and decreased accumulation of dermal collagen in deficient mice compared to WT, indicating the presence of abnormalities in the formation and stability of connective tissues [248], [264].

In another study, Mep1 α was shown to completely degrade human collagen IV, whilst having no effect on collagen I. Also human FN and nidogen-1 were proteolytically cleaved, each at

two sites, by Mep1 α ; FN is cleaved at positions Tyr²⁹⁴-Gln²⁹⁵ and Asn⁷⁰⁹-Thr⁷¹⁰ and nidogen-1 is cleaved at positions Asp⁶⁷-Arg⁶⁸ and Gln³⁶³-His³⁶⁴ [241].

c) Role in kidney and nephritis:

Mep1 α plays an important role in kidney diseases such as acute renal failure. Mice strains C3H/He and CBA/Ca that do not express *MEP1A* in their renal proximal tubular cells develop less severe forms of injury-induced kidney diseases than the other mice with normal Mep1 α levels [228]. Moreover, mice lacking *MEP1A* exhibit less renal damage and bladder inflammation after LPS administration [265]. A redistribution of meprins from apical brush-border membranes of proximal tubule to the basolateral tubular basement membrane is observed in rodents during cisplatin-induced acute kidney injury (AKI) and ischemia/reperfusion (IR) injury [266], [267]. The latter is known to induce tissue damage by creating an inflammatory response. This is made possible due to the action of meprins at the basement membrane that will digest and cleave its components, along with cell-adhesion molecules, resulting eventually in leucocyte infiltration and thus inducing inflammation [241], [263].

d) Role in angiogenesis and cancer:

In the last decade, studies have investigated the role of Mep1 α in angiogenesis. Angiogenesis is a process by which new blood vessels form from pre-existing ones in order to maintain organ development and homeostasis. Any imbalance results in various diseases such as cancer and inflammation [268]. For instance, a Morpholino knockdown of meprin α in zebrafish embryos resulted in defective tissue differentiation and severe failures in vascular system formation. These results were similar to VEGF-A morphants indicating that meprin α plays a pro-angiogenic role in processing and activating VEGF-A, which is necessary for blood vessel formation during embryogenesis [239]. In colorectal cancer, increased meprin α activity promoted the tumor progression through its pro-migratory and pro-angiogenic activities *in vitro*. For example, the pro-migratory role of activated meprin α in MDCK kidney cells was dependent on the presence of hepatocyte growth factor (HGF) and its pro-angiogenic activity can be observed in rat aortic ring assay after the addition of meprin α which stimulated the outgrowth and branching of vessels [269].

Studies have earlier focused on the abnormal expression of *MEP1A* in many diseases such as IBD, colitis and Alzheimer's disease. Recently, they are focusing on the implication of Mep1 α

to stimulate cancer cell proliferation, migration and invasion such as in breast, colon, colorectal and hepatocellular carcinomas (HCC). In colon carcinoma cell line, Caco-2, meprin α is expressed and secreted at the basolateral and apical plasma membranes resulting in increased accumulation of meprin α activity in the ECM, thus breaking down the stromal structure and affecting the migration of tumor cells to nearby tissues. In colon tissues, it can only be detected in the carcinoma part and not in the normal tissue, where its enzymatic activity was tripled [270]. In the human breast cancer cells MDA-MB-435, which are highly metastatic and invasive, the inhibition of polyamine biosynthesis resulted in a significant decrease (~70%) of *MEP1A* expression at the mRNA and protein levels. Moreover, actinonin-treated cells showed significantly less invasiveness *in vitro* confirming the pro-invasive role played by Mep1 α in breast cancer cells [271]. In addition, the ability of Mep1 α to process ECM proteins like procollagen III highlighted its role in metastasis [248]. Taken altogether, these observations show that Mep1 α role in cancer development, progression and metastasis is more complex than that of the other metalloproteases. Further studies should be pursued to better evaluate these functions.

4. Meprin α and HCC

Almost 15% of human cancers are associated with chronic infection and unresolved inflammation [251]. Of interest is HCC whose progression involves inflammation at all four stages which correspond to cell degeneration, fibrosis, cirrhosis and formation of tumor. HCC has very recently been associated with the overexpression of meprin α metalloprotease [272]. Meprin α is capable of modulating the immune microenvironment by processing and alternatively activating different pro-inflammatory cytokines like ILs (e.g., IL-1 β) [256]. Moreover, it is also known to induce inflammation by trans-activating EGFR through the release of its ligands- TGF- α and EGF- from the cell surface, hence resulting in the activation of ERK1/2 signaling cascade [259], [273], [274]. Therefore, meprin α sustains the inflammatory reaction in HCC and aids the progression of the disease.

In the recent years, scientists discovered that Mep1 α plays an important role in the progression of HCC where *MEP1A* expression level is remarkably elevated compared to the adjacent non-neoplastic ones. It was detected by quantitative real time polymerase chain

reaction (q-RT PCR) and immunohistochemistry (IHC) analysis. The level of *MEP1A* expression varies from one stage of tumor to another, and higher levels are detected in the advanced stages of cancer than in the early ones. Moreover, *MEP1A* expression widely varies in tumor cell cytoplasm from different HCC specimen, allowing the classification of patient tumors into *MEP1A*⁻ group (negative expression in tumor cells) and *MEP1A*⁺ group (positive expression in tumor cells). This classification can serve as an independent prognostic factor for HCC. For instance, high expression levels of *MEP1A* in the tumor cells are significantly associated with aggressive clinicopathological features (i.e., microvascular invasion, portal vein tumor thrombus, poorly differentiated tumors, and late clinical stage), poorer surgical prognosis, shorter time to recurrence and worse overall survival (OS) as revealed by Kaplan-Meier survival analysis. These consequences are linked to the different tumor stages (0-A, B, C), subgroups of microvascular invasions, and sometimes to certain mutations (TP53) [272], [275].

- **Overexpression of Mep1 α in relation to reptin expression**

Interestingly, HCC tumor cells also show an elevated expression of an oncogenic ATPase known as reptin. It was shown to significantly and positively regulate the expression of *MEP1A* in HCC. For instance, silencing reptin in the HCC cell lines Huh7 and Hep3B strongly reduces the mRNA and protein levels of meprin α , hence significantly reducing its proteolytic activity in conditioned medium. In addition, meprin α can serve as a mediator of reptin as, in the case of reptin silencing, overexpression of meprin α can restore normal cell migration [275].

- **Effect of Mep1 α on tumor cell proliferation**

The effect of meprin α on crucial cellular processes can be easily studied through gain of function and loss of function experiments. Gain of function can be achieved by the addition of the recombinant protein into the culture medium or the induction of overexpression of the gene by the cell lines of interest using transfection or transduction assays. The loss-of-function can be attained using different small interfering RNA (siRNA) or short hairpin RNA (shRNA) specific for a given protein.

Several evidences suggested that meprin α could induce cell proliferation and growth. However, this was not the case in a study by Osman and his team using HCC model cell lines. Neither the overexpression of meprin α nor its silencing had any effect on the proliferation of

cells as compared to the positive and negative controls. In the positive control, in which reptin has been silenced, significant decrease in the cell growth of HuH7 and Hep3B cells was observed, and this action was not reversed by the overexpression of *MEP1A*. Thus, little or no effect of meprin α on tumor cell proliferation was observed [275].

On the contrary, OuYang *et al.* showed that the overexpression of *MEP1A* in the HCC cell lines Hep3B and SMMC-7721 significantly enhanced their tumorigenicity *in vitro* and *in vivo* [272]. Functional assays have shown an increase in the growth rate and foci formation frequency in HCC cell lines. Moreover, the size and mass of the xenograft tumors from implanted *MEP1A* overexpressing cells in nude mice were significantly larger and heavier compared to the control group. All of these observations were reversed when *MEP1A* expression was knocked-down by shRNA in the corresponding cell lines, thus conferring a role for meprin α in cell proliferation [272].

- **Effect of Mep1 α on tumor cell migration and invasion**

Knowing that *MEP1A* overexpressing cells are associated with different clinicopathological features related to tumor metastasis, migration and invasion assays were performed *in vitro*. Cell lines stably expressing high levels of Mep1 α or even the addition of recombinant Mep1 α into the culture medium of cells (even when reptin was silenced by shRNA) enhanced the migratory activity of these cells by almost 3-fold compared to the control ones. This effect was abrogated when *MEP1A* was silenced using siRNA or shRNA. A role of Mep1 α in the invasive capacity of HCC cell lines has been confirmed in a study, where it is significantly increased in cells overexpressing *MEP1A* as examined by Matrigel assay [272], [275].

These *in vitro* results were further explored *in vivo* to check if Mep1 α has indeed an effect on tumor cell metastatic properties. For this purpose, nude mice were injected intravenously with HCC cell lines stably expressing high levels of *MEP1A*. After 6 weeks, the mice were sacrificed and the metastatic nodules in the lungs were counted. The overexpression of *MEP1A* significantly induced more metastatic nodules in the lungs as compared to the control mice. This result was further confirmed in BALB/c mice that were orthotopically transplanted with cells overexpressing *MEP1A*, and as expected, the liver tumors from *MEP1A* overexpressing mice was significantly larger and more metastatic nodules were found on the surface of the lungs as compared to the control [272].

Therefore, we can conclude that Mep1 α , a mediator of reptin, plays indeed a crucial role in the tumor cell migration and invasion, which promote metastasis, and hence, exacerbates their effect.

- **Induction of epithelial-mesenchymal transition (EMT) by Mep1 α in HCC cells**

The capacity of meprin to promote the metastasis of HCC cell lines is underlined by its ability to induce EMT. EMT is characterized by the shedding of the epithelial markers necessary for cell-cell adhesion and cell polarity and the acquisition of mesenchymal traits that aid in motility and migration [276]. For instance, overexpression of *MEP1A* induced a morphological change of cells that turn spindle-like shaped to resemble mesenchymal cells. It also increased the levels of mesenchymal proteins such as vimentin, matrix metalloproteinases (MMP-2, MMP-9) and led to a decrease in the level of E-cadherin. The latter was accompanied by an increase in zinc-finger E-box binding homeobox 1 (ZEB1) transcription factor which may have a role in the Mep1 α induced-EMT [272].

Noteworthy, EMT is induced by a signaling cascade involving the EGF through the activation of extracellular signal-related kinase (ERK) signaling pathway [277], [278]. Plus, in human colorectal adenocarcinoma cells, meprin α was shown to increase phosphorylation of EGF-R, probably after shedding of EGF or TGF β by meprin α , and consequently, the phosphorylation level of ERK1/2 was increased [32]. Interestingly, in HCC cell lines and in xenograft tumor models, *MEP1A* overexpression had the same effect on the phosphorylation levels of ERK1/2. Therefore, this suggests that Mep1 α can probably promote EMT and hence migration of tumor cells through the activation of ERK/ZEB1 pathway. Indeed, the inhibition of the ERK pathway by a specific inhibitor (U0126), in cells overexpressing *MEP1A*, resulted in a decreased level of ERK and ZEB1 phosphorylation and blocked the effect of Mep1 α on the migration of cells. So, these data emphasize the link between Mep1 α and the activation of ERK/ZEB1 pathway to promote EMT [272] (Figure 16).

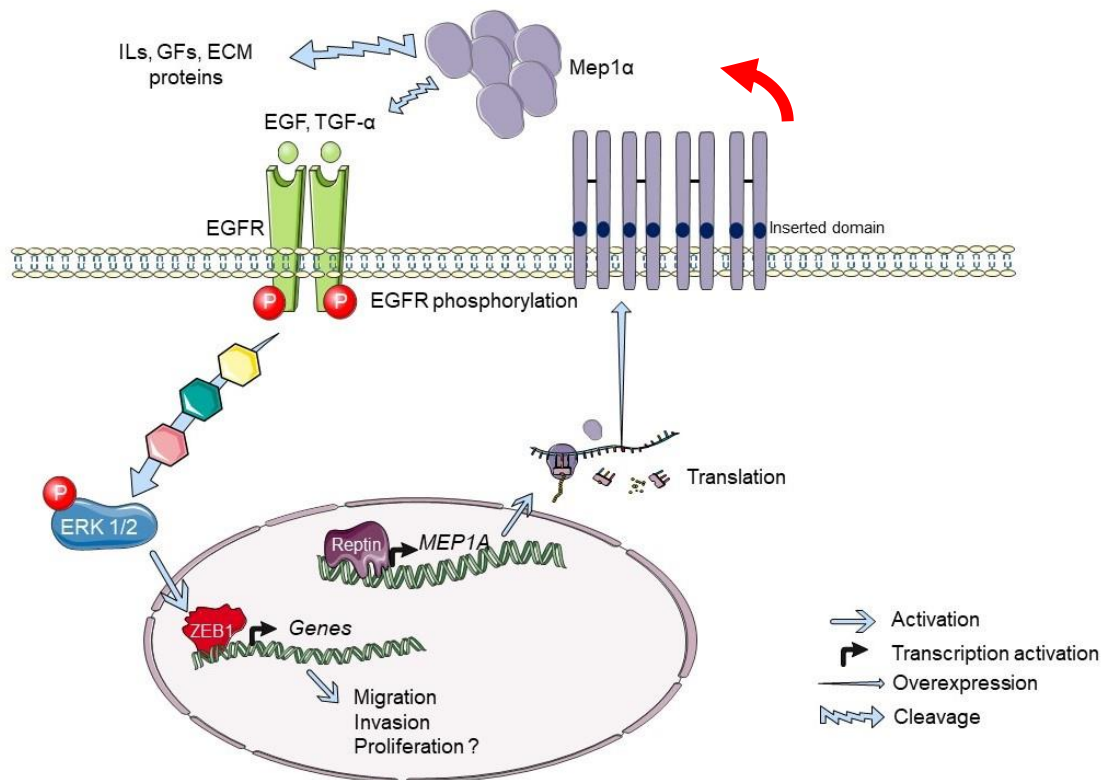


Figure 16: Mep1α overexpression by reptin induces metastasis.

Reptin significantly and positively induces the increased expression of Mep1α, which forms large oligomers in the extracellular environment after being cleaved at the inserted domain. Mep1α in turn can cleave a wide range of substrates including ILs, GFs and other proteins present in the environment. On top of that, Mep1α increases the level of EGFR phosphorylation, which allows the binding of its ligands and hence the triggering of the ERK1/2 pathway to activate the ZEB1 transcription factor. ZEB1 promotes the transcription of different proteins essential for EMT, such as vimentin, N-cadherin and MMPs. This induces the cells to detach from their surroundings, adopt a spindle-like shape and undergo metastasis.

Mep1α: meprin 1 alpha; ILs: interleukins; GFs: growth factors; ECM: extracellular matrix; EGFR: epidermal growth factor receptor; EGF: epidermal growth factor; TGF-α: transforming growth factor alpha; ERK1/2: extracellular regulated kinase 1/2; ZEB1: zinc-finger E-box binding homeobox 1; P: phosphorylated; MMPs: matrix metalloproteases

On the contrary, controversial data were recently published from a prospective cross-sectional study that has been done between January 2013 and June 2019 in the United Kingdom and Germany and that involved 195 patients. This study included 57 HCC patients with a background of liver cirrhosis (LC) and 138 non-HCC controls divided into 72 patients with LC, 57 with non-cirrhotic liver disease and 9 with normal liver function [279]. By applying capillary electrophoresis mass spectrometry (CE-MS) on urine samples of the HCC study cohort, 31 specific urinary peptide markers for HCC were discovered and then incorporated into a multivariate classification model, which had high sensitivity and specificity for HCC. After identifying these peptide sequences, and because it is known that they are the product of proteolytic cleavage, *in silico* mapping was done and revealed many potential protease

candidates, of our interest is the MEP1A. The activity of MEP1A was found to be decreased in HCC as compared to non-HCC liver diseases. This was further confirmed by IHC staining performed on normal liver tissue, cirrhotic tissue and HCC. Only moderate staining for MEP1A was observed in normal liver tissue, whilst there was no staining in cirrhosis and HCC [279].

5. Regulation of Meprin α expression

Mep1 α is highly regulated at the transcriptional and post-translational levels by transcription factors (TFs), microRNAs (miRs) and RNA binding proteins (HuR, TTP) [231], [280]. The 5' region upstream the transcription initiation site of *MEP1A* gene contains apparent binding sites for TFs, which are expressed at different stages of development and by different cell types. GATA TFs binding sites have been identified in the promoter region of human *MEP1A* gene. This family of TFs are usually expressed in developing and adult intestine and they function in activating a number of intestinal genes [281]. For instance, in Caco-2 human colon carcinoma cells that endogenously express meprin α , several GATA-binding sites were identified and are recognized by GATA-4, GATA-5 and more specifically GATA-6, which is expressed in these cells. This indicates that GATA TFs may play a role in tissue-specific expression of meprin α [282]. In addition, the *Mep1a* promoter region may also contain possible binding sites for steroid receptors, which could be of great importance especially if meprin plays a role in steroid-treated inflammatory diseases [231].

Another TF that is necessary to maintain *MEP1A* gene expression is CDX2 (*Caudal*-related homeobox transcription factor). CDX2 is an intestine-specific TF responsible for regulating several genes related to intestinal epithelial functions, thus controlling the balance between intestinal epithelial cell differentiation and proliferation [283], [284]. Mice lacking the *Mep1a* gene or heterozygous for the *Cdx2* gene (*Cdx2*^{+/-}) developed more serious inflammation than the WT ones during DSS-induced colitis [237], [253], [285]. In Caco-2 cells, knockdown of *Cdx2* by shRNA resulted in 40% loss of *MEP1A* expression and a 5-fold decrease in its promoter activity [284]. Similar observations were made later when *MEP1A* expression was established to be dependent on CDX2 activity [286]. Moreover, in active ulcerative colitis (UC), one form of IBD, the correlation between *CDX2* and *MEP1A* expression and *TNF- α* expression was negative, at both mRNA and protein levels. The levels of CDX2 and MEP1A were significantly

decreased compared to the healthy and inactive UCs, whilst *TNF- α* expression was increased. Similar observations were recorded in Caco-2 cells in response to *TNF- α* upregulation indicating that this cytokine plays a major role in suppressing *CDX2* and *MEP1A* expression during inflammation. The mechanism by which *TNF- α* acts is by interfering with the transcriptional activity of *CDX2* and DNA-binding of *CDX2* protein, hence suppressing its expression along with downregulating *MEP1A* expression. All of these actions played by *TNF- α* can be overridden using anti-*TNF- α* treatment with infliximab (IFX) [286].

Moreover, during fetal-to-adult and crypt-to-villus differentiation of small intestine epithelial cells, the expression of *MEP1A* gene is upregulated. The promoter region of this gene contains high fraction of hepatocyte nuclear factor-4 α (HNF-4 α) binding sites, indicating for the first time that this promoter is a target of HNF-4 α and in this case its expression depends on HNF-4 expression [287]. In addition, the expression of *HNF-4 α* is decreased during IBD, which could explain the decrease observed in *MEP1A* expression. Indeed, using *Hnf4 α* -null mice that are susceptible to colitis, it was shown that in the absence of HNF-4 α , the expression of *Mep1 α* was decreased [288]. Interestingly, there exists a close relationship between *CDX2* and HNF-4 α regulation, as these TFs serve as targets for one another in Caco-2 cells [284]. *TNF- α* is also capable of suppressing the expression of *HNF-4 α* via NFKB activation [289]. Altogether, we can deduce that during IBD, the inhibition observed at the level of *MEP1A* expression by the cytokine is due to the suppression of two TFs: *CDX2* and HNF-4 α [286].

During silicosis, macrophages and fibroblasts are activated and the expression of meprin α is diminished. The exogenous addition of meprin α reduces the activation of these cells, hence indicating an anti-fibrotic effect for meprin α . Similarly, the inhalation of silica by rats leads to a significant decrease in the level of Mep1 α in lungs, which is accompanied with an increase in pro-fibrotic factors and miR-155-5p. The latter is known to be crucial for fibrosis development. miR-155-5p can bind to the 3' UTR (untranslated region) of *Mep1 α* and negatively regulates its expression, hence decreasing the mRNA and protein levels of Mep1 α and promoting macrophage and fibroblast activation. The negative regulation of miR-155-5p by antagomir can reverse fibrotic lung disease [290].

Furthermore, Mep1 α can be regulated post-transcriptionally by some RNA-binding proteins (RBPs) like Hu antigen R (HuR) and tristetraprolin (TTP). These RBPs have opposing functions where HuR stabilizes the transcripts by promoting adenylation, competing for binding sites or

even enhancing translation, while TTR often functions to counteract the stabilization by promoting the deadenylation of transcripts. HuR can bind to and stabilize the 3'-UTR of *MEP1A* transcript, thus increasing its baseline expression. The levels of HuR or even its binding to 3'-UTR of *MEP1A* are not affected by PMA (propidium monoazide) induced destabilization of Mep1 α . On the other hand, TTP mRNA expression and protein levels were increased and its binding to 3'-UTR of *MEP1A* was strongly enhanced by PMA treatment. The binding of TTP decreases the mRNA stability of *MEP1A* and is required for PMA-induced destabilization of Mep1 α . This difference between these two RBPs in response to PMA can be explained by their different binding sites on the 3'-UTR [280].

It is newsworthy that potent pharmacological inhibitors of Mep1 α have been identified including actinonin (naturally occurring zinc-chelating hydroxamate), galardin, NNGH [N-isobutylN-(4-methoxyphenylsulphonyl)glycyl hydroxamic acid] and batimastat. Actinonin is the strongest inhibitor among others, with an inhibition constant (K_i) of 20nM [241]. In addition, one study has focused on designing novel selective inhibitors of Mep1 α based on a recently identified tertiary amine scaffold [291]. They showed that these inhibitors exhibited similar inhibitory potency to NNGH and galardin, but are less active than actinonin. However, the major advantage from designing these novel tertiary-amine-based inhibitors is their selectivity against off-target metalloproteases.

Part 1: PCSK9

(article submitted to JHEP Reports)

Objective

The profound increase in the global incidence of liver cancer is estimated to reach one million cases per year in the next decade. Moreover, it used to rank the 4th leading cause of cancer-related deaths worldwide, but it has now jumped to rank 3 based on the new GLOBOCAN 2020 [6], [11]. This raises up the awareness to the aggressiveness of this disease and the urgent need to develop new therapeutic treatments that are indeed effective, less toxic and affordable as compared to the already established ones, like chemotherapy.

In this context, dysregulation in the energetic metabolism is a new emerging hallmark of cancer cells. We have high interest in lipid metabolism and particularly in understanding the addictive relationship of cancer cells to lipids in order to find out the best therapeutic approach of rewiring the oncometabolic pathways to make cancer cells more vulnerable.

We analyzed the expression level of enzymes and proteins involved in this metabolic network using our transcriptomic data as well as the available transcriptomic databases (Transcriptomic data from Wu *et al.*, and Lopez Terrada *et al.* that are available online on R2: Genomic analysis and visualization platform). Our analysis highlighted a specific feature of liver cancer cells, which is translated by enhanced lipogenesis and impairment of lipid uptake. Indeed, we have observed an increase in the mRNA expression level of PCSK9, HMGCR and their transcription factor SREBP2 but a downregulation in the expression of LDL receptor in HCC and HBL tumors, which were further confirmed in hepatic cancer cell lines.

The expression of PCSK9 is also deregulated in other types of cancers, including neuroglioma, breast cancer, colorectal cancer. This raises a major question about the therapeutic strategies that could be adopted to target PCSK9 in these cancers. Very recent data in HCC showed that high expression of PCSK9 in tumor tissues is correlated with poor prognosis, being an independent risk factor for overall and disease-free survivals.

The present study aims at assessing the interest of inhibiting PCSK9 in liver cancer using different *in vitro* and *in vivo* experimental approaches. More specifically, we aim at:

Aim 1: Evaluating the anti-tumoral effects of pharmacological and genetic inhibiting of PCSK9 *in vitro* and *in vivo*. We will be using 3 cell lines (Huh6, Huh7, and HepG2) and 2 xenograft *in vivo* models, the chick embryo and the zebrafish.

Aim 2: Assessing the potential antitumoral mechanisms of action of PCSK9 by evaluating its lipid metabolism related functions as well as other activities.

Rewiring lipid metabolism by inactivating PCSK9 triggers lipid peroxide-induced cell death of ferroptosis in liver cancer cells

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Authors' contributions

Data acquisition, analyses, and interpretation, and drafting of paper: M.A., H.F., V.T., A.J.M.

Data analyses and interpretation: F.D.D., P.M., N.D.A., R.R., H.R, S.G. B.L., A-M.K. Critical revision of the paper for important intellectual content: H.F.K, R.R., H.R.R., C.F.G., B.B., H.K.F.

Writing of the paper: A.J.M. Obtained funding: A.J.M.

Highlights

- Liver cancers feature high lipogenesis versus lipid uptake
- Disruption of PCSK9 rewires lipid metabolism and impairs oncogenesis
- PCSK9 deficiency brings in oxidation-prone fatty acids
- PCSK9 targeting disrupts iron dependent anti-oxidant defense mechanisms
- Silencing of PCSK9 triggers liver cancer cell death by ferroptosis

Lay summary:

Liver cancers selectively orchestrate their dependency on lipids while gearing up the necessary protective measures against damages from metabolic stress. Inhibiting PCSK9 deprives tumor cells from this balancing posture, outpouring abundant lipid droplets and oxidation-prone fatty acids with concomitant impairment of antioxidant defenses. The increased oxidative burden and the failure of protective measures trigger fatal cell insults.

Keywords: ferroptosis, hepatocellular carcinoma, hepatoblastoma, lipid metabolism

Abstract

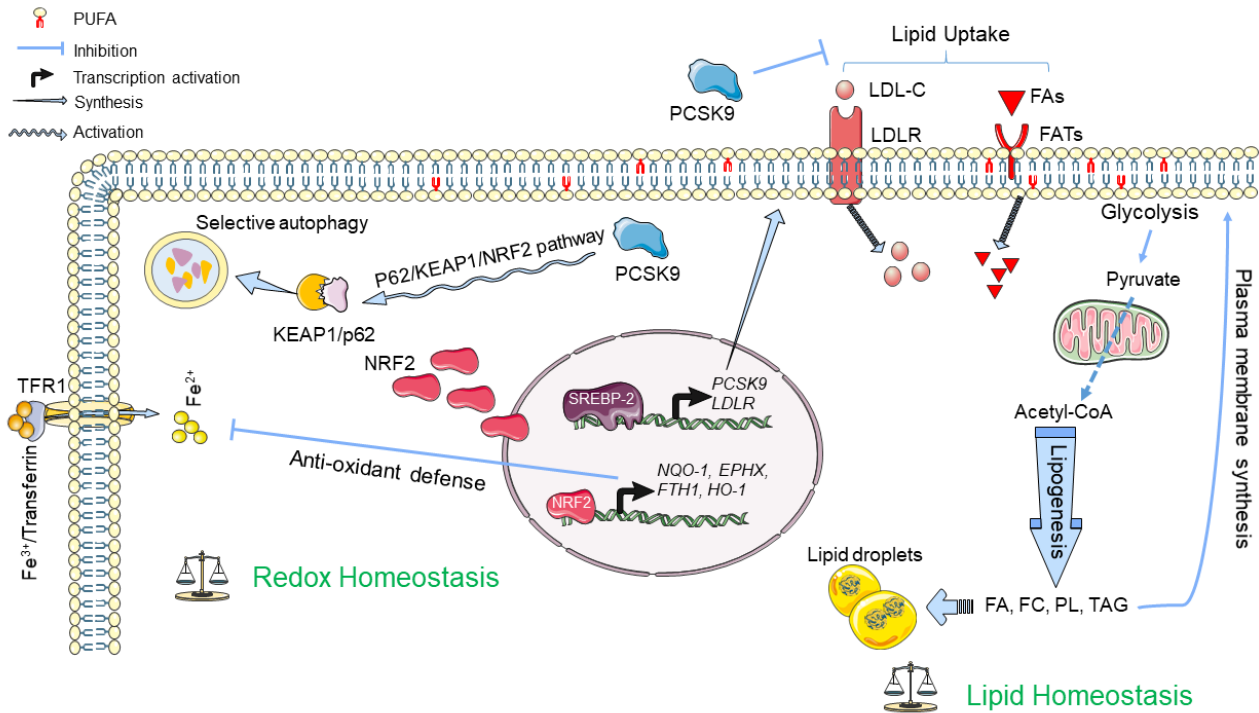
Background & Aims: Liver cancers are characterized by enhanced lipid biosynthesis, which is needed to sustain cell growth and tumorigenesis. We aim at taking advantages of this vulnerability within the oncogenic metabolic hub by rewiring the key enzymes triggering irreversible cell breakdown.

Methods: We targeted the key metabolic player PCSK9 using a pharmacological inhibitor as well as siRNA/shRNA alone or in combination with HMGCR inhibitors e.g., statins. We assessed the effect of these treatments using 3 hepatoma cell lines, Huh6, Huh7 and HepG2 and validated the results using the zebrafish *in vivo* model.

Results: Pharmacological treatment targeting PCSK9 has led to dose dependent inhibition of cell proliferation in all cell lines, which is associated with cytotoxicity and decreased cell migration. Co-treatment with statins such as simvastatin presented synergetic anti-proliferative effects. PCSK9 inhibition by siRNA was effective in reducing cellular growth and sensitizing tumoral cells to the action of statins and translated with increase of intracellular lipids and higher lipid hydroperoxide. Molecular signaling of cell death involved the disruption of the p62/KEAP1/Nrf2 antioxidative axis leading to ferroptosis whose morphological features were confirmed by electron and confocal microscopies. The anti-tumoral effects of PCSK9 deficiency was validated *in vivo* using xenograft experiments in zebra fish.

Conclusions: Beyond its critical role in lipid modulation, our findings herein underscore a novel functional involvement of PCSK9 in maintaining cell redox homeostasis and a new player of the specific cell death of ferroptosis. The inhibition of PCSK9 was effective in disrupting the oncometabolic process, enhancing the vulnerability of cancer cells to iron-triggered lipid peroxidation. We provide strong evidences supporting drug repositioning of anti-PCSK9 approaches to treat liver cancers.

Graphical abstract



Introduction

Cancer cells are highly dependent on different metabolic pathways among them enhanced *de novo* lipogenesis, which is becoming as a hallmark of aggressive cancers. This metabolic adaptation ensures the energetic needs of the cells, the building blocks for membranes as well as the signaling molecules to drive oncogenesis. Interestingly, unlike most normal cells even high proliferative ones, tumoral counterparts mostly display preference to increased endogenous fatty acids (FA) biosynthesis in spite of dietary lipid abundance [1]. This *de novo* pathway has a significant impact on the qualitative composition of the membranes *via* the enrichment of phospholipids with saturated and/or mono-unsaturated fatty acid chains since mammalian cells have a limited ability to synthesize polyunsaturated FAs (PUFAs) [2]. Because saturated FAs are less prone to lipid peroxidation than the polyunsaturated ones, by shifting lipid acquisition from external sources toward *de novo* lipogenesis, cancer cells acquire specific protective feature in their membranes to resist oxidative stress-induced cell insults. Since defects in hepatic lipid metabolism rewire many cellular pathways involved in oncogenesis and metastasis, interfering with lipid metabolism within the tumor and surrounding microenvironment becomes an attractive therapeutic approach for treating liver cancer patients. Because of the flexibility in the metabolic needs of cancer cells and the complex interplay among these key players of lipid metabolism, some factors may be more valuable and more relevant therapeutic targets.

This context highlights the importance of the proprotein convertase subtilisin/kexin type 9, or PCSK9. Targeting PCSK9 to treat cancer becomes attractive not only because of its critical role in lipid metabolism, but also because of its growing place in recent literature, which has reported its tight association with incidence and progression of several cancers [3]. Moreover, there are a diversity of therapeutic approaches targeting this enzyme that have been evaluated and, in some cases, approved for clinical use to lower cholesterol levels in patients suffering from hyperlipidemia.

Indeed, PCSK9 is a serine protease that belongs to the family of proprotein convertases, mainly involved in the degradation of the hepatic low density lipoprotein receptor (LDLR) present on hepatocytes' surface. This function interferes with the primary pathway of LDL-cholesterol (LDL-C) uptake from the circulation, thus leading to the increase of blood cholesterol levels (Figure 1A). PCSK9 can target other members of LDLRs, like very low-density lipoprotein

receptor (VLDLR), apolipoprotein E receptor 2 (ApoER2), cluster of differentiation 36 (CD36) and CD81 [4].

The promoters of *PCSK9* and *LDLR* genes contain a functional sterol regulatory element (SRE) that is targeted by transcription factor called sterol-responsive element binding protein 2 (SREBP2) in response to any change in the intracellular levels of cholesterol (Figure 1A) [5]. SREBP2 regulates the synthesis and absorption of cholesterol as well by targeting the gene expression of HMG-CoA (3-hydroxy 3-methylglutaryl- Coenzyme A) reductase or HMGCR, HMG-CoA synthase, farnesyl diphosphate synthase and squalene synthase [6].

Because of its essential role in lipid metabolism, many therapeutic approaches targeting PCSK9 have been implemented in combination with other hypolipidemic drugs such as statins to treat patients with hyperlipidemia and cardiovascular diseases.

Besides monoclonal antibodies (mAbs), one of the most recent approaches is the first-in-class chemically synthesized siRNA against PCSK9, inclisiran (Leqivo®; Novartis) that has been approved for the use in adults suffering from heterozygous familial hypercholesterolemia (FH) or non-familial, and mixed dyslipidaemia in Europe on December 2020 [7]–[9]. Different approaches called anti-secretagogue were developed to inhibit PCSK9 translation (by stalling of human 80S ribosomal subunit) and secretion [10]. Two molecules have been discovered, R-IMPP [(R)-N-(isoquinolin-1-yl)-3-(4-methoxyphenyl)-N-(piperidin-3-yl) propanamide] and PF-06446846, which underscore the therapeutic potential behind the use of selective inhibitors of mRNA translation.

The expression of PCSK9 is deregulated between different types of cancers, including HCC neuroglioma, breast cancer, colorectal cancer and others [3], [11], highlighting a major question about the therapeutic strategies that could be adopted to target PCSK9 in these cancers.

More specifically, a very recent study reported high expression of PCSK9 in tumor tissues in HCC patients. In this research, the expression of PCSK9 in tumors correlated with poor prognosis after curative resection and was an independent risk factor for overall and disease-free survivals. This illustrates the potential of PCSK9 as a prognostic marker for HCC [12].

The present study aims at assessing the interest of inhibiting PCSK9 in liver cancer using different *in vitro* and *in vivo* experimental approaches. We showed the critical role of this enzyme in controlling the homeostasis of lipid biosynthesis and traffic but also and for the first

time, its place in maintaining the redox homeostasis. The interference with this protein will lead to aberrant lipid metabolism, high lipid peroxidation and death of liver cancer cells by ferroptosis.

Materials and Methods

Patient samples

All patients were recruited in accordance with European and French law and institutional ethical guidelines. Non-tumoral, tumor and adjacent non-tumoral livers were collected from patients treated at French university hospitals, from the French HEPATOBIO network or the SIOPEL Liver Tumor and Tissue Bank (www.siopep.org).

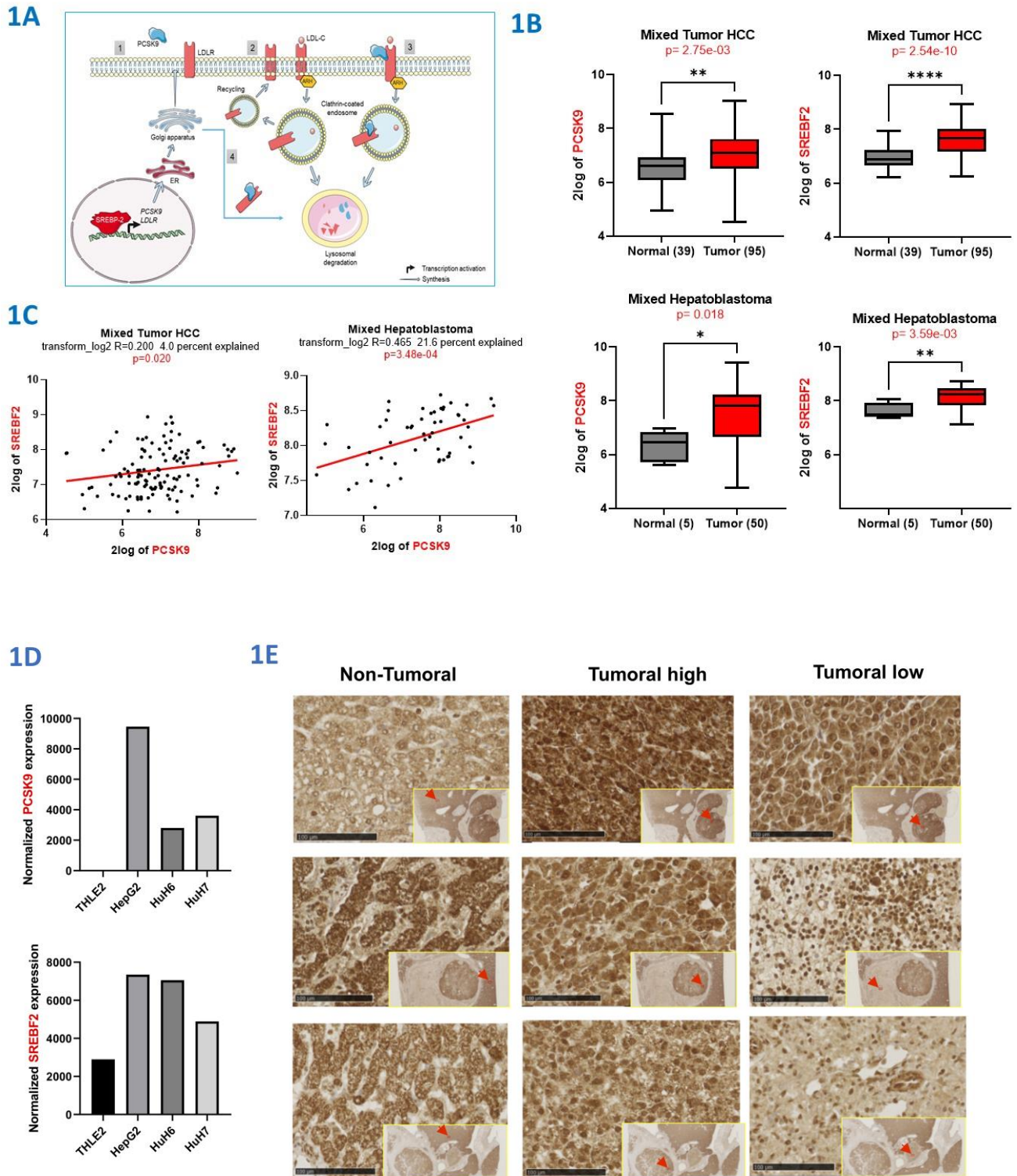
See Supplementary Material for additional experimental procedures and data.

Results

PCSK9 is overexpressed in liver cancers

Using available transcriptomic databases, we showed elevated gene expression of many genes, and among them *PCSK9* and its transcription factor *SREBF2* (Figure 1A). Both genes are significantly upregulated in both adult (HCC) and pediatric liver cancers according to the data from Wu *et al.*, and Lopez-Terrada *et al.*, posted in R2: Genomics Analysis and Visualization Platform (Figure 1B). Since SREBP2 transcription factor is the main regulator of lipid metabolism genes (Figure 1A), the expression of its coding gene *SREBF2* is correlated with that of *PCSK9*. The correlation was stronger in HB than HCC (Figure 1C and Figure 1S). The expression of other lipid metabolism genes in HB showed reduction in LDLR and increase in HMGCR levels with strong correlations of all these genes among each others (Figure 1S). We confirmed the same tendencies in 3 liver cancer cell lines (e.g., HepG2, Huh6 and Huh7) in comparison with the normal liver cell line THLE2 (Figure 1D). IHC analyses of PCSK9 in tumoral tissues indicate more heterogeneous expression pattern, with low and high expression levels (Figure 1E and 1F). Interestingly, unlike the rather diffuse cytoplasmic expression of PCSK9 observed in adjacent non-tumoral tissues and normal liver, the expression in tumoral tissues appeared to be more perinuclear and nuclear (Figure 1E and 1F). This later observation was

noticed in both low and high PCSK9 expressing tumoral tissues. These findings suggest some differential type of regulation and/or impact of this enzyme in the context of liver cancer cells. We next ask how the inhibition of PCSK9 would affect the whole oncogenic process.



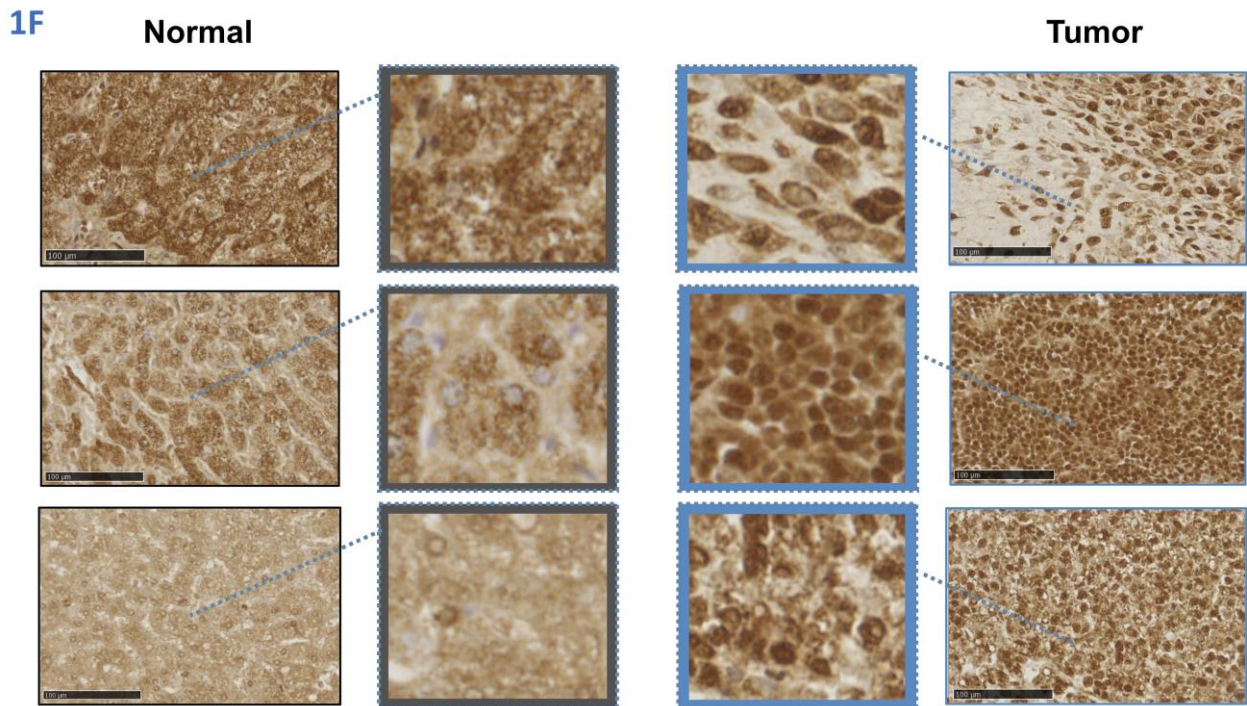


Figure 1: Lipid metabolism defects in liver cancers.

- (A) PCSK9 regulation and function. (1) The synthesis of PCSK9 and LDLR is regulated transcriptionally by SREBP-2. Once translated, they will travel through the ER and Golgi apparatus for maturation before being secreted. (2) LDLR expressed on the cell surface will bind to LDL-C and will be internalized in an AHR-dependent manner *via* clathrin-coated endosomes. Inside the endosomes, LDL-C will dissociate from the receptor and will be directed toward lysosomal degradation, whilst LDLR will be recycled back to the cell surface. (3) Extracellular and (4) intracellular pathway regulation of LDLR by PCSK9: in both pathways, PCSK9 binds to the EGF-like domain of LDLR and targets it to lysosomal degradation instead of recycling.
- (B) *PCSK9* and *SREBF2* gene expression upregulation in 2 transcriptomic datasets generated from R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>). Upper histograms, hepatocellular carcinoma (HCC): Wu – 134 – MAS 5.0 – u133p2. Lower histograms, hepatoblastoma (HB): López-Terrada – 55- fRMA – u133p2. Unpaired t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.
- (C) Two-tailed Pearson correlation shows a positive correlation between *PCSK9* and *SREBF2* gene expression in the same datasets as in B. Left, HCC. Right, HB.
- (D) Normalized gene expression of *PCSK9* and *SREBF2* in three liver cancer cell lines (HepG2, Huh6 and Huh7) and one normal cell line (TLHE2).
- (E) IHC labeling of *PCSK9* in HB tumoral and adjacent non-tumoral tissues. Staining of the whole tissue sections is shown in included subsets in which red squares and arrows indicate the depicted enlarged area of staining. Non-tumoral tissue is normal liver area adjacent to the tumor, as indicated. Samples were collected as described in materials and methods. The black bars represent 100 μm .
- (F) *PCSK9* staining of HB tumoral tissues was compared to normal liver tissues from the same patients.

Targeting of PCSK9 inhibits cells growth and migration without disrupting bioenergetics

To inhibit PCSK9 pharmacologically, we used the anti-secretagogue R-IMPP [10]. First, we confirmed the strong inhibition of PCSK9 by this molecule in the Huh7 cell line, which was 81 and 92% at 10 μ M and 30 μ M, respectively, after 72 hours of treatment (Figure 2S). Then, we assessed the effect of this drug on cell proliferation using the MTS assay (Figure 2A). Our analysis showed a dose dependent inhibition of cell growth in all 3 cell lines, HepG2, Huh6 and Huh7. Huh6 was the most sensitive to the drug with an IC_{50} (at day 3) of 10 μ M vs 14 μ M and 24 μ M for Huh7 and HepG2, respectively. Similar to the clinical approach of combining anti-PCSK9 with statins, we treated our cells with R-IMPP combined with different concentration of simvastatin e.g., 25 μ M for Huh7 and HepG2 and 5 μ M for Huh6. These values were chosen in accordance with the specific sensitivity to this statin determined for each cell line (Figure 3S). The MTS analyses showed synergetic effects on the inhibition of cell proliferation in the presence of both drugs for the Huh7 and HepG2 cell lines but not for the Huh6 cell line, for which R-IMPP appeared to have no significant effect in the presence of SV on cell proliferation (Table 1, Figure 2B, Figure 3S).

Table 1: Proliferation inhibition by R-IMPP or/and simvastatin (SV) of various liver cancer cell lines.

	Huh6	Huh7	HepG2
R-IMPP	10 μ M	14 μ M	24 μ M
SV	2 μ M	17 μ M	39 μ M
R-IMPP + SV^a	8 μ M	11 μ M	17 μ M

The IC_{50} values are given in μ M.

^aThe SV concentration used in the presence of R-IMPP was 5 μ M for Huh6 and 25 μ M for Huh7 and HepG2.

To find out more about the effects on cancer-relevant hallmarks e.g., cell migration, we performed a wound-healing assay with the Huh7 cell line using the IncuCyte system (Figure 2C). R-IMPP treatment at 10 μ M (lower than the IC_{50}) was effective in reducing cell migration by 26% after 24 hours (Figure 2C). This effect was comparable to the action of 25 μ M simvastatin (33% after 24 hours, Figure 2C, Figure 4S). By combining both drugs, inhibition

rate reached 36% after 24 hours, indicating no significant gain compared to simvastatin alone (Figure 4S).

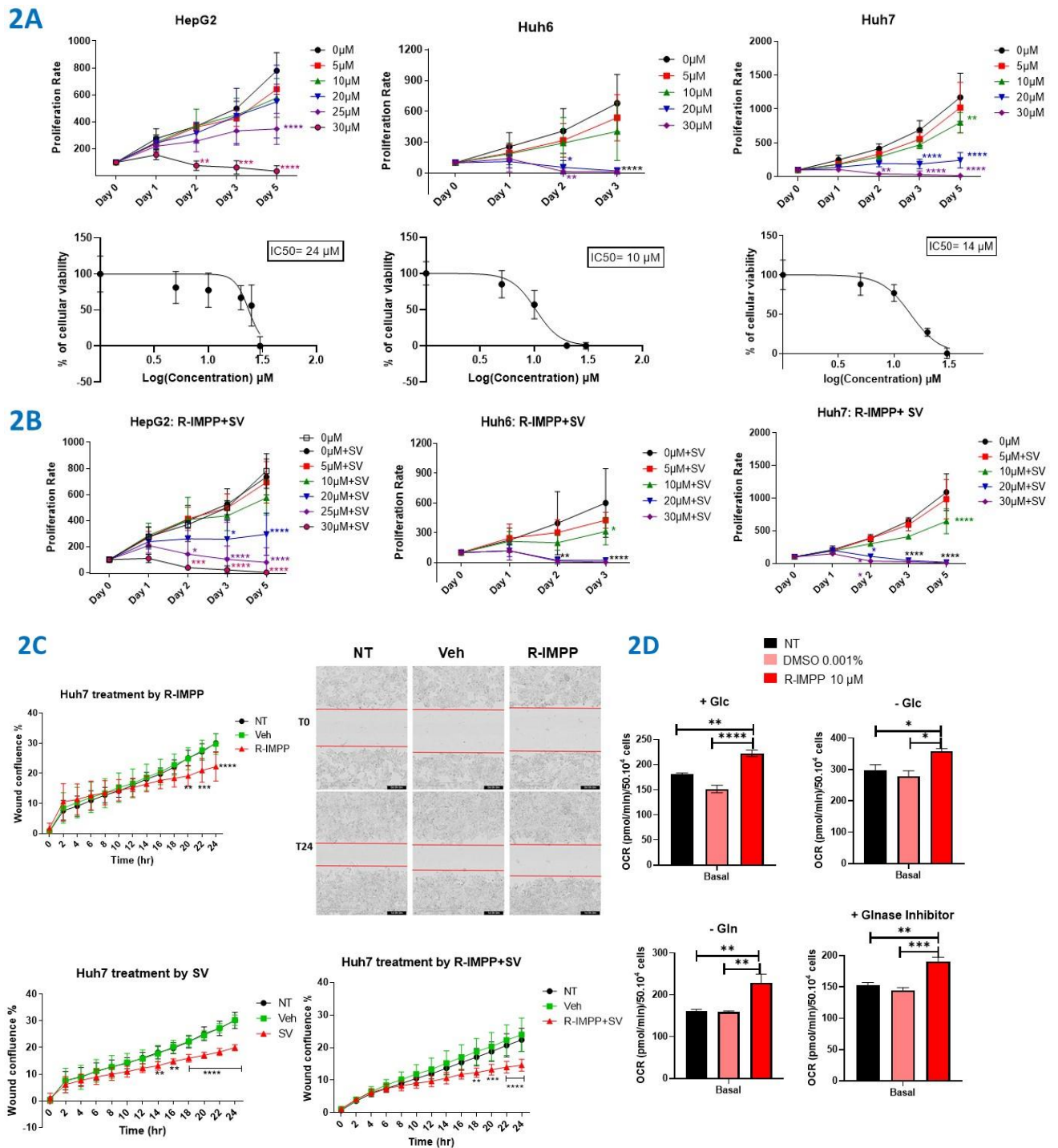


Figure 2: Effect of pharmacological inhibition of PCSK9 by R-IMPP on cellular proliferation, migration and mitochondrial respiration.

(A) Dose dependent inhibition of cell growth by R-IMPP as evaluated by MTS analyses. IC_{50} at day 3 is given for each cell line. *Two-way anova test.

(B) Effect of R-IMPP treatment in combination with simvastatin (SV). The concentrations of SV were selected based on separate analyses of the statin effect alone (25 μ M SV for Huh7 and HepG2 and 5 μ M for Huh6). *Two-way anova test.

(C) Effect of inhibition of PCSK9 by R-IMPP and/or HMGCR by simvastatin (SV) on Huh7 cell migration in a wound healing assay using IncuCyte. Wound-healing confluence was followed for up to 24 hours after treatment with 10 μ M R-IMPP or 25 μ M SV or a combination of both. NT refers to non-treated cells. Veh stands for R-IMPP (0.001% DMSO) and SV (0.001% ethanol) solvents. *Two-way anova test.

(D) Oxygen consumption rate (OCR) was evaluated by the Seahorse assay in Huh7 cells, which were incubated in different media settings, as indicated. NT: non-treated. Ordinary one-way anova test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

To explore energy metabolism, an emerging hallmark of cancer, we measured the oxygen consumption rate (OCR) of the Huh7 cell line using the Seahorse XF96 technology (Agilent). The results revealed a stimulation of OCR in complete medium, in the absence of glucose (mitochondrial respiration independent of glycolysis) or in the presence of a glutaminase inhibitor (Figure 2D). These bioenergetic data indicate a stimulation of oxidative phosphorylation independent of glucose or glutamine as energy substrates in cells that do not secrete PCSK9.

In addition to the pharmacological inhibition of PCSK9, we sought more specific way of targeting the enzyme e.g., by siRNA approach. We designed 2 different specific sequences of siRNA (si1 and si2), which were validated for PCSK9 production inhibition by western blot (Figure 3A). Three days after transfection, 96% and 99% of PCSK9 were lost in Huh7 and Huh6, respectively with the 2 tested siRNAs, while in HepG2, 87% and 95% of PCSK9 were lost with si1 and si2, respectively. Next, we verified whether this approach was effective as well in inhibiting cell proliferation (Figure 3B). Transfection with both siRNAs was comparatively effective in slowing down proliferation of all cell lines tested, with some fluctuations in Huh7 where si2 exhibited stronger inhibition effect than si1 (Figure 3B). Interestingly, lower doses of simvastatin were needed to inhibit growth in these Huh7 and HepG2 cell lines deficient for PCSK9 (Figure 3C). Indeed, 10 μ M instead of 25 μ M were enough to significantly slow down cell proliferation of untreated Huh7 and HepG2 cells (Figure 3S). Huh6 was the best responder to this statin in this condition (1 μ M) as well as in the presence of PCSK9 (2 μ M) (Figure 3S).

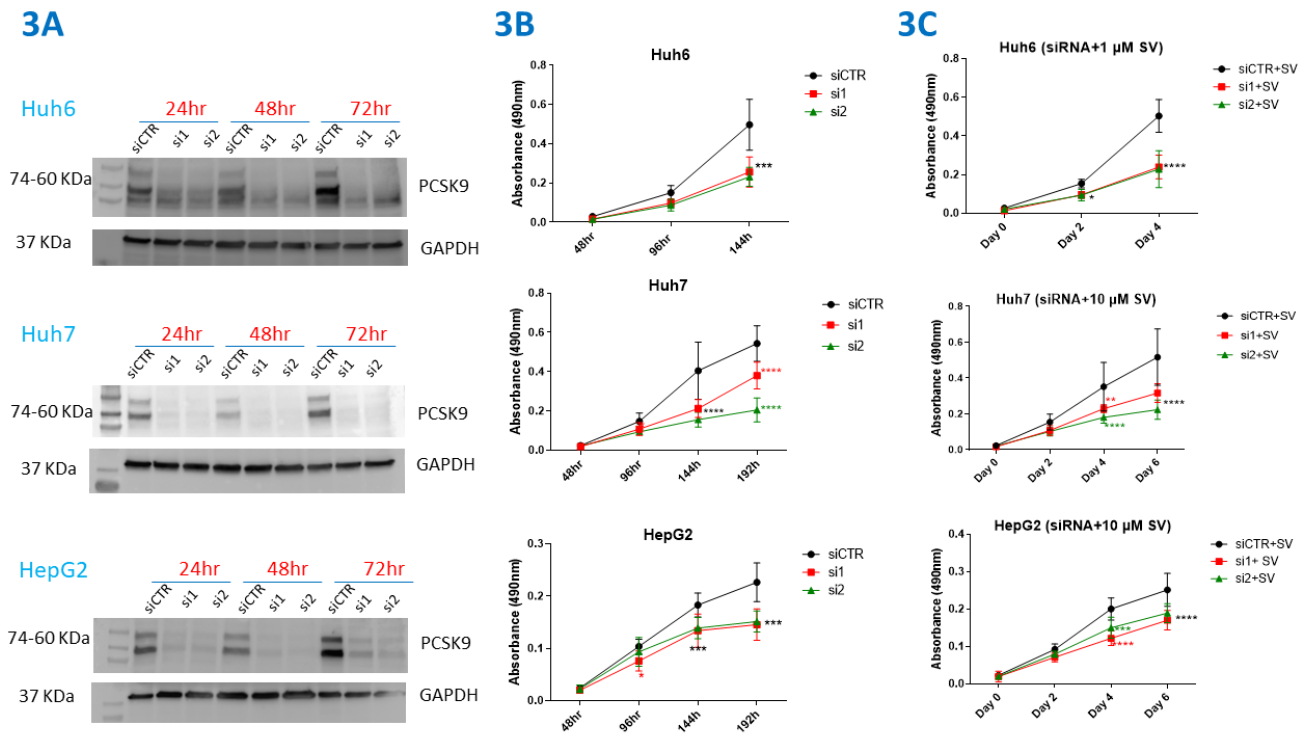


Figure 3: Effect of PCSK9 silencing by siRNA on liver cancer cell proliferation.

(A) Validation of depletion of PCSK9 by siRNA silencing. Cell extracts were prepared 24, 48 and 72 hours after transfection with control siRNA (siCTR) or targeting PCSK9 (si1 and si2) of Huh6 or Huh7 or HepG2 cells. 40 μ g of cell proteins were loaded per lane on an SDS-PAGE. After electrophoresis and transfer, the membrane was analyzed by western blot using anti-PCSK9 antibodies and GAPDH antibodies for comparison of loading. The two bands observed for PCSK9 correspond to the pro-PCSK9 (74 kDa) and cleaved PCSK9 (60 kDa).

(B) Evaluation of liver cancer cell proliferation by MTS after transfection with siRNA targeting PCSK9 (si1 and si2) in comparison with control siRNA (siCTR).

(C) Effect of siRNA silencing of PCSK9 in combination with 1 μ M (Huh6) or 10 μ M (Huh7 and HepG2) simvastatin (SV).

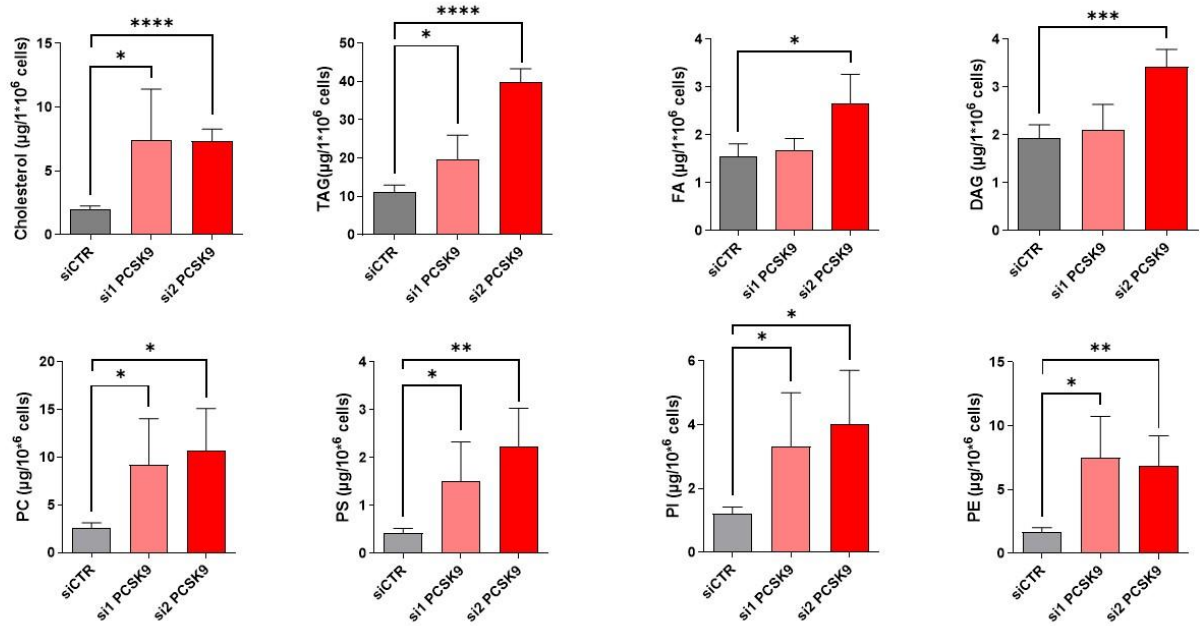
Two-way anova test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

PCSK9 silencing disrupts lipid metabolism and leads to excessive accumulation of lipids in cancer cells

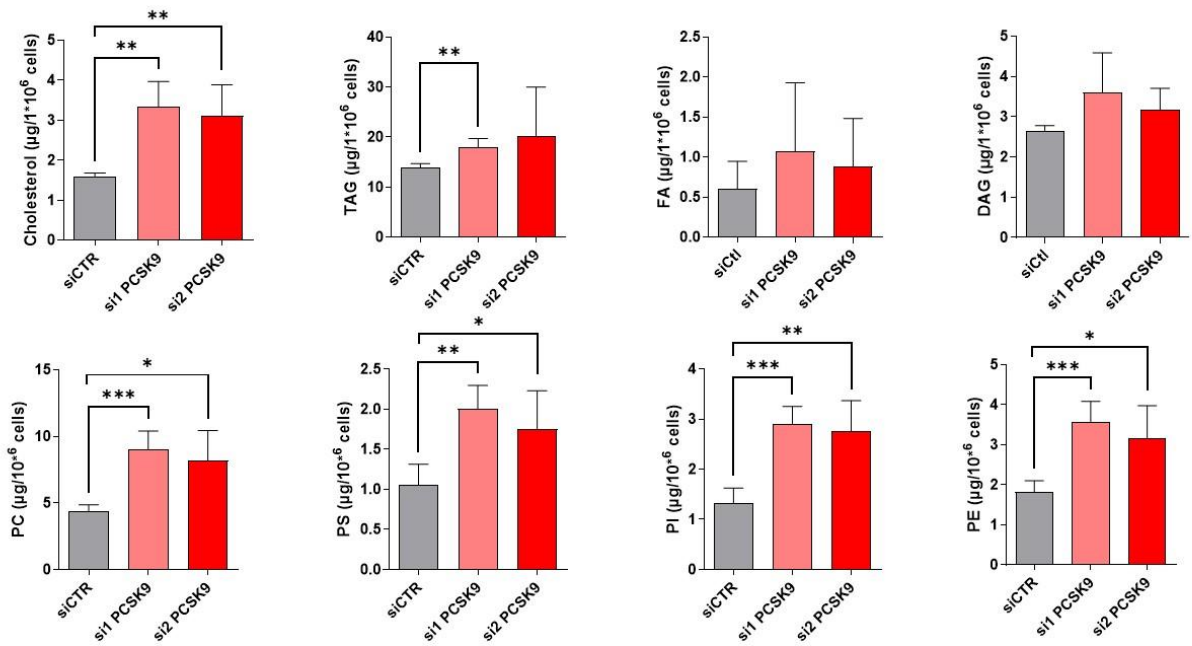
PCSK9 is essentially involved in maintaining adequate cellular lipid fluxes by modulating several lipid receptors at the cell surface. Because of this important role in lipid metabolism, we quantified major neutral lipid species and phospholipids in Huh7 and HepG2 cells in the presence or absence of PCSK9 expression. The extent of changes in lipid amount among both types of cells was really important (Figure 4A & 4B). We observed some variations between both siRNA and cell types. Nonetheless, most of the lipid entities were significantly higher in PCSK9-inactivated cells, with a 3 to 5-fold increase for some lipids such as cholesterol and phospholipids in HepG2 cells (Figure 4A). These data highlight a major disruption of the lipid

homeostasis. To investigate possible changes in lipogenesis, we fed the HepG2 cells with [1-¹⁴C] acetate to measure the enrichment in different lipid metabolites (Figure 5S). The results showed a more active and elevated lipogenesis in the absence of PCSK9 after 4 hours. By examining the saturation levels of FA (Figure 4C), we observed a borderline increase in total unsaturated FA ($p=0.06$) after siRNA treatment, although the increase was significant globally for PUFA and for C18:2 FA as an example. The increase of PUFA amount in the absence of PCSK9 probably resulted from enhanced uptake of external sources of lipids and thereby leading to the enrichment in peroxidation-prone intracellular FA. In order to verify whether lipid peroxidation is enhanced in this case, we measured the levels of lipid hydroperoxide in HepG2 cells 4, 6 and 8 days after transfection with PCSK9 siRNA or control siRNA (Figure 4D). Indeed, inhibition of PCSK9 led to significant increase of the amount of lipid hydroperoxide at all time points which is probably the consequence of the presence of higher oxidation sensitive PUFA in the absence of PCSK9. Globally, when PCSK9 is silenced, cells seem to be gearing up all machineries to take up and synthesize more lipids, generating quantitative and qualitative imbalances in intracellular lipids. Because of the toxicity of lipids such as free cholesterol, cells store the excess of fat in lipid droplets. For these reasons, we used the high-resolution electron microscopy to take a closer look at the cellular constituents, searching for any possible abnormalities. Indeed, the presence of lipid droplets was very obvious at lower and higher magnification by tomography electron microscopy (Figure 4E).

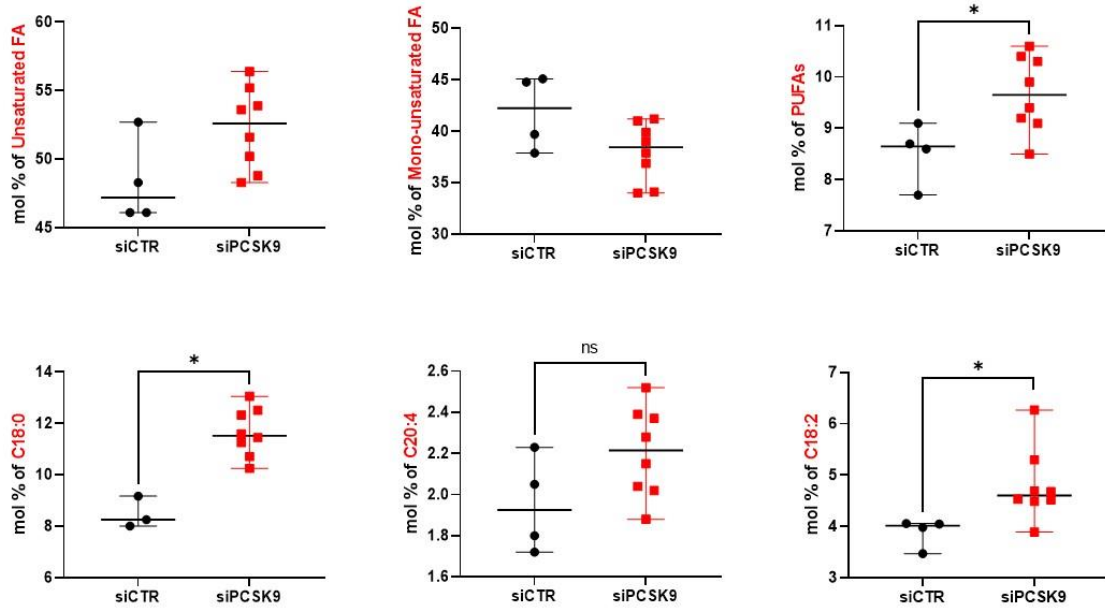
4A HepG2



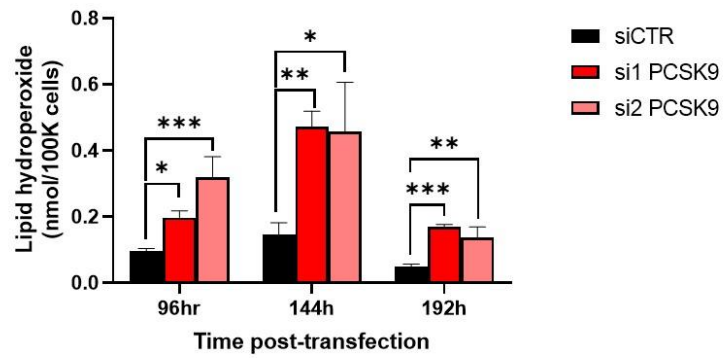
4B Huh7



4C



4D



4E

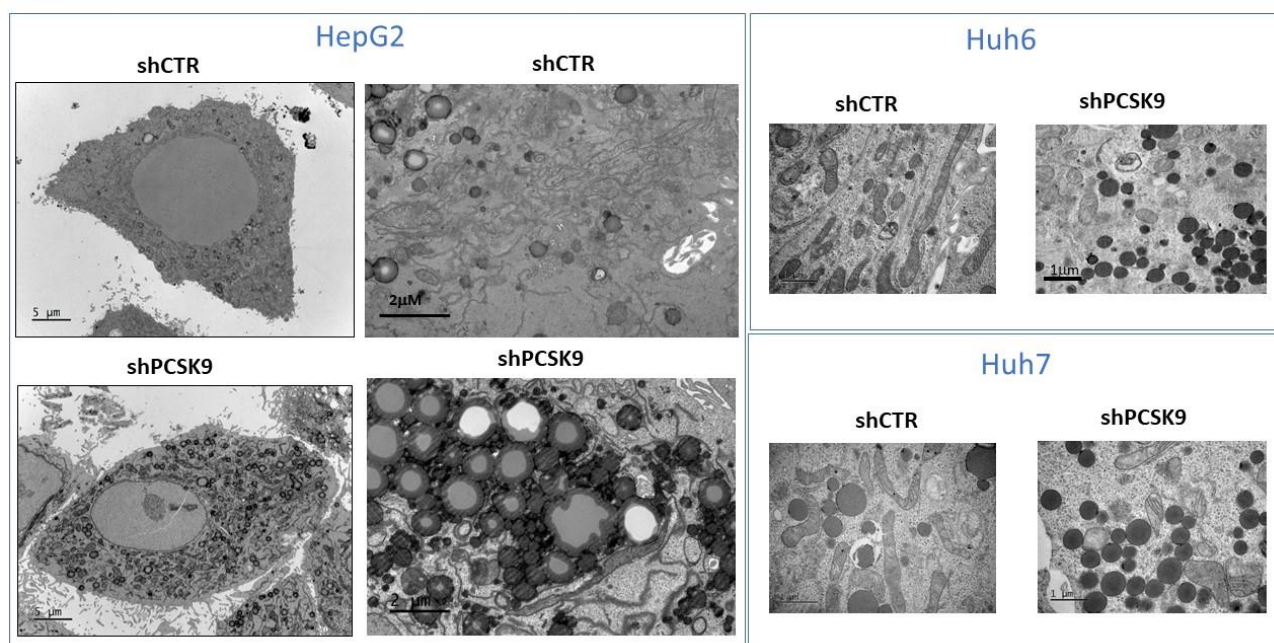


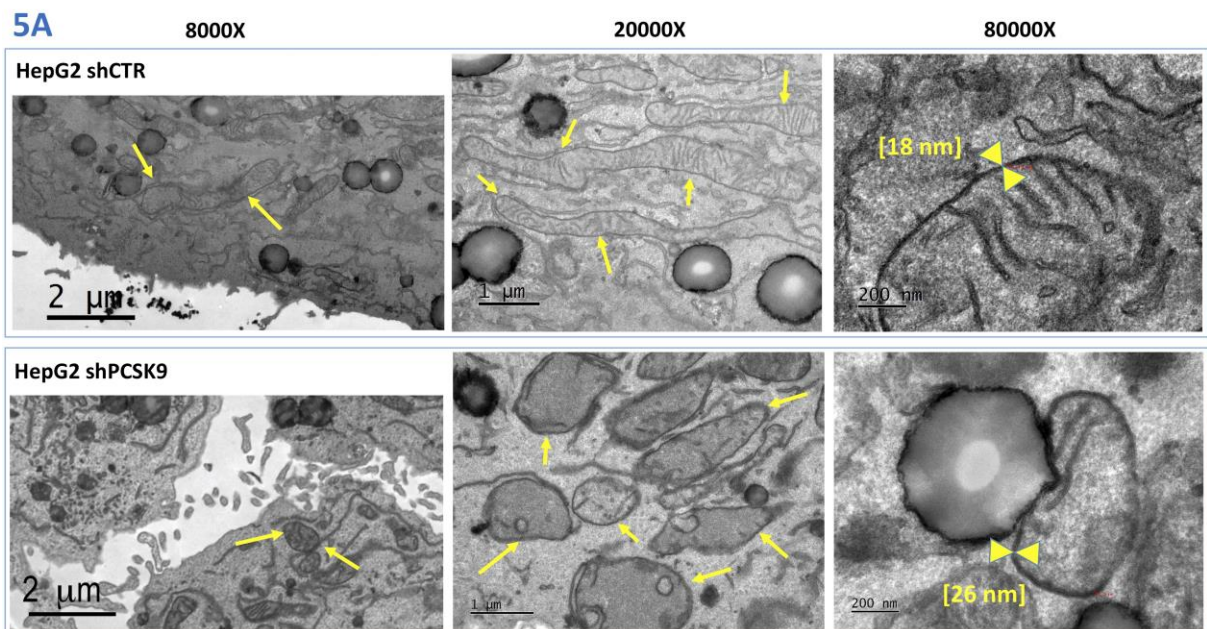
Figure 4: Quantification of neutral lipids, phospholipids, lipid peroxidation and lipid droplets after PCSK9 silencing.

(A and B) Cholesterol, triacyl glycerol (TAG), fatty acids (FA) diacyl glycerol (DAG), phosphatidyl -choline (PC), -serine (PS), -inositol (PI), and -ethanolamine (PE) were measured in HepG2 (A) and Huh7 (B) after transfection with PCSK9 siRNA or control for 144 hours. Unpaired T-test where each siRNA PCSK9 group is compared to CTR group alone, (C) FA composition and saturation in HepG2 cells silenced or not for PCSK9. *Mann-Whitney t-test. (D) Lipid hydroperoxide quantification in HepG2 cells after transfection with PCSK9 siRNA (si1 and si2) or control for 96h, 144h and 192h. Quantities are represented in nmol per 100,000 cells. *Ordinary one-way anova test. (E) Transmission electron microscopy (TEM) photomicrographs of liver cancer cell lines transduced with specific PCSK9 shRNA (shPCSK9) or control shRNA (shCTR) (Transduction efficiency in [Figure 6S](#)). Dark and dense particles seen at lower and higher magnification are lipid droplets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

PCSK9 inhibition fails to activate the anti-oxidative p62/Keap1/Nrf2 pathway triggering ferroptosis

In the absence of any confirmed sign of cell death by apoptosis/necrosis and senescence (data not shown), we investigated features of other mechanisms. Taking into account the lipid phenotype of PCSK9 inhibition, the high level of lipid hydroperoxide and the possible induction of cell toxicity, we turned our attention to the potential occurrence of the lipid-peroxide triggered cell death by ferroptosis. Electron microscopy observations revealed obvious changes of the mitochondrial morphology consisting in size shrinkage, thickening of the double bilayer membranes and disappearance of mitochondrial cristae ([Figure 5A](#)). This last observation was confirmed by measuring the thickness of the mitochondrial double

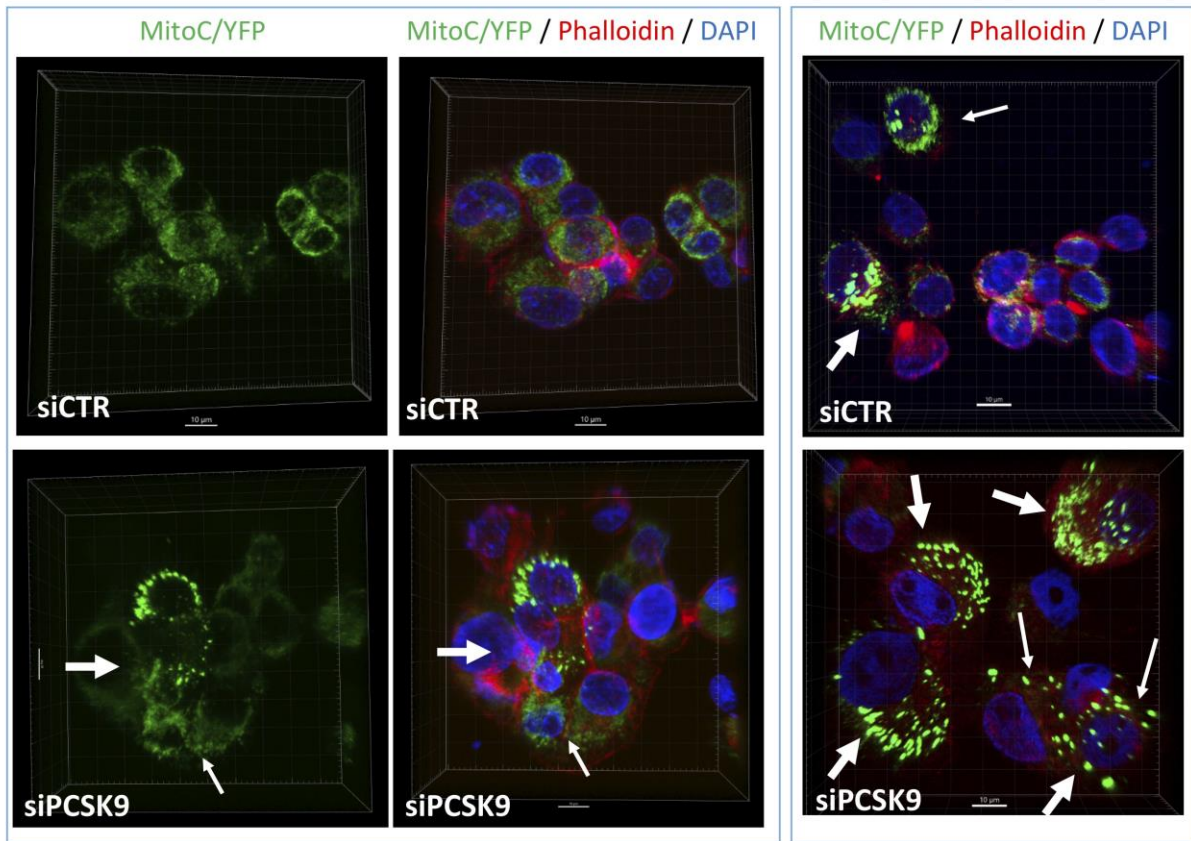
membrane, which was larger in liver cells lacking PCSK9 (Figure 5A, right panels). For optimal visualization of the mitochondria, we created a lentivirus that expresses a chimeric CFP/YFP protein, which was initially designed to visualize mitochondrial ATP levels in living cells [13]. We observed, using confocal microscopy, that some cells presented mitochondria with brighter and denser fluorescence (Figure 5B), indicating the probe was accumulating into these mitochondria. The frequencies of these events were much higher in cells depleted for PCSK9 48 and 72 hours after siRNA transfections. The fluorescence signal was more diffuse in cells transfected with the control siRNA, indicating different mitochondria morphology and potentially higher ATP levels in PCSK9 deleted cells. This has to be considered in regard to the increased OCR capacity observed in the absence of PCSK9 (Figure 2D).



5B

48 hrs

72 hrs



5C

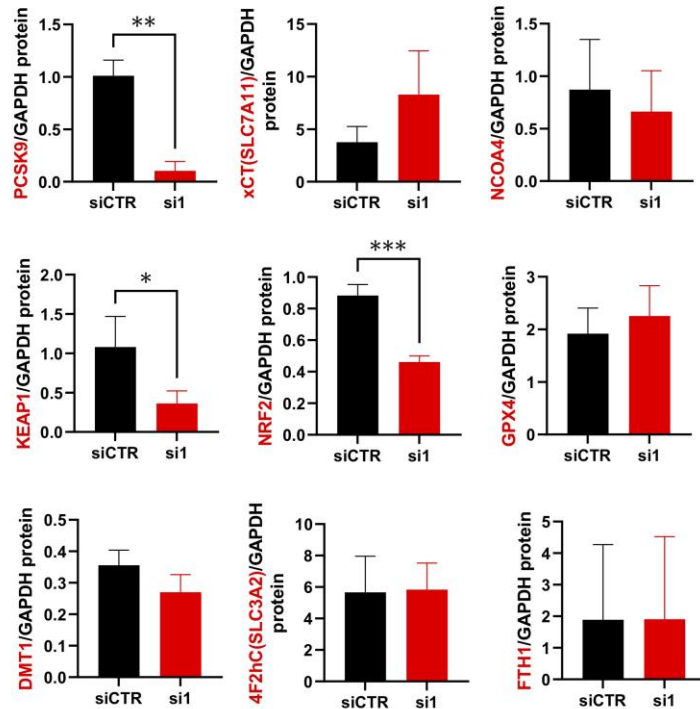
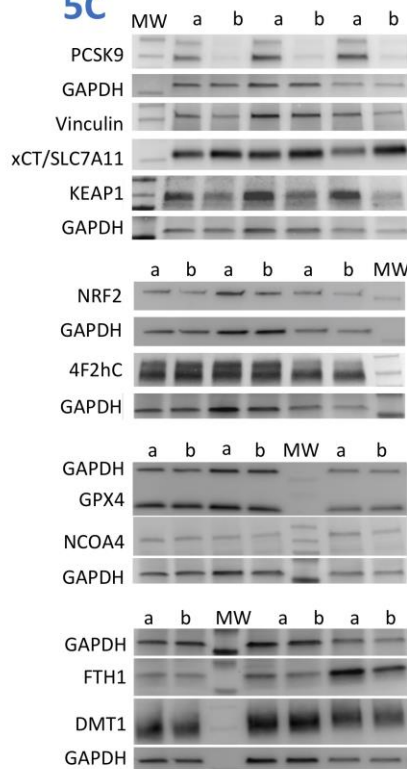


Figure 5: Mitochondria morphological changes induced by PCSK9 silencing and analyses of cell signaling typical of ferroptosis.

(A) TEM photomicrographs of HepG2 cells transduced with PCSK9 shRNA (top panels) and control shRNA (bottom panels) at different magnifications. Arrows point to mitochondria. The mitochondrial membranes were measured at the higher magnification of 80,000 (between yellow head arrows) and the results are indicated between brackets.

(B) Confocal microscopy observation of HepG2 cells performed 48 and 72 hours after transfection with PCSK9 siRNA (si1) and siCTR. Mitochondria were labelled with C/YFP using a specific lentivirus targeting system (MitoC/YFP). Phalloidin (red) and DAPI (blue) were used for microfilament and nuclear DNA staining, respectively. Arrows indicate bright mitochondria network observed more frequently when PCSK9 is silenced.

(C) Western blot analyses of proteins involved in different signaling pathways of ferroptosis. Membrane images of each protein and its relative housekeeping gene were combined/fused for comparison purposes. Wells were loaded alternatively with samples from siCTR (a) and siPCSK9 (b) transfected cells (from left to right). MW stands for molecular weight markers. GAPDH or vinculin were used for protein normalization. Unpaired T-test, * $p < 0,05$; ** $p < 0,01$, *** $p < 0.001$ (n=3).

To investigate the potential occurrence of ferroptosis at the molecular level, we assessed the changes of some proteins of the main cell signaling pathways described for this process e.g., the Xc/GPX4, MVA, the sulfur transfer pathway, the P62-Keap1-NRF2 pathway, the P53/SLC7A11, ATG5-ATG7-NCOA4 pathway, the P53-SAT1-ALOX15 pathway, and the HSPB1-TRF1, FSP1-COQ10-NAD(P)H pathway (Figure 7S). Western blot analyses of protein extracts from cells transfected by one of the PCSK9 specific siRNA, si1 and the control siRNA were performed on the following proteins: xCT/SLC7A11, GPX4, Keap1, NRF2, FTH1, NCOA4, 4F2hC / CD98 and DMT1 (Figure 5C). The only significant changes were observed in the Keap1 and Nrf2 of the p62/keap1/NRF2 pathway with a 2.75-fold decrease for Keap1 ($p=0.04$) and a 2-fold decrease for NRF2 ($p=0.0008$) in cells transfected with anti-PCSK9 siRNA as compared to controls.

Blocking PCSK9 has anti-tumoral effects in zebrafish and CAM *in vivo* models

To validate the effect of blocking PCSK9 *in vivo*, we used an established model e.g., the zebrafish model xenografted with HepG2 labeled with tomato red fluorescence by lentivirus transduction. After sorting by FACS, fluorescent cells were transduced with an empty lentivirus or encoding an shRNA against PCSK9 (shPCSK9). As can be seen in Figure 6, the cell proliferation as followed by *in vivo* fluorescence labeling was significantly reduced 24 hours and 48 hours after injection of the cells transduced with shPCSK9.

6

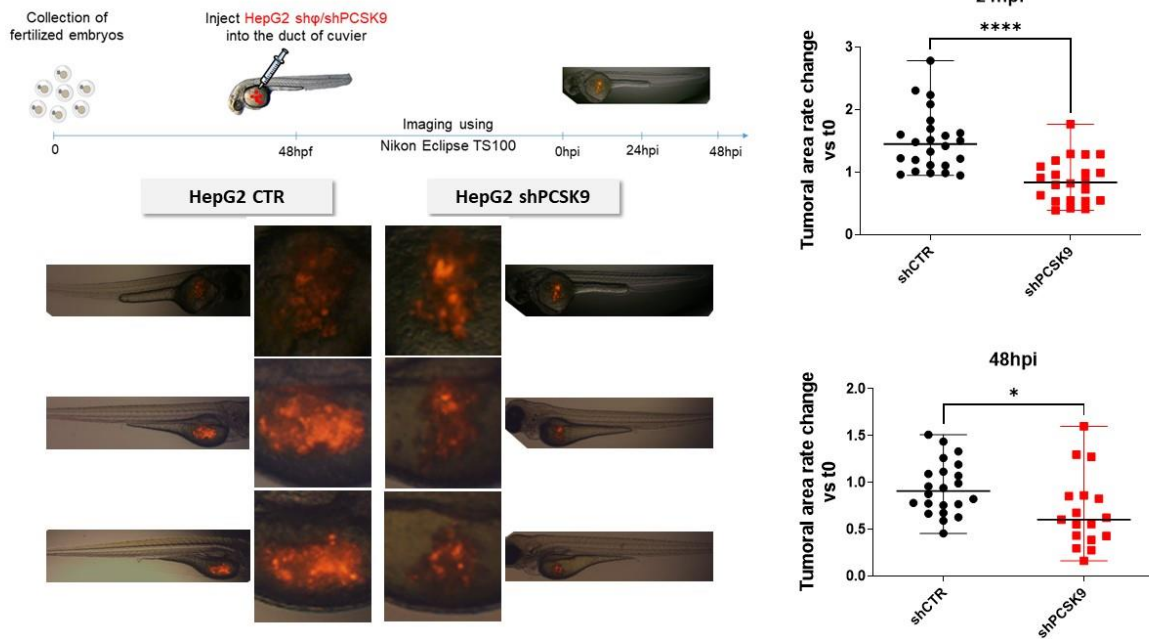


Figure 6: Depleting PCSK9 impairs liver tumor development in vivo.

In zebrafish experiments: tomato red fluorescent labeled HepG2 with shPCSK9 or control were xenografted in zebrafish embryos. Tumoral cell growth was evaluated by the quantification of the fluorescence 24 and 48 hours post injection (hpi).

Unpaired t- test, *: $p < 0.05$, ****: $p < 0.0001$

Discussion

Lipid metabolism plays a central role in liver oncogenesis and the important role of PCSK9 in lipid homeostasis places it at the center of the stage as an attractive target in liver cancers. PCSK9 modulation of lipid metabolism arises not only from LDLR binding and targeting for lysosomal destruction, but also from the degradation of other lipoprotein receptors such as VLDLR, ApoER2, CD36, and LRP1. Therefore, it was not unconceivable to expect the alteration of lipid homeostasis we have observed in liver cancer cells in the absence of PCSK9; lipid receptors madly play when PCSK9 is away.

We report in this paper many anti-oncogenic features of anti-PCSK9 approaches including the reduction of cell proliferation and migration and the triggering of lipid cytotoxicity in liver tumor cells. Lipid metabolism was strongly affected by this approach, whose disruption probably leads to massive accumulation of lipid droplets, peroxidation of fatty acids and probably generation of overwhelming oxidative stress. In the meantime, anti-oxidative

defenses failed to reactivate. The increased oxidative burden and the failure of protective measures trigger irreversible cell damages and death by ferroptosis.

Ferroptosis mode of cell death was discovered in recent years. It is linked to iron-dependent lipid peroxidation triggering the death process [14]. Unlike other modes of cell death, the distinctive morphological features of ferroptosis are obvious at the mitochondrial level with shrinkage of the organelle, increased membrane density and reduction or disappearance of mitochondrial cristae. The integrity of cell membrane is not affected and the nucleus is normal in size with no chromatin condensation [14]–[16]. Intriguingly, no bioenergetics failure is seen during this process unlike other cell death types. To the contrary, intracellular ATP content seems to be even higher in cells undergoing ferroptosis [14].

Herein, we recognize all these ferroptosis morphological features in cells silenced for PCSK9 in parallel to a higher bioenergetics' status. Specific targeting and fluorescent labeling of mitochondria allowed to visualize mitochondria undergoing a heavy and concentrated organelle clustering in accordance with the structure of the mitochondrial network during ferroptosis (Figure 5B).

Moreover, our search for a potential ferroptosis signaling pathways singles out the Keap1/Nrf2 axis. Indeed, PCSK9 depletion led to significant reduction of Nrf2, which is the chief coordinator of the antioxidant response machinery [17]. In normal conditions, Keap1 controls the basal cytoplasmic level of Nrf2 by promoting its degradation by the ubiquitin-proteasome system. Under oxidative stress conditions, Keap1 -as a redox sensor- gets oxidized and dissociates from Nrf2, which becomes activated and translocates into the nucleus, where it can induce the transcription of different branches of the antioxidant defense system. In HCC, Nrf2 is hyperactivated promoting the survival of cancer cells and also conferring cellular resistance to chemotherapeutic drugs [18]–[20].

In our experimental context, although Nrf2 is downregulated in the absence of PCSK9, its dissociation from Keap1 and its nuclear translocation indicate that the oxidative sensor system is still functional albeit unable to launch effective antioxidative responses, as seen by the lack of activation of FTH1, one of Nrf2 downstream targets.

It is of note that among the main mechanisms accounting for the biological effects of sorafenib, the only approved first-line drug for advanced HCC, is the induction of ferroptosis

[21], probably by inhibiting the SLC7A11 transporter [14]. Although sorafenib treatment improves the survival to some extent, unfortunate severe adverse effects and emerging resistance make it an unsatisfactory therapeutic approach [22]. Interestingly, many strategies to improve sorafenib resistance are aimed at modulating ferroptosis, which is thus emerging as a potential new weapon in the fight against cancer. Some of these strategies are targeting NRF2 itself. Indeed, genetic or pharmacological inhibition of NRF2 in HCC cells makes them more sensitive to the action of sorafenib and even overcomes chemoresistance through the induction of ferroptosis [23].

Above all, since disruption of PCSK9 inhibits the anti-ferroptosis p62-Keap1-NRF2 pathway, one could speculate that a combination therapy of anti-PCSK9 with sorafenib would alleviate drug resistance and improve prognosis.

In parallel to its involvement in ferroptosis through the p62-Keap1-NRF2 pathway, PCSK9 may have impact on this death process through a mechanism that depends on its cholesterol-regulating functions. Indeed, by enhancing lipid/lipoprotein uptake, PCSK9 deficiency may enrich the membrane in PUFAs, which are sensitive to lipid peroxidation and are one of the essential elements for ferroptosis [24]. Free PUFAs can be esterified into membrane phospholipids before being oxidized and thereby inducing ferroptosis signals. Moreover, among some of highly produced/imported phospholipids following PCSK9 depletion phosphatidylethanolamine (PE) was increased 2 to 4 times (Figure 4). Oxidized PEs are the key phospholipids acting as death signals to induce ferroptosis in cells [25]. In this process, 15-lipoxygenase is an important contributor to pro-ferroptosis PE peroxidation capable of generating doubly and triply-oxygenated diacylated PE species. Tocopherols and vitamin E suppress this oxygenation and protect against ferroptosis [25]. Therefore, PCSK9 inhibition may trigger ferroptosis in lipid dependent and independent fashions.

Apart from its role in lipid homeostasis, PCSK9 is involved in various signaling pathways including antiviral activity, apoptosis and more recently anti-tumor immune responses. Indeed, Liu *et al.* [26] demonstrated that the blockade of PCSK9 can boost immunotherapeutic efficacy of anti PD-1 approach. The research involved PCSK9 in the degradation of MHC1, hindering its recycling at the cell surface. Hence depletion of this enzyme results in high cellular expression of MHC1, leading to massive infiltration of cytotoxic T cells.

As per our results, PCSK9 may now modulate cancer survival and resistance by an additional mechanism consisting in exerting anti-oxidative housekeeping activities. The additional benefit of blocking PCSK9 would be to create stressful conditions related to lipid peroxidation, impair the safeguarding anti-oxidation system, and therefore sensitize cancer cells to ferroptosis.

Taking into account all these anti-tumoral effects of anti-PCSK9 approaches and the existence of a wide variety of therapeutic strategies of PCSK9 blockade (monoclonal antibodies, small molecule and peptide inhibitors, antisense oligonucleotides, siRNA, etc.), we believe this enzyme is a very valuable and attractive target for the potential treatment of liver cancers.

Overall, the analyses of the lipid metabolism signature of hepatic cancers cells led us to target a specific metabolic network controlled by PCSK9. Beside its critical role in modulating lipid metabolism and fluxes, our research brings out a previously unknown function of this intriguing enzyme e.g., maintaining the redox homeostasis *via* the p62/keap1/Nrf2 axis. The inhibition of PCSK9 led to excessive lipid accumulation and created a void in the defense against oxidative stress thereby enhancing the vulnerability of cancer cells to ferroptosis. Specific targeting of PCSK9 in hepatic cancer cells showed strong beneficial outcomes and novel mechanistic insights highlighting a new player of cancer cell death by ferroptosis. Taken altogether, our experimental evidences support the argument of using available drugs targeting this enzyme in a repositioning approach to treat liver cancers.

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Abbreviations

A

Alox15: arachidonate 15-lipoxygenase
ApoB: apolipoprotein B
ApoER2: apolipoprotein E receptor 2
ATG5: autophagy related 5
ATG7: autophagy related 7

C

CAM: chick chorioallantoic membrane
CD36: cluster of differentiation 36
CD81: cluster of differentiation 81
CoQ₁₀: coenzyme Q₁₀

F

FA: fatty acid
FH: familial hypercholesterolemia
FSP1: ferroptosis suppresson protein 1

G

GPX4: glutathione peroxidase 4

H

HB(L): hepatoblastoma
HCC: hepatocellular carcinoma
HMGR: 3-hydroxy 3-methylglutaryl Coenzyme A reductase
HSPB1: heat shock protein beta 1

I

IC₅₀: half-maximal inhibitory concentration
IHC: immunohistochemistry

K

Keap1: kelch-like ECH-associated protein 1

L

LDL-C: low density lipoprotein cholesterol
LDLR: low density lipoprotein receptor
LRP1: LDLR related protein 1

M

mAbs: monoclonal antibodies
MHC1: major histocompatibility complex 1
mRNA: messenger ribonucleic acid
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MVA: mevalonate pathway

N

NAD(P)H: nicotinamide adenine dinucleotide (phosphate) + hydrogen
NCOA4: nuclear receptor coactivator 4
Nrf2: nuclear factor erythroid 2-related factor 2

O

OCR: oxygen consumption rate

P

PCSK9: proprotein convertase subtilisin/kexin type 9
PE: phosphatidylethanolamine
PUFA: polyunsaturated fatty acid
P53: tumor protein p53
P62: also called sequestosome 1

R

R-IMPP: (R)-N-(isoquinolin-1-yl)-3-(4-methoxyphenyl)-N-(piperidin-3-yl) propanamide

S

SAT1: spermidine/spermine N1-acetyltransferase 1
shRNA: short hairpin ribonucleic acid
siRNA: small interfering ribonucleic acid
SLC7A11: solute carrier family 7 member 11
SRE: sterol regulatory element
SREBP2/SREBF2: sterol regulatory element binding transcription factor 2

T

TFR1: transferrin receptor 1

V

VLDLR: very low-density lipoprotein receptor

X

Xc⁻: System Xc⁻ cysteine/glutamate antiporter
xCT: alternative name of SLC7A1

References

- [1] T. Mashima, H. Seimiya, and T. Tsuruo, "De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy," *Br. J. Cancer*, vol. 100, no. 9, pp. 1369–1372, May 2009, doi: 10.1038/sj.bjc.6605007.
- [2] E. Rysman *et al.*, "De novo Lipogenesis Protects Cancer Cells from Free Radicals and Chemotherapeutics by Promoting Membrane Lipid Saturation," *Cancer Res.*, vol. 70, no. 20, pp. 8117–8126, Oct. 2010, doi: 10.1158/0008-5472.CAN-09-3871.
- [3] K. Mahboobnia *et al.*, "PCSK9 and cancer: Rethinking the link," *Biomed. Pharmacother.*, vol. 140, p. 111758, Aug. 2021, doi: 10.1016/j.biopha.2021.111758.
- [4] M. Wiciński, J. Żak, B. Malinowski, G. Popek, and G. Grzešek, "PCSK9 signaling pathways and their potential importance in clinical practice," *EPMA J.*, vol. 8, no. 4, pp. 391–402, Dec. 2017, doi: 10.1007/s13167-017-0106-6.
- [5] R. Schulz, K.-D. Schlüter, and U. Laufs, "Molecular and cellular function of the proprotein convertase subtilisin/kexin type 9 (PCSK9)," *Basic Res. Cardiol.*, vol. 110, no. 2, p. 4, Jan. 2015, doi: 10.1007/s00395-015-0463-z.
- [6] J. D. Horton, J. L. Goldstein, and M. S. Brown, "SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver," *J. Clin. Invest.*, vol. 109, no. 9, pp. 1125–1131, May 2002, doi: 10.1172/JCI15593.
- [7] Y. N. Lamb, "Inclisiran: First Approval," *Drugs*, vol. 81, no. 3, pp. 389–395, 2021, doi: 10.1007/s40265-021-01473-6.
- [8] "Novartis receives EU approval for Leqvio®* (inclisiran), a first-in-class siRNA to lower cholesterol with two doses a year**," *Novartis*. <https://www.novartis.com/news/media-releases/novartis-receives-eu-approval-leqvio-inclisiran-first-class-sirna-lower-cholesterol-two-doses-year> (accessed Aug. 31, 2021).
- [9] F. J. Raal *et al.*, "Inclisiran for the Treatment of Heterozygous Familial Hypercholesterolemia," *N Engl J Med*, p. 11, 2020.
- [10] D. N. Petersen *et al.*, "A Small-Molecule Anti-secretagogue of PCSK9 Targets the 80S Ribosome to Inhibit PCSK9 Protein Translation," *Cell Chem. Biol.*, vol. 23, no. 11, pp. 1362–1371, Nov. 2016, doi: 10.1016/j.chembiol.2016.08.016.
- [11] M. Bhat *et al.*, "Decreased PCSK9 expression in human hepatocellular carcinoma," *BMC Gastroenterol.*, vol. 15, Dec. 2015, doi: 10.1186/s12876-015-0371-6.
- [12] S.-Z. Zhang *et al.*, "PCSK9 promotes tumor growth by inhibiting tumor cell apoptosis in hepatocellular carcinoma," *Exp. Hematol. Oncol.*, vol. 10, p. 25, Mar. 2021, doi: 10.1186/s40164-021-00218-1.
- [13] H. Imamura *et al.*, "Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 37, pp. 15651–15656, Sep. 2009, doi: 10.1073/pnas.0904764106.
- [14] S. J. Dixon *et al.*, "Ferroptosis: An Iron-Dependent Form of Non-Apoptotic Cell Death," *Cell*, vol. 149, no. 5, pp. 1060–1072, May 2012, doi: 10.1016/j.cell.2012.03.042.
- [15] W. S. Yang and B. R. Stockwell, "Synthetic Lethal Screening Identifies Compounds Activating Iron-Dependent, Nonapoptotic Cell Death in Oncogenic-RAS-Harboring Cancer Cells," *Chem. Biol.*, vol. 15, no. 3, pp. 234–245, Mar. 2008, doi: 10.1016/j.chembiol.2008.02.010.
- [16] N. Yagoda *et al.*, "RAS–RAF–MEK-dependent oxidative cell death involving voltage-dependent anion channels," *Nature*, vol. 447, no. 7146, pp. 864–868, Jun. 2007, doi: 10.1038/nature05859.

- [17] H. Motohashi and M. Yamamoto, "Nrf2–Keap1 defines a physiologically important stress response mechanism," *Trends Mol. Med.*, vol. 10, no. 11, pp. 549–557, Nov. 2004, doi: 10.1016/j.molmed.2004.09.003.
- [18] A. Raghunath, K. Sundarraj, F. Arfuso, G. Sethi, and E. Perumal, "Dysregulation of Nrf2 in Hepatocellular Carcinoma: Role in Cancer Progression and Chemoresistance," *Cancers*, vol. 10, no. 12, p. 481, Dec. 2018, doi: 10.3390/cancers10120481.
- [19] J. D. Hayes, M. McMahon, S. Chowdhry, and A. T. Dinkova-Kostova, "Cancer Chemoprevention Mechanisms Mediated Through the Keap1–Nrf2 Pathway," *Antioxid. Redox Signal.*, vol. 13, no. 11, pp. 1713–1748, Dec. 2010, doi: 10.1089/ars.2010.3221.
- [20] X.-J. Wang *et al.*, "Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2," *Carcinogenesis*, vol. 29, no. 6, pp. 1235–1243, Jun. 2008, doi: 10.1093/carcin/bgn095.
- [21] C. Louandre *et al.*, "The retinoblastoma (Rb) protein regulates ferroptosis induced by sorafenib in human hepatocellular carcinoma cells," *Cancer Lett.*, vol. 356, no. 2, Part B, pp. 971–977, Jan. 2015, doi: 10.1016/j.canlet.2014.11.014.
- [22] J. Nie, B. Lin, M. Zhou, L. Wu, and T. Zheng, "Role of ferroptosis in hepatocellular carcinoma," *J. Cancer Res. Clin. Oncol.*, vol. 144, no. 12, pp. 2329–2337, Dec. 2018, doi: 10.1007/s00432-018-2740-3.
- [23] X. Sun *et al.*, "Activation of the p62-Keap1-NRF2 Pathway Protects against Ferroptosis in Hepatocellular Carcinoma Cells," *Hepatol. Baltim. Md*, vol. 63, no. 1, pp. 173–184, Jan. 2016, doi: 10.1002/hep.28251.
- [24] W. S. Yang and B. R. Stockwell, "Ferroptosis: death by lipid peroxidation," *Trends Cell Biol.*, vol. 26, no. 3, pp. 165–176, Mar. 2016, doi: 10.1016/j.tcb.2015.10.014.
- [25] V. E. Kagan *et al.*, "Oxidized Arachidonic/Adrenic Phosphatidylethanolamines Navigate Cells to Ferroptosis," *Nat. Chem. Biol.*, vol. 13, no. 1, pp. 81–90, Jan. 2017, doi: 10.1038/nchembio.2238.
- [26] X. Liu *et al.*, "PCSK9 inhibition potentiates cancer immune checkpoint therapy," *Nature*, vol. 588, no. 7839, pp. 693–698, Dec. 2020, doi: 10.1038/s41586-020-2911-7.

Supplementary Material

Supplementary Experimental Procedures

Transcriptomic data acquisition

The R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) was used to generate the gene expression data from different available datasets. In this study, three different datasets were selected: Mixed Hepatoblastoma - López-Terrada – 55- fRMA – u133p2 (GEO ID: [gse75271](#)) [1], Mixed Tumor HCC – Wu – 134 – MAS50 (GEO ID: [gse45436](#)) [2] and Mixed Hepatoblastoma - Raymond - 62 - deseq2_rlog - ensh38e82 (GEO ID: [gse104766](#)) [3]. The expression of lipid-related genes such as, *PCSK9*, *LDLR*, *SREBF2* and *HMGCR* and the correlation between them were checked. Numeric data of gene expression were downloaded in excel files and graphs were generated using GraphPad Prism 9 software (GraphPad Software, Inc.). In addition, the level of expression of lipid-related genes in hepatic cell lines was generated by referring to the transcriptomic data done on these cell lines by Hooks *et al.* [3].

RNA sequencing analysis

Total RNA from Huh7 and THLE-2 cell lines was extracted using the *mirVana* kit (Thermo Fisher Scientific) according to the supplier's protocol and the analysis was done by Hooks *et al.* [3] in a manner similar to what they've done for the other cell lines and patient tissues.

Cell Culture

Human HCC (Huh7) and HBL (HepG2, Huh6) derived cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM GlutaMAX™ supplemented, with high (4.5g/L) for Huh7 and HepG2 or low (1g/L) D-glucose for Huh6) (Gibco, Invitrogen), supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cell line authentication was performed on April 2021 using short tandem repeats (LGC, Molsheim, France) and the absence of mycoplasma contamination was tested on a monthly basis.

Lentivirus production and transduction

Lentivirus vector production was done by the Vect'UB service platform, (INSERM US 005-CNRS UMS 3427- TBM-Core, Université de Bordeaux, France). Lentiviral particles were produced by

transient transfection of HEK293T (human embryonic kidney cells) according to standard protocols. In brief, subconfluent HEK293T cells were co-transfected with lentiviral genome (psPAX2) (gift from Didier Trono (Addgene plasmid # 12260), with an envelope coding plasmid (pMD2G-VSVG) and with vector constructs (305 pLKO-sh886 or 306 pLKO-shCTR) by calcium phosphate precipitation. LVs were harvested 48 hours post-transfection and concentrated by ultrafiltration, Viral titers of VSV-g pseudotype pLV lentivectors were determined by transducing HEK293T cells with serial dilutions of viral supernatant and lentiviral integration was evaluated by quantitative-PCR using RRE primers. The following forward (F) and reverse (R) sequences of shPCSK9-886 were used:

F-5' CCGGGGGTCATGGTCACCGACTTCGCTCGAGCGAAGTCGGTGACCATGACCCTTTTT-3' and R-5' AATTCAAAAAGGGTCATGGTCACCGACTTCGCTCGAGCGAAGTCGGTGACCATGACCC 3'. The hairpin sequence of negative control shRNA is:

CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG

(<http://www.addgene.org/pgvec1?f=c&identifier=1864&atqx=plko&cmd=findpl>)

HepG2 cells stably expressing tomato transgene were generated by lentivirus transduction at a multiplicity of infection (MOI) of 10 with an MND-Tomato-265 virus (donated by the Vect'UB platform). Red fluorescent cells were sorted by Fluorescence-Activated Cell Sorting (FACS). Stable inhibition of PCSK9 expression was induced by cell transduction with the lentivirus 305 pLKO-sh886 (shPCSK9) or the control (306 pLKO-shCTR) at an MOI of 10. Transduced cells were selected using puromycin (P8833, Sigma) at 3µg/mL.

siRNA transfection

Small interfering siRNAs (si1 {sense: 5' GUGCUC AACUGCCAAGGGA[dT][dT] 3'; anti-sense: 5' UCCCUUGGCAGUUGAGCAC[dT][dT] 3'}) and si2 {sense: 5' GGGUCAUGGUCACCGACUU[dT][dT] 3'; anti-sense: 5' AAGUCGGUGACCAUGACCC[dT][dT] 3'}) against PCSK9 (Sigma Aldrich) were diluted in 1X siMAX dilution buffer (30 mM HEPES, 100 mM KCl, 1 mM MgCl₂, pH 7.3, Eurofins). Hepatic cancer cells were transfected independently with 20 nM si1 or 2 or control siCTR (AllStars Negative Control siRNA, Qiagen) using lipofectamine RNAi MAX transfection reagent (Invitrogen) according to manufacturer's instructions of reverse transfection. For transfection, Lipofectamine RNAi MAX was diluted 1/100th in transfection medium (OptiMEM, Gibco).

Chemical Inhibitors

Different inhibitors that regulate lipid metabolism pathways were bought from SelleckChemicals (Houston, USA), including one HMGCR inhibitor that blocks the mevalonate pathway simvastatin (S1796), and one PCSK9 inhibitor called R-IMPP (S8420). The drugs were dissolved in Dimethyl sulfoxide (DMSO), except for simvastatin, and were stored at -20°C. All of these drugs were tested at multiple doses in the 3 cell lines. Simvastatin requires to be manually activated by dissolving 50 mg in 1 mL of warm (50°C) ethanol and adding 0.813 mL of 1 N NaOH. It is left for 30 min to allow the conversion of simvastatin to the active acid form. Finally, pH is adjusted to 7.2 using small quantities of 1 N HCl.

Proliferation assay

Cells were seeded into 96-well plates in triplicates at various densities (3000 C/well for Huh7 and HepG2; 700-2000 C/well for Huh6) and then treated with various concentrations of simvastatin (0-100 µM) and R-IMPP (0-30 µM). The proliferation of cells was assessed for 5 days using CellTiter 96® AQueous One Solution Reagent (Promega) and the absorbance was recorded at 490 nm using ClarioStar (BMG Labtech, Champigny sur-Marne, France).

Western Blot

Cells were lysed in RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics) and centrifuged at 13,000 rpm for 15 min at 4°C. Protein concentration was determined using the Pierce™ BCA protein assay kit (Thermo Scientific). Approximately 40 µg of proteins were loaded per lane for western blot analyses in 4-15% precast polyacrylamide gel (BioRad) and blotted onto 0.2 µm nitrocellulose membrane (BioRad). The membranes were blocked in 5% BSA in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20), then incubated with each of the following specific primary antibodies: sheep anti-PCSK9 (1 µg/mL, AF3888, R&D systems), ferroptosis antibody sampler kit (Cell signaling, 29650), rabbit anti-HO-1/HMOX1 (1:3000, 10701-1-AP, ProteinTech), mouse anti-NQO1 (1:7000, 67240-1-Ig, ProteinTech), mouse anti-GAPDH HRP conjugated (1:10000, BLE649203, BioLegend) and goat anti-vinculin (1:1000, sc-7649, Santa Cruz Biotechnology) overnight at 4°C. After incubation with the appropriate secondary antibody coupled with horseradish peroxidase (goat anti-mouse IgG (H+L) HRP, 1:3000, 170-6516, BioRad; rabbit anti-goat IgG HRP, 1:1000, HAF017, R&D Systems; goat anti-rabbit IgG HRP, 1:5000, A0545, Sigma; rabbit

anti-sheep IgG HRP, 1:3000, 402100, Calbiochem), all blots were revealed with Fusion FX (Vilber Lourmat) following incubation with the ECL reagents from BioRad. Quantification was performed using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Migration Assay

2×10^4 Huh7 cells were seeded per well in an IncuCyte® ImageLock 96-well plate in the late afternoon (confluence ~90%). In the morning, scratch wounds of 700-800 micron wide were made using the IncuCyte® WoundMaker, a 96-pin wound making tool. The cells were washed twice with 1X PBS, and a fresh medium containing the different drugs was added into the corresponding wells. The migration assay was monitored by the IncuCyte S3 live-cell analysis system (Essen BioScience, Ltd, Royston Hertfordshire, United Kingdom) up to 24 hours, where images were taken every 2 hours.

Seahorse XF Cell Mito Stress Test

Huh7 cells treated with 10 μ M R-IMP for 48 hours were seeded (4 replicates) in XFe96 Cell Culture Microplate (Agilent technologies, #102416-100) at 80-90% confluency in DMEM GlutaMAX™ supplemented with 10% FBS. They were incubated overnight at 37°C in 5% CO₂ atmosphere. XFe96 Sensor cartridge was hydrated in calibration solution overnight at 37°C in a non-CO₂ incubator. On the day of the experiment, the medium was removed and replaced with 160 μ L of Seahorse XF DMEM Medium pH 7.4 (Agilent Technologies, #103575-100) supplemented with: i) 1 mM pyruvate, 2 mM glutamine, 5 mM glucose [**+Glc**]; ii) 2 mM glutamine [**-Glc**]; iii) 1 mM pyruvate, 5 mM glucose, [**-Gln**], iv) 2 mM glutamine, 40 μ M BPTES (glutaminase inhibitor, Sigma, SML0601) [**+Glnase Inhibitor**]. Cells were incubated in a CO₂ free incubator at 37°C for 1 hour. During that time, the compound working solutions were prepared from stocks at the following concentrations: 7.5 μ M oligomycin (O4876, Sigma), 2 μ M rotenone (R8875, Sigma), 8 μ M antimycin (A8674, Sigma) and 7.5 μ M CCCP (C2759, Sigma). 20 μ L of the solutions are then loaded into the sensor cartridge in their respective ports A, B and C.

To run the assay, prepare the software with the necessary information and plate map, also indicate the number and order of injections. Start by inserting the sensor cartridge to calibrate it in Agilent Seahorse XFe/XF Analyzer before replacing the calibration plate with cell culture

plate. The oxygen consumption rate (OCR) was measured upon the injection of prepared compound solutions into cells, based on the designed protocol.

Lipidomics

The extraction of lipids from cell pellets was performed by chloroform:methanol (2:1, v/v) following the original Folch method with a CHCl_3 :MeOH:H₂O ratio of 8:4:3 (v/v). For this purpose, 0.5 mL methanol and 1 mL chloroform were added directly to the cell pellet. The suspension was incubated for 30 min on ice with repeated vortexing. After the addition of water to separate the aqueous and organic phases, the mixture was incubated on ice for an additional 10 min. The samples were then centrifuged at 1000 x g for 5 min. The organic phase was transferred to a new tube. The aqueous layer was re-extracted with 2 mL chloroform:methanol (2:1, v/v). The chloroform layers were combined, evaporated to dryness and resuspended in 100 μL chloroform:methanol (1:1, v/v).

Phospholipids were analyzed by loading 25 μL of total lipids onto HPTLC plates (60F254, Merck, Germany) and developed with methyl acetate/n-propanol/chloroform/methanol/0.25% aqueous KCl (5:5:5:2:1.8, v/v) as solvent. Neutral lipids were analyzed by loading 25 μL of total lipids onto HPTLC plates and developed with hexane/ethyl ether/formic acid (10:5:0.5, v/v).

For lipid quantification, the plates were then immersed in a copper acetate solution (3% copper acid + 8% phosphoric acid in distilled water) and heated at 115°C for 30 min. Lipids were identified by co-migration with known standards and quantified by densitometric analysis using a TLC scanner (CAMAG, Switzerland).

Radiolabeling experiment

For radiolabeling experiments, the counted cells of each sample were transferred to a glass tube in 6 mL of DMEM medium. To start the reaction, 200 nmol (10 μCi) of [$1\text{-}^{14}\text{C}$] acetate (PerkinElmer Life Sciences) were added to each tube and the tubes were incubated at 37°C in 5% CO₂. The uptake of acetate was studied for each sample at 3 different time points (1 hour, 2 hours and 4 hours). To stop the reaction, the samples were centrifuged at 1000xg for 5 minutes and the supernatants were removed. After addition of 2 mL chloroform/methanol (2:1, v/v), the cells were incubated overnight at -20°C. To separate the aqueous and organic phases, 1 mL of 0.9% NaCl was added, the mixtures were centrifuged at 1000xg for 5 minutes.

The organic phases were transferred to a new tube. The aqueous layer was re-extracted with 2 mL chloroform/methanol (2:1, v/v). The chloroform layers were combined and washed one time with 1 mL 0.9% NaCl. The organic phases were evaporated to dryness, re-suspended in 100 µL chloroform/methanol (2:1, v/v) and stored at -20 °C. Radiolabeled products were analyzed by thin-layer chromatography using HPTLC Silica Gel 60 plates (Merck). Phospholipids were separated with methyl acetate/n-propanol/chloroform/methanol/0.25% aqueous KCl (5:5:5:2:1.8, v/v) as solvent and to separate neutral lipids, a mixture of hexane/ether/formic acid (10:5:0.5, v/v/v) was used as solvent. They were identified by co-migration with unlabeled standards, and quantification was done by autoradiography using a Storm 860 molecular imager (GE Healthcare).

FA Saturation and Lipid Peroxide Analysis

Cell pellets of counted cells were directly used for fatty acid analysis. Fatty acid methyl esters were obtained by transmethylation at 90 °C for 1 h with 0.5 M sulfuric acid in methanol containing 2% (v/v) dimethoxypropane and 50 µg of heptadecanoic acid (C17:0) as internal standards. After cooling, 1 mL of NaCl (2.5%, w/v) was added, and fatty acyl chains were extracted with 1 mL hexane. Samples were subsequently analyzed by GC-MS as described by Domergue *et al.* [4]. The measurement of lipid hydroperoxide was performed using the Lipid Hydroperoxide (LPO) Assay Kit (Cayman Chemical) as instructed by the manufacturer.

Immunofluorescence

For mitochondria fluorescent labeling, cells were transduced by the lentivirus MitoC/YFP at MOI 10 for 24 hours before siRNA transfection. We created this lentivirus by using the pcDNA-MitA1.03 plasmid with a cassette containing a chimera consisting of variants of CFP (mseCFP) and YFP (cp173-mVenus) connected by the Epsilon subunit of *Bacillus subtilis* F₀F₁-ATP synthase and designed to be targeted to mitochondria and to report ATP levels by FRET [5]. Next, 48 and 72-hours transfected HepG2 cells (siCTR and si1) were seeded in duplicate in Nunc™ Lab-Tek™ II Chamber Slide™ System (154534, ThermoFisher Scientific) at 30,000-40,000 cells per well in complete DMEM for 6 hours before fixing them using 4% PFA (paraformaldehyde, 15710, Electron Microscopy Sciences) for 10 min at room temperature (RT). After the removal of PFA, cells were washed three times with PBS (phosphate-buffered saline) and permeabilized with 0,25% Triton® X-100 (Ref. 2000, Euromedex) for 3 min,

followed again by a 3-step wash. Next, blocking was done using 5% BSA (bovine serum albumin fraction V, 10735094001, Roche) dissolved in PBS for 30 min at RT, then followed by incubation for 1-1.5 hour with Phalloidin-FluoProbes® 556 (1:500, FP-BV4620, Interchim). Finally, the slide was washed with PBS and distilled water before putting mounting medium with DAPI (VECTASHIELD®) for nuclear DNA staining and covering it with a cover slip.

The slides are observed with confocal microscope model Leica DM6000 TCS SP5 MP at 20X or 40X magnification in the Photonic Unit of Bordeaux Imaging Center (BIC).

Immunohistochemistry

The 3.5- μ m thick sections of hepatoblastoma tumors were de-paraffinized, rehydrated and antigen retrieval was performed in 0.01 M citrate buffer pH 6 solution. All staining procedures were performed by an autostainer (Dako-Agilent Clara, United States) using standard reagents provided by the manufacturer. The sections were blocked using EnVision™ Flex peroxidase-blocking reagent (SM801, Dako-Agilent) to block endogenous peroxidase, then washed and incubated with rabbit anti-PCSK9 (1:100, 55206-1-AP, ProteinTech). Incubation in horseradish peroxidase (EnVision Flex/HRP, SM802, Dako-Agilent) was used for signal amplification. 3,3'-Diamino-benzidine (DAB, Dako) development was used for detecting primary antibodies by producing a crisp brown end product at the site of the target antigen. The slides were counterstained with hematoxylin, dehydrated and mounted. Each immunohistochemical run contained a negative control (buffer, no primary antibody). Sections were visualized with a Hamamatsu NANOZOOMER 2.0 HT at 20X magnification in the Photonic Unit of Bordeaux Imaging Center (BIC).

Transmission Electron Microscopy

HepG2 cells transduced with shCTR and shPCSK9 were seeded in Nunc™ Lab-Tek™ 8-chamber slide system (ThermoFisher) to a confluence of 80%. The cells were fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) during 2 hours at room temperature (RT), washed in 0.1 M phosphate buffer (pH 7.4) and then post-fixed in 1% osmium tetroxide in water during 1hr. Then samples were washed in water, dehydrated through a series of graded ethanol and embedded in a mixture of pure ethanol and epoxy resin (Epon 812; Delta Microscopy, Toulouse, France) 50/50 (v/v) during 2 hours and then in 100% resin overnight at RT. The polymerization of the resin was carried out over

a period of 48 hours at 60°C. Samples were then sectioned using a diamond knife (Diatome, Biel-Bienne, Switzerland) on an ultramicrotome (EM UC7, Leica Microsystems, Vienna, Austria). Ultrathin sections (70 nm) were picked up on copper grids. Grids were examined with a Transmission Electron Microscope (H7650, Hitachi, Tokyo, Japan) at 80kV.

***In vivo* Zebra fish model**

Zebrafish were maintained at 28°C and in light cycle conditions (12 hours). The *casper* mutant fish line was purchased from the Zebrafish International Resource Center (ZIRC). For zebrafish xenotransplantation, 48 hpf (hour post-fertilization) zebrafish embryos were dechorionated and anaesthetized in egg water solution containing 0.04 mg/mL tricaine (Sigma-Aldrich) before human cell injection. Approximately 200 to 500 fluorescent cells were injected (Eppendorf® Femtojet® microinjector) into the duct of Cuvier of each embryo, and zebrafish were maintained in 0.3X Danieau's solution for 1 hour at 28°C. After confirmation of a visible cell mass at the injection site, zebrafish were transferred to a 24-well plate in 500 µL of 0.3X Danieau's solution incubator and maintained at 34°C. The zebrafish with already formed metastasis at 1 hpi (hour post-injection) were discarded.

After 24 hpi and 48 hpi, living zebrafish embryos were anesthetized using 0.04 mg/mL tricaine and were observed under a fluorescence inverted microscope (Nikon Eclipse TS100). Low magnification (X4 objective) was used to provide an overview of the tumor cell metastasis pattern throughout the fish. Pictures were taken by using the Archimed (Microvision Instruments) software. The Fiji software was used for automated tumor area evaluation. Briefly, a 30-225 intensity threshold was set to select cells and the 'analyze' particle tool was used with default selection of the cell size and cell shape during counting. A Fiji macro was generated using the 'record' function to streamline analyses.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, Inc.). For two-group comparison, we used the *t* test when values are ≥ 15 , otherwise Mann-Whitney rank sum test was used. For quantitative comparisons of more than two samples, One-way ANOVA test was used followed by Bonferroni post-test. Two-way ANOVA followed by Bonferroni post-test was used for experiments containing three groups or more at different time points. For correlation graphs, two-tailed Pearson correlation test was used. The

experiments were carried out, independently, at least 3 times unless otherwise stated. In this case, n = number of independent experiments. A p-value of <0.05 was considered to be statistically significant. For all data in figures, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ or exact p-values were indicated. All tests were two-sided.

Supplementary figures

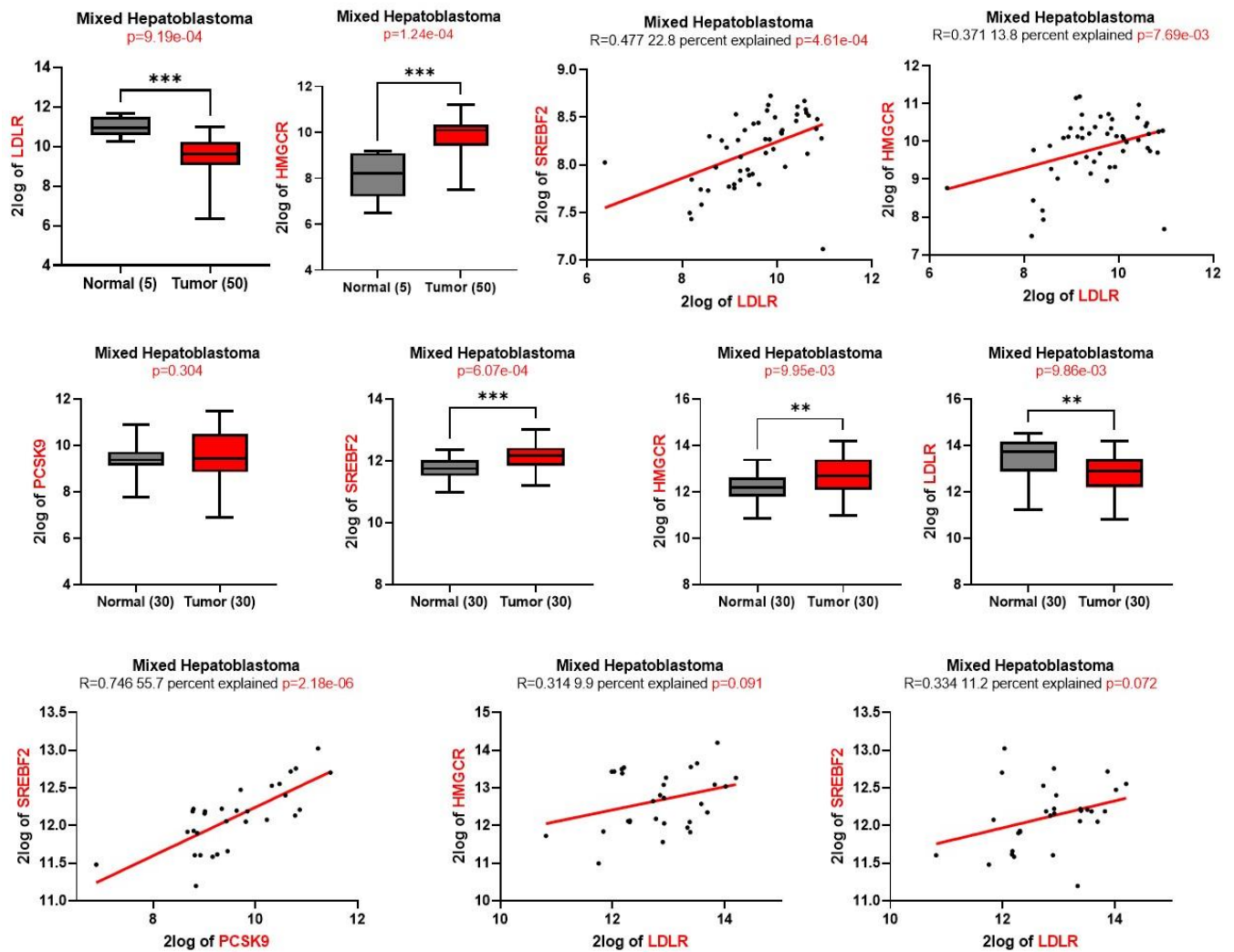


Figure 174S: Additional lipid metabolism defects in liver cancers.

All data shown here are generated from R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>). (*upper panel*)

LDLR and *HMGCRCR* gene expression in hepatoblastoma (HB): López-Terrada – 55- fRMA – u133p2 and the positive

correlation between lipid-related genes. (*middle panel*) *PCSK9*, *SREBF2*, *LDLR* and *HMGCRCR* gene expression in

Hepatoblastoma: Raymond - 62 - deseq2_rlog - ensh38e82 and (*lower panel*) the correlation between the different lipid-related genes. Unpaired t-test was used for gene expression and two-tailed Pearson correlation test was used between the

genes. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001

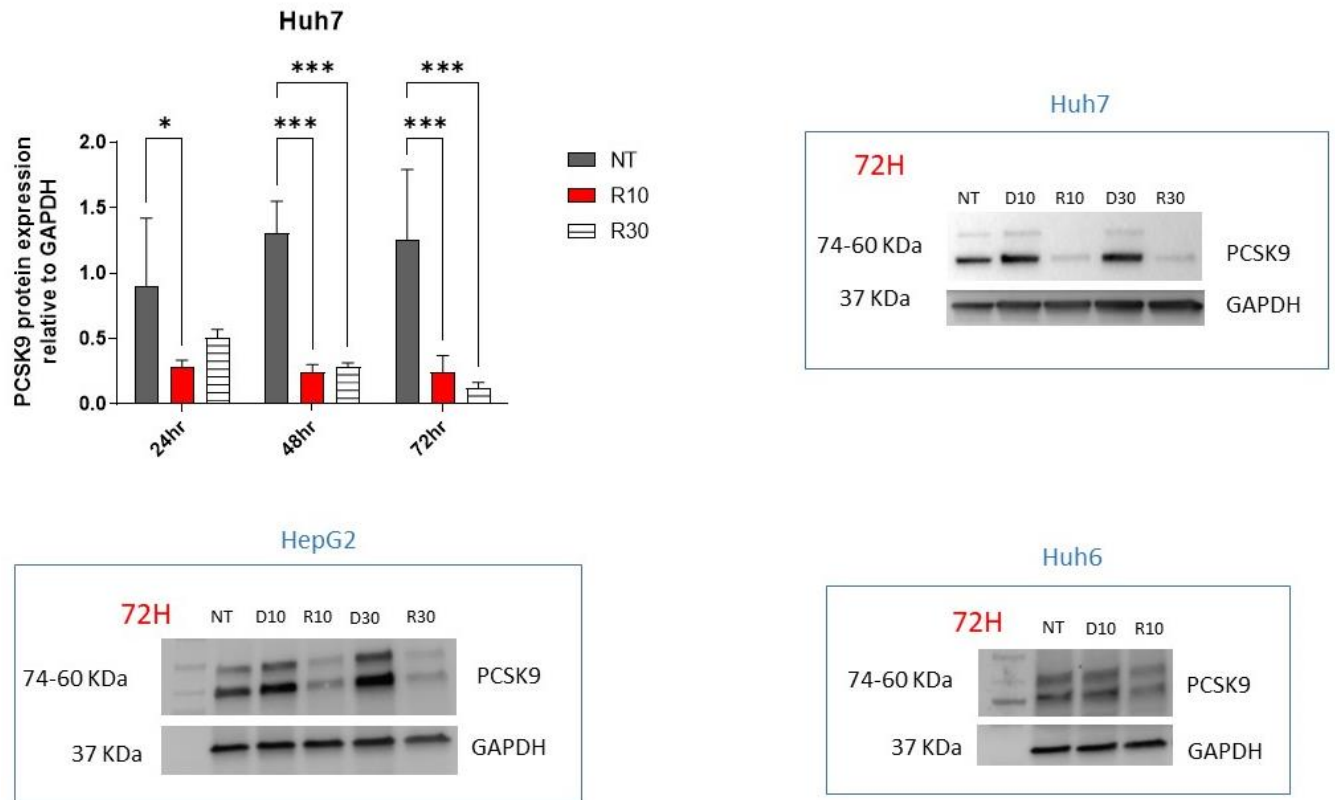


Figure 182: Inhibition of PCSK9 protein level by R-IMPP.

Huh7, HepG2 and Huh6 cells were treated with 10 and 30 μM of R-IMPP. PCSK9 protein level was evaluated by western blot in these 2 conditions 24, 48 and 72 hours after treatment. Internal GAPDH was used for protein normalization (n=3). NT: non-treated; D: DMSO; R: R-IMPP. Two-way anova test, * p<0.05; ** p<0.01; *** p<0.001.

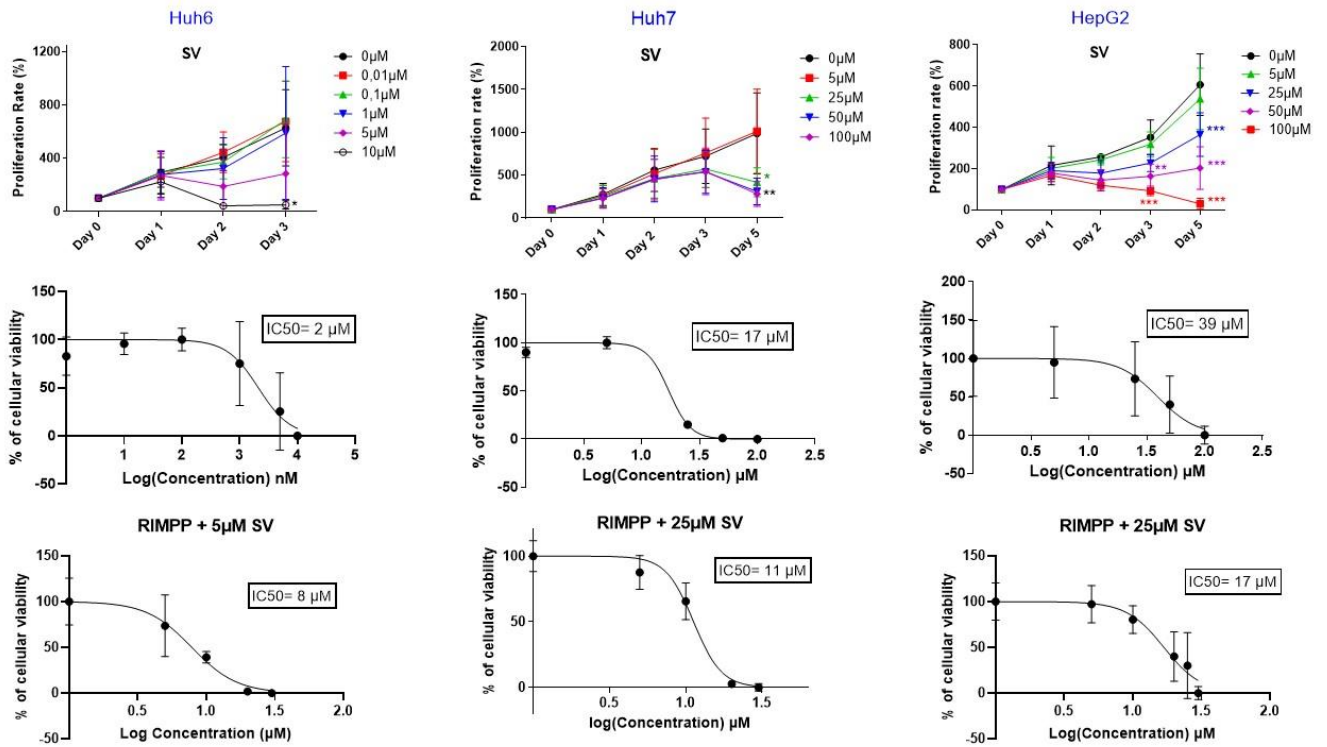


Figure 193S: Effect of Simvastatin on cellular proliferation.

(upper panel) cell growth was evaluated by MTS analysis using 3 liver cancer cell lines, Huh6, Huh7 and HepG2. Proliferation was evaluated up to 5 days after SV treatment except for Huh6 where the experiment was stopped at day 3 because of the extensive cell death at this time point. (middle panel) IC50 of SV treatment at day 3 are shown for the 3 cell lines. (lower panel) IC50 of R-IMP and SV treatment at day 3 are shown for the 3 cell lines. Two-way anova test, * $p < 0.05$; ** $p < 0.01$;

*** $p < 0.001$.

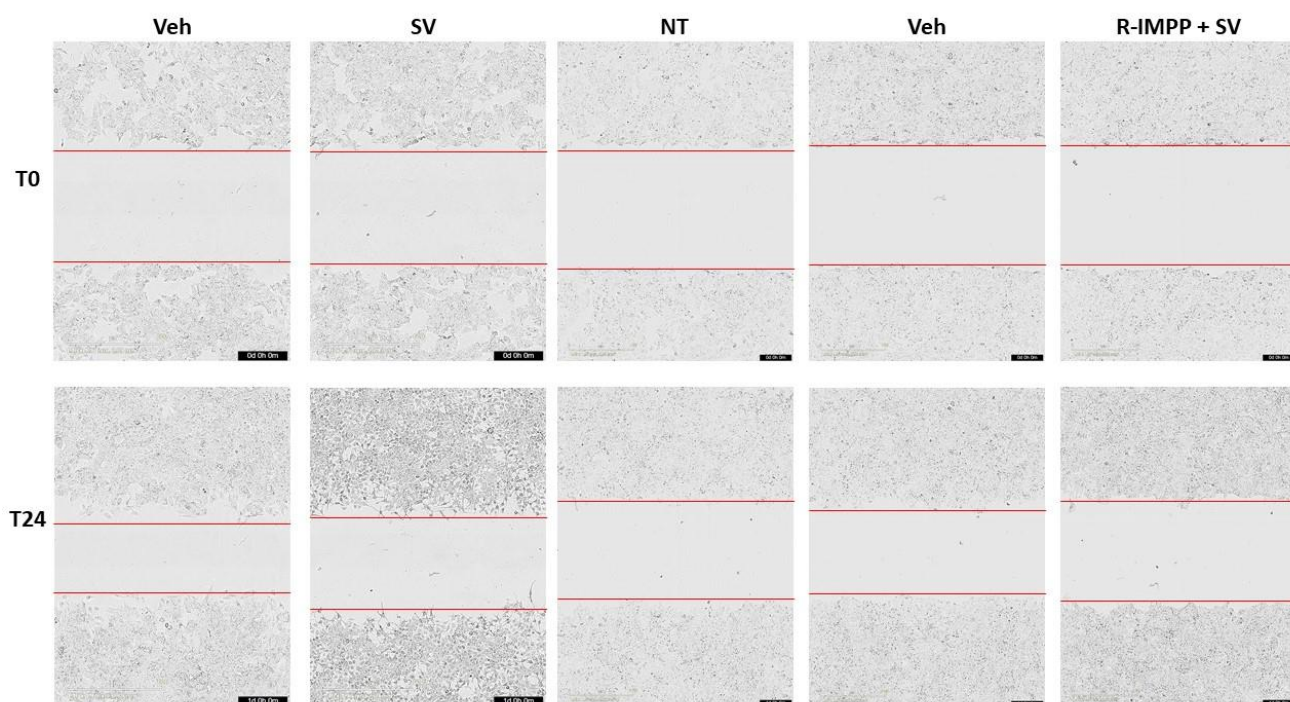


Figure 204S: Effect of Simvastatin alone or in combination with R-IMPP on cellular migration.

Images of wound healing assay taken by IncuCyte. Wound-healing confluence was followed for up to 24 hours after treatment with 25 μ M SV or a combination of 10 μ M R-IMPP and 25 μ M SV. NT refers to non-treated cells. Veh stands for R-IMPP (0.001% DMSO) and SV (0.001% ethanol) solvents.

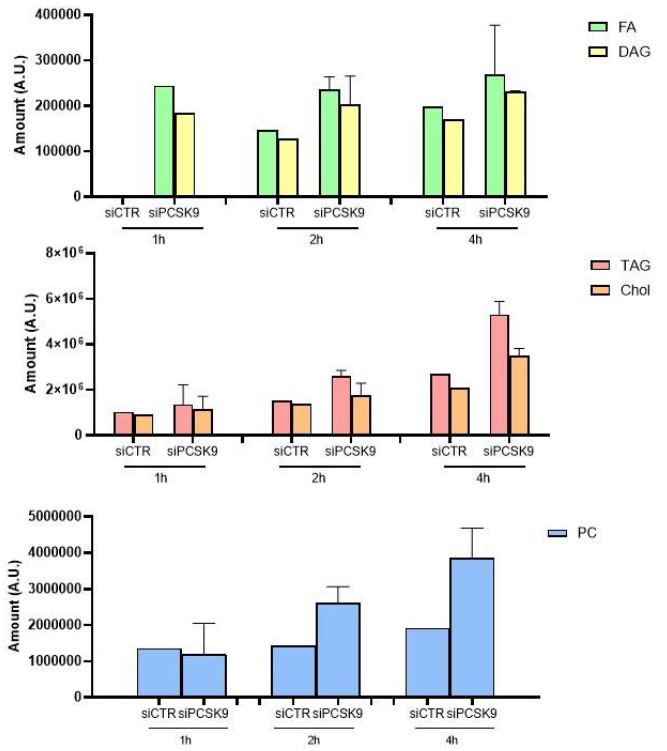
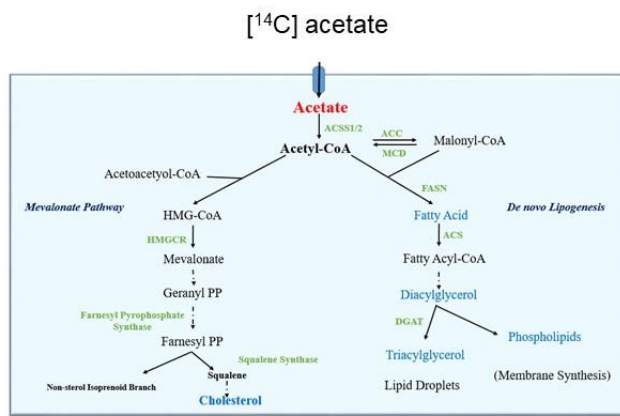


Figure 215S: ^{14}C enrichment of different neutral lipids and phospholipids after cell feeding with $[1-^{14}\text{C}]$ acetate.

Cells were transfected with siRNA (si1 and si2) for 72 hours then fed with $[^{14}\text{C}]$ acetate. Lipid extraction was performed 1, 2 and 4 hours after acetate feeding started. Average values were obtained from both PCSK9 siRNA.

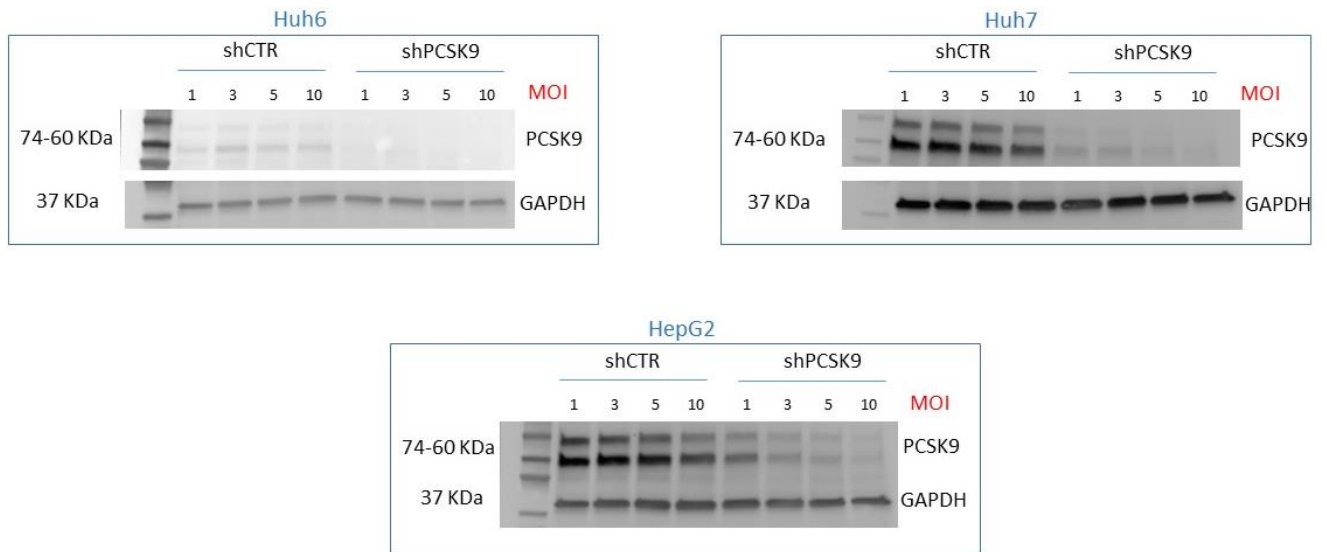


Figure 226S: Inhibition of PCSK9 protein level by shRNA lentivirus transduction.

Validation of depletion of PCSK9 by shRNA silencing. Cell extracts were prepared after transduction with control shRNA (shCTR) or targeting PCSK9 (shPCSK9) of Huh6 or Huh7 or HepG2 cells. Different MOIs (multiplicity of infection) were tested: 1, 3, 5 and 10, the latter was picked for further experiments. 40 µg of cell proteins were loaded per lane on an SDS-PAGE. After electrophoresis and transfer, the membrane was analyzed by western blot using anti-PCSK9 antibodies and GAPDH antibodies for comparison of loading. The two bands observed for PCSK9 correspond to the pro-PCSK9 (74 kDa) and cleaved PCSK9 (60 kDa).

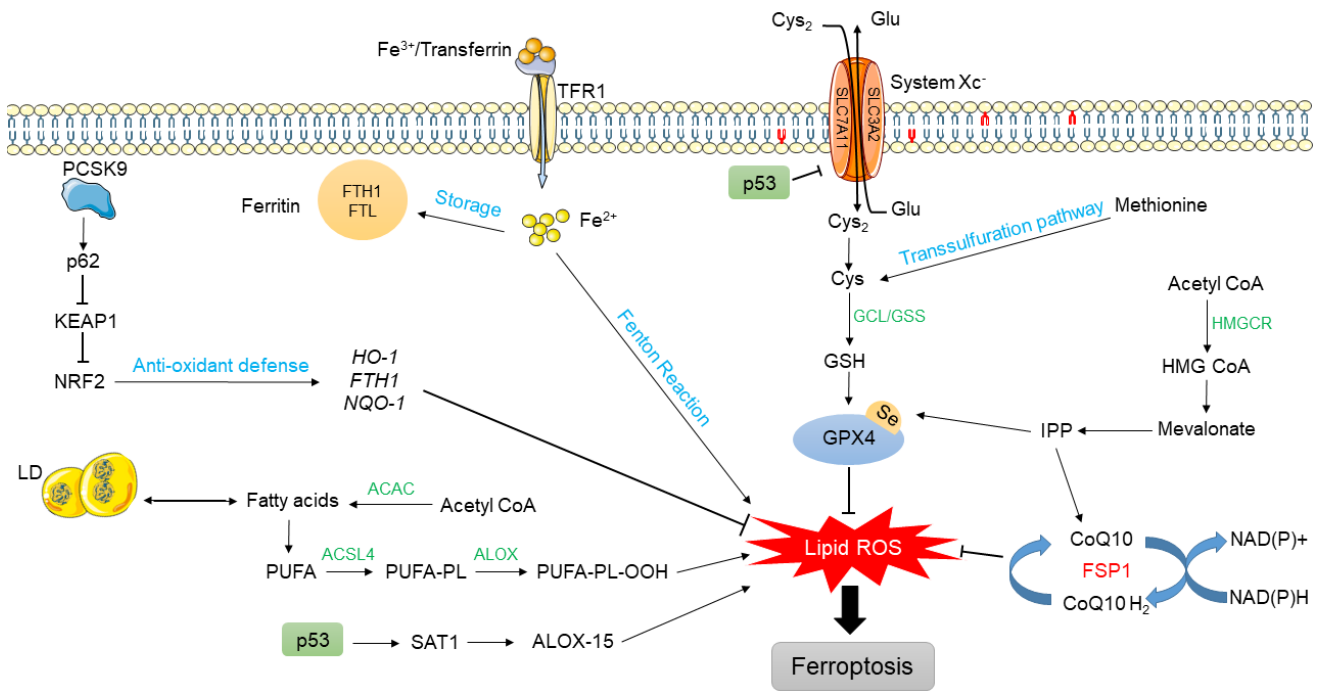


Figure 237S: Regulation of pathways leading to ferroptosis.

The central regulator that inhibits ferroptosis is the selenoprotein GPX4, which together with GSH have antioxidant capacity against lipid ROS, and thus blocking ferroptosis (*right side*). The pathways that lead to the activation and synthesis of GPX4/GSH and hence ferroptosis inhibition include System Xc⁻ mediated import of cystine, production of cysteine by transsulfuration pathway, and finally production of selenocysteine by mevalonate pathway. Another important product of the mevalonate pathway is the ubiquinone/coenzyme Q10 (CoQ10), which can inhibit ferroptosis independently of GPX4. The oxidoreductase FSP1 reduces CoQ10 to ubiquinol (CoQ10 H₂) that can trap lipid ROS, and then regenerates CoQ10 using NAD(P)H. (*left side*) The p62/Keap1/NRF2 pathway plays an important role in inhibiting ferroptosis by activating the expression of anti-oxidant defense genes, which as we found is regulated by PCSK9.

Supplementary References

- [1] P. Sumazin *et al.*, “Genomic analysis of hepatoblastoma identifies distinct molecular and prognostic subgroups,” *Hepatol. Baltim. Md*, vol. 65, no. 1, pp. 104–121, Jan. 2017, doi: 10.1002/hep.28888.
- [2] “Forfeited hepatogenesis program and increased embryonic stem cell traits in young hepatocellular carcinoma (HCC) comparing to elderly HCC.” <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3826595/> (accessed Jan. 19, 2022).
- [3] K. B. Hooks *et al.*, “New insights into diagnosis and therapeutic options for proliferative hepatoblastoma,” *Hepatology*, vol. 68, no. 1, pp. 89–102, 2018, doi: 10.1002/hep.29672.
- [4] F. Domergue *et al.*, “Three Arabidopsis Fatty Acyl-Coenzyme A Reductases, FAR1, FAR4, and FAR5, Generate Primary Fatty Alcohols Associated with Suberin Deposition1[C][W][OA],” *Plant Physiol.*, vol. 153, no. 4, pp. 1539–1554, Aug. 2010, doi: 10.1104/pp.110.158238.
- [5] H. Imamura *et al.*, “Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 37, pp. 15651–15656, Sep. 2009, doi: 10.1073/pnas.0904764106.

Part 2: Meprin α

Novel lipid-modulating roles of meprin α in liver cancer

Introduction:

Being the third leading cause of cancer-related mortality worldwide and with an incidence that continues to rise, liver cancer is gaining more attention than ever. The two most studied primary liver cancers in adults and children are hepatocellular carcinoma (HCC) and hepatoblastoma (HBL), respectively. HCC is the most common primary liver tumor that develops mostly in patients with underlying chronic liver disease, like cirrhosis. Other risk factors may lead to the development of HCC including HBV/HCV infection, excessive alcohol consumption and non-alcoholic fatty liver diseases. The incidence rate of HCC has been increasing over the last decade, with over 740,000 new cases/year being recorded. HBL, on the other hand, represents the majority of pediatric liver tumors, and not only it is rare, but also its incidence is rising worldwide. The etiology of HBL is still mysterious, and in 80% of the cases, HBL occurs sporadically. In some instances, it can be associated with premature birth, very low birth weight (less than 1500 g) and genetic/epigenetic alterations. The available methods of treatment for HCC and HBL like pre-operative chemotherapy and efficient surgical resections have enhanced the final outcomes. However, in some patients, such methods may not work because in some instances, tumors could be unresectable, metastatic and /or chemo-resistant. Hence, the search for novel therapeutic strategies provided by new biologic and molecular investigations is needed more than ever to treat these patients.

Meprin α is a metalloprotease that belongs to the astacin family of zinc endopeptidases and the metzincin superfamily of metalloproteinases. Furin-mediated cleavage of meprin α from the plasma membrane followed by non-covalent oligomerization in the extracellular environment leads to its activation. Once activated, it can cleave a wide variety of substrates including receptors, cytokines, extracellular matrix (ECM) proteins, etc. In HCC, meprin α was found to be overexpressed and associated with aggressive clinicopathological features, faster recurrence, worse overall survival and hence poor prognosis. No studies have been done yet with regard to the role of meprin α in HBL. That's why we were interested in investigating whether or not meprin α has a role in HBL and what its possible oncogenic functions in liver cancer might be.

Objective

In addition to PCSK9 inhibition approach to fight liver cancer, we investigated meprin α , a multifunctional metalloprotease, because it was reported to be involved in PCSK9 processing and to be highly expressed in adult liver cancers.

Indeed, our transcriptomic analysis of tumoral tissues derived from HBL patients showed significant overexpression in tumors (specifically the most proliferative subtype) when compared to normal liver tissues. We confirmed the same expression pattern using online available transcriptomic data from Lopez-Terrada *et al.*, Raymond *et al.* and Buendia *et al.* (available on R2: Genomic analysis and visualization platform).

Since Mep1a is known for its protumoral activities, we asked whether PCSK9-mediated connection of Mep1a opens up lipid-dependent activities of this metalloprotease in liver cancers.

The specific aims of this part of the project are:

Aim 1: to create cell line models to mimic the tumor profile by overexpressing Mep1a in Huh7 and HepG2 cells and analyze the impact of high levels of this metalloprotease on the overall composition of the cellular secretome.

Aim 2: to conduct functional assays in these cell lines with specific focus on lipid metabolism and to validate the results *in vivo* using liver tissues from the available *MEP1A* KO mice.

Materials and Methods:

Same materials and methods as in the first part were used in this second part of the project, with the exception of some that are mentioned below.

Meprin α expression in Huh7 and HepG2

The overexpression of meprin α was induced following lentivirus transduction of both cell lines using pLX304 expression-ready lentiviral system (Addgene®) containing the *MEP1A* gene (pLX304-MEP1A), followed by the V5 epitope tag (9 amino acids: IPNPLLGLD) at the C-terminus. Additional transductions were performed using the empty pLX304 construct (pLX304-Mock) in order to obtain a control cell line. Cells containing the plasmid DNA were selected with blasticidin (20 $\mu\text{g/ml}$) selection. The obtained cell lines were named accordingly, Huh7/HepG2 Mock and Huh7/HepG2 MepV5.

It is important to note that this lentivirus didn't work in Huh6 cell lines because the CMV promotor was deactivated in these cells, so no expression of meprin α was detected after transduction. For that purpose, new cloning and new lentivirus production were done (refer to annex for detailed protocol).

Conditioned media collection

The extracellular mediators produced by HepG2 cells overexpressing meprin α (MepV5) and their control (Mock) were collected in the form of conditioned media (CM). The cells were plated in Falcon® culture flasks of 75 cm^2 at a density of 1.10^6 cells. Once they reached 80% confluency, DMEM medium was removed and replaced with X-VIVO 15™ (BE02-060F, Lonza®) for 48 h. The CM (supernatants) were then collected and filtered at 0.22 μm (Millex® GP Millipore Express® PES Membrane) then stored at -20°C for later experiments. The cells were then counted to ensure that the CM came from comparable number of cells ($\pm 15\%$).

Western Blot

Protein extracts from the different cell lines were prepared using RIPA buffer (NaCl 138 mM, NP-40/Igepal 1%, EDTA pH 8 2 mM, glycerol 10%, Tris-HCl pH 7.5 20 mM, SDS 0.1 %) supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and centrifuged at 13,000 rpm for 15 min at 4°C . Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, USA) was used to measure the protein contents of cell

extracts at 562 nm. 40 µg of protein lysates were then mixed with Laemmli buffer 1X (BioRad, Hercules, California, USA) and DTT 100 mM (Dithiothreitol, GE Healthcare LifeSciences), denatured at 95°C for 10 minutes then separated by precast polyacrylamide gel (4-15%) (Mini-PROTEAN Stain Free™ TGX Gels, BioRad®) for 2 hours at 80 V in a migration buffer (Tris-Glycine 1X SDS 1% pH 8.6). Proteins were then semi-dry transferred to a 0.2 µm nitrocellulose membrane (Transblot Turbo, BioRad®) in Trans-Blot Turbo Buffer 1X (BioRad®), containing 20% ethanol. After blocking with 5% BSA for 2 hours at room temperature, the membrane was incubated overnight with different primary antibodies (**Table 4**) at 4°C. The membrane was washed three times with TBS-Tween-20, 0.1% and incubated in the dark for 60 min with the respective secondary antibody (**Table 5**) at room temperature. The membrane was subsequently washed three additional times and scanned using Fusion FX (Vilber Lourmat). Quantification was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Table 4: Primary antibodies

Antibody	Origin	Dilution	Reference	Type
Anti-Mepri α	Goat	1/1000	R&D (AF3220)	Polyclonal
Anti-PCSK9	Sheep	1 µg/ml	R&D (AF3888)	Polyclonal
Anti-LDLR	Rabbit	0.5 µg/ml	Novus Biologicals (NBP1-06709)	Polyclonal
Anti-GAPDH HRP	Mouse	1/10000	BioLegend (BLE649203)	Monoclonal

Table 5: Secondary antibodies

Antibody	Origin	Dilution	Reference
Anti-mouse IgG (H+L)-HRP	Goat	1/3000	Biorad (170-6516)
Anti-goat IgG HRP	Rabbit	1/1000	R&D (HAF017)
Anti-rabbit IgG HRP	Goat	1/5000	Sigma (A0545)
Anti-sheep IgG HRP	Rabbit	1/3000	Calbiochem (402100)

Quantitative real time RT-qPCR

Total RNA was extracted from the different cell lines using the Nucleospin RNA[®] isolation kit (Macherey-Nagel[™]) according to the manufacturer's protocol. The RNA was quantified using Xpose. Total RNA was subjected to DNase treatment (DNase I, amplification grade [100U]; Invitrogen[™]) prior to reverse transcription. Following that, RNA samples were reverse transcribed by Maxima reverse transcriptase [200U/ μ L] (Thermo-Scientific[™]) according to the following protocol:

1. Reaction components are added to a sterile, nuclease-free tube on ice in the indicated order:

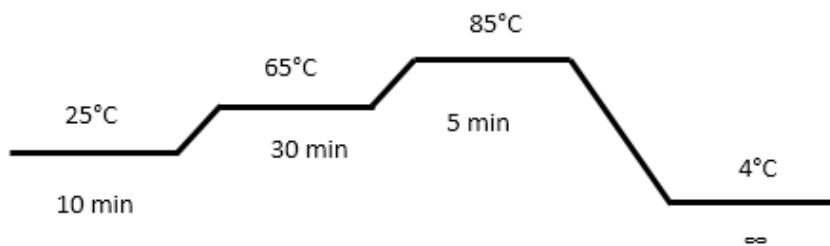
Template RNA	Total RNA	1 μ g
Primer	Random Hexamer (#SO142)	1 μ L (100 pmol)
dNTP mix, 10 mM each (#R0191)		1 μ L (0.5 mM final concentration)
Water, nuclease-free		To 14.5 μ L

2. If the RNA template is GC-rich or is known to contain secondary structures, it is gently mixed, briefly centrifuged and incubated at 65°C for 5 min. After chilling on ice, it is briefly centrifuged and placed again on ice
3. The following reaction components are then added in the indicated order:

5X RT Buffer	4 μ L
Thermo Scientific™ RiboLock RNase Inhibitor (#EO0381)	0.5 μ L (20 U)
Maxima Reverse Transcriptase	1 μ L (200 U)
Total volume	20 μ L

After gentle mixing they are briefly centrifuged.

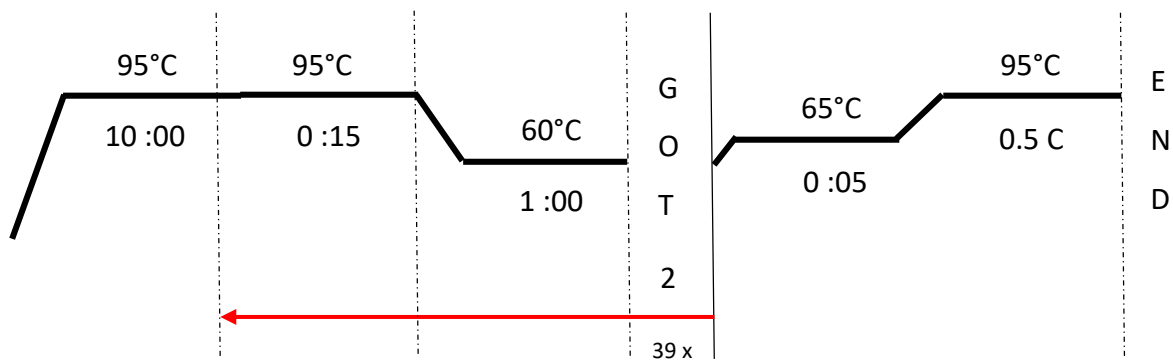
4. Incubation period:



The samples are either used directly for qPCR or stored at -20°C for up to one week.

Messenger RNA levels are determined by real time qPCR using SYBR qPCR Premix Ex Taq (Tli R4NaseH Plus) (TAKRR420W) with CFX Touch™ real-time qPCR Detection System (BioRad™).

1. A total of 1 μ l cDNA (50 ng/ μ l) is used as a template. It is mixed with a solution of 6 μ l SYBR Takara with 2.5 μ l of each primer (300 nM). The total reaction volume is 12 μ l.
2. Then cycles are run as illustrated in the following diagram



Experiments were performed in triplicate for each sample. The data were analyzed by using CFX Manager™ software (BioRad™). mRNA levels were normalized to stable housekeeping genes β -actin and GAPDH, as confirmed by geNorm software.

The RNA sequences of studied genes were found using the UCSC Genome Browser and primer pairs were designed with Primer-BLAST (NCBI®) software or primer bank (Table 6) and purchased from Eurofins®.

Table 6: Forward and reverse primers used for real time qPCR

Gene	Forward primer 5'→3'	Reverse Primer 5'→3'
β -Actin	ATTGCCGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
GAPDH	CAAGGAGTAAGACCCCTGGA	AGGGGAGATTCAGTGTGCTG
LDLR	GCTACCCCTCGAGACAGATG	CACTGTCCGAAGCCTGTTCT
HMGCR	GAGCGTGCGTAAGGTGAGG	ACAGAATCCTTGGATCCTCCAG
PCSK9	CTGGTGAAGATGAGTGGCCGA	CCCGGTGGTCACTCTGTATG
SREBF2	CCCTTCAGTGCAACGGTCATTAC	TGCCATTGGCCGTTTGTGTC

Fluorescence microscopy

Huh7 and HepG2 cells (Mock and MepV5) were seeded in chamber slides (8 well glass slide) at 5000 cell per well in complete DMEM (10% FBS + 1% streptomycin/penicillin) for 48 hours. The medium was then changed to DMEM that was not supplemented with any sera (incomplete DMEM) in order to do starvation for 24 hours. After that, fluorescently labeled native LDL (Low Density Lipoprotein from Human Plasma, Dil complex or Dil-LDL from Invitrogen™, L3482) was added into each well at a final concentration of 5 μ g/mL. The slide chamber was then incubated at 37°C for 48 hours in a humidified, 5% CO₂ atmosphere. Once the incubation period was over, the medium was discarded and wells were washed 4 times with PBS before fixing them using 4% paraformaldehyde (PFA) for 15 minutes. Finally, the chambers were washed again with PBS and distilled water (dH₂O) before adding mounting medium for fluorescence with DAPI (VECTASHIELD®) and covering it with a cover slip. The slide was then observed under fluorescence microscope.

Flow Cytometry

For the purpose of measuring the uptake of DiI-LDL by HCC cell lines, Huh7 cells (Mock and MepV5) were seeded in a 6-well plate at 50,000 cells per well in complete DMEM. The cells were left for 48 hours in complete medium. They were then starved by changing the medium to incomplete DMEM for 12-24 hours. Finally, DiI-LDL (C_i 1 mg/mL / C_f 5 μ g/mL) was added to the wells -except the negative control ones. The plate was then incubated at 37°C for 2-4 hours in a humidified, 5% CO₂ atmosphere. The measurement of the uptake was done by flow cytometry.

Label-free quantitative proteomics

Proteomic analysis was performed on proteins that were secreted into a 48-hour conditioned-media (X-VIVO 15™, Lonza) derived from HepG2 Mock and HepG2-MepV5 cells. Samples were loaded on a 10% acrylamide SDS-PAGE gel. Migration was stopped when the samples entered the resolving gel and the proteins were visualized by colloidal blue staining. Each SDS-PAGE band was cut into 1 mm x 1 mm gel pieces. Gel pieces were de-stained in 25 mM ammonium bicarbonate (NH₄HCO₃), 50% acetonitrile (ACN) and shrunk in ACN for 10 minutes.

After ACN removal, the gel pieces were dried at room temperature (RT). The proteins were first reduced in 10 mM dithiothreitol, 100 mM NH₄HCO₃ for 30 minutes at 56°C then alkylated in 100 mM iodoacetamide, 100 mM NH₄HCO₃ for 30 minutes at RT and shrunk in ACN for 10 minutes. After ACN removal, the gel pieces were rehydrated with 100 mM NH₄HCO₃ for 10 minutes at RT. Before protein digestion, the gel pieces were shrunk in ACN for 10 minutes and dried at RT. The proteins were digested by incubating each gel slice with 10 ng/ μ L of trypsin (T6567, Sigma-Aldrich) in 40 mM NH₄HCO₃, 10% ACN, rehydrated at 4°C for 10 minutes, and were finally incubated overnight at 37°C. The resulting peptides were extracted from the gel in three steps: the first incubation was in 40 mM NH₄HCO₃, 10% ACN for 15 minutes at RT and the two subsequent incubations were in 47.5% ACN, 5% formic acid for 15 minutes at RT. The three collected extractions were pooled with the initial digestion supernatant, dried in SpeedVac, and re-suspended with 25 μ L of 0.1% formic acid before nanoLC-MS/MS analysis.

Online nanoLC-MS/MS analyses were performed using an Ultimate 3000 RSLC Nano-UPHLC system (Thermo Scientific, USA) coupled to a nanospray Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, USA). 10 μ L of each peptide extract were

loaded on a 300 μm ID x 5 mm PepMap C18 precolumn (Thermo Scientific, USA) at a flow rate of 20 $\mu\text{L}/\text{min}$. After 5 minutes desalting, peptides were separated on a 75 μm ID x 25 mm C18 Acclaim PepMao[®] RSLC column (Thermo Scientific, USA) with a 4-40% linear gradient of solvent B (0.1% formic acid in 80% ACN) in 108 min. The separation flow rate was set at 300 nL/min. The mass spectrometer operated in positive ion mode at a 1.8 kV needle voltage. Data were acquired using Xcalibur 3.1 software in a data-dependent mode. MS scans (m/z 350-1600) were recorded at the resolution of $R=70000$ (@ m/z 200) and an AGC target of 3×10^6 ions collected within 100ms. Dynamic exclusion was set to 30 s and top 12 ions were selected from fragmentation in HCD mode. MS/MS scans with a target value of 1×10^5 ions were collected with a maximum fill time of 100ms and a resolution of $R=17500$. Additionally, only +2 and +3 charged ions were selected for fragmentation. The other settings were as follows: no sheath and no auxiliary gas flow, heated capillary temperature, 200°C; normalized HCD collision energy of 27% and an isolation width of 2 m/z .

For protein identification, we used the Mascot 2.5 algorithm available with Proteome Discoverer 1.4 Software (ThermoFischer Scientific Inc.). It was used in batch mode by searching against the UniProt Homo sapiens database (70,709 entries, Reference Proteome Set, release 2017_04) from <http://www.uniprot.org/> website. Two missed enzyme cleavage were allowed. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.02 Da. Oxidation of methionine, acetylation of lysine and deamidation of asparagine and glutamine were looked for dynamic modifications. Carbamidomethylation on cysteine was searched as static modification. Raw LC-MS/MS data were imported in Proline Studio (<http://proline.profiroteomics.fr/>) for feature detection, alignment and quantification. Protein identification was only accepted with at least 2 specific peptides with a pretty rank=1 and with a protein FDR value less than 1.0% calculated using the “decoy” option in Mascot.

Protein intensity levels were considered to be variable if the ratio of HepG2 MepV5/HepG2 Mock was less than or equal to ≤ 0.66 or greater than or equal to 1.5 (Fold Change -1.5/1.5). The significance of these variations was verified by a Limma t-test ($p < 0.05$).

Lipidomic analysis

WT and Mep1 α KO mice liver tissues were donated by Institut Für Biochemie-Universität Kiel, Germany by Prof. Dr. Christoph Becker-Pauly. The extraction of lipids from approximately 50

mg of liver tissues was done by homogenizing the tissue using the TissueLyser II (Qiagen). Tissues were disrupted with stainless steel beads 5 mm (Qiagen #69989). For homogenization, 1 mL of ice cold isopropanol was added and homogenization was performed twice for 30 sec. The homogenates were transferred to glass tubes and the TissueLyser tube was washed with 1 mL isopropanol. The samples were then heated at 85°C for 30 min.

After cooling, 2 mL CHCl₃:MeOH (2:1) were added and the mixture was stirred at 220 rpm for 2 hours at room temperature on a rotary shaker. After separation of the two layers by centrifugation at 1000 x g for 5 min, the organic phase was collected and transferred to a new tube. The upper aqueous layer was re-extracted with 2 mL of CHCl₃ under stirring on a rotary shaker for another 12 hours, centrifuged off and the two organic phases were combined. After washing with 1.5 mL of 0.9% NaCl, the samples were centrifuged at 1000 x g for 10 min. The resulting organic phases were dried on hydrophilic cotton that had been previously washed with 1 mL of CHCl₃, 1 mL of MeOH and 1 mL of CHCl₃:MeOH (1:1) to remove any remaining tissue debris. Finally, samples were evaporated to dryness and resuspended in 1 mL.

Results

1. MEP1A overexpression in HBL

RNAseq analysis of 22 patient-derived HBL tumors vs non-tumor counterparts made in our lab revealed the deregulation of the expression of many genes [96]. Interestingly, *MEP1A* expression was elevated in the tumor region compared to non-tumor counterparts (Figure 17, A). [For detailed expression in HBL subtypes, refer to figure S1, A in annex]. Moreover, using the R2: Genomic analysis and visualization platform, we also observed an upregulation in the expression of *MEP1A* in HBL tumors compared to normal tissues according to the data from López-Terrada *et al.* and Raymond *et al.* (Figure 17, B) as well as Buendia *et al.* (Figure S1, A). In addition, it was interesting to check whether the profile was similar in hepatic cancer cell lines *in vitro*. Indeed, in the RNAseq data derived from these cells, *MEP1A* expression was higher in Huh7 (HCC cell line) and HepG2 (HBL cell line) cells, while almost no expression was detected in THLE2 (normal liver cell line) and Huh6 (HBL cell line) cells (Figure 17, C). In order to confirm this expression at the protein level, western blot analysis was done and surprisingly, we couldn't detect any expression of meprin α in Huh7, HepG2 and Huh6 cells (Figure S1, B).

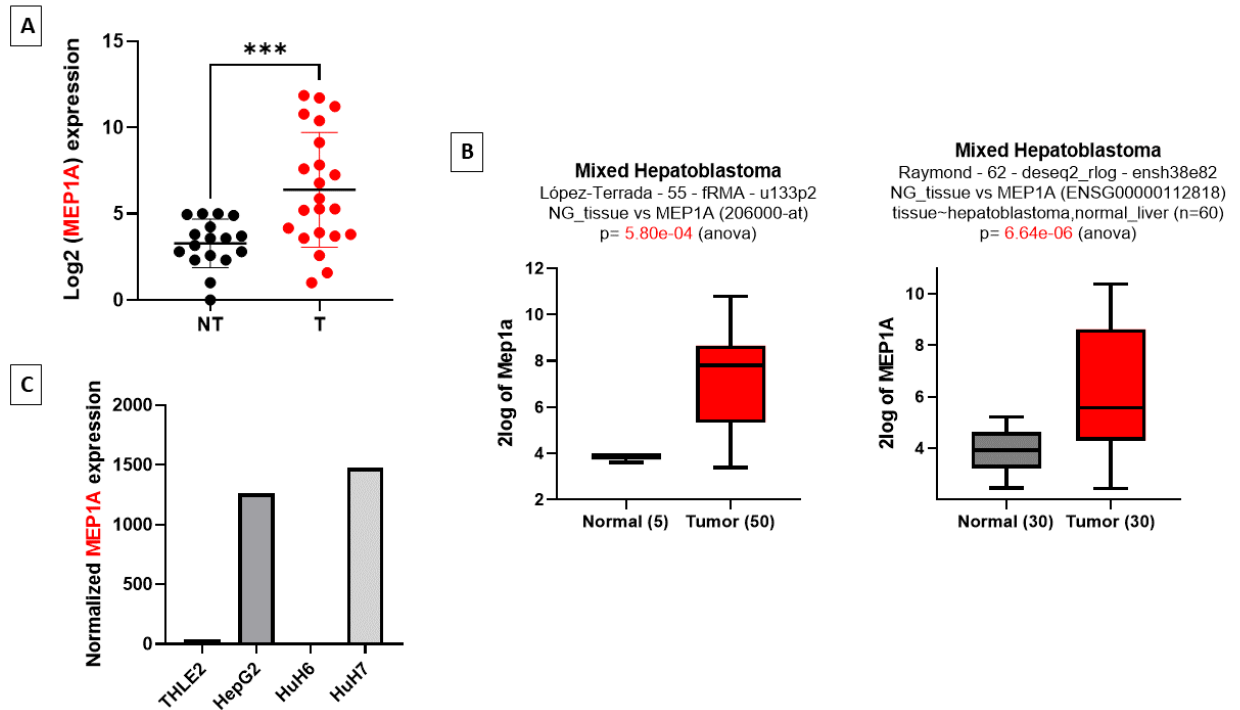


Figure 2417: Transcript level of MEP1A.

Transcript level of MEP1A in (A) HBL tumors compared to non-tumoral counterparts as revealed by our RNAseq analysis, (B) in mixed hepatoblastoma from López-Terrada *et al.* and Raymond *et al.*, (C) in our different liver tumor cell lines (HepG2, Huh6 and Huh7) vs normal THLE-2 cells. *Mann-Whitney test* *** $p=0.0008$.

2. Meprin α overexpression and proteomic analysis

Since our cells didn't show any expression of meprin α at the protein level, we aimed at overexpressing it using a lentivirus system. Huh7 and HepG2 cells were transduced with lentivirus pLX-304-MEP1A-V5 to create cell lines that are overexpressing *MEP1A* or with pLX-304-Mock as a control (Figure 18, A). Since it is known that meprin α is activated after cleavage and secretion, we next did proteomic analysis by LC-MS/MS on the CM obtained from HepG2 Mock and HepG2 MepV5 cells and compared the relative intensities using label-free approach in order to identify the proteins that could be deregulated upon meprin α expression. The criteria followed to determine significance includes: (i) validation if proteins are identified with at least 2 peptides and an FDR rate <1%; (ii) considered as variant if the ratio HepG2 MepV5/HepG2 Mock ≤ 0.6 or ≥ 1.5 . As a result, this approach allowed us to identify 111 proteins, of which 18 were significant variants according to the above criteria. Among these 18 variants, 6 proteins were upregulated (red dots) including meprin α and 12 proteins were

downregulated (green dots) in HepG2 MepV5 versus HepG2 Mock (Figure 18, B). **Table 7** summarizes the significantly deregulated proteins, some of which are known substrates for meprin α , such as PCSK9, SPARC, PAI-1 and SERPIN A3. Looking at these deregulated proteins, we noticed the possible implication of meprin α in two aspects: immunomodulation (SPARC/PAI-1 pathway) and metabolism (PCSK9 and its related proteins). Since the metabolism is our main interest, we proceeded in this direction.

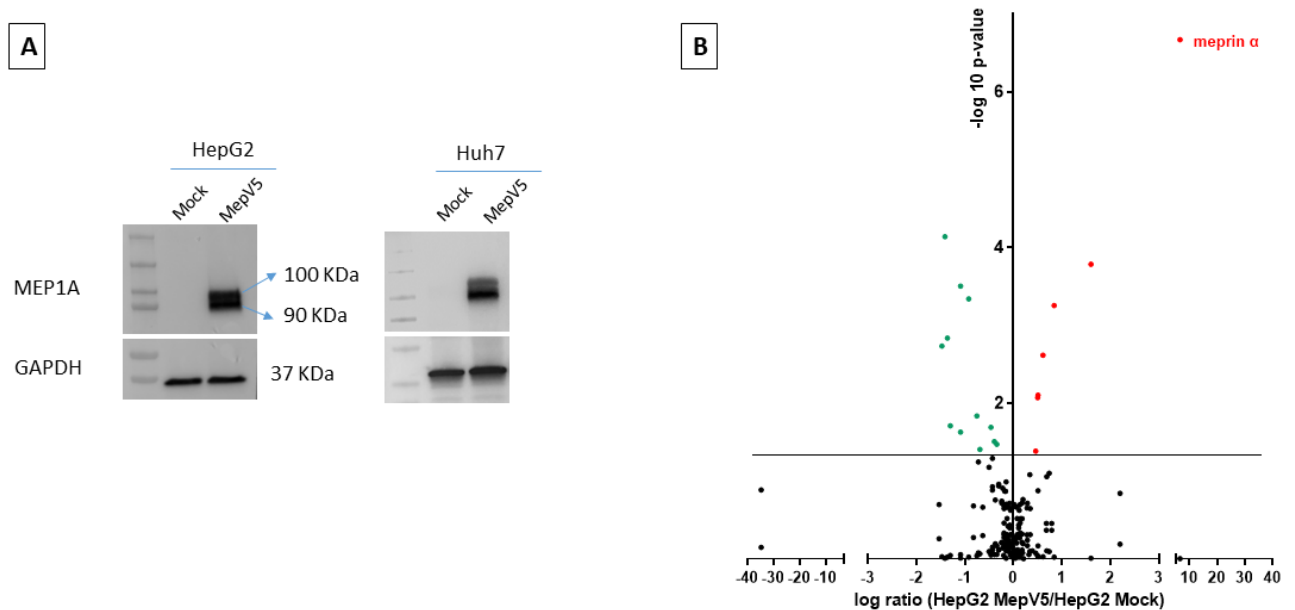


Figure 25-18: MEP1A overexpression and proteomic analysis

(A) Successful overexpression of MEP1A in HepG2 and Huh7 cells shows two bands at 100 and 90 kDa representing pro-protein and cleaved MEP1A respectively, (B) volcano plot shows $-\log_{10}$ p-value (Y-axis) vs log ratio HepG2 MepV5/Mock (X-axis) of all analyzed proteins, green dots represent significant downregulated proteins, while red dots represent significant upregulated proteins.

Table 7: Deregulated proteins in meprin overexpressing cells versus control

For instance, PCSK9 is a known regulator of lipid metabolism; LDHA is involved in anaerobic respiration and is responsible for the accumulation of lactate instead of pyruvate; SERPIN A3, also known as α -1-antichymotrypsin, is a protease inhibitor whose expression is downregulated in HCC. It was shown to control chromatin condensation and proliferation in HCC [291].

Upregulated		Downregulated	
IGFBP1	Matrilin-3	SPARC	EF2
PAI-1	SERPIN A3	Gastricsin	IGHG1
AFP	SERPIN F1	RACK1	SERPIN A5
LDHA	GAL3BP	HBB	C3/C5 convertase
PCSK9			

3. Altered lipid metabolism in the presence of meprin α

From our RNAseq data of HBL tumors, we observe that meprin α overexpression is accompanied by a deregulation in the expression of proteins and enzymes involved in lipid metabolism including, PCSK9, LDLR, HMGCR, and squalene synthase (FDFT1) (Figure 19, A). Indeed, meprin α overexpression is accompanied by an increase in PCSK9 and decrease in LDLR expression, which may lead to higher potential in lipoprotein uptake by cells. Besides, the observed increase in the expression of rate limiting enzymes of the mevalonate pathway indicates that the cells seem to rather depend on their own production of cholesterol (Figure 19, B). In order to check if we have the same gene expression profile in our meprin overexpressing cells, RT-qPCR was performed and the mRNA expression level of PCSK9, LDLR, HMGCR and SREBF2 was checked. The results didn't show any significant changes, but rather tendencies (Figure S2, A). Similarly, checking the protein expression level of PCSK9 and LDLR didn't reveal any significant changes as well (Figure S2, B). The absence of any relationship between the mRNA levels of these genes and the higher expression of Mep1a indicates that the crosstalk between Mep1a and the other genes is not at the transcriptional level. Taking into account the results from the proteomic analysis, it is conceivable that meprin α may act *via* its enzymatic activities to modulate the secretion/maturation of these varying proteins. The next step is to find out whether the change in the amount of some of these lipid

metabolism related proteins may be translated by functional changes at the lipid metabolism level.

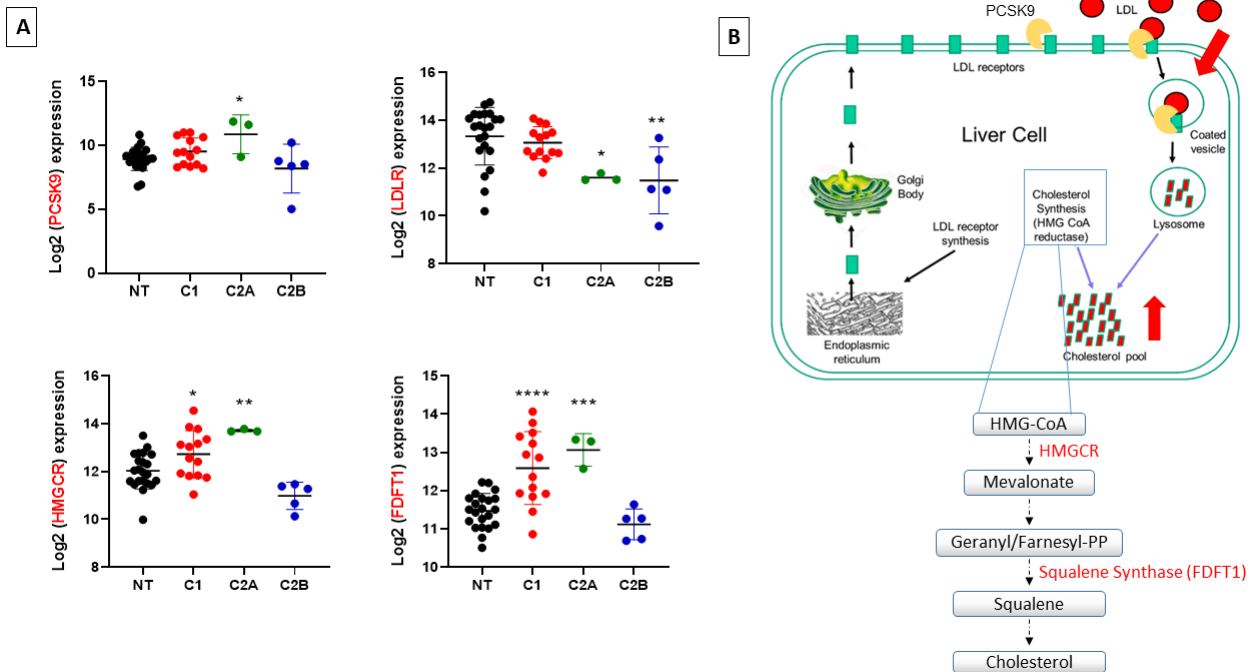


Figure 2649: mRNA expression level of genes involved in lipid uptake and cholesterol synthesis.

(A) The expression level of PCSK9, LDLR, HMGCR and squalene synthase (FDFT1) is increased (FDFT1) in HBL tumor as revealed by our RNAseq data [96], (B) figure summarizing the degradation of LDLR by PCSK9, thus decreasing lipid uptake, and the increase in the cholesterol synthesis by HMGCR and squalene synthase represented in red. *Ordinary one-way ANOVA*, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4. Lipid uptake by meprin overexpressing cells

Since PCSK9 is a potential substrate of Mep1a, we hypothesized that the higher PCSK9 observed in meprin-overexpressing cells would induce LDLR degradation, resulting in less uptake of lipid particles. In order to confirm this hypothesis, lipid uptake was measured by flow cytometry using native labeled Dil-LDL. The results revealed a significant decrease in the uptake of Dil-LDL by Huh7-MepV5 cells as compared to control (Figure 20). Microscopic examination of these cells confirmed that Mep1a-overexpressing Huh7 cells accumulate less Dil-LDL. These results bring the first functional argument linking meprin α to lipoprotein uptake and lipid metabolism.

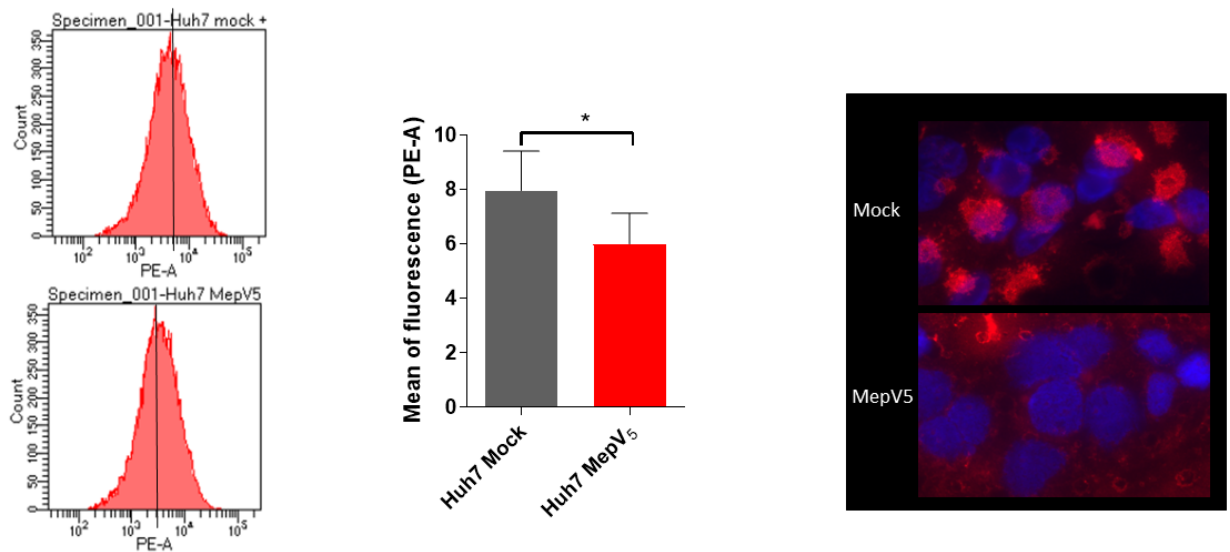


Figure 2729: Lipid uptake by meprin overexpressing cells.

Dil-LDL uptake measured by flow cytometry in meprin overexpressing Huh7 vs their control reveals a significant decrease, which we can see also in the immunofluorescence images of cells labeled with Dil-LDL (right panel). *Wilcoxon matched-pairs signed rank test* * $p=0.0313$.

5. Does meprin overexpression modulate mitochondrial respiration?

Next, we asked whether the overexpression of meprin affects the global energetic metabolism and mitochondrial activities. For this purpose, the mitochondrial oxygen consumption rate (OCR) was measured under different feeding conditions (see materials and methods section) using SeaHorse assay (Figure 21, A). The results show that the overexpression of meprin increases the mitochondrial OCR in Huh7 cells in all feeding conditions, both at the basal and maximal respiratory capacity (Figure 21, B & C), which were not modified by glucose. In Mock and MepV5 cells, the basal OCR is significantly higher in the absence of glucose, whilst it becomes significantly lower in the absence of glutamine or in the presence of a glutaminase inhibitor. Similarly, the maximal respiratory capacity of Huh7 Mock and MepV5 cells show a significant decrease in the presence of a glutaminase inhibitor. This indicates that Huh7 cells seem to be dependent on glutamine metabolism for respiration (check figure S3 for detailed OCR measurements in Huh7 Mock/MepV5 in the presence of oligomycin). Concerning HepG2 cells, the OCR was measured under two feeding conditions, presence or absence of glucose (Figure S3). The results show that meprin overexpressing cells have higher maximum

respiration than their control. The basal respiration was further significantly increased in the absence of glucose by Mep1a overexpression. The increase we have observed in both Huh7 and HepG2 cells could be due to an increase in lipid metabolism. This could be evaluated by targeting lipid metabolism enzymes and measuring again the respiration and proliferation of cells.

Globally, these respiration assays demonstrated that meprin α contributes to the global change in metabolism at least by increasing the addiction to glutamine and maybe by modulating the lipid metabolism.

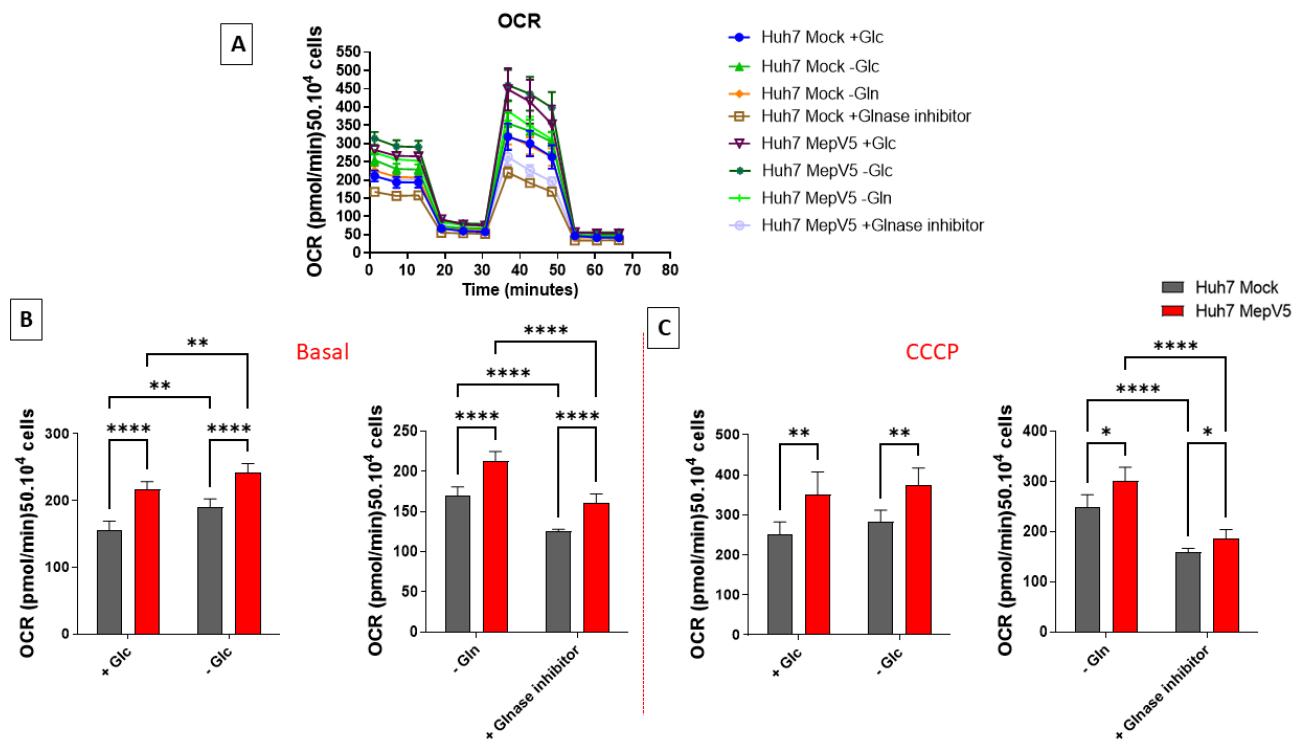


Figure 2824: OCR in Huh7 Mock versus MepV5 cells.

(A) Mitochondrial OCR in Huh7 Mock vs MepV5 cells under four different feeding conditions, (B) Basal OCR and (C) maximal OCR (CCCP) in the presence of glucose (+Glc), absence of glucose (-Glc), absence of glutamine (-Gln) or presence of glutaminase inhibitor (+Glnase inhibitor). The OCR at the basal level was also measured in the presence of oligomycin to inhibit ATP synthase and thus determine the mitochondrial OCR, and in the presence of CCCP to measure the maximal respiratory capacity. The different feeding conditions (\pm Glc, \pm Gln) were used to evaluate whether the cell respiration depends on one of the nutrients more than the other i.e., glucose over glutamine or not. The glutaminase inhibitor prevents glutamine metabolism and the cells need to switch to glucose for their respiration. Ordinary two-way ANOVA, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

6. Does overexpression of MEP1A modulate the cellular lipid composition?

Because of the change in lipid uptake and mitochondrial activity, which we hypothesized to be due to an increase in lipid metabolism, we assessed the amount of lipids e.g., neutral lipids (NL) and phospholipids (PL) in our meprin overexpressing cells. For *in vivo* validation of these data, we measured these lipids in mouse liver tissues (Wild type vs *MEP1A* KO mice) obtained from Institut Für Biochemie-Universität Kiel, Germany by Prof. Christoph Becker-Pauly. Lipidomics analysis revealed no significant difference in NL and PL composition in meprin overexpressing cells as compared to control when grown in complete medium (Figure 22, A & B) or in starvation condition (Figure S4). Since the overall lipid content of cells did not vary during the steady state, we cannot exclude that the decrease in lipid uptake observed earlier (Figure 20) may be compensated by an increased *de novo* lipogenesis. Further fluxomics studies may better address these metabolic aspects. Interestingly, data analysis of liver tissues derived from WT or *MEP1A* KO mice showed some lipid variations (figure 22 C & D). Indeed, cholesterol and FA content of liver is significantly lower in the absence of Mep1a while TAG was significantly higher in these samples. We reported no change in the amount of phospholipids. One explanation for these findings is that meprin α is involved in the activation of lipogenesis and hence its absence leads to lower synthesis of cholesterol and FA. This hypothesis is supported by the transcriptomic data associating high lipogenesis with high expression of this metalloprotease. These *in vivo* data clearly show that Mep1a can modulate to some extent the lipid metabolism in liver. However, the exact metabolic pathways involved need to be further clarified using more advanced metabolomic and fluxomic studies after adding labeled substrates and metabolites.

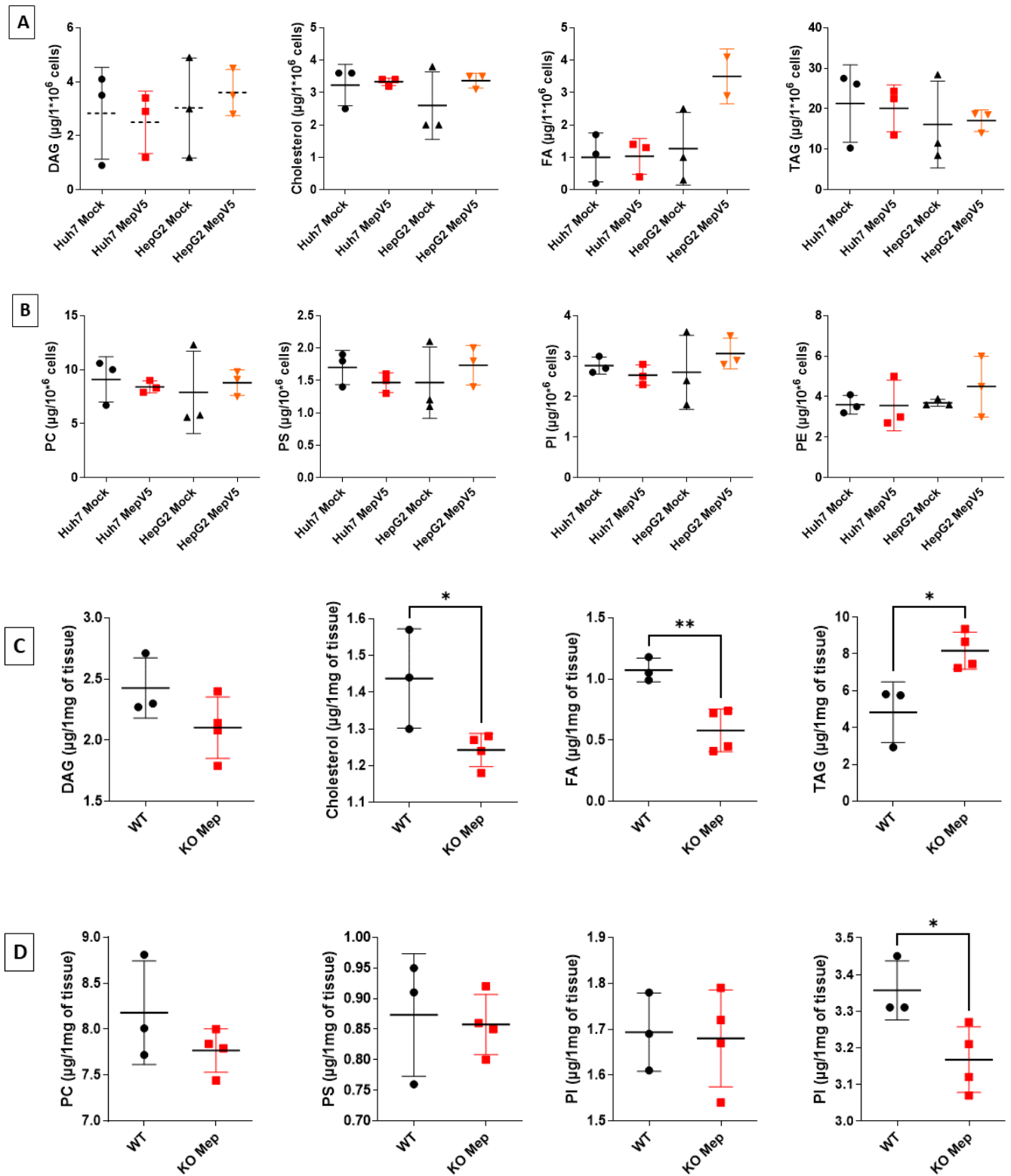


Figure 2922: Composition of NL and PL in meprin overexpressing cells and mice liver tissue.

(A & C) NL are neutral lipids i.e., diacylglycerol (DAG), cholesterol, fatty acid (FA) and triacylglycerol (TAG). (B & D) PL are phospholipids i.e., phosphatidyl-choline (PC), -serine (PS), -inositol (PI) and -ethanolamine (PE). (A & B) Meprin overexpressing cells vs their controls when cultured in complete medium; (C & D) Wild type (WT) and *MEP1A* KO mouse liver tissues. Unpaired T-test * $p < 0.05$; ** $p < 0.01$

7. Does statin treatment reverse lipid profile?

Since we have shown that Mep1a is implicated in lowering lipid uptake and activating lipogenesis, we asked next how the pharmacological inhibition of cholesterol synthesis would affect the lipid metabolism in meprin overexpressing cancer liver cells. Indeed, we used simvastatin, an inhibitor that targets the rate-limiting enzyme of the mevalonate pathway and cholesterol synthesis e.g., HMGCR. In this case, Huh7 and HepG2 Mock/MepV5 cells were treated with 25 μ M simvastatin for 3 days. First, we verified the levels of LDLR and PCSK9 proteins, which are known to be modulated by statins (Figure 23, A & B). We observed almost no change in the amount of LDLR induced by the statin treatment, and regardless of meprin overexpression, while it was expected to be higher with statin treatment. By looking at its negative regulator, PCSK9, which is known to be augmented by statin treatment, our results showed borderline increase in the expression of this protein when Mep1a is overexpressed. These high levels of PCSK9 probably lead to faster turnover and degradation rates of LDLR, hence preventing any upregulation of this receptor. This is despite the fact that our preliminary data of lipid uptake by Huh7 Mock/MepV5 cells seemed to reveal a marked increase in the uptake of DiI-LDL by cells with the statin treatment (Figure 23, C). If confirmed by further analysis, these data may be explained by a high activity and turnover of LDLR. However, the higher amount of PCSK9 prevented the recycling of this receptor to the cell membrane and rather promoted its continuous degradation by the lysosomes. The presence of meprin α didn't seem to modulate this process under the treatment conditions by simvastatin.

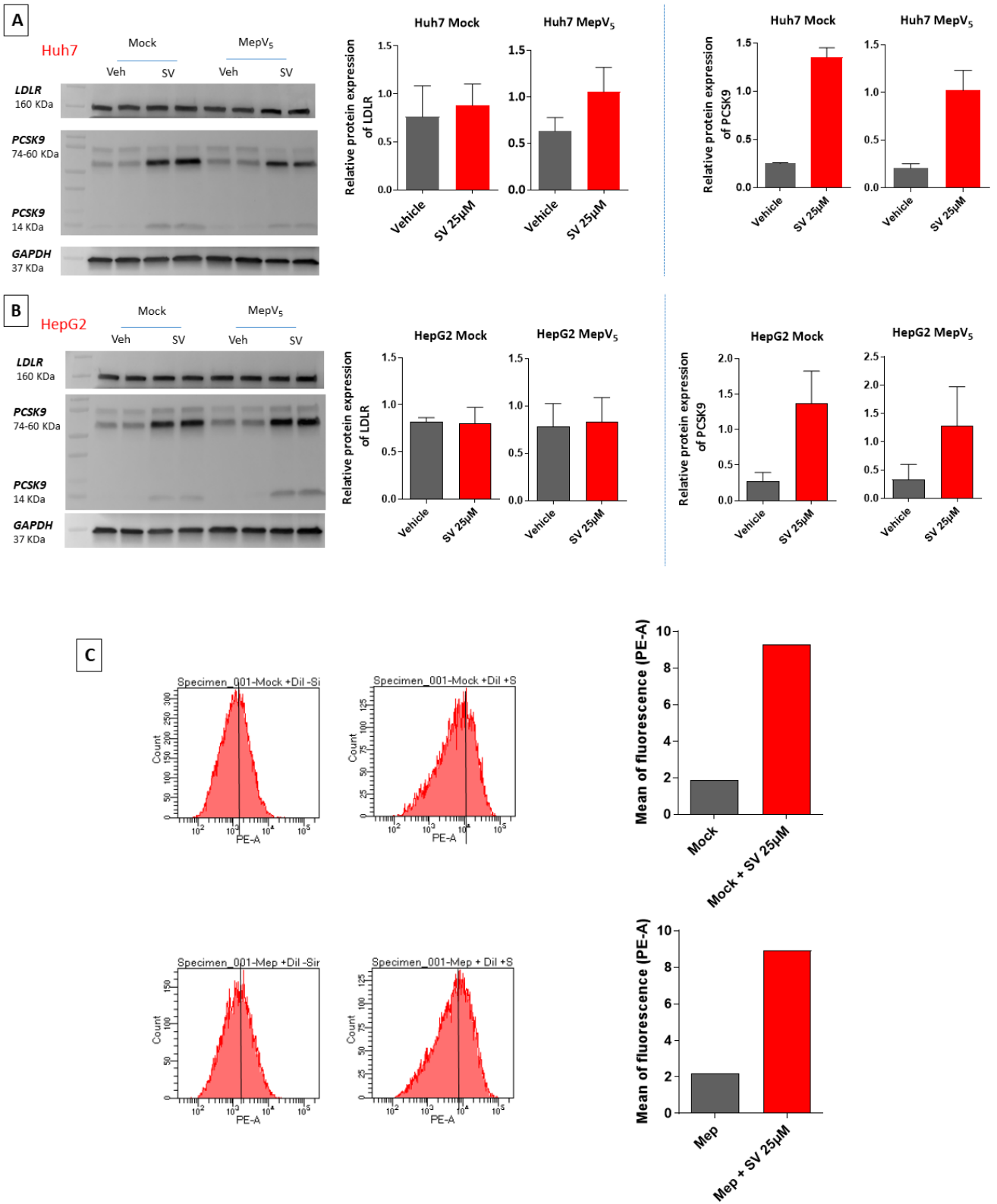


Figure 3023: Effect of simvastatin (SV) treatment on protein expression levels and DiI-LDL uptake.

Western blot showing the protein expression levels of LDLR and PCSK9 in Huh7 (A) and HepG2 (B) Mock and MepV₅ cells treated with 25µM SV for 3 days along their respective quantification; GAPDH represents the house-keeping gene (C) Flow cytometry of DiI-LDL uptake by Huh7 Mock and MepV₅ cells that are treated with 25µM SV for 3 days.

Conclusion and discussion

In this project, we have shown so far that *MEP1A* expression is increased in HBL tumors compared to the non-tumor tissues, and this goes in accordance with the other genomic analysis of hepatoblastoma tumors done by different groups [96], [292]. This increase was confirmed by RNAseq analysis of hepatic cancer cell lines, which showed a marked expression in HepG2 (HBL cell line) and Huh7 (HCC cell line), whilst no expression was detected in Huh6 (HBL cell line) and THLE-2 (normal cells). Surprisingly, by looking at the protein expression level of MEP1A in these cell lines, no expression was detected at all. For this reason and to be more relevant to the *in vivo* context, it was necessary to overexpress meprin in these cells using a lentivirus in order to mimic the real situation. This lentivirus system was only effective in HepG2 and Huh7 cells, but not in Huh6 due to the lack of activity of the CMV (cytomegalovirus) promoter in these cells [293] and it will be essential in the future to create such a model in this cell line. More lately, our team was able to subclone *MEP1A* under the control of EF1a (elongation factor 1 alpha) promoter and a new lentivirus was produced (see Annex). Future investigations with this additional cell line are needed to strengthen and validate our present findings.

Proteomic analysis of the secretome derived from HepG2 meprin overexpressing cells vs their control highlighted the potential role of this enzyme in lipid metabolism, through alterations in the expression of PCSK9 and its related proteins. This was confirmed through the significant decrease in the uptake of DiI-LDL in meprin overexpressing cells, which was reversed with statin treatment. Moreover, I have also shown that in the presence of meprin α , the mitochondrial respiration is significantly enhanced, and seems to be dependent on the presence of glutamine.

Of particular note in the proteomic data is the deregulation of proteins involved in angiogenesis, immunomodulation and tumor progression of HCC, like SPARC and PAI-1 [294], [295]. I believe it is necessary to explore this path in order to unravel the possible immunoncogenic roles of meprin α and there are some points that must be addressed. First, in addition to exploring cell secretome, proteomic analysis should be carried out in cell extracts as well, in order to uncover potential intracellular changes in proteins and pathways. Second, the overall analyses should be conducted in all liver cancer cell lines available in the laboratory

to have a better understanding of the role of meprin α in different cellular and pathological contexts. Third, it is true that we have induced higher expression of meprin in our cell lines, but what's even more important is to check the activity of this protein. I have conducted preliminary assessment of the enzymatic activity of meprin in the different cell lines, without conclusive results. Additional optimization of protocols is needed to finalize this part of the project.

Lipidomic analysis of male WT and *MEP1A* KO mice-derived tissues, as well as meprin overexpressing cells and their control, underlined an important role of meprin in the lipid metabolism of the liver both *in vitro* and *in vivo*. In addition, having these WT and *MEP1A* KO mice-derived liver tissues (male and female) as well as their blood samples could be useful to draw more complete pictures of changes in the lipid pattern at the systemic level, and whether the gender could affect such changes. For instance, regardless of the gender, the expression level of PCSK9 and its related proteins in these tissues should be checked by western blot and immunohistochemistry and the plasma level of PCSK9 by ELISA. Also, the female tissues can be included in lipidomic analysis to determine the lipid content and Oil Red O stain can be done on tissue sections to detect neutral triglycerides and lipids, hence monitoring the lipid droplets under meprin KO conditions. All of these would give us better understanding of the changes occurring at the lipidic level.

As for the cellular levels, more in depth analyses of lipidomic as well as fluxomics are needed to clarify the exact metabolic changes affected by meprin α to better understand how this enzyme affects the crosstalk and feedback regulation between lipid uptake and lipogenesis.

In conclusion, this part of the project has some promising results, but more analyses and exploration are needed to further understand the novel and complex involvement of meprin α in lipid metabolism, which seems to be partly linked to PCSK9. Modification of the energetic metabolism by meprin α may also be the tip of the iceberg of more complex and critical involvement of this enzyme in a wider pattern of metabolic pathways, which exploration may lead to novel functions of meprin in different physiopathological contexts.

General Conclusion and Perspectives

From my project, I have unraveled novel functions of PCSK9 including its involvement in maintaining cell redox homeostasis and serving as a new player in ferroptosis. Inhibition of PCSK9 was effective in breaking up the oncometabolic process and seemed to weaken the cellular response to iron-triggered lipid peroxidation and hence diminished cell growth. Moreover, I also identified that meprin α , which is overexpressed in HCC and HBL, has some novel and complex involvement in lipid metabolism that could be partly linked to PCSK9.

The main focus of my work was to have a deep understanding of the lipid metabolism, which deregulation has gained attention due to its critical role in liver oncogenesis in both HCC and HBL. I was aiming at finding alternative therapeutic approaches that are less toxic and widely available. The metabolism of lipids is a network of interconnected pathways finely regulated and where any malfunction could drive many pathological processes including cancers. Many key players have been described before as regulators of lipid metabolism including some metabolites like acetyl-CoA and acetate, enzymes and transcription factors like ACLY, ACSS2 and SREBP2 [296].

We have been specifically interested in a proprotein convertase called PCSK9 whose function and involvement in lipid metabolism have been described in section V of the introduction. The increased levels of PCSK9 that we have observed in multiple databases in HCC and HBL indicated that this protein could have oncogenic roles in tumor development. Alongside PCSK9, we have also seen an increase in meprin α , HMGCR and FDFT1, which are key rate-limiting enzymes of *de novo* lipogenesis. It is important to mention that meprin α is also overexpressed in HCC and promotes the aggressiveness of the disease resulting in bad prognosis. No one has ever studied meprin α in HBL though we have observed a significant overexpression of this metalloprotease in several databases related to this cancer.

Our overall analyses revealed deregulation of lipid metabolism with a shift towards *de novo* synthesis instead of lipoprotein uptake by receptors. This observed profile is seen in patients suffering from hypercholesterolemia (familial or non-familial) and CVD. In such patients, the first-line drugs used to treat them includes statins, which can be combined at certain stages (especially when statins are no longer efficient by themselves to lower cholesterol levels, or when patients develop resistance to statins) with PCSK9 inhibitors (at this stage, mAbs: evolocumab and alirocumab) (see section V.5 and V.8). An interesting consideration about

these drugs is that they are already FDA approved and have passed multiple clinical trials. In addition, statins have been studied for their anti-tumor effects in many cancers and are currently under investigation in more than 20 clinical trials [296], [297]. This highlights the need of testing the efficacy of these treatments, alone or in combination, in inhibiting tumor growth of HCC and HBL, hence serving as alternative approach to chemotherapy.

Indeed, my results show that targeting PCSK9 with pharmacological or genetic approaches resulted in inhibition of cell proliferation *in vitro* and decrease in tumor aggressiveness and size *in vivo*. The combination of such a treatment with statins revealed a synergetic effect. Moreover, the massive accumulation of peroxidation-prone lipids observed with PCSK9 inhibition by lipidomics and TEM revealed a new mechanism by which PCSK9 inhibition triggers cell death, which is ferroptosis, through Keap1/Nrf2 pathway. Ferroptosis is activated upon overwhelming accumulation of lipid peroxides triggering irreversible cell damages. Moreover, we have shown that the overexpression of meprin α resulted in the deregulation in the expression of many proteins involved in lipid metabolism and inflammation. Indeed, meprin α overexpression was accompanied by increased PCSK9 expression and decrease in lipid uptake.

These results open the way to several perspectives in the context of the novel involvement of PCSK9 in the control of ferroptosis. To validate these conclusions, additional experiments are needed.

First, it is important to complete the analysis by measuring the level of lipid peroxidation in cells and to identify the PUFA-phospholipids that are oxidized upon PCSK9 silencing. For instance, Kagan *et al.* [298] discovered doubly and triply oxygenated diacyl arachidonoyl and adrenoyl PE generated by lipooxygenase (LOX) as ferroptosis death signals. Experiments are currently under way to determine the extent of lipid peroxidation. Moreover, lipidomics will allow to verify the level of free and esterified FAs in this experimental setting.

Second, we need to verify whether the use of some inhibitors of ferroptosis such as ferrostatin-1 will be able to reverse some of the antitumoral effects of anti-PCSK9 approaches. Treatment by erastin will serve as positive control of ferroptosis.

Third, the synergism between PCSK9 inhibition and statin treatment that we have observed *in vitro* should be translated to *in vivo* models like CAM. In this case, different combinations and monotreatments can be tried to find the best conditions before considering a mammalian model like the murine one. Even though European regulations are now tending toward excluding animal experimentations, if such experimentation is allowed in the future, we could test the effectiveness of different formulations of our therapeutic agents (nanoparticle, liposomes or adeno-associated virus) and the mode of administration of drugs or siRNA (oral, bloodstream, peritumoral, etc.). Ideally, it would be essential to identify an easy delivery system that can bring the drug directly into the liver cancer cells. The dose of the delivery system, the mode of administration, immune responses and off-target effects leading to cytotoxicity could be studied in parallel in mice. Such strong *in vivo* data would reveal more preclinical information related to angiogenesis, metastasis and tumor growth taking into account the microenvironment.

As for meprin, proteomics analysis should be first extended to different cell lines and performed not only in cell supernatant but also in cell extract. Because meprin is a metalloprotease that needs to be cleaved and form oligomers in the ECM to become activated, it's essential to measure the activity of this protein in our cell lines to confirm its activation status and that it's functional. This can be done by using a fluorogenic peptide substrate specific for meprin α (mca-HVANDPIW-K-dnp), which was given by Pr. Christoph Becker-Pauly. After confirming that meprin α is active, and because PCSK9 has already been identified as a substrate of meprin α , which can cleave it at multiple sites [246], we must control in our conditions the different maturation forms of PCSK9.

Second, to further explore the connection between PCSK9 and meprin α and to verify the extent of PCSK9-dependent protumoral activities of meprin α , additional experiments are needed as well. One option is to overexpress meprin α in cells lacking or not PCSK9 (pre-transduced by shRNA) and see the impact of such treatment on tumor cell growth.

Finally, one intriguing question one could ask is whether targeting both genes would have additive synergetic effect on oncogenesis. We may answer this question by using meprin inhibitors (e.g., actinonin, galardin, etc.) in combination with PCSK9 siRNA in Mep1a overexpressing cell lines.

All these experiments will allow to better understand the role of PCSK9 and meprin α in lipid metabolism whose deregulation is an emerging hallmark in cancer cells. This understanding will allow us to offer more therapeutic options targeting both enzymes to treat liver cancers more effectively and with less side effects.

References

- [1] A. Sudhakar, "History of Cancer, Ancient and Modern Treatment Methods," *J. Cancer Sci. Ther.*, vol. 01, no. 02, pp. i–iv, 2009, doi: 10.4172/1948-5956.100000e2.
- [2] P. Anand *et al.*, "Cancer is a Preventable Disease that Requires Major Lifestyle Changes," *Pharm. Res.*, vol. 25, no. 9, pp. 2097–2116, Sep. 2008, doi: 10.1007/s11095-008-9661-9.
- [3] "Types of cancer," *Cancer Research UK*, Oct. 28, 2014. <https://www.cancerresearchuk.org/what-is-cancer/how-cancer-starts/types-of-cancer> (accessed Sep. 26, 2019).
- [4] D. Hanahan and R. A. Weinberg, "The Hallmarks of Cancer," *Cell*, vol. 100, no. 1, pp. 57–70, Jan. 2000, doi: 10.1016/S0092-8674(00)81683-9.
- [5] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, Mar. 2011, doi: 10.1016/j.cell.2011.02.013.
- [6] H. Sung *et al.*, "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries," *CA. Cancer J. Clin.*, vol. 71, no. 3, pp. 209–249, 2021, doi: 10.3322/caac.21660.
- [7] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2019," *CA. Cancer J. Clin.*, vol. 69, no. 1, pp. 7–34, 2019, doi: 10.3322/caac.21551.
- [8] "What Is Liver Cancer?" <https://www.cancer.org/cancer/liver-cancer/about/what-is-liver-cancer.html> (accessed Sep. 30, 2019).
- [9] "Critical appraisal of clinical practice guidelines for diagnosis and treatment of hepatocellular carcinoma - Schmidt - 2011 - Journal of Gastroenterology and Hepatology - Wiley Online Library." <https://onlinelibrary-wiley-com.proxy.insermbiblio.inist.fr/doi/full/10.1111/j.1440-1746.2011.06891.x> (accessed Mar. 18, 2020).
- [10] J. Ferlay *et al.*, "Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012," *Int. J. Cancer*, vol. 136, no. 5, pp. E359–E386, 2015, doi: 10.1002/ijc.29210.
- [11] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA. Cancer J. Clin.*, vol. 68, no. 6, pp. 394–424, 2018, doi: 10.3322/caac.21492.
- [12] Y. Li, H. Li, J. M. Spitsbergen, and Z. Gong, "Males develop faster and more severe hepatocellular carcinoma than females in krasV12 transgenic zebrafish," *Sci. Rep.*, vol. 7, Jan. 2017, doi: 10.1038/srep41280.
- [13] I. Shimizu and S. Ito, "Protection of estrogens against the progression of chronic liver disease," *Hepatol. Res.*, vol. 37, no. 4, pp. 239–247, 2007, doi: 10.1111/j.1872-034X.2007.00032.x.
- [14] J. M. Llovet *et al.*, "Hepatocellular carcinoma," *Nat. Rev. Dis. Primer*, vol. 2, no. 1, Art. no. 1, Apr. 2016, doi: 10.1038/nrdp.2016.18.
- [15] J. Bruix *et al.*, "Clinical Management of Hepatocellular Carcinoma. Conclusions of the Barcelona-2000 EASL Conference," *J. Hepatol.*, vol. 35, no. 3, pp. 421–430, Sep. 2001, doi: 10.1016/S0168-8278(01)00130-1.

- [16] S. J. Yu, "A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010-2016," *Clin. Mol. Hepatol.*, vol. 22, no. 1, pp. 7–17, Mar. 2016, doi: 10.3350/cmh.2016.22.1.7.
- [17] P. Song *et al.*, "The management of hepatocellular carcinoma around the world: a comparison of guidelines from 2001 to 2011," *Liver Int.*, vol. 32, no. 7, pp. 1053–1063, 2012, doi: 10.1111/j.1478-3231.2012.02792.x.
- [18] P. A. Farazi and R. A. DePinho, "Hepatocellular carcinoma pathogenesis: from genes to environment," *Nat. Rev. Cancer*, vol. 6, no. 9, Art. no. 9, Sep. 2006, doi: 10.1038/nrc1934.
- [19] R. Dhanasekaran, S. Bandoh, and L. R. Roberts, "Molecular pathogenesis of hepatocellular carcinoma and impact of therapeutic advances," *F1000Research*, vol. 5, May 2016, doi: 10.12688/f1000research.6946.1.
- [20] D. Lavanchy, "Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures," *J. Viral Hepat.*, vol. 11, no. 2, pp. 97–107, 2004, doi: 10.1046/j.1365-2893.2003.00487.x.
- [21] T. M. Block, A. S. Mehta, C. J. Fimmel, and R. Jordan, "Molecular viral oncology of hepatocellular carcinoma," *Oncogene*, vol. 22, no. 33, Art. no. 33, Aug. 2003, doi: 10.1038/sj.onc.1206557.
- [22] T. Tokino, H. Tamura, N. Hori, and K. Matsubara, "Chromosome deletions associated with hepatitis B virus integration," *Virology*, vol. 185, no. 2, pp. 879–882, Dec. 1991, doi: 10.1016/0042-6822(91)90564-R.
- [23] Y. Murakami *et al.*, "Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas," *Gut*, vol. 54, no. 8, pp. 1162–1168, Aug. 2005, doi: 10.1136/gut.2004.054452.
- [24] "HCV core protein and virus assembly: what we know without structures." <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6659715/> (accessed Mar. 19, 2020).
- [25] B. Rehmann and M. Nascimbeni, "Immunology of hepatitis B virus and hepatitis C virus infection," *Nat. Rev. Immunol.*, vol. 5, no. 3, Art. no. 3, Mar. 2005, doi: 10.1038/nri1573.
- [26] K. Melén, R. Fagerlund, M. Nyqvist, P. Keskinen, and I. Julkunen, "Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs," *J. Med. Virol.*, vol. 73, no. 4, pp. 536–547, 2004, doi: 10.1002/jmv.20123.
- [27] M. Majumder *et al.*, "Hepatitis C Virus NS5A Protein Impairs TNF-Mediated Hepatic Apoptosis, but Not by an Anti-FAS Antibody, in Transgenic Mice," *Virology*, vol. 294, no. 1, pp. 94–105, Mar. 2002, doi: 10.1006/viro.2001.1309.
- [28] K.-J. Park *et al.*, "Hepatitis C Virus NS5A Protein Modulates c-Jun N-terminal Kinase through Interaction with Tumor Necrosis Factor Receptor-associated Factor 2," *J. Biol. Chem.*, vol. 278, no. 33, pp. 30711–30718, Aug. 2003, doi: 10.1074/jbc.M209623200.
- [29] P. Ramadori, F. J. Cubero, C. Liedtke, C. Trautwein, and Y. A. Nevzorova, "Alcohol and Hepatocellular Carcinoma: Adding Fuel to the Flame," *Cancers*, vol. 9, no. 10, Sep. 2017, doi: 10.3390/cancers9100130.
- [30] C. Scoccianti *et al.*, "European Code against Cancer 4th Edition: Alcohol drinking and cancer," *Cancer Epidemiol.*, vol. 45, pp. 181–188, Dec. 2016, doi: 10.1016/j.canep.2016.09.011.
- [31] A. S. HAMID, I. G. TEFAMARIAM, Y. ZHANG, and Z. G. ZHANG, "Aflatoxin B1-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention," *Oncol. Lett.*, vol. 5, no. 4, pp. 1087–1092, Apr. 2013, doi: 10.3892/ol.2013.1169.

- [32] J. Martin and J.-F. Dufour, "Tumor suppressor and hepatocellular carcinoma," *World J. Gastroenterol. WJG*, vol. 14, no. 11, pp. 1720–1733, Mar. 2008, doi: 10.3748/wjg.14.1720.
- [33] C. R. Madden, M. J. Finegold, and B. L. Slagle, "Altered DNA Mutation Spectrum in Aflatoxin B1-Treated Transgenic Mice That Express the Hepatitis B Virus X Protein," *J. Virol.*, vol. 76, no. 22, pp. 11770–11774, Nov. 2002, doi: 10.1128/JVI.76.22.11770-11774.2002.
- [34] E. Dhamija, S. B. Paul, and S. Kedia, "Non-alcoholic fatty liver disease associated with hepatocellular carcinoma: An increasing concern," *Indian J. Med. Res.*, vol. 149, no. 1, pp. 9–17, Jan. 2019, doi: 10.4103/ijmr.IJMR_1456_17.
- [35] W. K. K. Wu, L. Zhang, and M. T. V. Chan, "Autophagy, NAFLD and NAFLD-Related HCC," *Adv. Exp. Med. Biol.*, vol. 1061, pp. 127–138, 2018, doi: 10.1007/978-981-10-8684-7_10.
- [36] V. W.-S. Wong, L. A. Adams, V. de Lédinghen, G. L.-H. Wong, and S. Sookoian, "Noninvasive biomarkers in NAFLD and NASH — current progress and future promise," *Nat. Rev. Gastroenterol. Hepatol.*, vol. 15, no. 8, Art. no. 8, Aug. 2018, doi: 10.1038/s41575-018-0014-9.
- [37] S. Singh, A. M. Allen, Z. Wang, L. J. Prokop, M. H. Murad, and R. Loomba, "Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies," *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.*, vol. 13, no. 4, pp. 643-654.e1–9; quiz e39-40, Apr. 2015, doi: 10.1016/j.cgh.2014.04.014.
- [38] N. Rivlin, R. Brosh, M. Oren, and V. Rotter, "Mutations in the p53 Tumor Suppressor Gene," *Genes Cancer*, vol. 2, no. 4, pp. 466–474, Apr. 2011, doi: 10.1177/1947601911408889.
- [39] T. Zhan, N. Rindtorff, and M. Boutros, "Wnt signaling in cancer," *Oncogene*, vol. 36, no. 11, Art. no. 11, Mar. 2017, doi: 10.1038/onc.2016.304.
- [40] Y. Ito *et al.*, "Expression and clinical significance of erb-B receptor family in hepatocellular carcinoma," *Br. J. Cancer*, vol. 84, no. 10, pp. 1377–1383, May 2001, doi: 10.1054/bjoc.2000.1580.
- [41] Y. Liu *et al.*, "ERBB4 acts as a suppressor in the development of hepatocellular carcinoma," *Carcinogenesis*, vol. 38, no. 4, pp. 465–473, Apr. 2017, doi: 10.1093/carcin/bgx017.
- [42] H. Ni *et al.*, "ErbB4 acts as a suppressor in colitis and its associated carcinoma by negatively regulating cholesterol metabolism," *Carcinogenesis*, vol. 40, no. 5, pp. 680–686, Jul. 2019, doi: 10.1093/carcin/bgy164.
- [43] K. J. Turner, V. Vasu, and D. K. Griffin, "Telomere Biology and Human Phenotype," *Cells*, vol. 8, no. 1, Jan. 2019, doi: 10.3390/cells8010073.
- [44] C.-M. Lee *et al.*, "Telomerase activity and telomerase catalytic subunit in hepatocellular carcinoma," *Hepatology*, vol. 51, no. 57, pp. 796–800, Jun. 2004.
- [45] "Recent updates of genetic and genomic alterations in hepatocellular carcinoma." <https://hrjournal.net/article/view/1303> (accessed Mar. 25, 2020).
- [46] J. M. Llovet, R. Montal, D. Sia, and R. S. Finn, "Molecular therapies and precision medicine for hepatocellular carcinoma," *Nat. Rev. Clin. Oncol.*, vol. 15, no. 10, Art. no. 10, Oct. 2018, doi: 10.1038/s41571-018-0073-4.
- [47] S. Caruso *et al.*, "Analysis of Liver Cancer Cell Lines Identifies Agents With Likely Efficacy Against Hepatocellular Carcinoma and Markers of Response," *Gastroenterology*, vol. 157, no. 3, pp. 760–776, Sep. 2019, doi: 10.1053/j.gastro.2019.05.001.

- [48] S. Boyault *et al.*, “Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets,” *Hepatology*, vol. 45, no. 1, pp. 42–52, 2007, doi: 10.1002/hep.21467.
- [49] Y. Hoshida *et al.*, “Integrative Transcriptome Analysis Reveals Common Molecular Subclasses of Human Hepatocellular Carcinoma,” *Cancer Res.*, vol. 69, no. 18, pp. 7385–7392, Sep. 2009, doi: 10.1158/0008-5472.CAN-09-1089.
- [50] D. A. Wheeler and L. R. Roberts, “Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma,” *Cell*, vol. 169, no. 7, pp. 1327–1341.e23, Jun. 2017, doi: 10.1016/j.cell.2017.05.046.
- [51] “EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma,” *J. Hepatol.*, vol. 69, no. 1, pp. 182–236, Jul. 2018, doi: 10.1016/j.jhep.2018.03.019.
- [52] M.-H. Chang *et al.*, “Decreased Incidence of Hepatocellular Carcinoma in Hepatitis B Vaccinees: A 20-Year Follow-up Study,” *JNCI J. Natl. Cancer Inst.*, vol. 101, no. 19, pp. 1348–1355, Oct. 2009, doi: 10.1093/jnci/djp288.
- [53] J. Bruix and M. Sherman, “Management of hepatocellular carcinoma,” *Hepatology*, vol. 42, no. 5, pp. 1208–1236, 2005, doi: 10.1002/hep.20933.
- [54] M. S. Grandhi, A. K. Kim, S. M. Ronnekleiv-Kelly, I. R. Kamel, M. A. Ghasebeh, and T. M. Pawlik, “Hepatocellular carcinoma: From diagnosis to treatment,” *Surg. Oncol.*, vol. 25, no. 2, pp. 74–85, Jun. 2016, doi: 10.1016/j.suronc.2016.03.002.
- [55] D. Li, T. Mallory, and S. Satomura, “AFP-L3: a new generation of tumor marker for hepatocellular carcinoma,” *Clin. Chim. Acta*, vol. 313, no. 1, pp. 15–19, Nov. 2001, doi: 10.1016/S0009-8981(01)00644-1.
- [56] R. Tateishi, H. Yoshida, Y. Matsuyama, N. Mine, Y. Kondo, and M. Omata, “Diagnostic accuracy of tumor markers for hepatocellular carcinoma: a systematic review,” *Hepatol. Int.*, vol. 2, no. 1, pp. 17–30, Mar. 2008, doi: 10.1007/s12072-007-9038-x.
- [57] “Pathologic diagnosis of early hepatocellular carcinoma: A report of the international consensus group for hepatocellular neoplasia,” *Hepatology*, vol. 49, no. 2, pp. 658–664, 2009, doi: 10.1002/hep.22709.
- [58] J. M. Llovet, C. Brú, and J. Bruix, “Prognosis of hepatocellular carcinoma: the BCLC staging classification,” *Semin. Liver Dis.*, vol. 19, no. 3, pp. 329–338, 1999, doi: 10.1055/s-2007-1007122.
- [59] A. Forner, M. Reig, and J. Bruix, “Hepatocellular carcinoma,” *The Lancet*, vol. 391, no. 10127, pp. 1301–1314, Mar. 2018, doi: 10.1016/S0140-6736(18)30010-2.
- [60] A. Villanueva, “Hepatocellular Carcinoma,” *N. Engl. J. Med.*, vol. 380, no. 15, pp. 1450–1462, Apr. 2019, doi: 10.1056/NEJMra1713263.
- [61] J. M. Llovet *et al.*, “Sorafenib in Advanced Hepatocellular Carcinoma,” *N. Engl. J. Med.*, vol. 359, no. 4, pp. 378–390, Jul. 2008, doi: 10.1056/NEJMoa0708857.
- [62] J. Bruix *et al.*, “Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial,” *Lancet Lond. Engl.*, vol. 389, no. 10064, pp. 56–66, 07 2017, doi: 10.1016/S0140-6736(16)32453-9.
- [63] O. B. Gbolahan, M. A. Schacht, E. W. Beckley, T. P. LaRoche, B. H. O’Neil, and M. Pyko, “Locoregional and systemic therapy for hepatocellular carcinoma,” *J. Gastrointest. Oncol.*, vol. 8, no. 2, pp. 215–228, Apr. 2017, doi: 10.21037/jgo.2017.03.13.
- [64] A. Raza and G. K. Sood, “Hepatocellular carcinoma review: Current treatment, and evidence-based medicine,” *World J. Gastroenterol. WJG*, vol. 20, no. 15, pp. 4115–4127, Apr. 2014, doi: 10.3748/wjg.v20.i15.4115.

- [65] R. A. Smith *et al.*, "Discovery of heterocyclic ureas as a new class of raf kinase inhibitors: identification of a second generation lead by a combinatorial chemistry approach," *Bioorg. Med. Chem. Lett.*, vol. 11, no. 20, pp. 2775–2778, Oct. 2001, doi: 10.1016/S0960-894X(01)00571-6.
- [66] S. M. Wilhelm *et al.*, "BAY 43-9006 Exhibits Broad Spectrum Oral Antitumor Activity and Targets the RAF/MEK/ERK Pathway and Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis," *Cancer Res.*, vol. 64, no. 19, pp. 7099–7109, Oct. 2004, doi: 10.1158/0008-5472.CAN-04-1443.
- [67] S. M. Wilhelm, L. Adnane, P. Newell, A. Villanueva, J. M. Llovet, and M. Lynch, "Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling," *Mol. Cancer Ther.*, vol. 7, no. 10, pp. 3129–3140, Oct. 2008, doi: 10.1158/1535-7163.MCT-08-0013.
- [68] F. Zustovich *et al.*, "Clinical experience and critical evaluation of the role of sorafenib in renal cell carcinoma," *Open Access J. Urol.*, vol. 3, pp. 69–82, May 2011, doi: 10.2147/OAJU.S7230.
- [69] A. Gauthier and M. Ho, "The Role of Sorafenib in the Treatment of Advanced Hepatocellular Carcinoma: An Update," *Hepatol. Res. Off. J. Jpn. Soc. Hepatol.*, vol. 43, no. 2, pp. 147–154, Feb. 2013, doi: 10.1111/j.1872-034X.2012.01113.x.
- [70] Z. Ezzoukhry *et al.*, "EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib," *Int. J. Cancer*, vol. 131, no. 12, pp. 2961–2969, 2012, doi: 10.1002/ijc.27604.
- [71] K.-F. Chen *et al.*, "Activation of Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Mediates Acquired Resistance to Sorafenib in Hepatocellular Carcinoma Cells," *J. Pharmacol. Exp. Ther.*, vol. 337, no. 1, pp. 155–161, Apr. 2011, doi: 10.1124/jpet.110.175786.
- [72] T. F. Greten and B. Sangro, "Targets for immunotherapy of liver cancer," *J. Hepatol.*, Sep. 2017, doi: 10.1016/j.jhep.2017.09.007.
- [73] P. Sharma, S. Hu-Lieskovan, J. A. Wargo, and A. Ribas, "Primary, Adaptive and Acquired Resistance to Cancer Immunotherapy," *Cell*, vol. 168, no. 4, pp. 707–723, Feb. 2017, doi: 10.1016/j.cell.2017.01.017.
- [74] A. B. El-Khoueiry *et al.*, "Impact of antitumor activity on survival outcomes, and nonconventional benefit, with nivolumab (NIVO) in patients with advanced hepatocellular carcinoma (aHCC): Subanalyses of CheckMate-040.," *J. Clin. Oncol.*, vol. 36, no. 4_suppl, pp. 475–475, Feb. 2018, doi: 10.1200/JCO.2018.36.4_suppl.475.
- [75] A. Garnier, M. Ilmer, R. Kappler, and M. Berger, "Therapeutic Innovations for Targeting Hepatoblastoma," *Anticancer Res.*, vol. 36, no. 11, pp. 5577–5592, Nov. 2016.
- [76] J. B. Litten and G. E. Tomlinson, "Liver Tumors in Children," *The Oncologist*, vol. 13, no. 7, pp. 812–820, 2008, doi: 10.1634/theoncologist.2008-0011.
- [77] S. Zhong, Y. Zhao, and C. Fan, "Hepatoblastoma with pure fetal epithelial differentiation in a 10-year-old boy," *Medicine (Baltimore)*, vol. 97, no. 2, Jan. 2018, doi: 10.1097/MD.00000000000009647.
- [78] P. Czauderna and H. Garnier, "Hepatoblastoma: current understanding, recent advances, and controversies," *F1000Research*, vol. 7, Jan. 2018, doi: 10.12688/f1000research.12239.1.
- [79] A. D. Baheti, A. Luana Stanescu, N. Li, and T. Chapman, "Contrast-enhanced CT features of hepatoblastoma: Can we predict histopathology?," *Clin. Imaging*, vol. 44, pp. 33–37, Jul. 2017, doi: 10.1016/j.clinimag.2017.03.023.

- [80] D. C. Aronson, P. Czauderna, R. Maibach, G. Perilongo, and B. Morland, "The treatment of hepatoblastoma: Its evolution and the current status as per the SIOPEL trials," *J. Indian Assoc. Pediatr. Surg.*, vol. 19, no. 4, pp. 201–207, 2014, doi: 10.4103/0971-9261.142001.
- [81] P. Czauderna *et al.*, "The Children's Hepatic tumors International Collaboration (CHIC): Novel global rare tumor database yields new prognostic factors in hepatoblastoma and becomes a research model," *Eur. J. Cancer Oxf. Engl. 1990*, vol. 52, pp. 92–101, Jan. 2016, doi: 10.1016/j.ejca.2015.09.023.
- [82] L. G. Spector and J. Birch, "The epidemiology of hepatoblastoma," *Pediatr. Blood Cancer*, vol. 59, no. 5, pp. 776–779, 2012, doi: 10.1002/pbc.24215.
- [83] S. Aretz *et al.*, "Should children at risk for familial adenomatous polyposis be screened for hepatoblastoma and children with apparently sporadic hepatoblastoma be screened for APC germline mutations?," *Pediatr. Blood Cancer*, vol. 47, no. 6, pp. 811–818, 2006, doi: 10.1002/pbc.20698.
- [84] A. Sparago *et al.*, "Mechanisms causing imprinting defects in familial Beckwith–Wiedemann syndrome with Wilms' tumour," *Hum. Mol. Genet.*, vol. 16, no. 3, pp. 254–264, Feb. 2007, doi: 10.1093/hmg/ddl448.
- [85] T. T. Lai and C. F. Bearer, "Iatrogenic Environmental Hazards in the Neonatal Intensive Care Unit," *Clin. Perinatol.*, vol. 35, no. 1, pp. 163–ix, Mar. 2008, doi: 10.1016/j.clp.2007.11.003.
- [86] D. Pang, R. McNally, and J. M. Birch, "Parental smoking and childhood cancer: results from the United Kingdom Childhood Cancer Study," *Br. J. Cancer*, vol. 88, no. 3, pp. 373–381, Feb. 2003, doi: 10.1038/sj.bjc.6600774.
- [87] J. D. Buckley *et al.*, "A case-control study of risk factors for hepatoblastoma. A report from the Childrens Cancer Study Group," *Cancer*, vol. 64, no. 5, pp. 1169–1176, Sep. 1989, doi: 10.1002/1097-0142(19890901)64:5<1169::aid-cnrc2820640534>3.0.co;2-i.
- [88] G. A. MacKinlay and J. Pritchard, "A common language for childhood liver tumours," *Pediatr. Surg. Int.*, vol. 7, no. 4, Jun. 1992, doi: 10.1007/BF00183998.
- [89] A. J. Towbin *et al.*, "2017 PRETEXT: radiologic staging system for primary hepatic malignancies of childhood revised for the Paediatric Hepatic International Tumour Trial (PHITT)," *Pediatr. Radiol.*, vol. 48, no. 4, pp. 536–554, Apr. 2018, doi: 10.1007/s00247-018-4078-z.
- [90] D. López-Terrada *et al.*, "Towards an international pediatric liver tumor consensus classification: proceedings of the Los Angeles COG liver tumors symposium," *Mod. Pathol.*, vol. 27, no. 3, Art. no. 3, Mar. 2014, doi: 10.1038/modpathol.2013.80.
- [91] D. C. Aronson and R. L. Meyers, "Malignant tumors of the liver in children," *Semin. Pediatr. Surg.*, vol. 25, no. 5, pp. 265–275, Oct. 2016, doi: 10.1053/j.sempedsurg.2016.09.002.
- [92] S. Cairo *et al.*, "Hepatic Stem-like Phenotype and Interplay of Wnt/ β -Catenin and Myc Signaling in Aggressive Childhood Liver Cancer," *Cancer Cell*, vol. 14, no. 6, pp. 471–484, Dec. 2008, doi: 10.1016/j.ccr.2008.11.002.
- [93] M. A. Buendia, "Unravelling the genetics of hepatoblastoma: Few mutations, what else?," *J. Hepatol.*, vol. 61, no. 6, pp. 1202–1204, Dec. 2014, doi: 10.1016/j.jhep.2014.09.016.
- [94] D. Jia *et al.*, "Exome sequencing of hepatoblastoma reveals novel mutations and cancer genes in the Wnt pathway and ubiquitin ligase complex," *Hepatology*, vol. 60, no. 5, pp. 1686–1696, 2014, doi: 10.1002/hep.27243.

- [95] G. E. Tomlinson and R. Kappler, "Genetics and epigenetics of hepatoblastoma," *Pediatr. Blood Cancer*, vol. 59, no. 5, pp. 785–792, 2012, doi: 10.1002/pbc.24213.
- [96] K. B. Hooks *et al.*, "New insights into diagnosis and therapeutic options for proliferative hepatoblastoma," *Hepatology*, vol. 68, no. 1, pp. 89–102, 2018, doi: 10.1002/hep.29672.
- [97] R. L. Meyers, P. Czauderna, and J.-B. Otte, "Surgical treatment of hepatoblastoma," *Pediatr. Blood Cancer*, vol. 59, no. 5, pp. 800–808, Nov. 2012, doi: 10.1002/pbc.24220.
- [98] D. von Schweinitz, "Management of liver tumors in childhood," *Semin. Pediatr. Surg.*, vol. 15, no. 1, pp. 17–24, Feb. 2006, doi: 10.1053/j.sempedsurg.2005.11.004.
- [99] S. Dasari and P. B. Tchounwou, "Cisplatin in cancer therapy: molecular mechanisms of action," *Eur. J. Pharmacol.*, vol. 0, pp. 364–378, Oct. 2014, doi: 10.1016/j.ejphar.2014.07.025.
- [100] P. Czauderna, D. Lopez-Terrada, E. Hiyama, B. Häberle, M. H. Malogolowkin, and R. L. Meyers, "Hepatoblastoma state of the art: pathology, genetics, risk stratification, and chemotherapy," *Curr. Opin. Pediatr.*, vol. 26, no. 1, pp. 19–28, Feb. 2014, doi: 10.1097/MOP.0000000000000046.
- [101] J. Zsiros *et al.*, "Dose-dense cisplatin-based chemotherapy and surgery for children with high-risk hepatoblastoma (SIOPEL-4): a prospective, single-arm, feasibility study," *Lancet Oncol.*, vol. 14, no. 9, pp. 834–842, Aug. 2013, doi: 10.1016/S1470-2045(13)70272-9.
- [102] P. R. Brock *et al.*, "Sodium Thiosulfate for Protection from Cisplatin-Induced Hearing Loss," *N. Engl. J. Med.*, vol. 378, no. 25, pp. 2376–2385, Jun. 2018, doi: 10.1056/NEJMoa1801109.
- [103] D. R. Freyer *et al.*, "EFFECTS OF SODIUM THIOSULFATE VERSUS OBSERVATION ON DEVELOPMENT OF CISPLATIN-INDUCED HEARING LOSS IN CHILDREN WITH CANCER: RESULTS FROM THE CHILDREN'S ONCOLOGY GROUP ACCL0431 RANDOMISED CLINICAL TRIAL," *Lancet Oncol.*, vol. 18, no. 1, pp. 63–74, Jan. 2017, doi: 10.1016/S1470-2045(16)30625-8.
- [104] A. A. Gupta *et al.*, "Critical review of controversial issues in the management of advanced pediatric liver tumors," *Pediatr. Blood Cancer*, vol. 56, no. 7, pp. 1013–1018, 2011, doi: 10.1002/pbc.22893.
- [105] G. M. Tiao *et al.*, "The current management of hepatoblastoma: A combination of chemotherapy, conventional resection, and liver transplantation," *J. Pediatr.*, vol. 146, no. 2, pp. 204–211, Feb. 2005, doi: 10.1016/j.jpeds.2004.09.011.
- [106] "Protease degradomics: A new challenge for proteomics | Nature Reviews Molecular Cell Biology." <https://www-nature-com.proxy.insermbiblio.inist.fr/articles/nrm858> (accessed Nov. 25, 2019).
- [107] N. G. Seidah *et al.*, "The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): Liver regeneration and neuronal differentiation," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 3, pp. 928–933, Feb. 2003, doi: 10.1073/pnas.0335507100.
- [108] M. Abifadel *et al.*, "Mutations in PCSK9 cause autosomal dominant hypercholesterolemia," *Nat. Genet.*, vol. 34, no. 2, pp. 154–156, Jun. 2003, doi: 10.1038/ng1161.
- [109] N. G. Seidah and A. Prat, "Precursor convertases in the secretory pathway, cytosol and extracellular milieu," *Essays Biochem.*, vol. 38, pp. 79–94, 2002, doi: 10.1042/bse0380079.
- [110] M. Wiciński, J. Żak, B. Malinowski, G. Popek, and G. Grześk, "PCSK9 signaling pathways and their potential importance in clinical practice," *EPMA J.*, vol. 8, no. 4, pp. 391–402, Dec. 2017, doi: 10.1007/s13167-017-0106-6.

- [111] S. Benjannet *et al.*, “NARC-1/PCSK9 and Its Natural Mutants ZYMOGEN CLEAVAGE AND EFFECTS ON THE LOW DENSITY LIPOPROTEIN (LDL) RECEPTOR AND LDL CHOLESTEROL,” *J. Biol. Chem.*, vol. 279, no. 47, pp. 48865–48875, Nov. 2004, doi: 10.1074/jbc.M409699200.
- [112] G. Lambert, F. Charlton, K.-A. Rye, and D. E. Piper, “Molecular basis of PCSK9 function,” *Atherosclerosis*, vol. 203, no. 1, pp. 1–7, Mar. 2009, doi: 10.1016/j.atherosclerosis.2008.06.010.
- [113] S. Naureckiene *et al.*, “Functional characterization of Narc 1, a novel proteinase related to proteinase K,” *Arch. Biochem. Biophys.*, vol. 420, no. 1, pp. 55–67, Dec. 2003, doi: 10.1016/j.abb.2003.09.011.
- [114] A. Zhou, S. Martin, G. Lipkind, J. LaMendola, and D. F. Steiner, “Regulatory Roles of the P Domain of the Subtilisin-like Prohormone Convertases,” *J. Biol. Chem.*, vol. 273, no. 18, pp. 11107–11114, May 1998, doi: 10.1074/jbc.273.18.11107.
- [115] J. D. Horton, J. C. Cohen, and H. H. Hobbs, “Molecular biology of PCSK9: its role in LDL metabolism,” *Trends Biochem. Sci.*, vol. 32, no. 2, pp. 71–77, Feb. 2007, doi: 10.1016/j.tibs.2006.12.008.
- [116] D. Cunningham *et al.*, “Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia,” *Nat. Struct. Mol. Biol.*, vol. 14, no. 5, pp. 413–419, May 2007, doi: 10.1038/nsmb1235.
- [117] E. N. Hampton, M. W. Knuth, J. Li, J. L. Harris, S. A. Lesley, and G. Spraggon, “The self-inhibited structure of full-length PCSK9 at 1.9 Å reveals structural homology with resistin within the C-terminal domain,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 37, pp. 14604–14609, Sep. 2007, doi: 10.1073/pnas.0703402104.
- [118] H. J. Kwon, T. A. Lagace, M. C. McNutt, J. D. Horton, and J. Deisenhofer, “Molecular basis for LDL receptor recognition by PCSK9,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 6, pp. 1820–1825, Feb. 2008, doi: 10.1073/pnas.0712064105.
- [119] T. A. Lagace *et al.*, “Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice,” *J. Clin. Invest.*, vol. 116, no. 11, pp. 2995–3005, Nov. 2006, doi: 10.1172/JCI29383.
- [120] Y.-W. Qian *et al.*, “Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis,” *J. Lipid Res.*, vol. 48, no. 7, pp. 1488–1498, Jul. 2007, doi: 10.1194/jlr.M700071-JLR200.
- [121] S. W. Park, Y.-A. Moon, and J. D. Horton, “Post-transcriptional Regulation of Low Density Lipoprotein Receptor Protein by Proprotein Convertase Subtilisin/Kexin Type 9a in Mouse Liver,” *J. Biol. Chem.*, vol. 279, no. 48, pp. 50630–50638, Nov. 2004, doi: 10.1074/jbc.M410077200.
- [122] V. M. Homer *et al.*, “Identification and characterization of two non-secreted PCSK9 mutants associated with familial hypercholesterolemia in cohorts from New Zealand and South Africa,” *Atherosclerosis*, vol. 196, no. 2, pp. 659–666, Feb. 2008, doi: 10.1016/j.atherosclerosis.2007.07.022.
- [123] K. N. Maxwell, E. A. Fisher, and J. L. Breslow, “Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 6, pp. 2069–2074, Feb. 2005, doi: 10.1073/pnas.0409736102.
- [124] T. Nishikido and K. K. Ray, “Non-antibody Approaches to Proprotein Convertase Subtilisin Kexin 9 Inhibition: siRNA, Antisense Oligonucleotides, Adnectins, Vaccination, and New Attempts at Small-Molecule Inhibitors Based on New Discoveries,” *Front. Cardiovasc. Med.*, vol. 5, 2019, doi: 10.3389/fcvm.2018.00199.

- [125] H.-D. Jang *et al.*, "Cyclase-associated protein 1 is a binding partner of proprotein convertase subtilisin/kexin type-9 and is required for the degradation of low-density lipoprotein receptors by proprotein convertase subtilisin/kexin type-9," *Eur. Heart J.*, vol. 41, no. 2, pp. 239–252, Jan. 2020, doi: 10.1093/eurheartj/ehz566.
- [126] R. Essalmani *et al.*, "In Vivo Evidence That Furin from Hepatocytes Inactivates PCSK9," *J. Biol. Chem.*, vol. 286, no. 6, pp. 4257–4263, Feb. 2011, doi: 10.1074/jbc.M110.192104.
- [127] B. Han *et al.*, "Isolation and characterization of the circulating truncated form of PCSK9," *J. Lipid Res.*, vol. 55, no. 7, pp. 1505–1514, Jul. 2014, doi: 10.1194/jlr.M049346.
- [128] L. Pisciotta *et al.*, "Additive effect of mutations in LDLR and PCSK9 genes on the phenotype of familial hypercholesterolemia," *Atherosclerosis*, vol. 186, no. 2, pp. 433–440, Jun. 2006, doi: 10.1016/j.atherosclerosis.2005.08.015.
- [129] K. N. Maxwell and J. L. Breslow, "Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 18, pp. 7100–7105, May 2004, doi: 10.1073/pnas.0402133101.
- [130] J. C. Cohen, E. Boerwinkle, T. H. Mosley, and H. H. Hobbs, "Sequence variations in PCSK9, low LDL, and protection against coronary heart disease," *N. Engl. J. Med.*, vol. 354, no. 12, pp. 1264–1272, Mar. 2006, doi: 10.1056/NEJMoa054013.
- [131] M. S. Brown and J. L. Goldstein, "Lowering LDL--Not Only How Low, But How Long?," *Science*, vol. 311, no. 5768, pp. 1721–1723, Mar. 2006, doi: 10.1126/science.1125884.
- [132] S. Benjannet, D. Rhainds, J. Hamelin, N. Nassoury, and N. G. Seidah, "The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications," *J. Biol. Chem.*, vol. 281, no. 41, pp. 30561–30572, Oct. 2006, doi: 10.1074/jbc.M606495200.
- [133] J. Mayne *et al.*, "Novel Loss-of-Function PCSK9 Variant Is Associated with Low Plasma LDL Cholesterol in a French-Canadian Family and with Impaired Processing and Secretion in Cell Culture," *Clin. Chem.*, vol. 57, no. 10, pp. 1415–1423, Oct. 2011, doi: 10.1373/clinchem.2011.165191.
- [134] P. F. Lebeau *et al.*, "The loss-of-function PCSK9Q152H variant increases ER chaperones GRP78 and GRP94 and protects against liver injury," *J. Clin. Invest.*, vol. 131, no. 2, p. e128650, doi: 10.1172/JCI128650.
- [135] R. Schulz, K.-D. Schlüter, and U. Laufs, "Molecular and cellular function of the proprotein convertase subtilisin/kexin type 9 (PCSK9)," *Basic Res. Cardiol.*, vol. 110, no. 2, p. 4, Jan. 2015, doi: 10.1007/s00395-015-0463-z.
- [136] M. S. Brown and J. L. Goldstein, "The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor," *Cell*, vol. 89, no. 3, pp. 331–340, May 1997, doi: 10.1016/S0092-8674(00)80213-5.
- [137] J. D. Horton, J. L. Goldstein, and M. S. Brown, "SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver," *J. Clin. Invest.*, vol. 109, no. 9, pp. 1125–1131, May 2002, doi: 10.1172/JCI15593.
- [138] J. D. Browning and J. D. Horton, "Fasting reduces plasma proprotein convertase, subtilisin/kexin type 9 and cholesterol biosynthesis in humans," *J. Lipid Res.*, vol. 51, no. 11, pp. 3359–3363, Nov. 2010, doi: 10.1194/jlr.P009860.
- [139] M. Wu, B. Dong, A. Cao, H. Li, and J. Liu, "Delineation of molecular pathways that regulate hepatic PCSK9 and LDL receptor expression during fasting in normolipidemic hamsters," *Atherosclerosis*, vol. 224, no. 2, pp. 401–410, Oct. 2012, doi: 10.1016/j.atherosclerosis.2012.08.012.

- [140] P. Costet *et al.*, "Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c," *J. Biol. Chem.*, vol. 281, no. 10, pp. 6211–6218, Mar. 2006, doi: 10.1074/jbc.M508582200.
- [141] D. Ai *et al.*, "Regulation of hepatic LDL receptors by mTORC1 and PCSK9 in mice," *J. Clin. Invest.*, vol. 122, no. 4, pp. 1262–1270, Apr. 2012, doi: 10.1172/JCI61919.
- [142] Z. Awan *et al.*, "The effect of insulin on circulating PCSK9 in postmenopausal obese women," *Clin. Biochem.*, vol. 47, no. 12, pp. 1033–1039, Aug. 2014, doi: 10.1016/j.clinbiochem.2014.03.022.
- [143] P. J. W. H. Kappelle, G. Lambert, and R. P. F. Dullaart, "Plasma proprotein convertase subtilisin-kexin type 9 does not change during 24h insulin infusion in healthy subjects and type 2 diabetic patients," *Atherosclerosis*, vol. 214, no. 2, pp. 432–435, Feb. 2011, doi: 10.1016/j.atherosclerosis.2010.10.028.
- [144] "Hepatocyte Nuclear Factor 1 α Plays a Critical Role in PCSK9 Gene Transcription and Regulation by the Natural Hypocholesterolemic Compound Berberine." <https://www.ncbi.nlm.nih.gov.proxy.insermbiblio.inist.fr/pmc/articles/PMC2781434/> (accessed Apr. 07, 2020).
- [145] S. Kourimate *et al.*, "Dual mechanisms for the fibrate-mediated repression of proprotein convertase subtilisin/kexin type 9," *J. Biol. Chem.*, vol. 283, no. 15, pp. 9666–9673, Apr. 2008, doi: 10.1074/jbc.M705831200.
- [146] Y. Duan *et al.*, "Peroxisome Proliferator-activated Receptor γ Activation by Ligands and Dephosphorylation Induces Proprotein Convertase Subtilisin Kexin Type 9 and Low Density Lipoprotein Receptor Expression," *J. Biol. Chem.*, vol. 287, no. 28, pp. 23667–23677, Jul. 2012, doi: 10.1074/jbc.M112.350181.
- [147] C. Langhi *et al.*, "Activation of the farnesoid X receptor represses PCSK9 expression in human hepatocytes," *FEBS Lett.*, vol. 582, no. 6, pp. 949–955, Mar. 2008, doi: 10.1016/j.febslet.2008.02.038.
- [148] T. Nozue, "Lipid Lowering Therapy and Circulating PCSK9 Concentration," *J. Atheroscler. Thromb.*, vol. 24, no. 9, pp. 895–907, Sep. 2017, doi: 10.5551/jat.RV17012.
- [149] B. Dong *et al.*, "Strong induction of PCSK9 gene expression through HNF1 α and SREBP2: mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters," *J. Lipid Res.*, vol. 51, no. 6, pp. 1486–1495, Jun. 2010, doi: 10.1194/jlr.M003566.
- [150] J. Mayne *et al.*, "Plasma PCSK9 levels are significantly modified by statins and fibrates in humans," *Lipids Health Dis.*, vol. 7, p. 22, Jun. 2008, doi: 10.1186/1476-511X-7-22.
- [151] P. Costet, M. M. Hoffmann, B. Cariou, B. Guyomarc'h Delasalle, T. Konrad, and K. Winkler, "Plasma PCSK9 is increased by fenofibrate and atorvastatin in a non-additive fashion in diabetic patients," *Atherosclerosis*, vol. 212, no. 1, pp. 246–251, Sep. 2010, doi: 10.1016/j.atherosclerosis.2010.05.027.
- [152] Y.-L. Guo *et al.*, "Short-term impact of low-dose atorvastatin on serum proprotein convertase subtilisin/kexin type 9," *Clin. Drug Investig.*, vol. 33, no. 12, pp. 877–883, Dec. 2013, doi: 10.1007/s40261-013-0129-2.
- [153] "High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. - PubMed - NCBI." <https://www.ncbi.nlm.nih.gov.proxy.insermbiblio.inist.fr/pubmed/20525997/> (accessed Apr. 07, 2020).
- [154] S. G. Lakoski *et al.*, "Indices of cholesterol metabolism and relative responsiveness to ezetimibe and simvastatin," *J. Clin. Endocrinol. Metab.*, vol. 95, no. 2, pp. 800–809, Feb. 2010, doi: 10.1210/jc.2009-1952.

- [155] H. K. Berthold, N. G. Seidah, S. Benjannet, and I. Gouni-Berthold, "Evidence from a randomized trial that simvastatin, but not ezetimibe, upregulates circulating PCSK9 levels," *PLoS One*, vol. 8, no. 3, p. e60095, 2013, doi: 10.1371/journal.pone.0060095.
- [156] Z. Awan *et al.*, "Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL cholesterol response: the JUPITER trial," *Clin. Chem.*, vol. 58, no. 1, pp. 183–189, Jan. 2012, doi: 10.1373/clinchem.2011.172932.
- [157] G. Dubuc *et al.*, "A new method for measurement of total plasma PCSK9: clinical applications," *J. Lipid Res.*, vol. 51, no. 1, pp. 140–149, Jan. 2010, doi: 10.1194/jlr.M900273-JLR200.
- [158] J. Davignon and G. Dubuc, "Statins and ezetimibe modulate plasma proprotein convertase subtilisin kexin-9 (PCSK9) levels," *Trans. Am. Clin. Climatol. Assoc.*, vol. 120, pp. 163–173, 2009.
- [159] T. Miyoshi, K. Nakamura, M. Doi, and H. Ito, "Impact of Ezetimibe Alone or in Addition to a Statin on Plasma PCSK9 Concentrations in Patients with Type 2 Diabetes and Hypercholesterolemia: A Pilot Study," *Am. J. Cardiovasc. Drugs Drugs Devices Interv.*, vol. 15, no. 3, pp. 213–219, Jun. 2015, doi: 10.1007/s40256-015-0119-2.
- [160] D. C. Chan *et al.*, "Fenofibrate concomitantly decreases serum proprotein convertase subtilisin/kexin type 9 and very-low-density lipoprotein particle concentrations in statin-treated type 2 diabetic patients," *Diabetes Obes. Metab.*, vol. 12, no. 9, pp. 752–756, Sep. 2010, doi: 10.1111/j.1463-1326.2010.01229.x.
- [161] J. S. Troutt, W. E. Alborn, G. Cao, and R. J. Konrad, "Fenofibrate treatment increases human serum proprotein convertase subtilisin kexin type 9 levels," *J. Lipid Res.*, vol. 51, no. 2, pp. 345–351, Feb. 2010, doi: 10.1194/jlr.M000620.
- [162] A. V. Khera, A. Qamar, M. P. Reilly, R. L. Dunbar, and D. J. Rader, "Effects of niacin, statin, and fenofibrate on circulating proprotein convertase subtilisin/kexin type 9 levels in patients with dyslipidemia," *Am. J. Cardiol.*, vol. 115, no. 2, pp. 178–182, Jan. 2015, doi: 10.1016/j.amjcard.2014.10.018.
- [163] H.-B. Xiao, Z.-L. Sun, H.-B. Zhang, and D.-S. Zhang, "Berberine inhibits dyslipidemia in C57BL/6 mice with lipopolysaccharide induced inflammation," *Pharmacol. Rep. PR*, vol. 64, no. 4, pp. 889–895, 2012, doi: 10.1016/s1734-1140(12)70883-6.
- [164] "Fumonisin B1 regulates LDL receptor and ABCA1 expression in an LXR dependent mechanism in liver (HepG2) cells," *Toxicol.*, vol. 190, pp. 58–64, Jan. 2021, doi: 10.1016/j.toxicol.2020.12.011.
- [165] Y. Osono, L. A. Woollett, J. Herz, and J. M. Dietschy, "Role of the low density lipoprotein receptor in the flux of cholesterol through the plasma and across the tissues of the mouse.," *J. Clin. Invest.*, vol. 95, no. 3, pp. 1124–1132, Mar. 1995.
- [166] S. Glerup, R. Schulz, U. Laufs, and K.-D. Schlüter, "Physiological and therapeutic regulation of PCSK9 activity in cardiovascular disease," *Basic Res. Cardiol.*, vol. 112, no. 3, 2017, doi: 10.1007/s00395-017-0619-0.
- [167] S. Poirier *et al.*, "The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2," *J. Biol. Chem.*, vol. 283, no. 4, pp. 2363–2372, Jan. 2008, doi: 10.1074/jbc.M708098200.
- [168] M. Mbikay *et al.*, "PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities," *FEBS Lett.*, vol. 584, no. 4, pp. 701–706, 2010, doi: 10.1016/j.febslet.2009.12.018.
- [169] J. Miao *et al.*, "The Role of Insulin in the Regulation of PCSK9," *Arterioscler. Thromb. Vasc. Biol.*, vol. 35, no. 7, pp. 1589–1596, Jul. 2015, doi: 10.1161/ATVBAHA.115.305688.

- [170] Z. Tang *et al.*, "PCSK9 siRNA suppresses the inflammatory response induced by oxLDL through inhibition of NF- κ B activation in THP-1-derived macrophages," *Int. J. Mol. Med.*, vol. 30, no. 4, pp. 931–938, Oct. 2012, doi: 10.3892/ijmm.2012.1072.
- [171] M. Ruscica *et al.*, "Suppressor of Cytokine Signaling-3 (SOCS-3) Induces Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) Expression in Hepatic HepG2 Cell Line," *J. Biol. Chem.*, vol. 291, no. 7, pp. 3508–3519, Feb. 2016, doi: 10.1074/jbc.M115.664706.
- [172] I. Giunzioni *et al.*, "Local effects of human PCSK9 on the atherosclerotic lesion," *J. Pathol.*, vol. 238, no. 1, pp. 52–62, Jan. 2016, doi: 10.1002/path.4630.
- [173] S. J. Korsmeyer, "BCL-2 gene family and the regulation of programmed cell death," *Cancer Res.*, vol. 59, no. 7 Suppl, pp. 1693s–1700s, Apr. 1999.
- [174] C.-Y. Wu, Z.-H. Tang, L. Jiang, X.-F. Li, Z.-S. Jiang, and L.-S. Liu, "PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax–caspase9–caspase3 pathway," *Mol. Cell. Biochem.*, vol. 359, no. 1, pp. 347–358, Jan. 2012, doi: 10.1007/s11010-011-1028-6.
- [175] K. Kysenius, P. Muggalla, K. Mätlik, U. Arumäe, and H. J. Huttunen, "PCSK9 regulates neuronal apoptosis by adjusting ApoER2 levels and signaling," *Cell. Mol. Life Sci.*, vol. 69, no. 11, pp. 1903–1916, Jun. 2012, doi: 10.1007/s00018-012-0977-6.
- [176] M.-X. Piao, J.-W. Bai, P.-F. Zhang, and Y.-Z. Zhang, "PCSK9 regulates apoptosis in human neuroglioma u251 cells via mitochondrial signaling pathways," *Int. J. Clin. Exp. Pathol.*, vol. 8, no. 3, pp. 2787–2794, Mar. 2015.
- [177] X. Sun, R. Essalmani, R. Day, A. M. Khatib, N. G. Seidah, and A. Prat, "Proprotein Convertase Subtilisin/Kexin Type 9 Deficiency Reduces Melanoma Metastasis in Liver," *Neoplasia N. Y. N.*, vol. 14, no. 12, pp. 1122–1131, Dec. 2012.
- [178] S.-Z. Zhang *et al.*, "PCSK9 promotes tumor growth by inhibiting tumor cell apoptosis in hepatocellular carcinoma," *Exp. Hematol. Oncol.*, vol. 10, p. 25, Mar. 2021, doi: 10.1186/s40164-021-00218-1.
- [179] M. Bhat *et al.*, "Decreased PCSK9 expression in human hepatocellular carcinoma," *BMC Gastroenterol.*, vol. 15, Dec. 2015, doi: 10.1186/s12876-015-0371-6.
- [180] M. He *et al.*, "Actinidia chinensis Planch root extract inhibits cholesterol metabolism in hepatocellular carcinoma through upregulation of PCSK9," *Oncotarget*, vol. 8, no. 26, pp. 42136–42148, Feb. 2017, doi: 10.18632/oncotarget.15010.
- [181] S.-S. Gan *et al.*, "Inhibition of PCSK9 protects against radiation-induced damage of prostate cancer cells," *OncoTargets Ther.*, vol. 10, pp. 2139–2146, Apr. 2017, doi: 10.2147/OTT.S129413.
- [182] X. Xu, Y. Cui, L. Cao, Y. Zhang, Y. Yin, and X. Hu, "PCSK9 regulates apoptosis in human lung adenocarcinoma A549 cells via endoplasmic reticulum stress and mitochondrial signaling pathways," *Exp. Ther. Med.*, vol. 13, no. 5, pp. 1993–1999, May 2017, doi: 10.3892/etm.2017.4218.
- [183] X. Liu *et al.*, "PCSK9 inhibition potentiates cancer immune checkpoint therapy," *Nature*, vol. 588, no. 7839, pp. 693–698, Dec. 2020, doi: 10.1038/s41586-020-2911-7.
- [184] J. C. Y. Chan *et al.*, "A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 24, pp. 9820–9825, Jun. 2009, doi: 10.1073/pnas.0903849106.
- [185] E. P. Navarese *et al.*, "Effects of Proprotein Convertase Subtilisin/Kexin Type 9 Antibodies in Adults With Hypercholesterolemia: A Systematic Review and Meta-analysis," *Ann. Intern. Med.*, vol. 163, no. 1, p. 40, Jul. 2015, doi: 10.7326/M14-2957.

- [186] J. G. Robinson *et al.*, “Efficacy and safety of alirocumab in reducing lipids and cardiovascular events,” *N. Engl. J. Med.*, vol. 372, no. 16, pp. 1489–1499, Apr. 2015, doi: 10.1056/NEJMoa1501031.
- [187] M. S. Sabatine *et al.*, “Efficacy and safety of evolocumab in reducing lipids and cardiovascular events,” *N. Engl. J. Med.*, vol. 372, no. 16, pp. 1500–1509, Apr. 2015, doi: 10.1056/NEJMoa1500858.
- [188] M. S. Sabatine *et al.*, “Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease,” *N. Engl. J. Med.*, vol. 376, no. 18, pp. 1713–1722, 04 2017, doi: 10.1056/NEJMoa1615664.
- [189] G. G. Schwartz *et al.*, “Alirocumab and Cardiovascular Outcomes after Acute Coronary Syndrome,” *N. Engl. J. Med.*, vol. 379, no. 22, pp. 2097–2107, 29 2018, doi: 10.1056/NEJMoa1801174.
- [190] A. A. Momtazi-Borojeni, M. R. Jaafari, A. Badiie, and A. Sahebkar, “Long-term generation of antiPCSK9 antibody using a nanoliposome-based vaccine delivery system,” *Atherosclerosis*, vol. 283, pp. 69–78, Apr. 2019, doi: 10.1016/j.atherosclerosis.2019.02.001.
- [191] K. Mahboobnia *et al.*, “PCSK9 and cancer: Rethinking the link,” *Biomed. Pharmacother.*, vol. 140, p. 111758, Aug. 2021, doi: 10.1016/j.biopha.2021.111758.
- [192] A. A. Momtazi-Borojeni, M. R. Jaafari, M. Afshar, M. Banach, and A. Sahebkar, “PCSK9 immunization using nanoliposomes: preventive efficacy against hypercholesterolemia and atherosclerosis,” *Arch. Med. Sci.*, vol. 17, no. 5, pp. 1365–1377, Mar. 2021, doi: 10.5114/aoms/133885.
- [193] A. A. Momtazi-Borojeni, M. R. Jaafari, A. Badiie, M. Banach, and A. Sahebkar, “Therapeutic effect of nanoliposomal PCSK9 vaccine in a mouse model of atherosclerosis,” *BMC Med.*, vol. 17, p. 223, Dec. 2019, doi: 10.1186/s12916-019-1457-8.
- [194] A. A. Momtazi-Borojeni, M. R. Jaafari, M. Banach, A. M. Gorabi, H. Sahraei, and A. Sahebkar, “Pre-Clinical Evaluation of the Nanoliposomal antiPCSK9 Vaccine in Healthy Non-Human Primates,” *Vaccines*, vol. 9, no. 7, p. 749, Jul. 2021, doi: 10.3390/vaccines9070749.
- [195] A. A. Momtazi-Borojeni, M. E. Nik, M. R. Jaafari, M. Banach, and A. Sahebkar, “Effects of immunization against PCSK9 in an experimental model of breast cancer,” *Arch. Med. Sci. AMS*, vol. 15, no. 3, pp. 570–579, May 2019, doi: 10.5114/aoms.2019.84734.
- [196] A. A. Momtazi-Borojeni, M. E. Nik, M. R. Jaafari, M. Banach, and A. Sahebkar, “Effects of immunisation against PCSK9 in mice bearing melanoma,” *Arch. Med. Sci. AMS*, vol. 16, no. 1, pp. 189–199, Dec. 2019, doi: 10.5114/aoms.2020.91291.
- [197] D. Lipovšek, “Adnectins: engineered target-binding protein therapeutics,” *Protein Eng. Des. Sel.*, vol. 24, no. 1–2, pp. 3–9, 2011, doi: 10.1093/protein/gzq097.
- [198] T. Mitchell *et al.*, “Pharmacologic profile of the Adnectin BMS-962476, a small protein biologic alternative to PCSK9 antibodies for low-density lipoprotein lowering,” *J. Pharmacol. Exp. Ther.*, vol. 350, no. 2, pp. 412–424, Aug. 2014, doi: 10.1124/jpet.114.214221.
- [199] E. A. Stein *et al.*, “LDL CHOLESTEROL REDUCTION WITH BMS-962476, AN ADNECTIN INHIBITOR OF PCSK9: RESULTS OF A SINGLE ASCENDING DOSE STUDY,” *J. Am. Coll. Cardiol.*, vol. 63, no. 12, Supplement, p. A1372, Apr. 2014, doi: 10.1016/S0735-1097(14)61372-3.
- [200] D. J. Craik, D. P. Fairlie, S. Liras, and D. Price, “The Future of Peptide-based Drugs,” *Chem. Biol. Drug Des.*, vol. 81, no. 1, pp. 136–147, 2013, doi: 10.1111/cbdd.12055.

- [201] C. I. Schroeder *et al.*, “Design and Synthesis of Truncated EGF-A Peptides that Restore LDL-R Recycling in the Presence of PCSK9 In Vitro,” *Chem. Biol.*, vol. 21, no. 2, pp. 284–294, Feb. 2014, doi: 10.1016/j.chembiol.2013.11.014.
- [202] Y. Zhang *et al.*, “Identification of a Small Peptide That Inhibits PCSK9 Protein Binding to the Low Density Lipoprotein Receptor,” *J. Biol. Chem.*, vol. 289, no. 2, pp. 942–955, Jan. 2014, doi: 10.1074/jbc.M113.514067.
- [203] N. G. Seidah *et al.*, “Annexin A2 Is a Natural Extrahepatic Inhibitor of the PCSK9-Induced LDL Receptor Degradation,” *PLoS ONE*, vol. 7, no. 7, Jul. 2012, doi: 10.1371/journal.pone.0041865.
- [204] K. S. Abdelwahed *et al.*, “Pseurotin A as a Novel Suppressor of Hormone Dependent Breast Cancer Progression and Recurrence by Inhibiting PCSK9 Secretion and Interaction with LDL Receptor,” *Pharmacol. Res.*, vol. 158, p. 104847, Aug. 2020, doi: 10.1016/j.phrs.2020.104847.
- [205] S. Luper, “A Review of Plants Used in the Treatment of Liver Disease: Part 1,” *Altern. Med. Rev.*, vol. 3, no. 6, p. 12, 1998.
- [206] K. Wellington and B. Jarvis, “Silymarin: A Review of its Clinical Properties in the Management of Hepatic Disorders,” *BioDrugs*, vol. 15, no. 7, pp. 465–489, Jul. 2001, doi: 10.2165/00063030-200115070-00005.
- [207] Z. Dong, W. Zhang, S. Chen, and C. Liu, “Silibinin A decreases statin-induced PCSK9 expression in human hepatoblastoma HepG2 cells,” *Mol. Med. Rep.*, vol. 20, no. 2, pp. 1383–1392, Aug. 2019, doi: 10.3892/mmr.2019.10344.
- [208] F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott, and F. Zhang, “Genome engineering using the CRISPR-Cas9 system,” *Nat. Protoc.*, vol. 8, no. 11, pp. 2281–2308, Nov. 2013, doi: 10.1038/nprot.2013.143.
- [209] X. Wang *et al.*, “CRISPR-Cas9 Targeting of PCSK9 in Human Hepatocytes In Vivo—Brief Report,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 36, no. 5, pp. 783–786, May 2016, doi: 10.1161/ATVBAHA.116.307227.
- [210] Q. Ding *et al.*, “Permanent Alteration of PCSK9 With In Vivo CRISPR-Cas9 Genome Editing,” *Circ. Res.*, vol. 115, no. 5, pp. 488–492, Aug. 2014, doi: 10.1161/CIRCRESAHA.115.304351.
- [211] S. Rashid *et al.*, “Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 15, pp. 5374–5379, Apr. 2005, doi: 10.1073/pnas.0501652102.
- [212] C. F. Bennett and E. E. Swayze, “RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform,” *Annu. Rev. Pharmacol. Toxicol.*, vol. 50, no. 1, pp. 259–293, 2010, doi: 10.1146/annurev.pharmtox.010909.105654.
- [213] M. J. Graham *et al.*, “Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice,” *J. Lipid Res.*, vol. 48, no. 4, pp. 763–767, Apr. 2007, doi: 10.1194/jlr.C600025-JLR200.
- [214] N. Gupta *et al.*, “A Locked Nucleic Acid Antisense Oligonucleotide (LNA) Silences PCSK9 and Enhances LDLR Expression In Vitro and In Vivo,” *PLoS ONE*, vol. 5, no. 5, May 2010, doi: 10.1371/journal.pone.0010682.
- [215] E. P. van Poelgeest *et al.*, “Antisense-mediated reduction of proprotein convertase subtilisin/kexin type 9 (PCSK9): a first-in-human randomized, placebo-controlled trial,” *Br. J. Clin. Pharmacol.*, vol. 80, no. 6, pp. 1350–1361, 2015, doi: 10.1111/bcp.12738.

- [216] E. P. van Poelgeest *et al.*, “Acute Kidney Injury During Therapy With an Antisense Oligonucleotide Directed Against PCSK9,” *Am. J. Kidney Dis.*, vol. 62, no. 4, pp. 796–800, Oct. 2013, doi: 10.1053/j.ajkd.2013.02.359.
- [217] M. Frank-Kamenetsky *et al.*, “Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 33, pp. 11915–11920, Aug. 2008, doi: 10.1073/pnas.0805434105.
- [218] Y. N. Lamb, “Inclisiran: First Approval,” *Drugs*, vol. 81, no. 3, pp. 389–395, 2021, doi: 10.1007/s40265-021-01473-6.
- [219] “Search the website,” *European Medicines Agency*. https://www.ema.europa.eu/en/search/search/type/ema_document (accessed Aug. 31, 2021).
- [220] “Novartis receives EU approval for Leqvio®* (inclisiran), a first-in-class siRNA to lower cholesterol with two doses a year**,” *Novartis*. <https://www.novartis.com/news/media-releases/novartis-receives-eu-approval-leqvio-inclisiran-first-class-sirna-lower-cholesterol-two-doses-year> (accessed Aug. 31, 2021).
- [221] F. J. Raal *et al.*, “Inclisiran for the Treatment of Heterozygous Familial Hypercholesterolemia,” *N Engl J Med*, p. 11, 2020.
- [222] “Novartis successfully completes acquisition of The Medicines Company, adding a potentially first-in-class, investigational cholesterol-lowering therapy inclisiran,” *Novartis*. <https://www.novartis.com/news/media-releases/novartis-successfully-completes-acquisition-medicines-company-adding-potentially-first-class-investigational-cholesterol-lowering-therapy-inclisiran> (accessed Aug. 31, 2021).
- [223] “FDA delays decision on Novartis cholesterol therapy,” *BioPharma Dive*. <https://www.biopharmadive.com/news/novartis-fda-complete-response-inclisiran-inspection/592554/> (accessed Aug. 31, 2021).
- [224] D. N. Petersen *et al.*, “A Small-Molecule Anti-secretagogue of PCSK9 Targets the 80S Ribosome to Inhibit PCSK9 Protein Translation,” *Cell Chem. Biol.*, vol. 23, no. 11, pp. 1362–1371, Nov. 2016, doi: 10.1016/j.chembiol.2016.08.016.
- [225] N. G. Lintner *et al.*, “Selective stalling of human translation through small-molecule engagement of the ribosome nascent chain,” *PLoS Biol.*, vol. 15, no. 3, Mar. 2017, doi: 10.1371/journal.pbio.2001882.
- [226] W. Li *et al.*, “Structural basis for selective stalling of human ribosome nascent chain complexes by a drug-like molecule,” *Nat. Struct. Mol. Biol.*, vol. 26, no. 6, pp. 501–509, Jun. 2019, doi: 10.1038/s41594-019-0236-8.
- [227] C. Gustafsen *et al.*, “The hypercholesterolemia-risk gene SORT1 facilitates PCSK9 secretion,” *Cell Metab.*, vol. 19, no. 2, pp. 310–318, Feb. 2014, doi: 10.1016/j.cmet.2013.12.006.
- [228] C. Broder and C. Becker-Pauly, “The metalloproteases meprin α and meprin β : unique enzymes in inflammation, neurodegeneration, cancer and fibrosis,” *Biochem. J.*, vol. 450, no. Pt 2, pp. 253–264, Mar. 2013, doi: 10.1042/BJ20121751.
- [229] R. J. Beynon, J. D. Shannon, and J. S. Bond, “Purification and characterization of a metallo-endoproteinase from mouse kidney,” *Biochem. J.*, vol. 199, no. 3, pp. 591–598, Dec. 1981.
- [230] E. E. Sterchi, J. R. Green, and M. J. Lentze, “Non-pancreatic hydrolysis of N-benzoyl-L-tyrosyl-p-aminobenzoic acid (PABA-peptide) in the human small intestine,” *Clin. Sci. Lond. Engl.* 1979, vol. 62, no. 5, pp. 557–560, May 1982, doi: 10.1042/cs0620557.

- [231] E. E. Sterchi, W. Stöcker, and J. S. Bond, "Meprins, membrane-bound and secreted astacin metalloproteinases," *Mol. Aspects Med.*, vol. 29, no. 5, pp. 309–328, Oct. 2008, doi: 10.1016/j.mam.2008.08.002.
- [232] C. M. Gorbea *et al.*, "Cloning, expression, and chromosomal localization of the mouse meprin beta subunit," *J. Biol. Chem.*, vol. 268, no. 28, pp. 21035–21043, Oct. 1993.
- [233] P. Saftig and K. Reiss, "The 'A Disintegrin And Metalloproteases' ADAM10 and ADAM17: novel drug targets with therapeutic potential?," *Eur. J. Cell Biol.*, vol. 90, no. 6–7, pp. 527–535, Jul. 2011, doi: 10.1016/j.ejcb.2010.11.005.
- [234] J. Scheller, A. Chalaris, C. Garbers, and S. Rose-John, "ADAM17: a molecular switch to control inflammation and tissue regeneration," *Trends Immunol.*, vol. 32, no. 8, pp. 380–387, Aug. 2011, doi: 10.1016/j.it.2011.05.005.
- [235] Q. Sun, H.-J. Jin, and J. S. Bond, "Disruption of the Meprin Alpha and Beta Genes in Mice Alters Homeostasis of Monocytes and Natural Killer Cells," *Exp. Hematol.*, vol. 37, no. 3, pp. 346–356, Mar. 2009, doi: 10.1016/j.exphem.2008.10.016.
- [236] J. M. Crisman, B. Zhang, L. P. Norman, and J. S. Bond, "Deletion of the mouse meprin beta metalloprotease gene diminishes the ability of leukocytes to disseminate through extracellular matrix," *J. Immunol. Baltim. Md 1950*, vol. 172, no. 7, pp. 4510–4519, Apr. 2004.
- [237] S. Banerjee *et al.*, "MEP1A allele for meprin A metalloprotease is a susceptibility gene for inflammatory bowel disease," *Mucosal Immunol.*, vol. 2, no. 3, pp. 220–231, May 2009, doi: 10.1038/mi.2009.3.
- [238] C. Becker-Pauly *et al.*, "The alpha and beta subunits of the metalloprotease meprin are expressed in separate layers of human epidermis, revealing different functions in keratinocyte proliferation and differentiation," *J. Invest. Dermatol.*, vol. 127, no. 5, pp. 1115–1125, May 2007, doi: 10.1038/sj.jid.5700675.
- [239] A. Schütte, J. Hedrich, W. Stöcker, and C. Becker-Pauly, "Let it flow: Morpholino knockdown in zebrafish embryos reveals a pro-angiogenic effect of the metalloprotease meprin alpha2," *PLoS One*, vol. 5, no. 1, p. e8835, Jan. 2010, doi: 10.1371/journal.pone.0008835.
- [240] A. Schütte, D. Lottaz, E. E. Sterchi, W. Stöcker, and C. Becker-Pauly, "Two alpha subunits and one beta subunit of meprin zinc-endopeptidases are differentially expressed in the zebrafish *Danio rerio*," *Biol. Chem.*, vol. 388, no. 5, pp. 523–531, May 2007, doi: 10.1515/BC.2007.060.
- [241] M.-N. Kruse *et al.*, "Human meprin alpha and beta homo-oligomers: cleavage of basement membrane proteins and sensitivity to metalloprotease inhibitors," *Biochem. J.*, vol. 378, no. Pt 2, pp. 383–389, Mar. 2004, doi: 10.1042/BJ20031163.
- [242] C. Becker *et al.*, "Differences in the activation mechanism between the alpha and beta subunits of human meprin," *Biol. Chem.*, vol. 384, no. 5, pp. 825–831, May 2003, doi: 10.1515/BC.2003.092.
- [243] A. Ohler, M. Debela, S. Wagner, V. Magdolen, and C. Becker-Pauly, "Analyzing the protease web in skin: meprin metalloproteases are activated specifically by KLK4, 5 and 8 vice versa leading to processing of proKLK7 thereby triggering its activation," *Biol. Chem.*, vol. 391, no. 4, pp. 455–460, Apr. 2010, doi: 10.1515/BC.2010.023.
- [244] J. Prox, P. Arnold, and C. Becker-Pauly, "Meprin α and meprin β : Procollagen proteinases in health and disease," *Matrix Biol. J. Int. Soc. Matrix Biol.*, vol. 44–46, pp. 7–13, Jul. 2015, doi: 10.1016/j.matbio.2015.01.010.

- [245] P. Arnold, A. Otte, and C. Becker-Pauly, "Meprin metalloproteases: Molecular regulation and function in inflammation and fibrosis," *Biochim. Biophys. Acta*, May 2017, doi: 10.1016/j.bbamcr.2017.05.011.
- [246] T. Jefferson *et al.*, "The substrate degradome of meprin metalloproteases reveals an unexpected proteolytic link between meprin β and ADAM10," *Cell. Mol. Life Sci.*, vol. 70, no. 2, pp. 309–333, Jan. 2013, doi: 10.1007/s00018-012-1106-2.
- [247] D. Hahn *et al.*, "Phorbol 12-myristate 13-acetate-induced ectodomain shedding and phosphorylation of the human meprin β metalloprotease," *J. Biol. Chem.*, vol. 278, no. 44, pp. 42829–42839, Oct. 2003, doi: 10.1074/jbc.M211169200.
- [248] D. Kronenberg *et al.*, "Processing of Procollagen III by Meprins: New Players in Extracellular Matrix Assembly?," *J. Invest. Dermatol.*, vol. 130, no. 12, pp. 2727–2735, Dec. 2010, doi: 10.1038/jid.2010.202.
- [249] G. P. Bertenshaw, M. T. Norcum, and J. S. Bond, "Structure of Homo- and Hetero-oligomeric Meprin Metalloproteases DIMERS, TETRAMERS, AND HIGH MOLECULAR MASS MULTIMERS," *J. Biol. Chem.*, vol. 278, no. 4, pp. 2522–2532, Jan. 2003, doi: 10.1074/jbc.M208808200.
- [250] J. L. Arolas *et al.*, "Structural basis for the sheddase function of human meprin β metalloproteinase at the plasma membrane," *Proc. Natl. Acad. Sci.*, vol. 109, no. 40, pp. 16131–16136, Oct. 2012, doi: 10.1073/pnas.1211076109.
- [251] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, Dec. 2002, doi: 10.1038/nature01322.
- [252] P. Arnold *et al.*, "Meprin Metalloproteases Generate Biologically Active Soluble Interleukin-6 Receptor to Induce Trans-Signaling," *Sci. Rep.*, vol. 7, Mar. 2017, doi: 10.1038/srep44053.
- [253] S. Banerjee *et al.*, "Balance of meprin A and B in mice affects the progression of experimental inflammatory bowel disease," *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 300, no. 2, pp. G273–G282, Feb. 2011, doi: 10.1152/ajpgi.00504.2009.
- [254] T. R. Keiffer and J. S. Bond, "Meprin Metalloproteases Inactivate Interleukin 6," *J. Biol. Chem.*, vol. 289, no. 11, pp. 7580–7588, Mar. 2014, doi: 10.1074/jbc.M113.546309.
- [255] R. N. Apte *et al.*, "The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions," *Cancer Metastasis Rev.*, vol. 25, no. 3, pp. 387–408, Sep. 2006, doi: 10.1007/s10555-006-9004-4.
- [256] C. Herzog, R. S. Haun, V. Kaushal, P. R. Mayeux, S. V. Shah, and G. P. Kaushal, "Meprin A and meprin alpha generate biologically functional IL-1 β from pro-IL-1 β ," *Biochem. Biophys. Res. Commun.*, vol. 379, no. 4, pp. 904–908, Feb. 2009, doi: 10.1016/j.bbrc.2008.12.161.
- [257] N. Kumar *et al.*, "The anti-inflammatory peptide Ac-SDKP is released from thymosin- β 4 by renal meprin- α and prolyl oligopeptidase," *Am. J. Physiol. - Ren. Physiol.*, vol. 310, no. 10, pp. F1026–F1034, May 2016, doi: 10.1152/ajprenal.00562.2015.
- [258] C. Herzog, R. S. Haun, S. V. Shah, and G. P. Kaushal, "Proteolytic processing and inactivation of CCL2/MCP-1 by meprins," *Biochem. Biophys. Rep.*, vol. 8, pp. 146–150, Aug. 2016, doi: 10.1016/j.bbrep.2016.08.019.
- [259] P. Minder, E. Bayha, C. Becker-Pauly, and E. E. Sterchi, "Meprin α transactivates the epidermal growth factor receptor (EGFR) via ligand shedding, thereby enhancing colorectal cancer cell proliferation and migration," *J. Biol. Chem.*, vol. 287, no. 42, pp. 35201–35211, Oct. 2012, doi: 10.1074/jbc.M112.368910.

- [260] S. Cosgrove, S. H. Chotirmall, C. M. Greene, and N. G. McElvaney, "Pulmonary Proteases in the Cystic Fibrosis Lung Induce Interleukin 8 Expression from Bronchial Epithelial Cells via a Heme/Meprin/Epidermal Growth Factor Receptor/Toll-like Receptor Pathway," *J. Biol. Chem.*, vol. 286, no. 9, pp. 7692–7704, Mar. 2011, doi: 10.1074/jbc.M110.183863.
- [261] "Extracellular matrix structure," *Adv. Drug Deliv. Rev.*, vol. 97, pp. 4–27, Feb. 2016, doi: 10.1016/j.addr.2015.11.001.
- [262] M. Trojanowska, E. Carwile LeRoy, B. Eckes, and T. Krieg, "Pathogenesis of fibrosis: type 1 collagen and the skin," *J. Mol. Med.*, vol. 76, no. 3–4, pp. 266–274, Feb. 1998, doi: 10.1007/s001090050216.
- [263] P. D. Walker, G. P. Kaushal, and S. V. Shah, "Meprin A, the major matrix degrading enzyme in renal tubules, produces a novel nidogen fragment in vitro and in vivo," *Kidney Int.*, vol. 53, no. 6, pp. 1673–1680, Jun. 1998, doi: 10.1046/j.1523-1755.1998.00949.x.
- [264] C. Broder *et al.*, "Metalloproteases meprin α and meprin β are C- and N-procollagen proteinases important for collagen assembly and tensile strength," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 35, pp. 14219–14224, Aug. 2013, doi: 10.1073/pnas.1305464110.
- [265] R. E. Yura, S. G. Bradley, G. Ramesh, W. B. Reeves, and J. S. Bond, "Meprin A metalloproteases enhance renal damage and bladder inflammation after LPS challenge," *Am. J. Physiol. - Ren. Physiol.*, vol. 296, no. 1, pp. F135–F144, Jan. 2009, doi: 10.1152/ajprenal.90524.2008.
- [266] C. Herzog, R. Seth, S. V. Shah, and G. P. Kaushal, "Role of meprin A in renal tubular epithelial cell injury," *Kidney Int.*, vol. 71, no. 10, pp. 1009–1018, May 2007, doi: 10.1038/sj.ki.5002189.
- [267] J. Bylander, Q. Li, G. Ramesh, B. Zhang, W. B. Reeves, and J. S. Bond, "Targeted disruption of the meprin metalloproteinase β gene protects against renal ischemia-reperfusion injury in mice," *Am. J. Physiol.-Ren. Physiol.*, vol. 294, no. 3, pp. F480–F490, Mar. 2008, doi: 10.1152/ajprenal.00214.2007.
- [268] P. Carmeliet, "Angiogenesis in life, disease and medicine," *Nature*, vol. 438, no. 7070, Art. no. 7070, Dec. 2005, doi: 10.1038/nature04478.
- [269] D. Lottaz *et al.*, "Enhanced Activity of Meprin- α , a Pro-Migratory and Pro-Angiogenic Protease, in Colorectal Cancer," *PLoS ONE*, vol. 6, no. 11, Nov. 2011, doi: 10.1371/journal.pone.0026450.
- [270] D. Lottaz, C. A. Maurer, D. Hahn, M. W. Büchler, and E. E. Sterchi, "Nonpolarized Secretion of Human Meprin α in Colorectal Cancer Generates an Increased Proteolytic Potential in the Stroma," *Cancer Res.*, vol. 59, no. 5, pp. 1127–1133, Mar. 1999.
- [271] G. L. Matters, A. Manni, and J. S. Bond, "Inhibitors of Polyamine Biosynthesis Decrease the Expression of the Metalloproteases Meprin α and MMP-7 in Hormone-independent Human Breast Cancer Cells," *Clin. Exp. Metastasis*, vol. 22, no. 4, pp. 331–339, Jun. 2005, doi: 10.1007/s10585-005-0660-5.
- [272] H.-Y. OuYang *et al.*, "MEP1A contributes to tumor progression and predicts poor clinical outcome in human hepatocellular carcinoma," *Hepatol. Baltim. Md*, vol. 63, no. 4, pp. 1227–1239, Apr. 2016, doi: 10.1002/hep.28397.
- [273] D. A. Bergin *et al.*, "Activation of the epidermal growth factor receptor (EGFR) by a novel metalloprotease pathway," *J. Biol. Chem.*, vol. 283, no. 46, pp. 31736–31744, Nov. 2008, doi: 10.1074/jbc.M803732200.
- [274] Y. Choudry and A. J. Kenny, "Hydrolysis of transforming growth factor- α by cell-surface peptidases in vitro," *Biochem. J.*, vol. 280 (Pt 1), pp. 57–60, Nov. 1991.

- [275] O. Breig *et al.*, “Metalloproteinase meprin α regulates migration and invasion of human hepatocarcinoma cells and is a mediator of the oncoprotein Reptin,” *Oncotarget*, vol. 8, no. 5, pp. 7839–7851, Jan. 2017, doi: 10.18632/oncotarget.13975.
- [276] Angadi, “Epithelial-mesenchymal transition - A fundamental mechanism in cancer progression: An overview.” <http://www.ijournalhs.org/article.asp?issn=2349-5006;year=2015;volume=8;issue=2;spage=77;epage=84;aui=Angadi> (accessed Nov. 01, 2017).
- [277] E. Lemieux, S. Bergeron, V. Durand, C. Asselin, C. Saucier, and N. Rivard, “Constitutively active MEK1 is sufficient to induce epithelial-to-mesenchymal transition in intestinal epithelial cells and to promote tumor invasion and metastasis,” *Int. J. Cancer*, vol. 125, no. 7, pp. 1575–1586, Oct. 2009, doi: 10.1002/ijc.24485.
- [278] S. Shin, C. A. Dimitri, S.-O. Yoon, W. Dowdle, and J. Blenis, “ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events,” *Mol. Cell*, vol. 38, no. 1, pp. 114–127, Apr. 2010, doi: 10.1016/j.molcel.2010.02.020.
- [279] A. Bannaga *et al.*, “Pathophysiological Implications of Urinary Peptides in Hepatocellular Carcinoma,” *Cancers*, vol. 13, no. 15, p. 3786, Jul. 2021, doi: 10.3390/cancers13153786.
- [280] A. N. Roff, R. P. Panganiban, J. S. Bond, and F. T. Ishmael, “Post-transcriptional regulation of meprin α by the RNA-binding proteins Hu antigen R (HuR) and tristetraprolin (TTP),” *J. Biol. Chem.*, vol. 288, no. 7, pp. 4733–4743, Feb. 2013, doi: 10.1074/jbc.M112.444208.
- [281] X. Gao, T. Sedgwick, Y.-B. Shi, and T. Evans, “Distinct Functions Are Implicated for the GATA-4, -5, and -6 Transcription Factors in the Regulation of Intestine Epithelial Cell Differentiation,” *Mol. Cell. Biol.*, vol. 18, no. 5, pp. 2901–2911, May 1998.
- [282] D. Hahn, R. Illisson, A. Metspalu, and E. E. Sterchi, “Human N-benzoyl-L-tyrosyl-p-aminobenzoic acid hydrolase (human meprin): genomic structure of the alpha and beta subunits,” *Biochem. J.*, vol. 346, no. Pt 1, pp. 83–91, Feb. 2000.
- [283] E. Suh and P. G. Traber, “An intestine-specific homeobox gene regulates proliferation and differentiation,” *Mol. Cell. Biol.*, vol. 16, no. 2, pp. 619–625, Feb. 1996, doi: 10.1128/MCB.16.2.619.
- [284] M. Boyd *et al.*, “Genome-wide Analysis of CDX2 Binding in Intestinal Epithelial Cells (Caco-2),” *J. Biol. Chem.*, vol. 285, no. 33, pp. 25115–25125, Aug. 2010, doi: 10.1074/jbc.M109.089516.
- [285] A. Calon *et al.*, “Different effects of the Cdx1 and Cdx2 homeobox genes in a murine model of intestinal inflammation,” *Gut*, vol. 56, no. 12, pp. 1688–1695, Dec. 2007, doi: 10.1136/gut.2007.125542.
- [286] M. Coskun *et al.*, “TNF- α -induced down-regulation of CDX2 suppresses MEP1A expression in colitis,” *Biochim. Biophys. Acta BBA - Mol. Basis Dis.*, vol. 1822, no. 6, pp. 843–851, Jun. 2012, doi: 10.1016/j.bbadis.2012.01.012.
- [287] A. Stegmann *et al.*, “Metabolome, transcriptome, and bioinformatic cis-element analyses point to HNF-4 as a central regulator of gene expression during enterocyte differentiation,” *Physiol. Genomics*, vol. 27, no. 2, pp. 141–155, Oct. 2006, doi: 10.1152/physiolgenomics.00314.2005.
- [288] S.-H. Ahn *et al.*, “Hepatocyte nuclear factor 4 α in the intestinal epithelial cells protects against inflammatory bowel disease,” *Inflamm. Bowel Dis.*, vol. 14, no. 7, pp. 908–920, Jul. 2008, doi: 10.1002/ibd.20413.

- [289] V. Nikolaidou-Neokosmidou, V. I. Zannis, and D. Kardassis, "Inhibition of hepatocyte nuclear factor 4 transcriptional activity by the nuclear factor κ B pathway," *Biochem. J.*, vol. 398, no. 3, pp. 439–450, Sep. 2006, doi: 10.1042/BJ20060169.
- [290] Y. Chen *et al.*, "Inhibition of miR-155-5p Exerts Anti-Fibrotic Effects in Silicotic Mice by Regulating Meprin α ," *Mol. Ther. Nucleic Acids*, vol. 19, pp. 350–360, Nov. 2019, doi: 10.1016/j.omtn.2019.11.018.
- [291] K. Tan, C. Jäger, D. Schlenzig, S. Schilling, M. Buchholz, and D. Ramsbeck, "Tertiary-Amine-Based Inhibitors of the Astacin Protease Meprin α ," *ChemMedChem*, vol. 13, no. 16, pp. 1619–1624, Aug. 2018, doi: 10.1002/cmdc.201800300.
- [292] P. Sumazin *et al.*, "Genomic analysis of hepatoblastoma identifies distinct molecular and prognostic subgroups," *Hepatol. Baltim. Md*, vol. 65, no. 1, pp. 104–121, Jan. 2017, doi: 10.1002/hep.28888.
- [293] S. W. Warmann *et al.*, "Adenovirus-mediated cytosine deaminase/5-fluorocytosine suicide gene therapy of human hepatoblastoma in vitro," *Pediatr. Blood Cancer*, vol. 53, no. 2, pp. 145–151, 2009, doi: 10.1002/pbc.21956.
- [294] C. Atorrasagasti *et al.*, "Overexpression of SPARC obliterates the in vivo tumorigenicity of human hepatocellular carcinoma cells," *Int. J. Cancer*, vol. 126, no. 11, pp. 2726–2740, 2010, doi: 10.1002/ijc.24966.
- [295] Y. Jin, Z.-Y. Liang, W.-X. Zhou, and L. Zhou, "Expression, clinicopathologic and prognostic significance of plasminogen activator inhibitor 1 in hepatocellular carcinoma," *Cancer Biomark.*, vol. 27, no. 3, pp. 285–293, Mar. 2020, doi: 10.3233/CBM-190560.
- [296] "Targeting Lipid Metabolism in Liver Cancer | Biochemistry." <https://pubs-acsc-org.proxy.insermbiblio.inist.fr/doi/10.1021/acs.biochem.0c00477> (accessed Oct. 07, 2021).
- [297] G. Alipour Talesh, V. Trézéguet, and A. Merched, "Hepatocellular Carcinoma and Statins," *Biochemistry*, vol. 59, no. 37, pp. 3393–3400, Sep. 2020, doi: 10.1021/acs.biochem.0c00476.
- [298] V. E. Kagan *et al.*, "Oxidized Arachidonic/Adrenic Phosphatidylethanolamines Navigate Cells to Ferroptosis," *Nat. Chem. Biol.*, vol. 13, no. 1, pp. 81–90, Jan. 2017, doi: 10.1038/nchembio.2238.
- [299] M. Haeussler *et al.*, "Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR," *Genome Biol.*, vol. 17, p. 148, Jul. 2016, doi: 10.1186/s13059-016-1012-2.
- [300] E. K. Brinkman, T. Chen, M. Amendola, and B. van Steensel, "Easy quantitative assessment of genome editing by sequence trace decomposition," *Nucleic Acids Res.*, vol. 42, no. 22, p. e168, Dec. 2014, doi: 10.1093/nar/gku936.
- [301] X. Yang *et al.*, "A public genome-scale lentiviral expression library of human ORFs," *Nat. Methods*, vol. 8, no. 8, pp. 659–661, Jun. 2011, doi: 10.1038/nmeth.1638.

Annex 1

Supplementary Figures

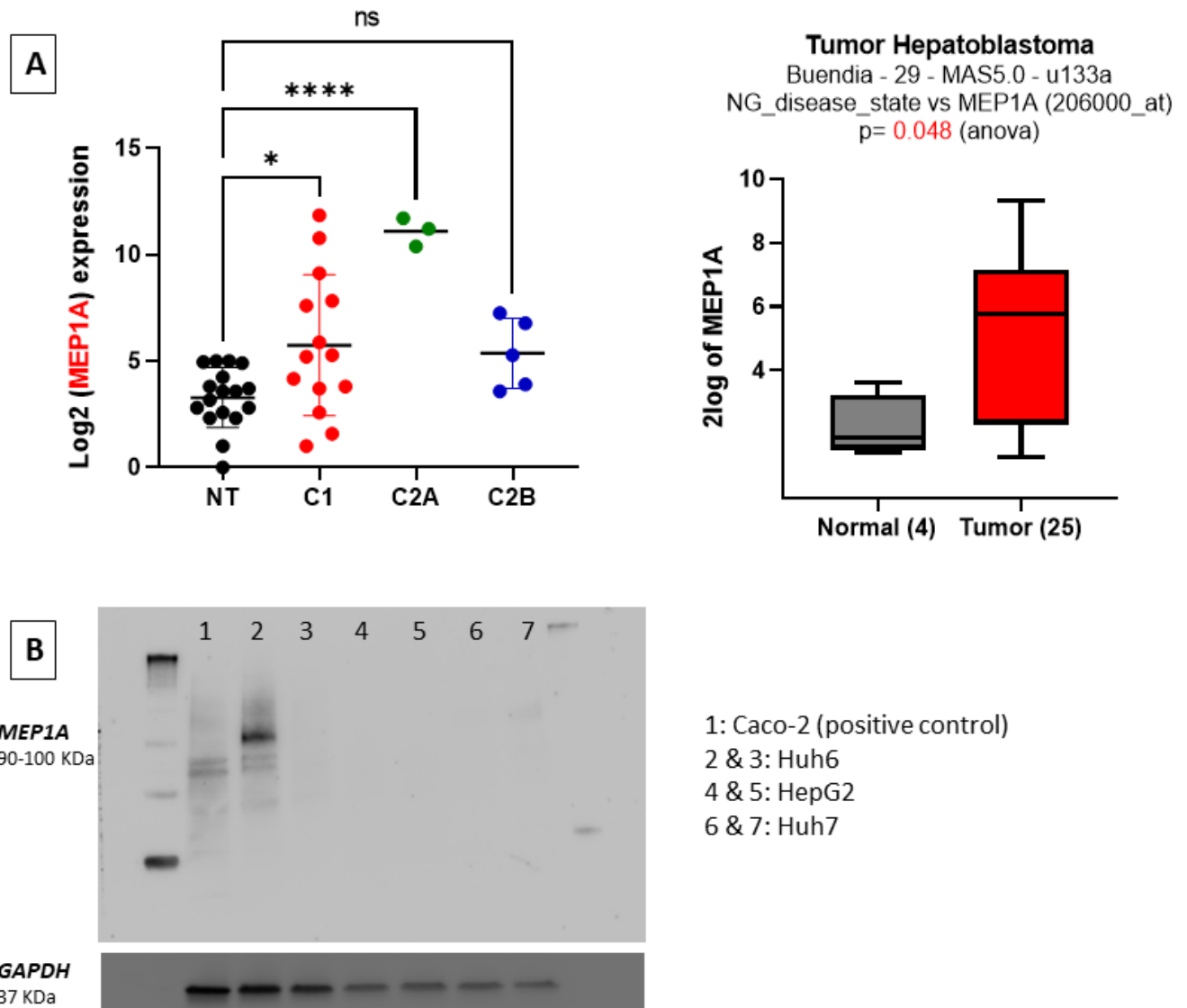


Figure S1: MEP1A mRNA expression and protein expression.

(A) mRNA expression from our RNAseq data in the different HBL subtypes and from Buendia et al. from R2 database; (B) protein expression level in our hepatic cancer cell lines.

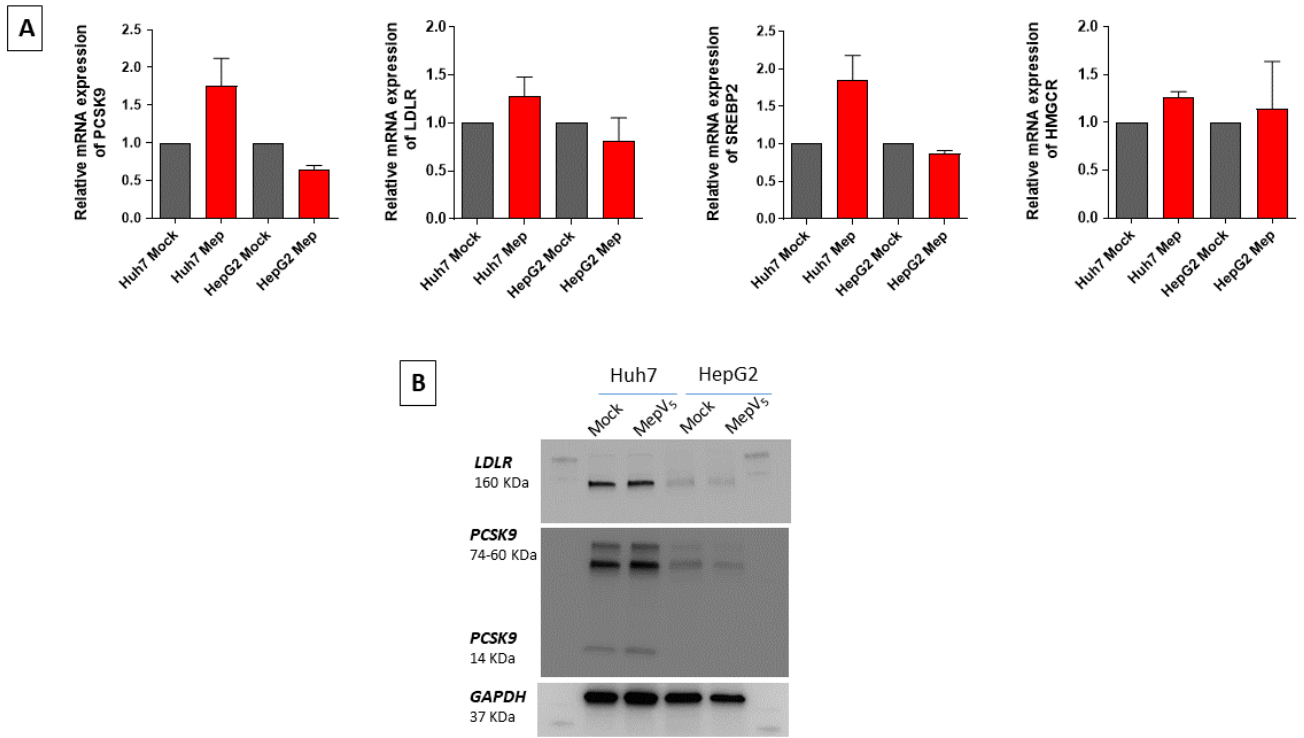
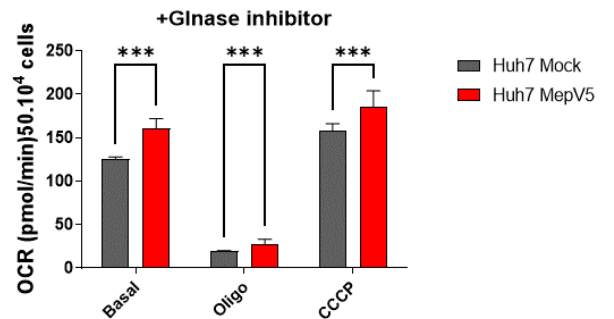
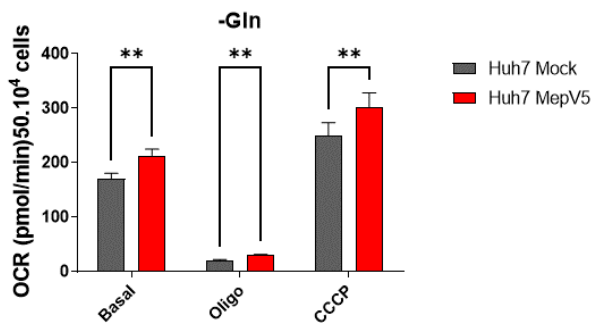
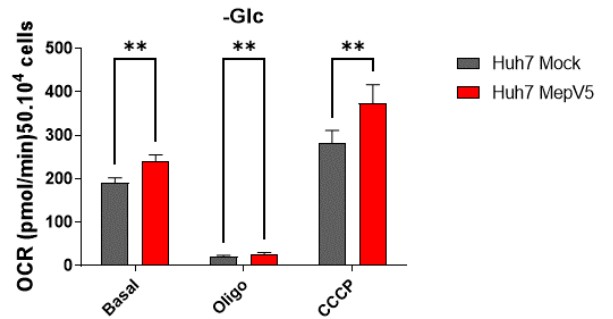
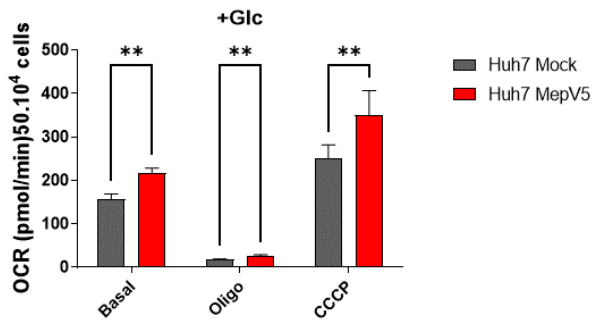
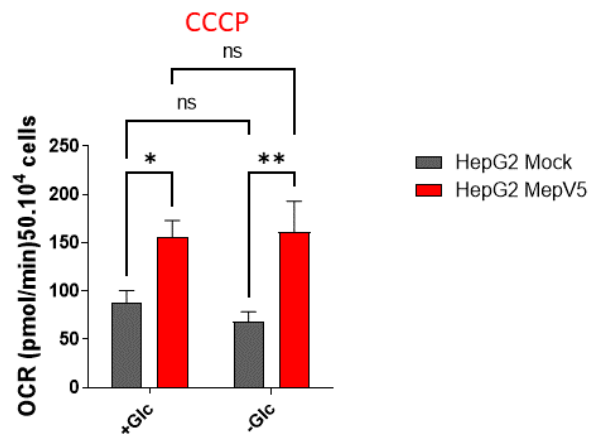
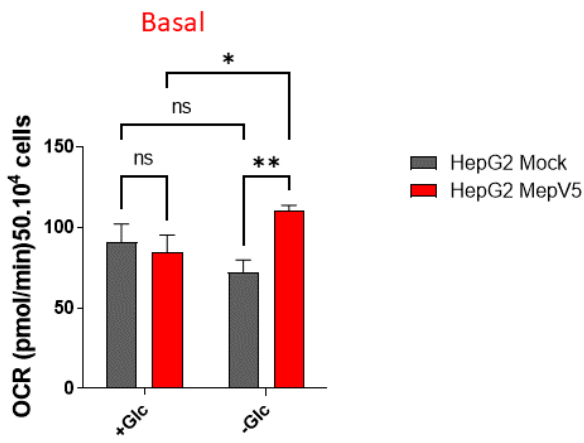
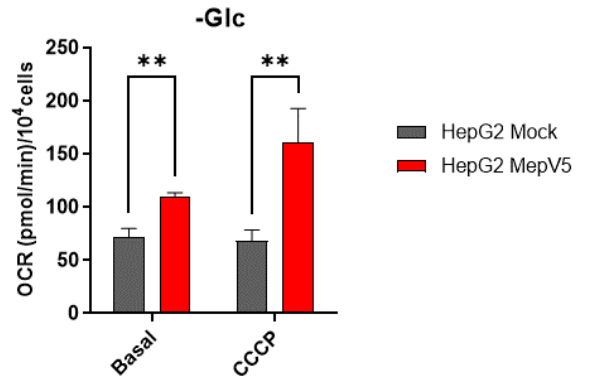
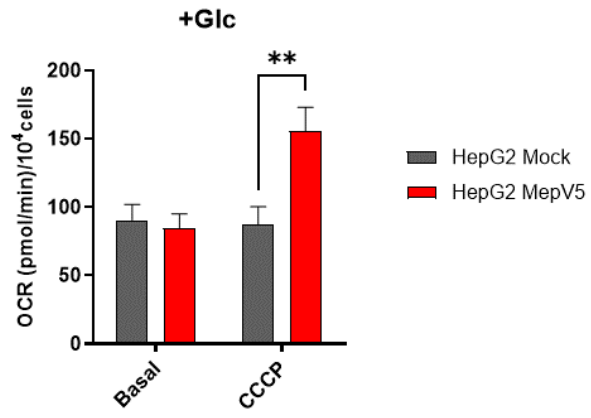


Figure S2: Expression of PCSK9 and its related genes.

(A) mRNA expression level of PCSK9, LDLR, SREBF2 and HMGCR in Mock and MepV5 cells; (B) protein expression level of PCSK9 and LDLR in Mock and MepV5 cells.



Oligomycin

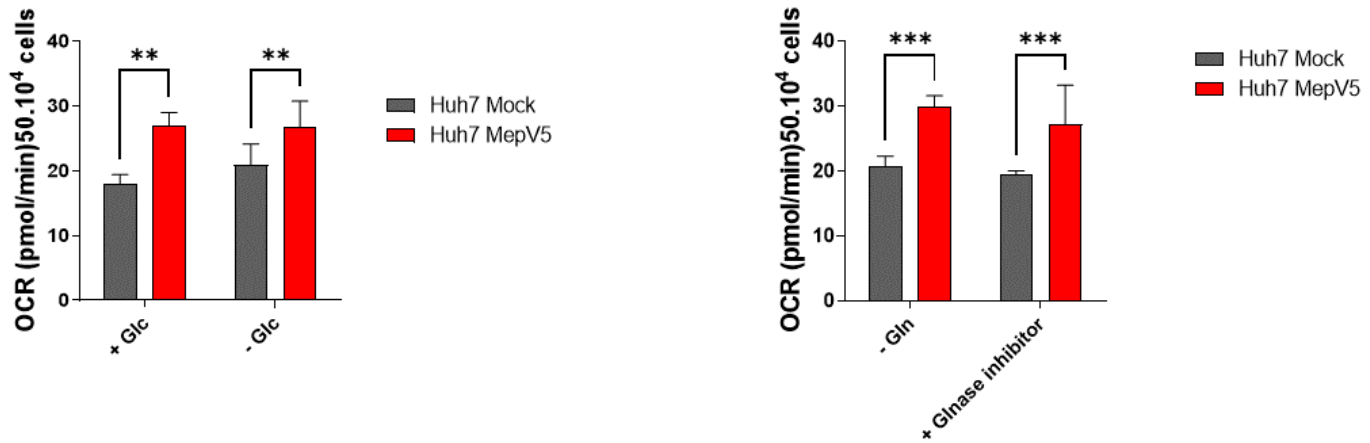
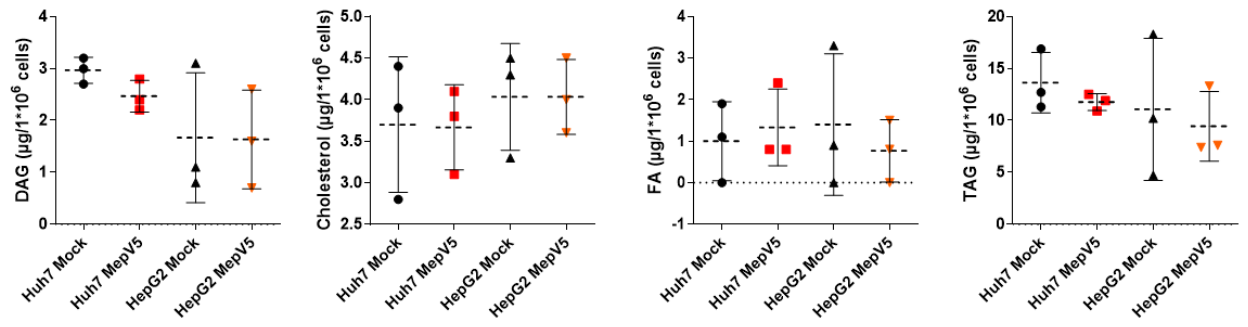


Figure S 3: OCR in HepG2 and Huh7 cells.

Top section shows the OCR in HepG2 cells in the presence of glucose (+Glc) and absence of glucose (-Glc) at basal and maximal respiratory capacity. Middle section shows the OCR in Huh7 cells at the basal level which was also measured in the presence of oligomycin to inhibit ATP synthase and thus determine the mitochondrial OCR, and in the presence of CCCP to measure the maximal respiratory capacity. Bottom figure shows the OCR in the presence of oligomycin between different feeding conditions

Neutral lipids



Phospholipids

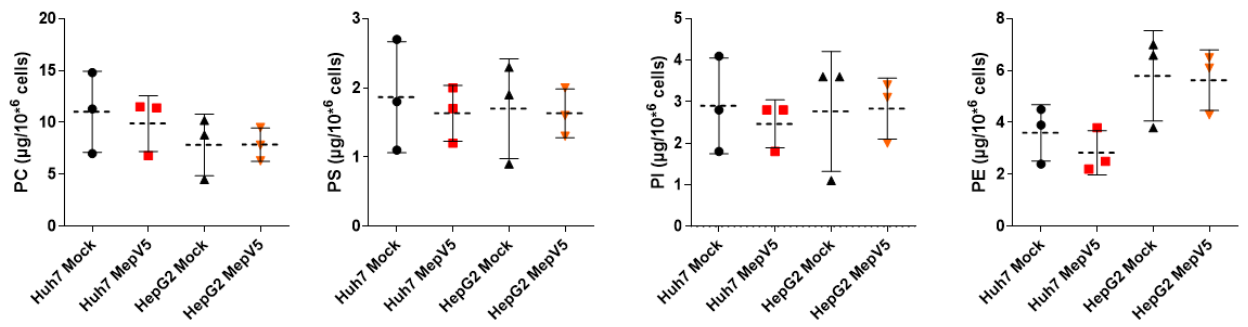


Figure S4: Lipidomics in Huh7 and HepG2 Mock/MepV5 cells under starvation conditions for 48 hours.

Annex 2

CRISPR/Cas9 mediated PCSK9 knock-out

Two sgRNA target DNA sequences (PCSK9#4: 5'- GTCGTGCTGGTCACCGCTGC -3'; PCSK9#5: 5'- CGGCTGTACCCACCCGCCAG -3') located in exon 6 of PCSK9 gene were designed using CRISPOR algorithm (crispor.tefor.net; [299]). Alt-R[®]-crRNA corresponding to target sequences were purchased from Integrated DNA Technologies (IDT) as well as human crRNA negative control and resuspended to 100 µM in IDTE buffer. They were then equally mixed with 100 µM Alt-R[®]-tracrRNA (IDT), annealed by heating for 5 minutes at 95°C and cooled to room temperature (RT). This dual gRNA was diluted to 1.2 µM in OPTI-MEM and 4.5 µL mixed with an equal volume of 1 µM OPTI-MEM diluted Alt-R[®] S.p-Cas9HIF1v3 (IDT) and with 1.5 µL of Cas9 Plus™ Reagent and 1 µL of Lipofectamine CRISPRmax reagent (ThermoFisher Scientific) to form ribonucleoprotein lipofection complex. After 10 minutes incubation at RT, the solution was added in a 96-well plate containing 10 000 cells/well plated the day before in 100 µL culture medium. At 2-3 days post transfection, cells were trypsinized and half of them were cultured while the other half was pelleted, lysed and used as PCR template using Phire Tissue Direct PCR Master Mix (ThermoFisher Scientific). PCR amplification of the targeted area was done following supplier instruction with primers 5'- TCTGTGCCTGTAAGGGAGGG -3' and 5'- ATGATGGAGGTTTCGAGCCC -3'. Sequencing of PCR products was done by Eurofins Genomics and Sanger data were used to quantify Indels reflecting gene KO with TIDE algorithm (tide.deskgen.com; [300]) or ICE algorithm from Synthego (ice.synthego.com).

Cells were FACS sorted to seed 1 cell/well in 96-well plates and let expand to obtain cellular clones, which were then characterized by western blot analysis.

Meprin α cloning

The *MEP1A* cDNA was amplified from the plasmid pLX304-MEP1A-V5 obtained from Thermo Scientific [301] and provided by Osman Breig [275]. It contains the sequence NM_005588.3 corresponding to the mRNA of the *Homo sapiens* meprin A subunit alpha (MEP1A, 2893 bp, Figure 24) fused to the V5 epitope sequence.

>NM_005588.3 Homo sapiens *mep1n* A subunit alpha (MEP1A), mRNA
 ACTTGCAGCAATGGCTTGGATTAGATCCACTTGCATTCTCTTTTTACCTTGCCTTTTGGCCACATAGCAGCTGTACCGA
 TTAAGTATCTTCTGAAGAAAATGTACATGATGCAGATTTGGTGAACAGAAGGATATTTAGAAATCAATTTAGCTGCA
 GGCTTGGACCTCTTTCAAGGGGACATCCTCTTGCAGAAATCCAGAAATGGCTGAGAGACCCAAACACCAGGTGGACGTT
 CCCCATTCTTACATCTTGGCTGATAATTTGGGGCTGAATGCTAAAGGAGCCATTCTGTATGCCTTTGAGATGTTCCGTC
 TCAAGTCTGTGTGGATTTCAAGCCCTATGAAGGAGAGAGCTCATATATCATATTTCAACAGTTTGTGGGTGCTGGTCT
 GAGGTTGGTGACCAACATGTGGGACAGAACATTTCCATTGGCCAAGGATGTGCCTATAAGCCATCATAGAACACGAGAT
 CCTGCATGCTTTGGGATTTTACCACGAGCAGTCAAGGACGGACCGGGATGATTATGTGAACATCTGGTGGGACCAATTC
 TTTCAGGTTACCAGCACAACCTTTGACACCTATGATGATAGCTTAATCACAGACCTCAATACACCCTATGATTATGAGTCT
 TTGATGCATACCAGCCTTTCTATTTAACAAGAATGCAAGTGTCCACCATCACAGCCAAGATCCCTGAGTTTAACTC
 CATTCTCGACAGAAACCTGGATTTCAAGTCCATTGATTTAGAGAGGCTGAACCGAATGTACAAATTGACACCAACTCACA
 CTCTTTTGGACCACTGTACTTTTGAAGAAGGCAAAATCTGTGGAATGATTACAGGGCACCAGAGATGACACTGACTGGGCC
 CATCAGGACAGTGTCTCAGGCTGGAGAAGTGGATCACACCTTGTGGGACAATGCACAGGTGCCGGCTACTTCATGCAGTT
 CAGCACCAGCTCGGGTCCGCGGAAGAGGCAGCCCTACTGGAGTCTCGGATTCTTTACCCAAAGAGGAAGCAGCAGTCC
 TGCAATTTTTCTATAAAATGACGGGAAGTCTTCCAGACAGACTCGTTGTCTGGGTGAGGAGGATGACAGCACAGCAAT
 GTTCGCAAGTTGGTGAAGGTGCAGACTTTTCAAGGAGATGATGACCACAATTGGAAAATTGCCCATGTGGTGTCAAAGA
 GGAACAGAAGTTTCGCTACTTTTCCAGGGCACAAGGGCAGCCCTCAGAATCACTGGGGGAATTTACCTAGATGACA
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 GGGGACAAGCTTCAGAGCCCTCGATTCTACAATTCGAGGGATATGGTTTTGGGGTAACTTTATACCCAAATAGCAGAGA
 AAGCTCTGGTTACTTGAGACTTGTCTTTTATGTGTGCAGTGGGGAGAACGATGCTATCCTGGAGTGGCCGGTAGAAAACA
 GACAGGTGATAATTACCATCCTTGACCAGGAGCCTGATGTCCGGAACAGGATGTCTCAAGCATGGTGTTCCTACTCCTCG
 AAGTCGCACACATCTCCAGCGATAAATGACACTGTCTGAGCAGGCGTCCAGGGTGGGAACCTATCATACGGACTG
 TAATTGTTTTAGAAGCATCGACTTGGGCTGGAGTGGTTTCAATTTCCACCAAATGCTGAAAAGGAGGAGTTTCTGAAAA
 ATGATGACCTCATCATATTTGTGGACTTTGAAGATATCACCCACTCAGCCAGACTGAAGTTCCCACTAAAGGCAAAAAGA
 CTGAGCCCCCAAGGCCCTCATTCTCAAGGCCAGGAGCAGCAGGTCTCCGAAGAAGGTTCCGGAAAAGGCCATGTTAGAGGA
 AGCCCTACCTGTCAGCCTGAGCCAGGGGACGCCAGCCGACAGAAGCGGTGCGTGGAGAACACAGGCCCCCTGGAGGACC
 ATAAGTGGCCACAGTACTTCAGAGACCCATGTGACCCAAACCTTGCAAAATGACGGCATCTGTGTGAACGTGAAGGGG
 ATGGCGAGCTGCAGGTGCATCTCTGGACATGCTTTCTTCTACACGGGGAGCGCTGTGAGCCGTGCAGGTGCACGGCAG
 TGTCTGGGCATGGTATCGGAGGCACGGCTGGCGTGATCTTCTGACCTTCTCCATCATCGCCATCCTTTCCAAAGGC
 CAAGGAAGTGACCTGCCTGCTGGCATTGGCCAGACCACAGCAGCACCTCCTCCATGCAGGCCCTAACTTTCCATGGTCA
 ATGCAGTTTTTATCAGCCTTGTCTTTGGATAGGACCTCAAGGACTAAGGCCTCCAGCCCCATGTGTGACCTTGTCTATCT
 CTCTGCCCCACATAAATCTGTTACTTTGTCTATGTGCTCCTAATGTATCTAGTGTGCTCTGACAACACTCATCACACTT
 CATTGTAATCACTTGTTTTATTGACTGTCTTCTATAGACTGTAAGTCCATGAGGGCAGGCACATGTTGTTCTCATT
 GACCGTGTGGCCCCAGTGCCTAGATGCATGGCTGGCACATTGTTGGCACTCAACAATGGTTGAATGAATAAAACAATAA
 ATGAATGAATAACTAAGATATAGAACTCTCATTATATTGCAGATTGAATATATATGATGAAATCTTATGTTGAATAT
 GTTGAATCAATACTCATTTTTTATTAGATACAGTAGTGTCTCACTCTTTTAAAGATCTTGTAAAGATTTCAAATAAA
 GGTACTTCTGGGCAGCCAGGCTGCACAGCATTGCTTCTCTGAGATTCTAAGAGAAGGCCCTTAATAAATTTAATAAA
 TATTGAGTTAGCA

Figure 3124: Nucleotide sequence of the MEP1A mRNA ref NM_005588.3 (GeneBank).

The two amplification primers were:

-on the 5' end, **GTGAGAATTCACCAT**ATGGCTTGGATTAGATC (the *Eco*RI site is written in bold and the ATG start codon is underlined)

-on the 3' end of the ORF, GTGAGGATCCTCACTTCTTGGCCTTTG (the TGA stop codon is underlined and the *Bam*HI site is written in bold).

The Q5[®] high fidelity DNA polymerase from New England Biolabs (ref M0491G) was used for the PCR with the following thermal cycles: 98°C, 30"; (98°C, 10"; 70°C, 30"; 72°C, 2'20) x 35; 72°C, 5'; 4°C, infinite, according to the manufacturer recommendations. The PCR fragment was purified on a column (Macherey Nagel kit, ref 740609) and subcloned into the pMiniT 2.0 plasmid from New England Biolabs (ref E1203S) (Figure 25). After ligation, the recombinant

plasmid was transformed into NEB10-beta cells from New England Biolabs (ref C3019H). The *EcoRI-BamHI* fragment was excised from the purified plasmids by restriction digest with *EcoRI*-HF® (ref R3101) and *BamHI*-HF® (ref R3136) from New England Biolabs.

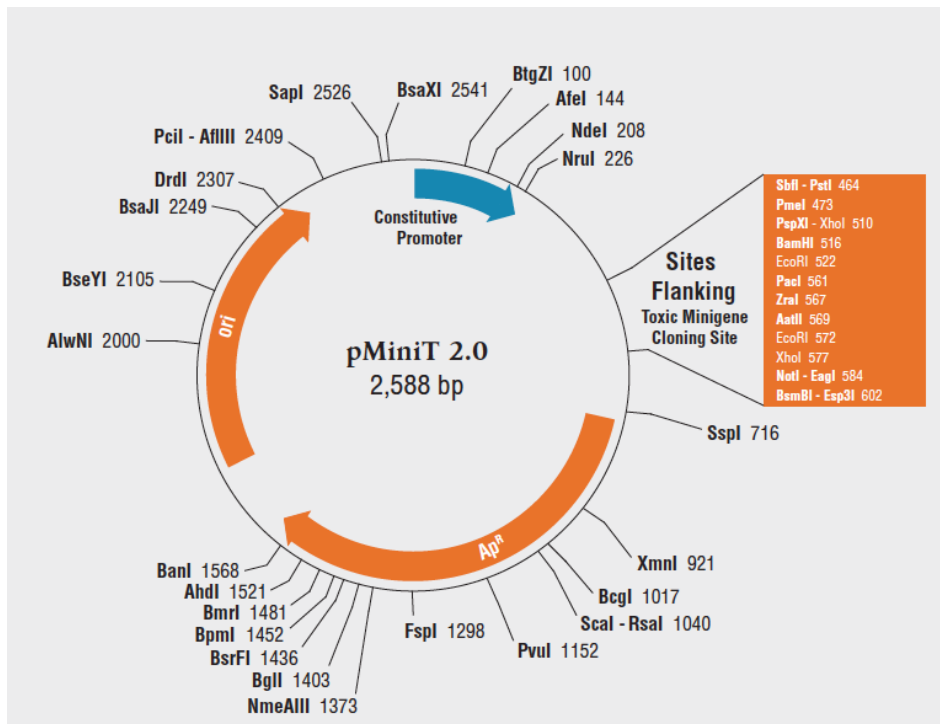


Figure 3225: The pMiniT 2.0 map (<https://www.neb.com/>)

The purified fragment was then subcloned into the pSin-EF1aL-MCS-IRES-Puro plasmid provided by the Vectorology platform from the University of Bordeaux (<https://www.tbmcore.u-bordeaux.fr/wp-content/uploads/2016/08/Fichierlentivecteurs.pdf>) digested with *EcoRI*-HF® (ref R3101) and *BamHI*-HF®. After ligation with the T4 DNA ligase from Thermofisher (ref EL0011), the reaction medium was transformed into NEB10-beta cells. The DNA sequence of the insert of the recombinant plasmids, named p412-MEP1A (Figure 26), was controlled by sequencing (Eurofins) with the primers shown in Table 8. The sequence was 100% consistent with that of NM_005588.3. A clone was further chosen for endotoxin-free DNA preparation (NucleoBond Xtra Midi EF kit from Macherey-Nagel, ref 740420). 70 µg were provided to the Vectorology platform for lentivirus production. A transient expression system, by co-transfection of three or four plasmids, is used to produce replication-defective recombinant virus particles.

Table 8: Names, sequences and locations of the sequencing primers used to sequence MEP1A insert into pSin-EF1aL-MCS-IRES-Puro.

The resulting plasmid was named p412-MEP1A. ^aLocation of the sequences on the p412-MEP1A plasmid.

	Sequence	Location ^a				
		start	end	Length (bp)	Tm (°C)	GC (%)
Forward						
277	AGTGGGTGGAGACTGAAGTTAG	3445	3466	22	57	50
SqFw1	GGCCATCATAGAACACGAGATCCTG	4047	4071	25	61	52
SqFw2	GTCCTTCAGACAGACTCGTTGTCTG	4655	4679	25	60	52
SqFw3	GTCCAGGGTGGGAACCTATCATAC	5238	5261	24	60	54
Reverse						
SqRv1	CGGAATTGGCCGCCCTAGATG	5850	5870	21	61	62
SqRv2	GGAAACTCCTCCTTTTCAGCATTGG	5316	5341	23	60	57
SqRv3	CATCATCTCCTTGAAAAGTCTGCACC	4725	4750	26	60	46
SqRv4	CACATAATCATCCCGGTCCGTCC	4103	4126	24	60	50

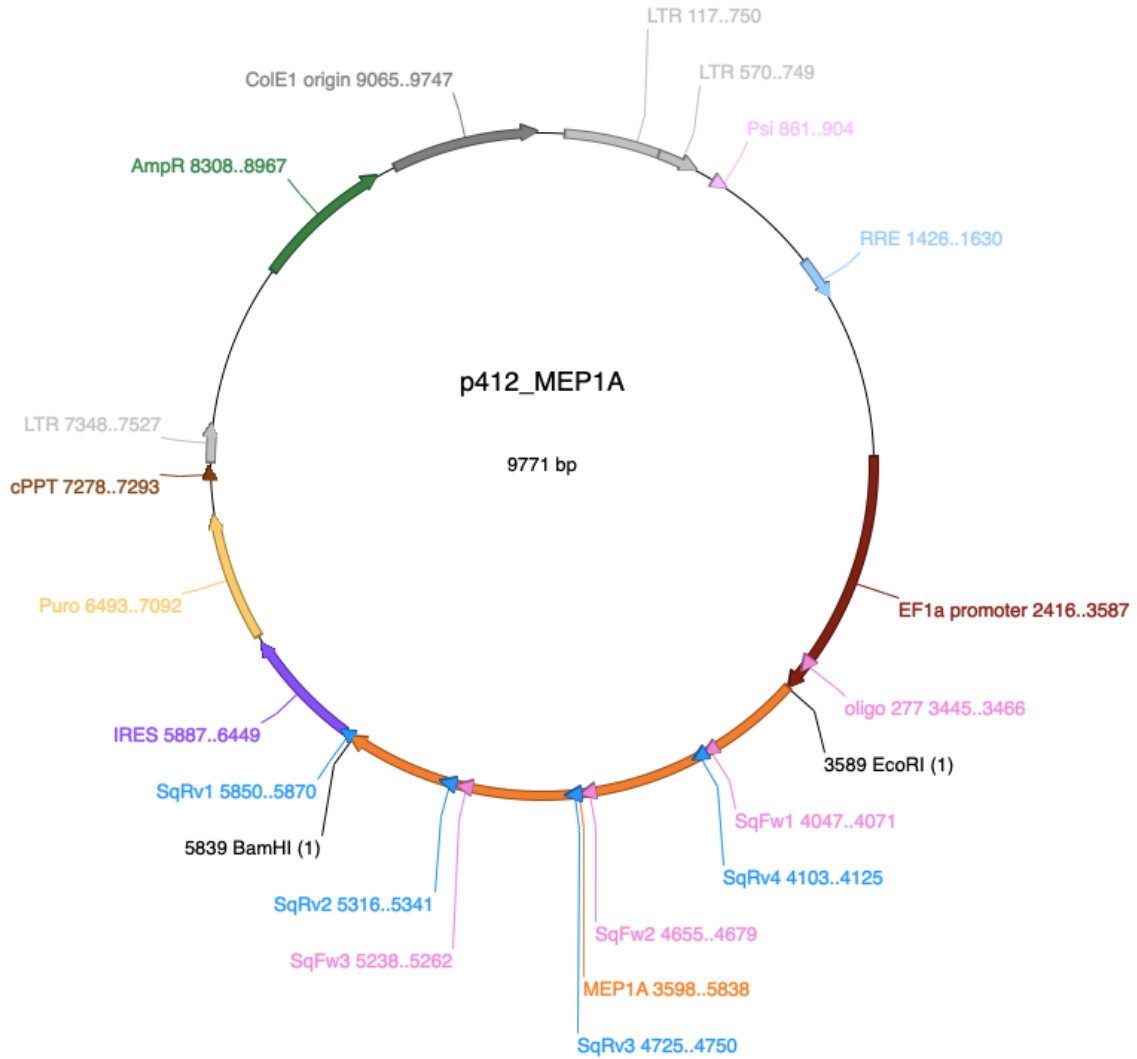


Figure 3326: The p412-MEP1A restriction map (drawn with the Ape program).

The *MEP1A* gene cloned between the *EcoRI* and *BamHI* sites (2241 bp) is under the control of the EF1a (Elongation factor 1-alpha 1) promoter (long form) and the puromycin resistance gene (Puro) under the control of an IRES (internal ribosome entry site). LTR, long terminal repeat; Amp^R, ampicillin resistance gene (bacterial selection); cPPT central PolyPurine tract (to enhance transduction efficiency); 277 and SqFw1 to 3, sequencing forward primers; SqRv1 to 4, sequencing reverse primers.

Annex 3

Published Review Article

Targeting Lipid Metabolism in Liver Cancer

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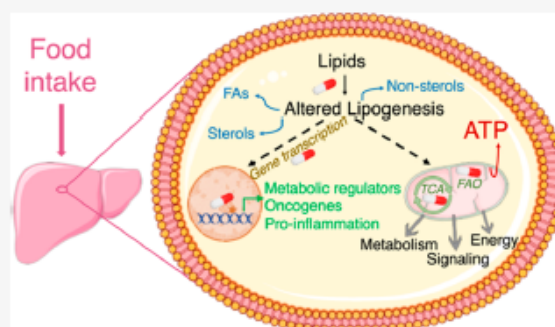
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ABSTRACT: Cancer cells are highly dependent on different metabolic pathways for sustaining their survival, growth, and proliferation. Lipid metabolism not only provides the energetic needs of the cells but also provides the raw material for cellular growth and the signaling molecules for many oncogenic pathways. Mainly processed in the liver, lipids play an essential role in the physiology of this organ and in the pathological progression of many diseases such as metabolic syndrome and hepatocellular carcinoma (HCC). The progression of HCC is associated with inflammation and complex metabolic reprogramming, and its prognosis remains poor because of the lack of effective therapies despite many years of dedicated research. Defects in hepatic lipid metabolism induce abnormal gene expression and rewire many cellular pathways involved in oncogenesis and metastasis, implying that interfering with lipid metabolism within the tumor and the surrounding microenvironment may be a novel therapeutic approach for treating liver cancer patients. Therefore, this review focuses on the latest advances in drugs targeting lipid metabolism and leading to promising outcomes in preclinical studies and some ongoing clinical trials.



Glucose and glutamine metabolism, tricarboxylic acid cycling, oxidative phosphorylation, the pentose phosphate pathway, and amino acid and lipid synthesis are all essential for the production of cellular components to cope with a faster growth rate. Indeed, the growth and expansion of tumors require a massive and complex system that coordinates to provide the necessary nutrients. They also depend on the microenvironment, including the extracellular matrix, the nearby cells, of which some are immune cells, and growth factors like cytokines. Cancer cells require more energy than normal cells to fulfill their needs. Therefore, their metabolism is expected to be modulated to meet their continuous demand for energy, which is provided in the form of ATP produced by the conversion of various metabolites such as glucose, lactate, pyruvate, glutamine, or fatty acids. Cancer cells usually originate from genetic mutations or epigenetic alterations, leading to reprogramming of their signaling and metabolic pathways.¹ In the recent past, there has been growing interest in reprogramming many metabolic pathways such as glucose, glutamine, or lipid metabolism. This last point will be the focus of this review.

Adult liver cancer ranks as the second most fatal cancer in the world.² It develops on a diseased liver, and its incidence rate has increased steadily over the past decade due to the increase in the frequency of viral hepatitis B or C infections or to environmental toxins as well as to the increased incidence of chronic liver diseases, such as non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD). It should be noted that NAFLD develops in patients with

metabolic syndromes such as type 2 diabetes mellitus, obesity, hypertension, and hyperlipidemia.^{3–5} Most of the primary liver cancers in adults are hepatocellular carcinomas (HCC), which account for 90% of all cases.

Liver cancers are characterized by modulation of the Warburg shift and upregulation of lipid catabolism.⁶ During the Warburg shift, a disproportionate relation exists between glucose uptake and respiration rate, where, even in the presence of adequate amounts of oxygen, cancer cells tend to generate energy from aerobic glycolysis more than from mitochondrial oxidative phosphorylation (OXPHOS), resulting in increased rates of glucose uptake, ATP generation, and lactate production.⁷ In this way, energy is supplied faster along with a carbon source, which is necessary for the anabolic synthesis of essential precursors. The exact role and significance of the Warburg effect in cancer cells are still being debated.⁸ The Warburg shift indirectly supports anabolic metabolism, presumably by feeding nonmitochondrial pathways, such as the pentose phosphate, hexosamine, and glycerol pathways, but it does not prevent the mitochondrial respiration that allows growth and proliferation by providing the

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tricarboxylic acid (TCA) cycle intermediates for anabolism. However, by reducing their OXPHOS, cancer cells can also reduce the amount of reactive oxygen species (ROS), which are highly reactive metabolites known to cause significant damage to cell structures.

In the following, we will dissect the main anabolic and catabolic pathways of lipid metabolism, e.g., lipogenesis and fatty acid oxidation with a primary focus on acetyl-CoA, which is a metabolic node located at the center of many metabolic pathways (Figure 1). In this context, we will show how

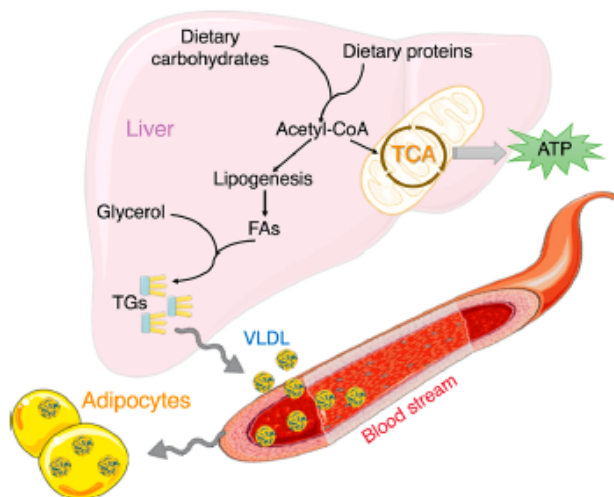


Figure 1. Overview of lipid metabolism. Carbohydrates and proteins that originate from the diet will be metabolized by the liver to produce acetyl-CoA, a main key player in the metabolic reactions. Acetyl-CoA can enter the TCA cycle in the mitochondria to produce energy. It can also be used to produce FAs by a process termed lipogenesis. These FAs together with glycerol will give rise to TGs that will be transferred through the bloodstream by very low density lipoproteins (VLDLs) to be stored in adipose tissues. During long fasting periods, these lipid particles will serve as a great source of energy through FAO and autophagy.

interfering with the production and fate of this key metabolite modulates oncogenesis. This will set the stage for the assessment of drugs targeting specific and critical steps of the complex lipid metabolic network in the particular context of the liver cancer.

■ ACETYL-COA, LIPID METABOLISM AND ONCOGENESIS

Acetyl-CoA is a central metabolite at the crossroads of many metabolic pathways and transformations (Figures 1 and 2). It is used not only in the synthesis of essential macromolecules needed by the cells but also in multiple catabolic and anabolic reactions, including synthesis of FAs, steroids, and non-sterol compounds.⁹ Indeed, acetyl-CoA is derived from multiple metabolic reactions that occur in the cell, such as glucose, fatty acid, and amino acid catabolism. The importance of this molecule resides in its different implications in many cellular processes, including energy production by the TCA cycle inside the mitochondria, serving as a building block molecule for the synthesis of macromolecules like FAs and sterols in the cytosol, as well as being an important nucleocytoplasmic molecule for the acetylation of histones and thus activation of gene expression.¹⁰

It is important to distinguish between the cytosolic and mitochondrial acetyl-CoA, as neither form can freely transfer between the cell compartments because there is no mitochondrial acetyl-CoA transporter. Acetyl-CoA shuttles between these two compartments by means of carnitine as discussed below. Mitochondrial acetyl-CoA (mAc-CoA) can be derived from pyruvate, FAO, and acetate, while cytosolic acetyl-CoA (cAc-CoA) can be derived from acetate, citrate, and ketone bodies.^{10,11} All of these reactions are catalyzed by different enzymes such as acetyl-CoA synthetase (ACSS) and ATP citrate lyase (ACL/ACLY)¹² (Figure 2).

Under nutrient deprivation conditions, the cells need to shift their metabolism from growth to survival to ensure their viability. Under this condition, cells need to direct acetyl-CoA into the mitochondria to produce energy, and hence, the level of the nucleocytoplasmic acetyl-CoA decreases. It is worth mentioning that, in the liver, mAc-CoA can be used to produce ketone bodies, such as acetoacetate and β -hydroxybutyrate, which serve as fuel sources for the heart and brain under starvation conditions.¹⁴

The critical uses of acetyl-CoA and its implication in the survival and growth of cancer cells in both nutrient-abundant and -deprived conditions led many researchers to focus on modulating the production and fates of this molecule (Table 1). Some of these approaches were achieved by blocking the transport of citrate from mitochondria to the cytosol through citrate carriers (CICs) by the action of the benzene tricarboxylate analogue (BTA). BTA has shown some anticancer effects in various tumors and in a xenograft mice model,¹⁵ indicating it can be used alone or in combination with other metabolic modulators as anticancer agents. Another way to block acetyl-CoA production is by using an inhibitor of the enzymes ACSS (ACSS 1–3) that catalyze the production of acetyl-CoA from acetate.

In a study of human HCC tumors and cell lines, those exhibiting low glucose and high acetate uptake presented a remarkable expression of ACSS1 and ACSS2. However, in one HCC cell line with the opposite features (high glucose and low acetate uptakes), only ACSS2 was expressed. The inhibition of these enzymes in cells with high acetate uptake using small interfering RNA (siRNA) resulted in decreased cell viability. This points to the importance of acetate as a carbon source for growth and lipid synthesis in liver cancer cells, notably those exhibiting a low-glycolysis phenotype.¹⁶ A recent study reported a high level of expression of ACSS2 in cisplatin-resistant bladder cancer patient tissues and showed that the use of an ACSS2 inhibitor leads to the abrogation of resistance in cisplatin-resistant bladder cancer cells.¹⁷ It remains to be seen whether this approach could be applied *in vivo* and clinically to other types of cancers such as liver cancers.

The lipid anabolic reactions termed the “de novo lipogenesis” and “mevalonate pathway” take place in the cell cytosol (Figure 3). A higher level of lipogenesis is a characteristic feature of cancer cells requiring the rate-limiting enzyme acetyl-CoA carboxylase (ACC), which catalyzes the irreversible carboxylation of acetyl-CoA to malonyl-CoA.¹⁸ The reverse decarboxylation reaction is catalyzed by yet another enzyme named malonyl-CoA decarboxylase (MCD), which in turn inhibits lipogenesis and stimulates β -oxidation.¹⁹ Then acetyl-CoA is combined with malonyl-CoA by fatty acid synthase (FASN) to produce fatty acids, which can be metabolized to produce TGs or phospholipids. TGs can be stored in lipid droplets as a reserve energy source, while

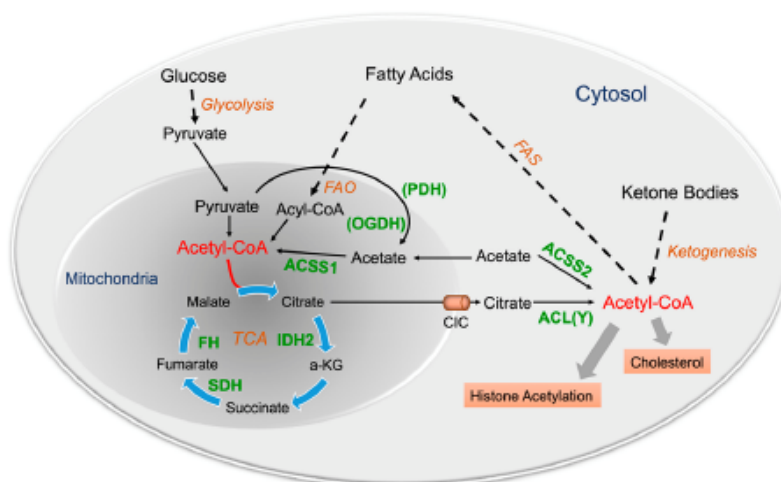


Figure 2. Metabolic pathways that generate and utilize acetyl-CoA. Acetyl-CoA can be produced by many metabolic pathways, two of which are mitochondrial and cytosolic acetyl-CoA. The enzymes involved in its production are colored green. The transport of acetate and citrate from and to the mitochondria is ensured by their respective carriers. Abbreviations: TCA, tricarboxylic acid cycle; FAO, fatty acid oxidation; FAS, fatty acid synthesis; α -KG, α -ketoglutarate; IDH2, isocitrate dehydrogenase 2; SDH, succinate dehydrogenase; FH, fumarate hydratase; ACS1, mitochondrial acetyl-CoA synthetase short chain family member 1; ACS2, cytosolic acetyl-CoA synthetase short chain family member 2; ACL(Y), ATP-citrate lyase; PDH, pyruvate dehydrogenase; OGDH, oxaloglutarate dehydrogenase (parentheses indicate the neomorphic function of the enzymes¹³).

Table 1. Some Potential Anticancer Drugs Targeting Lipid Metabolism

agent	target	developmental stage	observations	trial ID or ref
TVB-2640	fatty acid synthase (FASN)	phase I	colon and other resectable cancers	NCT02980029
TVB-2640 with paclitaxel, and trastuzumab		phase I	solid tumors	NCT02223247
TVB-2640 with bevacizumab		phase II	NSCLC with KRAS gene mutation	NCT03808558
		phase II	NASH	NCT03938246
		phase II	spread HER2+ breast cancer	NCT03179904
		phase II	astrocytoma	NCT03032484
hydroxycitrate, SB-204990	ATP citrate lyase (ACL/ACLY)	preclinical studies	anticancer effects <i>in vivo</i>	93
NDI-010976	acetyl-CoA carboxylase (ACC)	preclinical and clinical studies	anticancer effects <i>in vitro</i>	94
statins (e.g., simvastatin and atorvastatin)	HMG-CoA reductase	approved for treatment of hypercholesterolemia	anticancer effects currently under investigation in >20 clinical trials	95–97
terbinafine	squalene epoxidase (SQLE)	FDA-approved antifungal drug	inhibited SQLE-induced NAFLD-HCC cell growth	13
etomoxir, perhexiline	carnitine palmitoyltransferase 1 (CPT1)	perhexiline is approved for use as antianginal therapy	anticancer effects <i>in vitro</i> and <i>in vivo</i>	51, 98
CB-839, BPTES, and RNAi	glutaminase (GLS1)	preclinical and clinical studies	inhibited TCA anaplerosis; anticancer activities <i>in vitro</i> and <i>in vivo</i>	99, 100
metformin	mitochondrial complex I and ACC	approved for treating type 2 diabetes	phase II clinical trials in patients with breast cancer	NCT01266486
acetylsalicylic acid (aspirin) combined with sorafenib	analgesic		a prospective randomized control trial to prevent the recurrence in high-risk patients with HCC	NCT02748304

phospholipids are necessary for membrane synthesis and serve as signaling molecules (Figure 3). By using some specific inhibitors and other drugs inhibiting these enzymes and pathways, many studies have assessed their potential antitumoral involvement.

For instance, metformin, which is a known drug widely prescribed for the treatment of patients suffering from type 2 diabetes mellitus, was used in a mouse model with chemically induced liver tumors.²⁰ In this study, metformin was shown to protect mice from the induction of liver tumors by decreasing the level of expression of ACC, FASN, and ACLY. In addition, this drug was shown to have antineoplastic effects in breast

cancer cells that are associated with the mammalian target of rapamycin (mTOR) and S6 kinase activation.²¹ Last, in a large cohort study, metformin was reported to be a good selective protective agent against liver cancer.²²

Another family of drugs, soraphens (Sorafenib A), that are antifungal myxobacterial metabolites²³ was discovered to have an anticancer role by inhibiting the ACC enzyme.²⁴ This inhibition changes the phospholipid composition of cellular membranes and interferes with their dynamics.²⁵ Moreover, Sorafenib A was shown to impede the recycling process, dimerization, and localization of growth factor receptors, which

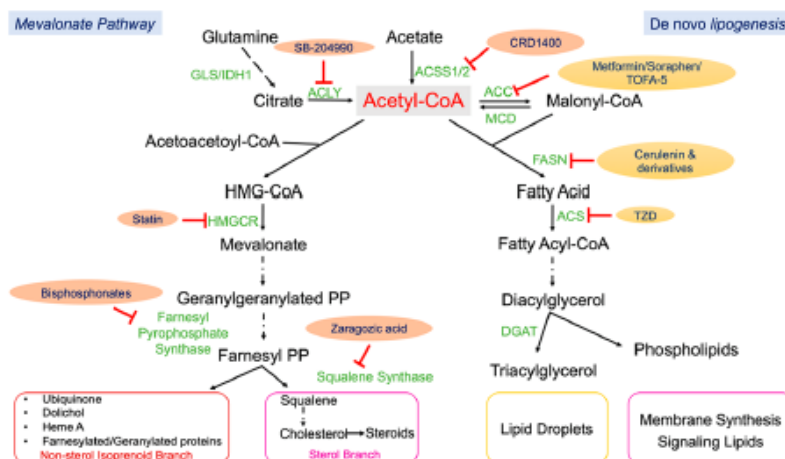


Figure 3. Drug targeting of lipid metabolic pathways. Acetyl-CoA can be incorporated into two anabolic pathways: mevalonate pathway (left side) to produce sterols and non-sterol compounds or fatty acid synthesis (right side) leading to the production of TGs and PLs. Enzymes catalyzing the reactions are colored green, and their respective inhibitors are shown in ovals.

are necessary for the pro-tumorigenic signaling cascade of cancer cells, notably in the case of the HCC cell line Huh7.²⁶

TOFA (5-tetradecyloxy-2-furoic acid) is yet another allosteric inhibitor of ACC that has proven to be cytotoxic for various cancer cell lines. It induces apoptosis in lung cancer cell line NCI-460, colon cancer cell lines HCT-8 and HCT-15, and prostate cancer cell line LNCaP.^{27,28} Moreover, it suppressed ovarian cancer cell proliferation and induced cell cycle arrest and apoptosis²⁹ *in vitro* and *in vivo*. It would be interesting to test this compound, first, on HCC cell lines.

The second anabolic pathway of lipid metabolism using acetyl-CoA is the mevalonate pathway, which leads to the production of sterol and non-sterol molecules, such as cholesterol and ubiquinone, respectively. This pathway is catalyzed by the rate-limiting enzyme β -hydroxy β -methylglutaryl-coenzyme A reductase (HMGCR), which is the direct target of statins (further discussed below), but other enzymes can also be targeted along this pathway. For example, the farnesyl pyrophosphate synthase can be inhibited by bisphosphonates (BPs), which are widely used as effective antiresorptive therapies in osteoporosis. The nitrogen-containing BPs prevent the prenylation of small GTPases (e.g., Ras) that are involved in tumorigenesis. In HCC cells, multiple BP-based drugs (e.g., zoledronic acid, pamidronate, and minodronate) exhibited anticancer effects such as the activation of pro-apoptotic cascades, induction of cell cycle arrest, inhibition of proliferation, migration, and overall tumorigenesis both *in vivo* and *in vitro*.^{30–34} Interestingly, the combination of BP, specifically zoledronic acid, with sorafenib treatment in HCC mouse models enhanced the latter's antitumor effects by depleting macrophages and decreasing tumor size and the levels of angiogenesis and metastasis.³⁵

Zaragozic acid, a natural polyketide isolated from fungi, inhibits squalene synthase and blocks the synthesis of cholesterol downstream of mevalonate formation. It also induces the death of the HCC cell line Huh7 as a result of an increased level of synthesis of geranylgeranoic acid and not cholesterol.³⁶ The potential antitumor role of zaragozic acid is highlighted by the fact that it also inhibited the growth of Rauscher virus-transformed lymphoma and Lewis lung carcinoma *in vivo*³⁷ and could potentiate the antitumor effects

of active and adoptive immunotherapy by prolonging the overall survival of tumor-bearing mice.

LIPOGENESIS IN LIVER CANCER CELLS

Fatty acids (FAs) are considered to be a great source of energy and can be used by cells for growth and proliferation. An increased level of lipogenesis is an emerging metabolic hallmark of cancer² because, together with lipolysis, it promotes tumor growth, proliferation, and survival.

The primary organ of lipid metabolism is the liver, which is its main function, through the maintenance of lipid homeostasis and energy balance. FAs are mainly synthesized in the liver and mostly from dietary carbohydrates, which are transformed into acetyl-CoA, leading to lipogenesis. Lipids can be stored in the adipocytes to be further used as an energy source during long periods of fasting.³⁸ Therefore, normal liver physiology depends largely on the balance of the lipid metabolism, any disruption of which can induce inflammation and fibrogenesis as well as fatty liver diseases and HCC.

Metabolic profiles of liver cancer cells have been characterized by a growing number of studies that aimed to improve our understanding of their behavior and possibly discover biomarkers related to hepatic oncogenesis and propose more adapted therapeutic approaches.³⁹ Carcinoma is developing in the liver through intermediate phases such as NAFLD/NASH or cirrhosis, depending on various alterations of metabolic pathways. Thus, the deregulation of bile acids and phospholipids is observed in the early stages of liver diseases, followed at the advanced stages by an increase in the Warburg shift and lipid catabolism.⁶ Metabolomics studies conducted on HCC have shown that the enhancement of the Warburg effect can be attributed to an increase in the level of glycolysis along with an increase in the level of β -oxidation, making them common metabolic hallmarks of the tumor.^{40–43} This metabolic reprogramming observed during the progression to HCC is considered a common feature of tumors.

Serum lipid profiling by ultrafast LC/IT-TOF MS has been effective in highlighting differences in the lipidome of healthy patients and patients with liver diseases.⁴⁴ Indeed, Chen et al.⁴⁴ were able to identify 96 serum lipids, 75 of which were differently regulated: eight sphingomyelins (SMs), ten polyunsaturated phosphatidylcholines (PCs), six lysophospha-

tidylcholines (LPCs), ten TGs, and two cholesterol esters (ChoEs) were downregulated for patients with cirrhosis and HCC, compared to healthy patients and patients with hepatitis. These analyses have revealed lipidomic differences according to the stage of HBV-induced liver disease. It is interesting to note that a saturated PC, PC (32:0), was upregulated for all diseased patients, but more extensively for those with cirrhosis and HCC. This could be related to its protective role against oxidative stress for cancer cells.²⁵ Li et al.⁴⁵ performed lipid analyses of HCC liver along with the corresponding paracarcinoma tissues and reported deregulation of 53 lipids. The level of TG deregulation was dependent on the number of double bonds. Downregulation was observed for TG with more than two double bonds, while those with two or fewer double bonds were more abundant. Furthermore, the authors found a positive correlation among the severity of the disease, the higher concentrations of saturated TGs, and the lower concentrations of polyunsaturated TGs in HCC tissues. PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) containing zero, one, and three double bonds were upregulated, and those presenting two, four, and six double bonds were downregulated. Lin et al.⁴⁶ analyzed the lipid content of HCC cells with various metastatic abilities and found an up to 25% reduction of the levels of palmitic acyl (C16:0)-containing phosphoglycerolipids, which was associated with the enhanced metastatic abilities of these cells. Therefore, they have investigated the antitumor activity of palmitic acid (C16:0). Treatment of highly metastatic HCC cells by palmitic acid showed inhibition of cell proliferation, migration, and invasion, a decrease in cell membrane fluidity, inhibition of glucose uptake, and thus affected malignant behaviors of cancer cells. Moreover, treatment of nude mice bearing cell carcinoma xenografts with palmitic acid led to the suppression of both subcutaneous tumor growth and metastatic nodule formation.¹³

In summary, the differential abundance of certain lipids in HCC can potentially be used to identify new biomarkers of HCC but could also pave the way to new therapeutic approaches in a lipid metabolism context.

■ FATTY ACIDS AND LIVER CANCER

Most of the cancer research focuses on the effect of glycolysis, glutaminolysis, and FA synthesis on cancer cell function. Less attention has been paid to the importance of lipid catabolism in HCC and other cancer types.⁴⁷ Lipid catabolism shares some common oncogenic functions with glycolysis as an important energy supplier supporting cell survival and growth, and providing the intermediate metabolites for biosynthesis processes in the fast-growing tumor.³⁹ The two main energy sources in the human body, glycogen and TG, are both synthesized in the liver. The very first to be consumed during short-time fasting is glycogen, and once it is depleted, cells can switch to TG, potentially generating 6 times as much ATP as glycogen and thus making it the preferred energy storage form.³⁹ The catabolism of fatty acids occurs through FAO, also known as β -oxidation, mostly in energy-demanding tissues such as the heart and skeletal muscles as well as in the liver (Figure 4). Mitochondrial FAO consists of a series of oxidation reactions that produce acetyl-CoA and especially energy in the form of ATP.⁴⁸

Highly proliferative tumor cells and especially poorly vascularized cells in solid tumors experience high levels of metabolic stress related to the lack of nutrients and oxygen and

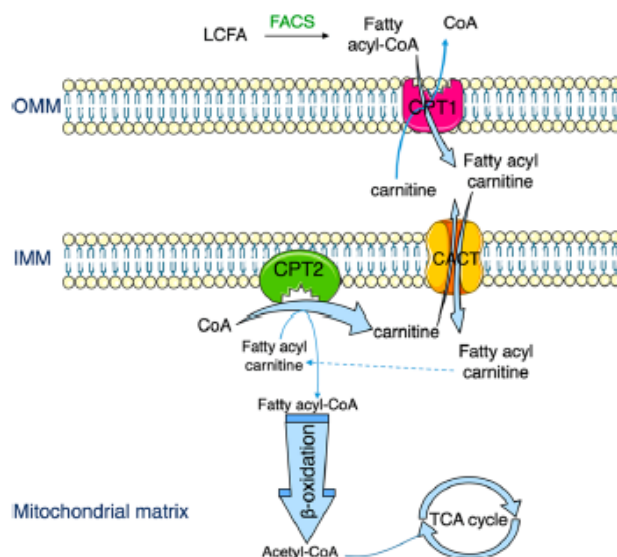


Figure 4. Fatty acid transport and oxidation. Long chain fatty acids (LCFAs) are transported from the bloodstream into the cells, where they are transformed into fatty acyl-CoA by the fatty acyl-CoA synthetase (FACS). Fatty acyl-CoA then enters the mitochondria only after being converted to fatty acyl carnitine by carnitine palmitoyltransferase 1 (CPT1) that is located in the outer mitochondrial membrane (OMM). Following that, FAs can cross the inner mitochondrial membrane (IMM) by means of the carnitine-acyl carnitine translocase (CACT), which facilitates the diffusion of one fatty acyl carnitine into the matrix in exchange for one carnitine molecule. Finally, in the matrix, carnitine palmitoyltransferase 2 (CPT2) regenerates fatty acyl-CoA from fatty acyl carnitine, and fatty acyl-CoA enters into a series of oxidation reactions to yield acetyl-CoA for OXPHOS.

may undergo apoptosis.³⁹ However, almost all cancer cells are resistant to programmed cell death through different metabolic and nonmetabolic processes. For instance, treatment-resistant HCC cells can counteract that effect by increasing the level of FAO, regardless of the availability of glucose or glycogen. Indeed, unlike sensitive cells, resistant cells can survive under starvation by activating lipid catabolism,⁴⁹ thus confirming the essential role of FAO in cell survival and tumor progression. This process is also involved during loss of attachment (LOA) when tumor cells lose contact with the nearby extracellular matrix. In anchorage-dependent cells, LOA causes anoikis, a unique mechanism by which cells undergo programmed cell death due to insufficient cell–matrix interactions within the microenvironment. However, metastatic tumor cells may escape from anoikis and invade different locations. Pharmacological inhibitors of FAO, initially developed to treat diabetes or cardiopathies, can induce cancer cell death *in vitro* as well as in mice models. For instance, HCC tumors and cell lines with aberrant activation of β -catenin do not exhibit the classical Warburg shift. Although these tumors are not glycolytic, they show an increase in their FAO activity that serves as an energy source during growth. Treating such cells with etomoxir, an irreversible inhibitor of the FAO rate-limiting enzyme CPT1, ceased the development of HCC due to FAO shutdown.⁵⁰

However, the development and use of etomoxir in clinical trials have been stopped due to its high toxicity to heart and skeletal muscles as well as increases in the levels of liver enzymes resulting in hepatotoxicity.⁵¹ Fortunately, a new reversible inhibitor that is highly selective for CPT1A (the liver

isoform) called ST1326 has been developed and was shown to possess antitumor effects in leukemia and lymphoma.^{52,53} Therefore, the use of this drug may be a relevant therapeutic approach for the treatment of HCC tumors.

LIPID METABOLISM IN RELATION TO INFLAMMATION

Altered lipid metabolism and local immunosuppression in the tumor microenvironment are other typical hallmarks of cancer. The main focus in this section is the crosstalk between these two features in the context of liver cancer. We have a particular interest in the metabolic signature and the role of specialized proresolving lipid mediators (SPM) in the resolution of inflammation and immunosuppression as related to tumor-associated macrophages (TAMs) and their immune modulation of the tumor microenvironment.

Control of Tumor-Associated Macrophage Polarization. Cancer cells recruit nearby cells to thrive. HCC emerges from chronic unresolved inflammation because its cells massively recruit TAMs as the main immunosuppressive cells in their microenvironment.⁵⁴ TAMs are known for their role in tumor development, angiogenesis, invasion, and metastasis. The shift in the role of TAMs is due to different metabolic changes in response to signals coming from the tumor itself or even from other immune cells. For this reason, two main isotypes of TAMs are distinguished: the classically activated M1 (antitumor-like macrophages) and the alternately activated M2 (protumor-like macrophages).⁵⁵ For instance, in HCC patients, high levels of M2 macrophages were detected in the intra- and peri-tumoral regions.⁵⁶ These levels were associated with an increased number of tumor nodules and venous infiltration, thus indicating poor prognosis in HCC patients. Moreover, M2 macrophages enhanced tumor growth and lung metastasis in an orthotopic nude mice model of liver cancer as compared to the control and M1 macrophage-injected mice, in which the latter significantly decreased the tumor volume. In addition, co-culturing the HCC cell line with M2 macrophages *in vitro* promoted cellular growth and migration. All of these effects were the result of a signaling pathway that involves a potent chemoattractant, the C-C motif chemokine 22 (CCL22) produced by M2 macrophages and its specific receptor, C-C chemokine receptor type 4 (CCR4), in HCC cells.⁵⁶

Cell metabolism is largely modulated by mTOR signaling, which is essential for many processes, including tumor development, and solid tumors are currently treated with rapamycin or its analogues. As depicted in Figure 5, two multiprotein complexes can be distinguished, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which function in regulating protein synthesis and cytoskeleton organization, respectively.^{57,58} These two complexes are activated through their cognate receptors, leading to the activation of class 1 phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinases (AKT 1/2/3), known as the PI3K/AKT/mTOR axis.^{59,60} mTORC1 is strongly linked to inflammation mediated by macrophages via regulating many inflammatory mediators such as NFκB (nuclear factor κB), IL-10 (interleukin 10), TGF-β (transforming growth factor β), and PD-L1 (programmed death ligand 1) expression.⁶¹ mTORC2 is also thought to play a great role in macrophage polarization [switch between M1 and M2 macrophages (Figure 6)] and inflammatory response regulation.⁶² For instance, the activation of mTORC1 sustains and promotes M2 activation of

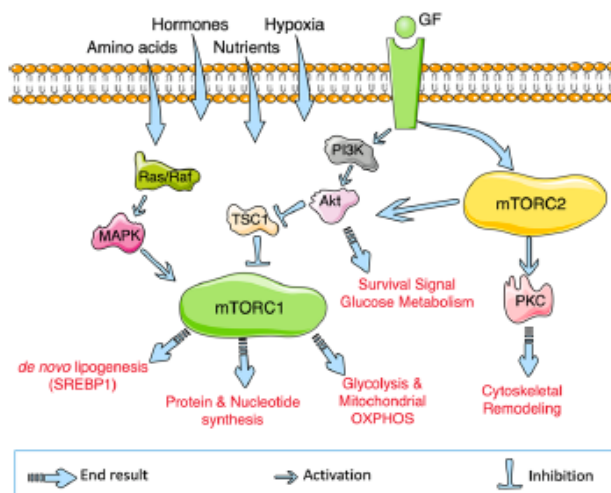


Figure 5. mTOR signaling. In response to multiple factors, mTOR complexes can be activated (mTORC1 in response to growth factors, nutrients, hormones, amino acids, and hypoxia and mTORC2 in response to growth factors).⁶⁶ For instance, mTORC1 is regulated by the PI3K/Akt/TSC1 and Ras/MAPK pathways. Once activated, it can promote the synthesis of proteins and nucleotides as well as regulate cell metabolism, especially by enhancing lipogenesis in a SREBP1-dependent manner, and stimulate glycolysis and mitochondrial oxidative metabolism. On the contrary, mTORC2 activation promotes cell survival, cytoskeletal reorganization, and glucose metabolism.

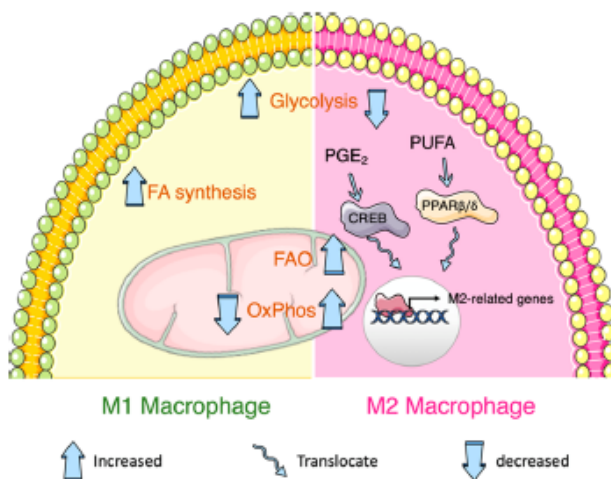


Figure 6. Summary of metabolic differences between M1 and M2 macrophages. The metabolic profiles of M1 and M2 polarized macrophages are completely opposite. A shift in the metabolism toward a high level of glycolysis and FA synthesis and a low level of OXPHOS is a feature of M1 macrophages (yellow side). However, a decrease in the level of glycolysis and an increase in the level of FAO and OXPHOS are characteristic of M2 macrophages (pink side).

macrophages in cases involving tuberous sclerosis complex 1 (TSC1) and AKT kinases.⁶³ TSC1 is a key modulator of macrophage polarization via mTOR-dependent and -independent pathways.^{64,65} However, in some cases in which hypoxia is induced in TAM, the deletion of an important inhibitor of mTORC1, named REDD1 for regulated in development and DNA damage responses 1, can switch macrophages to a new metabolic phenotype, increased

glycolysis, to inhibit the metastasis of the tumor.^{64,65} Therefore, the activation of the mTOR signaling pathway can have different effects, giving it a great role in regulating the differentiation of monocytes and thus polarization of TAMs.

Understanding how TAMs respond to mTOR signaling and thus polarize accordingly is still challenging. It is worth mentioning that M2-polarized macrophages enhance their metabolism in a direction that favors FAO to sustain the continuous energy demand^{67,68} unlike M1-like macrophages that promote the *de novo* synthesis of fatty acid⁶⁹ (Figure 6). For instance, the presence of polyunsaturated fatty acids (PUFAs) in the tumor microenvironment supports not only the survival and migration of cancer cells but also the polarization of TAM,^{55,70} where PUFAs function as potent agonists for peroxisome proliferator-activated receptor β/δ (PPAR β/δ). PPAR β/δ is a ligand-activated transcription factor (TF) that regulates the expression of genes involved in macrophage polarization toward the M2 phenotype⁷⁰ in the tumor microenvironment to further enhance tumor growth and development. Prostaglandin E₂ (PGE₂) also plays a role in M2-like polarization by binding to different cell factors to activate an important TF called cAMP response element binding protein (CREB) that mediates the transcription of genes involved in the M2 phenotype.⁷¹ Prostaglandin synthesis can be inhibited by targeting microsomal PGE₂ synthase 1 (mPGES1) and cyclooxygenase-2 (COX2), and as a result, M2 to M1 polarization of TAMs is promoted in different cancer models.^{72–74}

PGE₂ was also shown to increase the rate of growth of the HepG2 HCC cell line, and this effect was dependent on its selective EP1 receptor.⁷⁵ Conversely, inhibition of PGE₂ by (–)-epi-gallocatechin-3-gallate (EGCG) reduced the viability of HepG2 cells and hepatoma cells.⁷⁵ EGCG is the most abundant bioactive compound of green tea leaves known for its antioxidant and antitumor activities, although the underlying mechanisms are still not clearly understood. Clinical trials in HCC are needed for the identification of the optimal therapeutic dosage of EGCG and of more effective strategies for preventing EGCG-induced toxicity.⁷⁶ On the contrary, Kang et al.⁷⁷ have very recently reported that polarization of macrophages toward the M2-like phenotype is controlled by mTOR-Semaphorin 6D-PPAR γ reverse signaling through controlling fatty acid metabolism. Any defect in this signaling axis leads to impaired fatty acid uptake and metabolic reprogramming, and therefore dysregulation of polarization.

Finally, CG33, an antibody targeting the heparan sulfate proteoglycan glypican 3 (GPC3), is currently in clinical phase I trials for the treatment of advanced HCC.^{78,79} Indeed, in HCC membrane-associated GPC3 expression was shown to favor M2-polarized TAM recruitment.⁶⁸

Lipid Metabolism and Resolution of Inflammation. The second aspect of immunomodulation is related to the action of lipid mediators SPM, which make up a family of arachidonic acid- and ω -3 polyunsaturated fatty acid-derived lipid mediators generated via the actions of some enzymes such as the lipoxygenases (ALOX).⁸⁰ Their role is to counteract the action of prostaglandins and to limit the effects of inflammation under various pathogenic conditions, one of which is cancer. The synthesis of some SPM is triggered by the action of aspirin, e.g., aspirin-triggered lipoxins (AT-LX or epi-lipoxins) or AT-resolvins.

The mechanisms related to the potency of SPM to reduce the risk of cancer progression are starting to come into focus.⁸¹

We were among the first to show that the proresolution actions of SPMs are mediated by their potential to induce the phagocytic capabilities of macrophages.⁸⁰ In addition to modulating phagocytosis, these SPMs seem to alter the uptake of lipids by macrophages, and this probably modulates their immunosuppressive impact in the tumor microenvironment.⁸²

These properties are becoming more relevant in the context of cellular damage linked to cytotoxic treatment in cancer patients. In fact, therapy-generated cell debris could inadvertently stimulate the inflammation and growth of dormant tumor cells or small tumors. Debris-stimulated tumor cells were shown to be inhibited by some SPMs, namely, resolvin D1 (RvD1), RvD2, or RvE1, by enhancing the clearance of debris via macrophage phagocytosis in multiple tumor types.⁸¹ The results from this study highlight a novel approach for enhancing the endogenous clearance of tumor cell debris to prevent tumor growth and recurrence.

Moreover, the role of low-dose aspirin in the resolution of cancer was shown to be dependent on the resolving receptor, and treatment of mice carrying Lewis lung carcinoma (LLC) with AT-LXA4 and AT-RvD1 and -3 inhibited tumor growth by stimulating macrophage phagocytosis of the tumor cell debris generated by chemotherapy and by suppressing proinflammatory cytokine secretion by macrophages.⁸³ Interestingly, the doses of AT-SPM administered in this LLC mouse model were much smaller (>1000-fold) than the doses of aspirin necessary to trigger a similar *in vivo* response. This is why these AT-SPMs are a more powerful chemopreventive or antitumor treatment than aspirin, with potentially less risk, but this remains to be evaluated in humans.

In conclusion, using specialized proresolving lipid mediators to enhance endogenous clearance of tumor cell debris by macrophages may be a beneficial approach to complement cytotoxic cancer therapies. Also, altering lipid metabolism in TAMs may be a promising target in cancer therapy. Modulating specific metabolic enzymes to allow the switch from M2 to M1 macrophages may be a more adapted approach for obtaining antitumoral macrophages instead of eradicating these cells altogether. Thus, weakening the immunosuppressive ability of macrophages and enhancing their antitumoral activity led to a better response to chemotherapy or even immunotherapy.⁵⁵

■ NEW TREATMENT STRATEGIES TARGETING LIPID METABOLISM FOR HCC TREATMENT

Lipid metabolism is now considered as a major metabolic pathway involved in the progression of cancer. The production of lipids is necessary not only for DNA and proteins but also for promoting cancer cell growth and proliferation. Lipids are known for their important structural role as they are the building blocks for membranes. They also function as signaling molecules in cancer and can be used for post-translational modifications of proteins.⁸⁴ For these reasons, targeting lipid metabolism is considered a promising approach when seeking new therapeutic ways to cure cancer.

Several studies have focused on the inhibition of enzymes of the FA biosynthesis pathway in an attempt to block cancer cell growth, and they have been reviewed in refs 85–87 and are summarized in Table 1. Some are already on clinical trials. Among them, FASN, ACC, and the stearyl-CoA desaturase (SCD) have been pharmacologically targeted to inhibit lipid synthesis. For instance, FASN inhibitors (C75 and C247 or C93 and C95) used in mouse models exhibited chemo-

preventive effects in breast cancer⁸⁸ and reduced the level of induction of lung tumorigenesis.⁸⁹ Moreover, targeting ACC and SCD was efficient in limiting the growth and proliferation of cancer cells.^{24,90} ACLY catalyzes the production of acetyl-CoA from citrate during FA synthesis, and its inhibition by SB-204990 has decreased the rates of hepatic cholesterol and FA syntheses.⁹¹ ACLY also plays a key role in the regulation of histone acetylation, thereby linking lipid metabolism to the regulation of gene expression.⁹²

It has been known for a long time that cholesterol accumulates in cancerous tissues, in addition to an increased level of FA biosynthesis. Cholesterol, like TGs, is an essential lipid for cell viability, not only because it is a component of cell membrane fluidity but also because it is a precursor of many hormones (estrogens, androgens, progesterone, and vitamins) and steroids. It is supplied by exogenous diet and cellular synthetic pathways. Hypercholesterolemia is usually treated by inhibiting the rate-limiting step of the cholesterol biosynthesis catalyzed by the HMGCR with statins. In recent studies, rosuvastatin was proven to prevent the development of HCC associated with NAFLD in mice and improves the hepatic background and thus can be used as a preventive drug for HCC development.⁹⁵ Also, fluvastatin, together with sorafenib (a multikinase inhibitor), had a synergistic antitumor efficacy in HCC that resulted in the inhibition of cancer cell proliferation and the promotion of apoptosis.⁹⁶

There is more promising evidence that statins could decrease the risk of HCC. This is discussed in more detail in the review by Alipour et al.¹⁰¹ For instance, many observational studies performed in high-risk areas such as Taiwan^{102–105} show that the use of statins reduces the risk of HCC. El-Serag HB et al.,¹⁰⁶ who observed a cohort of patients with diabetes, also concluded that the use of statins significantly reduced the risk of HCC. The role of statins in reducing HCC risk was confirmed when evaluating patients in the United States, which is a low-risk HCC area.¹⁰⁷ Another study recorded very promising results in a randomized controlled trial of pravastatin in patients having advanced HCC.¹⁰⁸ The significant prolonged survival of these patients observed upon treatment with 40 mg of pravastatin per day compared to the nontreated ones (median survival of 18 months in the pravastatin group compared to 9 months in the control group; $P = 0.006$), suggests a promising value as an auxiliary treatment.

It is important to mention that inhibition of cholesterol synthesis by statins can lead to a restorative feedback loop mediated by sterol regulatory element binding protein 2 (SREBP2), making the anticancer effects of these drugs less effective. Indeed, SREBP is a TF and master regulator of lipid biogenesis.¹⁰⁹ It regulates the rate-limiting enzyme for cholesterol synthesis, HMGCR, and also the low-density lipoprotein receptor (LDLR) leading to cellular uptake of LDL and the depletion of serum cholesterol levels.¹¹⁰ Consequently, targeting SREBP2 was shown to effectively suppress the feedback activation loop triggered by statin treatment, widening the therapeutic window to include tumors otherwise unresponsive to statins.¹¹¹

Moreover, any atypical activation can lead to fatty liver disease and possibly cancer development.¹¹² During angiogenesis, lipid metabolism is further enhanced and hence SREBP is activated by endothelial cells in response to vascular endothelial growth factor (VEGF).¹¹³ However, treatment with a potent inhibitor of SREBP, 25-hydroxycholesterol (25-

HC), prevented the migration and proliferation induced by VEGF in these endothelial cells.¹¹³ Therefore, SREBP2 seems to be an attractive and novel target that can be used in combination therapies with statins.

Furthermore, the epigenetic inhibitor MI-2 exhibits anti-glioma activity,¹¹⁴ which could extend to tumors not harboring the H3K27M mutation of histone 3. Indeed, if MI-2 was originally described as inhibiting menin, an essential component of the histone methyltransferase complex, it was recently suggested that lanosterol synthase (LSS) would be the real target of MI-2 in the case of isolated diffuse intrinsic pontine glioma (DIPG) cell treatment.¹¹⁵ LSS catalyzes the complex conversion of (S)-2,3-oxidosqualene in lanosterol, the first steroidal intermediate of cholesterol biosynthesis. LSS can also catalyze the shunt pathway from (S)-2,3-oxidosqualene to 24(S),25-epoxycholesterol (24,25-EC), a liver X receptor (LXR) agonist. MI-2 induces 24,25-EC accumulation and upregulation of the LXR target, to the detriment of cholesterol synthesis, similarly to RO-48-8071, a known LSS inhibitor.¹¹⁶ The final outcome is cell death, and addition of cholesterol reverses the cell toxicity induced by RO-48-8071, thus confirming the role of cholesterol blocking in limiting cancer progression. LSS certainly deserves more attention in the context of drug research for the treatment of HCC.

Targeting lipid metabolism is a promising therapeutic approach that needs to be extensively studied and explored. Because of modern technology, different inhibitors for different pathways were brought to light and are being studied in preclinical and clinical trials. Some of them are already approved and being used for the treatment of diseases such as hyperlipidemia, diabetes, and other metabolic syndromes. However, more effort is needed to fully understand the behavior of cancer cells and the way in which they escape the various cell defense mechanisms.

■ ASPIRIN, LIVER CANCER CELL LINES, AND HCC

In addition to triggering the production of some specialized immunomodulatory SPMs, aspirin has been connected to cancer in different perspectives. A chemopreventive role has been attributed to aspirin initially in colorectal cancer (CRC),¹¹⁷ making it one of the promising anticancer drugs. This is supported by several studies, whether clinical or epidemiological, that were performed over the past several decades.^{118–120} Very recently, Yang et al.¹²¹ reported that aspirin inhibited cancer growth by targeting an important TF, NF κ B. Aspirin could suppress abnormal lipid metabolism in HepG2 and Huh7 liver cancer cell lines in a dose-dependent manner. This drug clearly acted by inhibiting an essential enzyme in lipid metabolism, acyl-CoA synthetase long chain family member 1 (ACSL1), at both mRNA and protein levels. Therefore, the potential role that could be played by aspirin in HCC would be through suppressing abnormal lipid metabolism by abrogating the NF κ B/ACSL1 signaling pathway.

Moreover, aspirin when combined with sorafenib for the treatment of sorafenib-resistant HCC cells (HCC-LM3 and Huh7-R) increased the level of cellular inhibition and apoptosis of these resistant cells *in vitro* and *in vivo*¹²² without having side effects such as weight loss, hepatotoxicity, or inflammation. Hence, the conclusion of this study is that aspirin can overcome the resistance to sorafenib and the combination of sorafenib and aspirin can be a promising therapeutic option for HCC. A similar effect has been observed in Navitodax-resistant HCC cell lines.¹²³ Navitodax is an

inhibitor of the antiapoptotic proteins Bcl-2 and Bcl-xL (B-cell leukemia/lymphoma-2 and -extra large, respectively) with strong antitumor activities in different cancers, but with a weak binding affinity for Mcl-1 (induced myeloid leukemia cell differentiation protein 1), a member of the Bcl-2 family, which is overexpressed in HCC.^{124,125} Aspirin treatment suppressed Mcl-1 expression and enhanced the inhibition of cell viability with induction of apoptosis in the HCC cell lines HepG2 and BEL-7402. Furthermore, other *in vitro* and *in vivo* studies ensured the role of aspirin in overcoming treatment resistances and enhancing the effect of IFN- α in the treatment of HCC through activating the STAT1 gene that is known to exert its antiproliferative and proapoptotic effect by mediating the tumor cell response to IFN- α .¹²⁶

Some of the HCC tumors are unresectable, and the only available method of treating such tumors is transarterial chemoembolization (TACE).¹²⁷ The response to this treatment differs among patients; for example, patients in the intermediate stage of the disease show better median survival (16–20 months) than those in the advanced stages of HCC (8 months).¹²⁸ However, when this treatment is repeated several times, its efficacy is diminished and the patients enter the state of TACE failure/refractoriness.¹²⁹ Therefore, in this case, multiple nodules will be scattered in the liver, and the nontumoral part of the liver will be damaged because of the multiple uses of TACE, leading in the end to a decrease in the survival time of the patients.¹³⁰ Because of all of these limitations associated with TACE, an alternative is urgently needed, probably combination therapies, to decrease the risks of failure and improve the survival outcome of patients in such treatments. Li et al.¹³¹ were the first to evaluate the combined effects of aspirin and TACE therapy in treating patients suffering from HCC. They observed that the auxiliary aspirin treatment with TACE in cases of unresectable HCC could improve the overall survival of such patients without having side effects like fatal bleeding.

CONCLUSION

Enhanced lipogenesis in highly proliferative tumor cells provides the building block materials, signaling molecules, and bioactive mediators to ensure their growth, proliferation, and persistence. The progression of HCC is associated with complex metabolic reprogramming, as well as immunosuppressive drive provided by TAMs and mediated by bioactive lipid mediators.

By dissecting the diverse lipid metabolic network and highlighting many of its cancer-associated signatures, we have discussed how the lipidome can be malleable to interfere with oncogenesis.

Several established drugs targeting lipid metabolism are showing promising anticancer properties and may emerge as highly valuable choices for new oncology indications. Other drugs could emerge to be used in a drug combination approach with the aim of either counteracting the adaptation of cancer cells to metabolic reprogramming or improving the effectiveness of current immunotherapeutic treatments. Finally, the bioactive lipids appear to hold great promise in our understanding of the involvement of lipids in immunosuppression and oncogenesis and can ultimately be considered as a new treatment modality that deserves to be assessed in cancer.

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ABBREVIATIONS

24,25-EC, 24(S),25-epoxycholesterol; ACC, acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; ACSL1, acyl-CoA synthetase long chain family member 1; ACSS1/2, acetyl-CoA synthetase short chain family member 1/2; AKT 1/2/3, protein kinase B 1/2/3; AT-LX, aspirin-triggered lipoxins; Bcl-2, B cell leukemia/lymphoma 2; Bcl-xL, B cell lymphoma-extra large; C16:0, palmitic acid; CCL22, C–C motif chemokine 22; CCR4, C–C chemokine receptor type 4; ChoEs, cholesterol esters; COX2, cyclooxygenase-2; CPT1, carnitine palmitoyltransferase 1; CPT1A, -B, or -C, carnitine palmitoyltransferase 1 isoform A, B, or C, respectively; CREB, cAMP response element binding protein; DGAT, diglyceride acyltransferase; DIPG, diffuse intrinsic pontine glioma; EGCG, (–)-epigallocatechin-3-gallate; ERKs, extracellular signal-regulated kinases; FA, fatty acid; FAO, fatty acid β -oxidation; FAS, tumor necrosis factor receptor superfamily member 6; FASN, fatty acid synthase; FOXP3, forkhead box P3; GPC3, glypican 3; HCC, hepatocellular carcinoma; HMG-CoA, β -hydroxy β -methylglutaryl-coenzyme A; HMGCR, HMG-CoA reductase; IL-10, interleukine 10; LOA, loss of attachment; LPC, lysophosphatidylcholine; LSS, lanosterol synthase; LXR, liver X receptor; M1 or M2, M1 or M2 type macrophage, respectively; Mcl-1, induced myeloid leukemia cell differentiation protein 1; mPGES1, microsomal PGE₂ synthase 1; mTOR, mammalian target of rapamycin; mTORC1 or -2, mTOR complex 1 or 2, respectively; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF κ B,

nuclear factor κ B; OXPHOS, mitochondrial oxidative phosphorylation; PC, phosphatidylcholine; PD-L1, programmed death ligand 1; PE, phosphatidylethanolamine; PGE₂, prostaglandin E₂; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PML, promyelocytic leukemia protein; PPAR β/δ , peroxisome proliferator-activated receptor β/δ ; PPP, pentose phosphate pathway; PUFA, polyunsaturated fatty acid; REDD1, regulated in development and DNA damage responses; ROS, reactive oxygen species; SCD, stearoyl-CoA desaturase; SM, sphingomyelin; SPM, specialized proresolving mediator; SREBP, sterol regulatory element binding protein; TAM, tumor-associated macrophage; TF, transcription factor; TG, triglyceride or triacylglycerol; TGF- β , transforming growth factor β ; Treg, regulatory immunosuppressive T cell; TSC1, tuberous sclerosis complex 1; TZD, thiazolidinedione; VEGF, vascular endothelial growth factor; VLDL, very low density lipoprotein.

REFERENCES

- (1) Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of Cancer: The Next Generation. *Cell* 144, 646–674.
- (2) International Agency for Research on Cancer (2014) World cancer report 2014.
- (3) de Martel, C., Maucourt-Boulch, D., Plummer, M., and Franceschi, S. (2015) World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma: HEPATOLOGY, Vol. XX, No. X. *Hepatology* 62, 1190–1200.
- (4) El-Serag, H. B. (2012) Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterology* 142, 1264–1273.e1.
- (5) Baffy, G., Brunt, E. M., and Caldwell, S. H. (2012) Hepatocellular carcinoma in non-alcoholic fatty liver disease: An emerging menace. *J. Hepatol.* 56, 1384–1391.
- (6) Beyoğlu, D., and Idle, J. R. (2013) The metabolomic window into hepatobiliary disease. *J. Hepatol.* 59, 842–858.
- (7) Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009) Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* 324, 1029–1033.
- (8) DeBerardinis, R. J., and Chandel, N. S. (2020) We need to talk about the Warburg effect. *Nature Metabolism* 2, 127–129.
- (9) Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J. M., Madeo, F., and Kroemer, G. (2015) Acetyl coenzyme A: a central metabolite and second messenger. *Cell Metab.* 21, 805–821.
- (10) Shi, L., and Tu, B. P. (2015) Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. *Curr. Opin. Cell Biol.* 33, 125–131.
- (11) Ma, Y., Temkin, S. M., Hawkrige, A. M., Guo, C., Wang, W., Wang, X.-Y., and Fang, X. (2018) Fatty acid oxidation: An emerging facet of metabolic transformation in cancer. *Cancer Lett.* 435, 92–100.
- (12) Kim, J.-A., and Yeom, Y. I. (2018) Metabolic Signaling to Epigenetic Alterations in Cancer. *Biomol. Ther.* 26, 69–80.
- (13) Liu, D., Wong, C. C., Fu, L., Chen, H., Zhao, L., Li, C., Zhou, Y., Zhang, Y., Xu, W., Yang, Y., Wu, B., Cheng, G., Lai, P. B.-S., Wong, N., Sung, J. J. Y., and Yu, J. (2018) Squalene epoxidase drives NAFLD-induced hepatocellular carcinoma and is a pharmaceutical target. *Sci. Transl. Med.* 10, No. eaap9840.
- (14) McGarry, J. D., and Foster, D. W. (1980) Regulation of Hepatic Fatty Acid Oxidation and Ketone Body Production. *Annu. Rev. Biochem.* 49, 395–420.
- (15) Catalina-Rodríguez, O., Kolukula, V. K., Tomita, Y., Preet, A., Palmieri, F., Wellstein, A., Byers, S., Giaccia, A. J., Glasgow, E., Albanese, C., and Avantiaggiati, M. L. (2012) The mitochondrial citrate transporter, CIC, is essential for mitochondrial homeostasis. *Oncotarget* 3, 1220–1235.
- (16) Yun, M., Bang, S.-H., Kim, J. W., Park, J. Y., Kim, K. S., and Lee, J. D. (2009) The importance of acetyl coenzyme A synthetase for 11C-acetate uptake and cell survival in hepatocellular carcinoma. *J. Nucl. Med.* 50, 1222–1228.
- (17) Wen, H., Lee, S., Zhu, W.-G., Lee, O.-J., Yun, S. J., Kim, J., and Park, S. (2019) Glucose-derived acetate and ACS2 as key players in cisplatin resistance in bladder cancer. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1864, 413–421.
- (18) Brownsey, R. W., Boone, A. N., Elliott, J. E., Kulpa, J. E., and Lee, W. M. (2006) Regulation of acetyl-CoA carboxylase. *Biochem. Soc. Trans.* 34, 223–227.
- (19) Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R. B., Newgard, C. B., Lopaschuk, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab.* 7, 45–56.
- (20) Bhalla, K., Hwang, B. J., Dewi, R. E., Twaddel, W., Goloubeva, O. G., Wong, K.-K., Saxena, N. K., Biswal, S., and Girnun, G. D. (2012) Metformin Prevents Liver Tumorigenesis by Inhibiting Pathways Driving Hepatic Lipogenesis. *Cancer Prev. Res.* 5, 544–552.
- (21) Zakikhani, M., Dowling, R., Fantus, I. G., Sonenberg, N., and Pollak, M. (2006) Metformin Is an AMP Kinase-Dependent Growth Inhibitor for Breast Cancer Cells. *Cancer Res.* 66, 10269–10273.
- (22) Memmott, R. M., Mercado, J. R., Maier, C. R., Kawabata, S., Fox, S. D., and Dennis, P. A. (2010) Metformin Prevents Tobacco Carcinogen-Induced Lung Tumorigenesis. *Cancer Prev. Res.* 3, 1066–1076.
- (23) Gerth, K., Bedorf, N., Irschik, H., Höfle, G., and Reichenbach, H. (1994) The soraphens: a family of novel antifungal compounds from *Sorangium cellulosum* (Myxobacteria). I. Soraphen A1 alpha: fermentation, isolation, biological properties. *J. Antibiot.* 47, 23–31.
- (24) Beckers, A., Organe, S., Timmermans, L., Scheys, K., Peeters, A., Brusselmans, K., Verhoeven, G., and Swinnen, J. V. (2007) Chemical Inhibition of Acetyl-CoA Carboxylase Induces Growth Arrest and Cytotoxicity Selectively in Cancer Cells. *Cancer Res.* 67, 8180–8187.
- (25) Rysman, E., Brusselmans, K., Scheys, K., Timmermans, L., Derua, R., Munck, S., Van Veldhoven, P. P., Waltregny, D., Daniëls, V. W., Machiels, J., Vanderhoydonc, F., Smans, K., Waelkens, E., Verhoeven, G., and Swinnen, J. V. (2010) De novo Lipogenesis Protects Cancer Cells from Free Radicals and Chemotherapeutics by Promoting Membrane Lipid Saturation. *Cancer Res.* 70, 8117–8126.
- (26) Stoiber, K., Naglo, O., Perpeintner, C., Zhang, S., Koeberle, A., Ulrich, M., Werz, O., Müller, R., Zahler, S., Lohmüller, T., Feldmann, J., and Braig, S. (2018) Targeting de novo lipogenesis as a novel approach in anti-cancer therapy. *Br. J. Cancer* 118, 43–51.
- (27) Guseva, N. V., Rokhlin, O. W., Glover, R. A., and Cohen, M. B. (2011) TOFA (5-tetradecyl-oxy-2-furoic acid) reduces fatty acid synthesis, inhibits expression of AR, neuropilin-1 and Mcl-1 and kills prostate cancer cells independent of p53 status. *Cancer Biol. Ther.* 12, 80–85.
- (28) Wang, C., Xu, C., Sun, M., Luo, D., Liao, D.-F., and Cao, D. (2009) Acetyl-CoA carboxylase- α inhibitor TOFA induces human cancer cell apoptosis. *Biochem. Biophys. Res. Commun.* 385, 302–306.
- (29) Li, S., Qiu, L., Wu, B., Shen, H., Zhu, J., Zhou, L., Gu, L., and Di, W. (2013) TOFA suppresses ovarian cancer cell growth in vitro and in vivo. *Mol. Med. Rep.* 8, 373–378.
- (30) Wada, A., Fukui, K., Sawai, Y., Imanaka, K., Kiso, S., Tamura, S., Shimomura, I., and Hayashi, N. (2006) Pamidronate induced anti-proliferative, apoptotic, and anti-migratory effects in hepatocellular carcinoma. *J. Hepatol.* 44, 142–150.
- (31) Kogure, T., Ueno, Y., Kimura, O., Kondo, Y., Inoue, J., Fukushima, K., Iwasaki, T., and Shimosegawa, T. (2009) A novel third generation bisphosphonate, minodronate (YM529), prevented proliferation and migration of hepatocellular carcinoma cells through inhibition of mevalonate pathway. *Hepatol. Res.* 39, 479–489.
- (32) Ilyas, A., Hashim, Z., Naeem, N., Haneef, K., and Zarina, S. (2014) The effect of alendronate on proteome of hepatocellular carcinoma cell lines. *Int. J. Proteomics* 2014, 532953.
- (33) Liu, Q., Tao, Y., Bai, R., Chang, S., and Hua, D. (2013) Zoledronic acid inhibits growth of hepatocellular carcinoma cells in vitro and in vivo. *Chin. Med. J.* 126, 1486–1490.

- (34) Honda, Y., Takahashi, S., Zhang, Y., Ono, A., Murakami, E., Shi, N., Kawaoka, T., Miki, D., Tsuge, M., Hiraga, N., Abe, H., Ochi, H., Imamura, M., Aikata, H., and Chayama, K. (2015) Effects of bisphosphonate zoledronic acid in hepatocellular carcinoma, depending on mevalonate pathway. *J. Gastroenterol. Hepatol.* 30, 619–627.
- (35) Zhang, W., Zhu, X.-D., Sun, H.-C., Xiong, Y.-Q., Zhuang, P.-Y., Xu, H.-X., Kong, L.-Q., Wang, L., Wu, W.-Z., and Tang, Z.-Y. (2010) Depletion of tumor-associated macrophages enhances the effect of sorafenib in metastatic liver cancer models by antimetastatic and antiangiogenic effects. *Clin. Cancer Res.* 16, 3420–3430.
- (36) Shidoji, Y., and Tabata, Y. (2019) Unequivocal evidence for endogenous geranylgeranoic acid biosynthesized from mevalonate in mammalian cells. *J. Lipid Res.* 60, 579–593.
- (37) Lanterna, C., Musumeci, A., Raccosta, L., Corna, G., Moresco, M., Maggioni, D., Fontana, R., Doglioni, C., Bordignon, C., Traversari, C., and Russo, V. (2016) The administration of drugs inhibiting cholesterol/oxysterol synthesis is safe and increases the efficacy of immunotherapeutic regimens in tumor-bearing mice. *Cancer Immunol. Immunother.* 65, 1303–1315.
- (38) Wang, Y., Viscarra, J., Kim, S.-J., and Sul, H. S. (2015) Transcriptional regulation of hepatic lipogenesis. *Nat. Rev. Mol. Cell Biol.* 16, 678–689.
- (39) Lu, G.-D., and Hooi, S. C. (2017) Lipid Metabolism in Liver Cancer. *Updates in Liver Cancer*, 49–67.
- (40) Beyoglu, D., Imbeaud, S., Maurhofer, O., Bioulac-Sage, P., Zucman-Rossi, J., Dufour, J.-F., and Idle, J. R. (2013) Tissue metabolomics of hepatocellular carcinoma: Tumor energy metabolism and the role of transcriptomic classification. *Hepatology* 58, 229–238.
- (41) Budhu, A., Roessler, S., Zhao, X., Yu, Z., Forgues, M., Ji, J., Karoly, E., Qin, L.-X., Ye, Q.-H., Jia, H.-L., Fan, J., Sun, H.-C., Tang, Z.-Y., and Wang, X. W. (2013) Integrated Metabolite and Gene Expression Profiles Identify Lipid Biomarkers Associated With Progression of Hepatocellular Carcinoma and Patient Outcomes. *Gastroenterology* 144, 1066–1075.e1.
- (42) Chen, T., Xie, G., Wang, X., Fan, J., Qui, Y., Zheng, X., Qi, X., Cao, Y., Su, M., Wang, X., Xu, L. X., Yen, Y., Liu, P., and Jia, W. (2011) Serum and Urine Metabolite Profiling Reveals Potential Biomarkers of Human Hepatocellular Carcinoma. *Mol. Cell. Proteomics* 10, A110.004945.
- (43) Zeng, J., Yin, P., Tan, Y., Dong, L., Hu, C., Huang, Q., Lu, X., Wang, H., and Xu, G. (2014) Metabolomics Study of Hepatocellular Carcinoma: Discovery and Validation of Serum Potential Biomarkers by Using Capillary Electrophoresis–Mass Spectrometry. *J. Proteome Res.* 13, 3420–3431.
- (44) Chen, S., Yin, P., Zhao, X., Xing, W., Hu, C., Zhou, L., and Xu, G. (2013) Serum lipid profiling of patients with chronic hepatitis B, cirrhosis, and hepatocellular carcinoma by ultra fast LC/IT-TOF MS. *Electrophoresis* 34, 2848–2856.
- (45) Li, Z., Guan, M., Lin, Y., Cui, X., Zhang, Y., Zhao, Z., and Zhu, J. (2017) Aberrant Lipid Metabolism in Hepatocellular Carcinoma Revealed by Liver Lipidomics. *Int. J. Mol. Sci.* 18, 2550.
- (46) Lin, L., Ding, Y., Wang, Y., Wang, Z., Yin, X., Yan, G., Zhang, L., Yang, P., and Shen, H. (2017) Functional lipidomics: Palmitic acid impairs hepatocellular carcinoma development by modulating membrane fluidity and glucose metabolism. *Hepatology* 66, 432–448.
- (47) Carracedo, A., Cantley, L. C., and Pandolfi, P. P. (2013) Cancer metabolism: fatty acid oxidation in the limelight. *Nat. Rev. Cancer* 13, 227–232.
- (48) Ma, Y., Temkin, S. M., Hawkrigde, A. M., Guo, C., Wang, W., Wang, X.-Y., and Fang, X. (2018) Fatty acid oxidation: An emerging facet of metabolic transformation in cancer. *Cancer Lett.* 435, 92–100.
- (49) Lu, G.-D., Ang, Y. H., Zhou, J., Tamilarasi, J., Yan, B., Lim, Y. C., Srivastava, S., Salto-Tellez, M., Hui, K. M., Shen, H.-M., Nguyen, L. N., Tan, B. C., Silver, D. L., and Hooi, S. C. (2015) CCAAT/enhancer binding protein α predicts poorer prognosis and prevents energy starvation-induced cell death in hepatocellular carcinoma. *Hepatology* 61, 965–978.
- (50) Senni, N., Savall, M., Cabrerizo Granados, D., Alves-Guerra, M.-C., Sartor, C., Lagoutte, I., Gougelet, A., Terris, B., Gilgenkrantz, H., Perret, C., Colnot, S., and Bossard, P. (2019) β -catenin-activated hepatocellular carcinomas are addicted to fatty acids. *Gut* 68, 322–334.
- (51) Holubarsch, C. J. F., Rohrbach, M., Karrasch, M., Boehm, E., Polonski, L., Ponikowski, P., and Rhein, S. (2007) A double-blind randomized multicentre clinical trial to evaluate the efficacy and safety of two doses of etomoxir in comparison with placebo in patients with moderate congestive heart failure: the ERGO (etomoxir for the recovery of glucose oxidation) study. *Clin. Sci.* 113, 205–212.
- (52) Ricciardi, M. R., Mirabilli, S., Allegretti, M., Licchetta, R., Calarco, A., Torrisi, M. R., Foà, R., Nicolai, R., Peluso, G., and Tafuri, A. (2015) Targeting the leukemia cell metabolism by the CPT1a inhibition: functional preclinical effects in leukemias. *Blood* 126, 1925–1929.
- (53) Pacilli, A., Calienni, M., Margarucci, S., D'Apolito, M., Petillo, O., Rocchi, L., Pasquinelli, G., Nicolai, R., Koverech, A., Calvani, M., Peluso, G., and Montanaro, L. (2013) Carnitine-Acyltransferase System Inhibition, Cancer Cell Death, and Prevention of Myc-Induced Lymphomagenesis. *J. Natl. Cancer Inst.* 105, 489–498.
- (54) Lewis, M., and Merched, A. (2014) Tumor-Associated Macrophages, Inflammation and Pathogenesis of Hepatocellular Carcinoma. *J. Mol. Genet. Med.* 08, 08.
- (55) Mazzone, M., Menga, A., and Castegna, A. (2018) Metabolism and TAM functions—it takes two to tango. *FEBS J.* 285, 700–716.
- (56) Yeung, O. W. H., Lo, C.-M., Ling, C.-C., Qi, X., Geng, W., Li, C.-X., Ng, K. T. P., Forbes, S. J., Guan, X.-Y., Poon, R. T. P., Fan, S.-T., and Man, K. (2015) Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *J. Hepatol.* 62, 607–616.
- (57) Zarogoulidis, P., Lampaki, S., Turner, J. F., Huang, H., Kakolyris, S., Syrigos, K., and Zarogoulidis, K. (2014) mTOR pathway: A current, up-to-date mini-review (Review). *Oncol. Lett.* 8, 2367–2370.
- (58) Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., Vander Heiden, M. G., MacKeigan, J. P., Finan, P. M., Clish, C. B., Murphy, L. O., and Manning, B. D. (2010) Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell* 39, 171–183.
- (59) Luo, L., Wall, A. A., Yeo, J. C., Condon, N. D., Norwood, S. J., Schoenwaelder, S., Chen, K. W., Jackson, S., Jenkins, B. J., Hartland, E. L., Schroder, K., Collins, B. M., Sweet, M. J., and Stow, J. L. (2014) Rab8a interacts directly with PI3K γ to modulate TLR4-driven PI3K and mTOR signalling. *Nat. Commun.* 5, 4407.
- (60) Shimobayashi, M., and Hall, M. N. (2014) Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat. Rev. Mol. Cell Biol.* 15, 155–162.
- (61) Katholnig, K., Linke, M., Pham, H., Hengstschläger, M., and Weichhart, T. (2013) Immune responses of macrophages and dendritic cells regulated by mTOR signaling. *Biochem. Soc. Trans.* 41, 927–933.
- (62) Huang, S. C.-C., Smith, A. M., Everts, B., Colonna, M., Pearce, E. L., Schilling, J. D., and Pearce, E. J. (2016) Metabolic Reprogramming Mediated by the mTORC2-IRF4 Signaling Axis Is Essential for Macrophage Alternative Activation. *Immunity* 45, 817–830.
- (63) Arranz, A., Doxaki, C., Vergadi, E., Martinez de la Torre, Y., Vaporidi, K., Lagoudaki, E. D., Ieronymaki, E., Androulidaki, A., Venihaki, M., Margioris, A. N., Stathopoulos, E. N., Tschlis, P. N., and Tsatsanis, C. (2012) Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. *Proc. Natl. Acad. Sci. U. S. A.* 109, 9517–9522.
- (64) Covarrubias, A. J., Aksoylar, H. I., and Hornig, T. (2015) Control of macrophage metabolism and activation by mTOR and Akt signaling. *Semin. Immunol.* 27, 286–296.
- (65) Zhu, L., Yang, T., Li, L., Sun, L., Hou, Y., Hu, X., Zhang, L., Tian, H., Zhao, Q., Peng, J., Zhang, H., Wang, R., Yang, Z., Zhang, L., and Zhao, Y. (2014) TSC1 controls macrophage polarization to prevent inflammatory disease. *Nat. Commun.* 5, 4696.

- (66) Mao, Z., and Zhang, W. (2018) Role of mTOR in Glucose and Lipid Metabolism. *Int. J. Mol. Sci.* 19, 2043–2054.
- (67) O'Neill, L. A. J., and Hardie, D. G. (2013) Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* 493, 346–355.
- (68) Huang, S. C.-C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A. M., Beatty, W., Love-Gregory, L., Lam, W. Y., O'Neill, C. M., Yan, C., Du, H., Abumrad, N. A., Urban, J. F., Artyomov, M. N., Pearce, E. L., and Pearce, E. J. (2014) Cell-intrinsic lysosomal lipolysis is essential for macrophage alternative activation. *Nat. Immunol.* 15, 846–855.
- (69) Kelly, B., and O'Neill, L. A. (2015) Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* 25, 771–784.
- (70) Schumann, T., Adhikary, T., Wortmann, A., Finkemagel, F., Lieber, S., Schnitzer, E., Legrand, N., Schober, Y., Nockher, W. A., Toth, P. M., Diederich, W. E., Nist, A., Stiewe, T., Wagner, U., Reinartz, S., Müller-Brüsselbach, S., and Müller, R. (2015) Deregulation of PPAR β/δ target genes in tumor-associated macrophages by fatty acid ligands in the ovarian cancer microenvironment. *Oncotarget* 6, 13416–13433.
- (71) Barminko, J. A., Nativ, N. L., Schloss, R., and Yarmush, M. L. (2014) Fractional Factorial Design to Investigate Stromal Cell Regulation of Macrophage Plasticity. *Biotechnol. Bioeng.* 111, 2239–2251.
- (72) Heusinkveld, M., de Vos van Steenwijk, P. J., van Goedemans, R., Ramwadhoebe, T. H., Gorter, A., Welters, M. J. P., van Hall, T., and van der Burg, S. H. (2011) M2 Macrophages Induced by Prostaglandin E2 and IL-6 from Cervical Carcinoma Are Switched to Activated M1 Macrophages by CD4⁺ Th1 Cells. *J. Immunol.* 187, 1157–1165.
- (73) Prima, V., Kaliberova, L. N., Kaliberov, S., Curiel, D. T., and Kusmartsev, S. (2017) COX2/mPGES1/PGE2 pathway regulates PD-L1 expression in tumor-associated macrophages and myeloid-derived suppressor cells. *Proc. Natl. Acad. Sci. U. S. A.* 114, 1117–1122.
- (74) Nakanishi, Y., Nakatsuji, M., Seno, H., Ishizu, S., Akitake-Kawano, R., Kanda, K., Ueo, T., Komekado, H., Kawada, M., Minami, M., and Chiba, T. (2011) COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in Apc Min/+ mouse polyps. *Carcinogenesis* 32, 1333–1339.
- (75) Jin, J., Chang, Y., Wei, W., He, Y., Hu, S., Wang, D., and Wu, Y. (2012) Prostanoid EP1 receptor as the target of (–)-epigallocatechin-3-gallate in suppressing hepatocellular carcinoma cells in vitro. *Acta Pharmacol. Sin.* 33, 701–709.
- (76) Bimonte, S., Albino, V., Piccirillo, M., Nasto, A., Molino, C., Palaia, R., and Cascella, M. (2019) Epigallocatechin-3-gallate in the prevention and treatment of hepatocellular carcinoma: experimental findings and translational perspectives. *Drug Des., Dev. Ther.* 13, 611–621.
- (77) Kang, S., Nakanishi, Y., Kioi, Y., Okuzaki, D., Kimura, T., Takamatsu, H., Koyama, S., Nojima, S., Nishide, M., Hayama, Y., Kinehara, Y., Kato, Y., Nakatani, T., Shimogori, T., Takagi, J., Toyofuku, T., and Kumanogoh, A. (2018) Semaphorin 6D reverse signaling controls macrophage lipid metabolism and anti-inflammatory polarization. *Nat. Immunol.* 19, 561–570.
- (78) Zhu, A. X., Gold, P. J., El-Khoueiry, A. B., Abrams, T. A., Morikawa, H., Ohishi, N., Ohtomo, T., and Philip, P. A. (2013) First-in-Man Phase I Study of GC33, a Novel Recombinant Humanized Antibody Against Glypican-3, in Patients with Advanced Hepatocellular Carcinoma. *Clin. Cancer Res.* 19, 920–928.
- (79) Ikeda, M., Ohkawa, S., Okusaka, T., Mitsunaga, S., Kobayashi, S., Morizane, C., Suzuki, I., Yamamoto, S., and Furuse, J. (2014) Japanese phase I study of GC33, a humanized antibody against glypican-3 for advanced hepatocellular carcinoma. *Cancer Science* 105, 455–462.
- (80) Merched, A. J., Ko, K., Gotlinger, K. H., Serhan, C. N., and Chan, L. (2008) Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. *FASEB J.* 22, 3595–3606.
- (81) Sulciner, M. L., Gartung, A., Gilligan, M. M., Serhan, C. N., and Panigrahy, D. (2018) Targeting lipid mediators in cancer biology. *Cancer Metastasis Rev.* 37, 557–572.
- (82) Baerched, A. J., Serhan, C. N., and Chan, L. (2011) Nutrigenetic disruption of inflammation-resolution homeostasis and atherogenesis. *J. Nutrigenet. Nutrigenomics* 4, 12–24.
- (83) Gilligan, M. M., Gartung, A., Sulciner, M. L., Norris, P. C., Sukhatme, V. P., Bielenberg, D. R., Huang, S., Kieran, M. W., Serhan, C. N., and Panigrahy, D. (2019) Aspirin-triggered proresolving mediators stimulate resolution in cancer. *Proc. Natl. Acad. Sci. U. S. A.* 116, 6292–6297.
- (84) Baenke, F., Peck, B., Miess, H., and Schulze, A. (2013) Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Dis. Models & Mech.* 6, 1353–1363.
- (85) Abramson, H. N. (2011) The Lipogenesis Pathway as a Cancer Target. *J. Med. Chem.* 54, 5615–5638.
- (86) Menendez, J. A., and Lupu, R. (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* 7, 763–777.
- (87) Santos, C. R., and Schulze, A. (2012) Lipid metabolism in cancer. *FEBS J.* 279, 2610–2623.
- (88) Alli, P. M., Pinn, M. L., Jaffee, E. M., McFadden, J. M., and Kuhajda, F. P. (2005) Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. *Oncogene* 24, 39–46.
- (89) Orita, H., Coulter, J., Tully, E., Kuhajda, F. P., and Gabrielson, E. (2008) Inhibiting Fatty Acid Synthase for Chemoprevention of Chemically Induced Lung Tumors. *Clin. Cancer Res.* 14, 2458–2464.
- (90) Fritz, V., Benfodda, Z., Rodier, G., Henriquet, C., Iborra, F., Avancès, C., Allory, Y., de la Taille, A., Culine, S., Blancou, H., Cristol, J. P., Michel, F., Sardet, C., and Fajas, L. (2010) Abrogation of De novo Lipogenesis by Stearoyl-CoA Desaturase 1 Inhibition Interferes with Oncogenic Signaling and Blocks Prostate Cancer Progression in Mice. *Mol. Cancer Ther.* 9, 1740–1754.
- (91) Pearce, N. J., Yates, J. W., Berkhout, T. A., Jackson, B., Tew, D., Boyd, H., Camilleri, P., Sweeney, P., Gribble, A. D., Shaw, A., and Groot, P. H. E. (1998) The role of ATP citrate-lyase in the metabolic regulation of plasma lipids. *Biochem. J.* 334, 113–119.
- (92) Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., and Thompson, C. B. (2009) ATP-Citrate Lyase Links Cellular Metabolism to Histone Acetylation. *Science* 324, 1076–1080.
- (93) Icard, P., Wu, Z., Fournel, L., Coquerel, A., Lincet, H., and Alifano, M. (2020) ATP citrate lyase: A central metabolic enzyme in cancer. *Cancer Lett.* 471, 125–134.
- (94) Chajès, V., Cambot, M., Moreau, K., Lenoir, G. M., and Joulin, V. (2006) Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. *Cancer Res.* 66, 5287–5294.
- (95) Yokohama, K., Fukunishi, S., Ii, M., Nakamura, K., Ohama, H., Tsuchimoto, Y., Asai, A., Tsuda, Y., and Higuchi, K. (2016) Rosuvastatin as a potential preventive drug for the development of hepatocellular carcinoma associated with non-alcoholic fatty liver disease in mice. *Int. J. Mol. Med.* 38, 1499–1506.
- (96) Cheng, Y., Luo, R. C., Zheng, H., Wang, B., Liu, Y., Liu, D. L., Chen, J., Xu, W., Li, A., and Zhu, Y. (2017) Synergistic anti-tumor efficacy of sorafenib and fluvastatin in hepatocellular carcinoma. *Oncotarget* 8, 23265–23276.
- (97) Kubatka, P., Kruzliak, P., Rotrekl, V., Jelinkova, S., and Mladovicova, B. (2014) Statins in oncological research: From experimental studies to clinical practice. *Critical Reviews in Oncology/Hematology* 92, 296–311.
- (98) Schlaepfer, I. R., Nambiar, D. K., Ramteke, A., Kumar, R., Dhar, D., Agarwal, C., Bergman, B., Graner, M., Maroni, P., Singh, R. P., Agarwal, R., and Deep, G. (2015) Hypoxia induces triglycerides accumulation in prostate cancer cells and extracellular vesicles supporting growth and invasiveness following reoxygenation. *Oncotarget* 6, 22836–22856.

- (99) Xiang, Y., Stine, Z. E., Xia, J., Lu, Y., O'Connor, R. S., Altman, B. J., Hsieh, A. L., Gouw, A. M., Thomas, A. G., Gao, P., Sun, L., Song, L., Yan, B., Slusher, B. S., Zhuo, J., Ooi, L. L., Lee, C. G. L., Mancuso, A., McCallion, A. S., Le, A., Milone, M. C., Rayport, S., Felsner, D. W., and Dang, C. V. (2015) Targeted inhibition of tumor-specific glutaminase diminishes cell-autonomous tumorigenesis. *J. Clin. Invest.* 125, 2293–2306.
- (100) Gross, M. L., Demo, S. D., Dennison, J. B., Chen, L., Chernov-Rogan, T., Goyal, B., Janes, J. R., Laidig, G. J., Lewis, E. R., Li, J., Mackinnon, A. L., Parlati, F., Rodriguez, M. L. M., Shwonek, P. J., Sjogren, E. B., Stanton, T. F., Wang, T., Yang, J., Zhao, F., and Bennett, M. K. (2014) Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. *Mol. Cancer Ther.* 13, 890–901.
- (101) Alipour Talesh, G., Trézéguet, V., and Merched, A. (2020) Hepatocellular Carcinoma and Statins. *Biochemistry* 59, 3393–3400.
- (102) Chiu, H.-F., Ho, S.-C. M., Chen, C.-C., and Yang, C.-Y. (2011) Statin Use and the Risk of Liver Cancer: A Population-Based Case-Control Study. *Am. J. Gastroenterol.* 106, 894–898.
- (103) Tsan, Y.-T., Lee, C.-H., Ho, W.-C., Lin, M.-H., Wang, J.-D., and Chen, P.-C. (2013) Statins and the Risk of Hepatocellular Carcinoma in Patients With Hepatitis C Virus Infection. *J. Clin. Oncol.* 31, 1514–1521.
- (104) Tsan, Y.-T., Lee, C.-H., Wang, J.-D., and Chen, P.-C. (2012) Statins and the Risk of Hepatocellular Carcinoma in Patients With Hepatitis B Virus Infection. *J. Clin. Oncol.* 30, 623–630.
- (105) Friis, S., Poulsen, A. H., Johnsen, S. P., McLaughlin, J. K., Fryzek, J. P., Dalton, S. O., Sorensen, H. T., and Olsen, J. H. (2005) Cancer risk among statin users: A population-based cohort study. *Int. J. Cancer* 114, 643–647.
- (106) El-Serag, H. B., Johnson, M. L., Hachem, C., and Morgana, R. O. (2009) Statins Are Associated With a Reduced Risk of Hepatocellular Carcinoma in a Large Cohort of Patients With Diabetes. *Gastroenterology* 136, 1601–1608.
- (107) McGlynn, K. A., Divine, G. W., Sahasrabudde, V. V., Engel, L. S., VanSlooten, A., Wells, K., Yood, M. U., and Alford, S. H. (2014) Statin use and risk of hepatocellular carcinoma in a U.S. population. *Cancer Epidemiol.* 38, 523–527.
- (108) Kawata, S., Yamasaki, E., Nagase, T., Inui, Y., Ito, N., Matsuda, Y., Inada, M., Tamura, S., Noda, S., Imai, Y., and Matsuzawa, Y. (2001) Effect of pravastatin on survival in patients with advanced hepatocellular carcinoma. A randomized controlled trial. *Br. J. Cancer* 84, 886–891.
- (109) Bengoechea-Alonso, M. T., and Ericsson, J. (2007) SREBP in signal transduction: cholesterol metabolism and beyond. *Curr. Opin. Cell Biol.* 19, 215–222.
- (110) Brown, M. S., and Goldstein, J. L. (2009) Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. *J. Lipid Res.* 50 (Suppl), S15–27.
- (111) Pandya, A. A., Mullen, P. J., Goard, C. A., Ericson, E., Sharma, P., Kalkat, M., Yu, R., Pong, J. T., Brown, K. R., Hart, T., Gebbia, M., Lang, K. S., Giaever, G., Nislow, C., Moffat, J., and Penn, L. Z. (2015) Genome-wide RNAi analysis reveals that simultaneous inhibition of specific mevalonate pathway genes potentiates tumor cell death. *Oncotarget* 6, 26909–26921.
- (112) Shao, W., and Espenshade, P. J. (2012) Expanding Roles for SREBP in Metabolism. *Cell Metab.* 16, 414–419.
- (113) Zhou, R.-H., Yao, M., Lee, T.-S., Zhu, Y., Martins-Green, M., and Shyy, J. Y.-J. (2004) Vascular Endothelial Growth Factor Activation of Sterol Regulatory Element Binding Protein. *Circ. Res.* 95, 471–478.
- (114) Grembecka, J., He, S., Shi, A., Purohit, T., Muntean, A. G., Sorenson, R. J., Showalter, H. D., Murai, M. J., Belcher, A. M., Hartley, T., Hess, J. L., and Cierpicki, T. (2012) Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. *Nat. Chem. Biol.* 8, 277–284.
- (115) Phillips, R. E., Yang, Y., Smith, R. C., Thompson, B. M., Yamasaki, T., Soto-Feliciano, Y. M., Funato, K., Liang, Y., Garcia-Bermudez, J., Wang, X., Garcia, B. A., Yamasaki, K., McDonald, J. G., Birsoy, K., Tabar, V., and Allis, C. D. (2019) Target identification reveals lanosterol synthase as a vulnerability in glioma. *Proc. Natl. Acad. Sci. U. S. A.* 116, 7957–7962.
- (116) Morand, O. H., Aebi, J. D., Dehmlow, H., Ji, Y. H., Gains, N., Lengsfeld, H., and Humber, J. (1997) Ro 48–8.071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys, and minipigs: comparison to simvastatin. *J. Lipid Res.* 38, 373–390.
- (117) Chan, A. T., Arber, N., Burn, J., Chia, W. K., Elwood, P., Hull, M. A., Logan, R. F., Rothwell, P. M., Schrör, K., and Baron, J. A. (2012) Aspirin in the Chemoprevention of Colorectal Neoplasia: An Overview. *Cancer Prev. Res.* 5, 164–178.
- (118) U.S. Preventive Services Task Force (2007) Routine aspirin or nonsteroidal anti-inflammatory drugs for the primary prevention of colorectal cancer: U.S. Preventive Services Task Force recommendation statement. *Ann. Intern. Med.* 146, 361–364.
- (119) Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett, W. C., and Speizer, F. E. (1995) Aspirin and the Risk of Colorectal Cancer in Women. *N. Engl. J. Med.* 333, 609–614.
- (120) Nan, H., Hutter, C. M., Lin, Y., Jacobs, E. J., Ulrich, C. M., White, E., Baron, J. A., Berndt, S. I., Brenner, H., Butterbach, K., Caan, B. J., Campbell, P. T., Carlson, C. S., Casey, G., Chang-Claude, J., Chanock, S. J., Cotterchio, M., Duggan, D., Figueiredo, J. C., Fuchs, C. S., Giovannucci, E. L., Gong, J., Haile, R. W., Harrison, T. A., Hayes, R. B., Hoffmeister, M., Hopper, J. L., Hudson, T. J., Jenkins, M. A., Jiao, S., Lindor, N. M., Lemire, M., Le Marchand, L., Newcomb, P. A., Ogino, S., Pflugeisen, B. M., Potter, J. D., Qu, C., Rosse, S. A., Rudolph, A., Schoen, R. E., Schumacher, F. R., Seminara, D., Slatery, M. L., Thibodeau, S. N., Thomas, F., Thornquist, M., Warnick, G. S., Zanke, B. W., Gauderman, W. J., Peters, U., Hsu, L., and Chan, A. T. (2015) Association of Aspirin and NSAID Use With Risk of Colorectal Cancer According to Genetic Variants. *JAMA, J. Am. Med. Assoc.* 313, 1133–1142.
- (121) Yang, G., Wang, Y., Feng, J., Liu, Y., Wang, T., Zhao, M., Ye, L., and Zhang, X. (2017) Aspirin suppresses the abnormal lipid metabolism in liver cancer cells via disrupting an NFκB-ACSL1 signaling. *Biochem. Biophys. Res. Commun.* 486, 827–832.
- (122) Li, S., Dai, W., Mo, W., Li, J., Feng, J., Wu, L., Liu, T., Yu, Q., Xu, S., Wang, W., Lu, X., Zhang, Q., Chen, K., Xia, Y., Lu, J., Zhou, Y., Fan, X., Xu, L., and Guo, C. (2017) By inhibiting PFKFB3, aspirin overcomes sorafenib resistance in hepatocellular carcinoma. *Int. J. Cancer* 141, 2571–2584.
- (123) Li, G., Zhang, S., Fang, H., Yan, B., Zhao, Y., Feng, L., Ma, X., and Ye, X. (2013) Aspirin overcomes Navitoclax-resistance in hepatocellular carcinoma cells through suppression of Mcl-1. *Biochem. Biophys. Res. Commun.* 434, 809–814.
- (124) Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., Johnson, E. F., Marsh, K. C., Mitten, M. J., Nimmer, P., Roberts, L., Tahir, S. K., Xiao, Y., Yang, X., Zhang, H., Fesik, S., Rosenberg, S. H., and Elmore, S. W. (2008) ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. *Cancer Res.* 68, 3421–3428.
- (125) van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., Willis, S. N., Scott, C. L., Day, C. L., Cory, S., Adams, J. M., Roberts, A. W., and Huang, D. C. S. (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 10, 389–399.
- (126) Li, T., Dong, Z.-R., Guo, Z.-Y., Wang, C.-H., Tang, Z.-Y., Qu, S.-F., Chen, Z.-T., Li, X.-W., and Zhi, X.-T. (2013) Aspirin enhances IFN-α-induced growth inhibition and apoptosis of hepatocellular carcinoma via JAK1/STAT1 pathway. *Cancer Gene Ther.* 20, 366–374.
- (127) Li, J.-H., Xie, X.-Y., Zhang, L., Le, F., Ge, N.-L., Li, L.-X., Gan, Y.-H., Chen, Y., Zhang, J.-B., Xue, T.-C., Chen, R.-X., Xia, J.-L., Zhang, B.-H., Ye, S.-L., Wang, Y.-H., and Ren, Z.-G. (2015) Oxaliplatin and 5-fluorouracil hepatic infusion with lipiodolized

chemoembolization in large hepatocellular carcinoma. *World Journal of Gastroenterology* 21, 3970–3977.

(128) Sanoff, H. K., Chang, Y., Stavas, J. M., Stürmer, T., and Lund, J. (2015) Effectiveness of Initial Transarterial Chemoembolization for Hepatocellular Carcinoma Among Medicare Beneficiaries. *J. Natl. Compr. Cancer Network* 13, 1102–1110.

(129) Raoul, J.-L., Gilibert, M., and Piana, G. (2014) How to Define Transarterial Chemoembolization Failure or Refractoriness: A European Perspective. *Liver Cancer* 3, 119–124.

(130) Kudo, M., Matsui, O., Izumi, N., Kadoya, M., Okusaka, T., Miyayama, S., Yamakado, K., Tsuchiya, K., Ueshima, K., Hiraoka, A., Ikeda, M., Ogasawara, S., Yamashita, T., and Minami, T. (2014) Transarterial Chemoembolization Failure/Refractoriness: JSH-LCSGJ Criteria 2014 Update. *Oncology* 87, 22–31.

(131) Li, J.-H., Wang, Y., Xie, X.-Y., Yin, X., Zhang, L., Chen, R.-X., and Ren, Z.-G. (2016) Aspirin in combination with TACE in treatment of unresectable HCC: a matched-pairs analysis. *Am. J. Cancer Res.* 6, 2109–2116.

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References 101–105 were misnumbered in the version published on October 2, 2020 and were corrected on October 7, 2020.

Remodelage du métabolisme lipidique en ciblant PCSK9 comme nouvelle approche thérapeutique dans les cancers du foie

La dérégulation du métabolisme énergétique, dont l'augmentation de la biosynthèse des lipides, est une caractéristique émergente de nombreux cancers, dont les cancers du foie adultes (le carcinome hépatocellulaire ou CHC) et pédiatriques (l'hépatoblastome ou HBL). Le CHC est la 3^{ème} cause mondiale de décès par cancer. L'HBL est un cancer embryonnaire rare dont l'incidence augmente. L'analyse des données transcriptomiques disponibles montre une dérégulation de l'expression de gènes impliqués dans l'absorption et le métabolisme des lipides dans les deux cancers. Ainsi sont surexprimées PCSK9, pro-protéine convertase qui régule négativement le LDLR, et HMGCR, enzyme de la voie de synthèse *de novo* du cholestérol. Le rôle de PCSK9 dans le cancer du foie est peu connu. Aussi, l'objectif de mon projet est de remodeler le métabolisme des lipides autour de PCSK9 en utilisant des médicaments disponibles et surexprimer une enzyme impliquée dans la maturation de PCSK9, la méprine alpha (Mep1a) dont l'expression est augmentée dans les cancers du foie. Mes résultats montrent que le ciblage pharmacologique ou génique de PCSK9 diminue croissance et migration cellulaires et augmente la consommation d'O₂. L'inhibition de l'expression génique de PCSK9 augmente les niveaux intracellulaires de certains lipides et perturbe l'axe p62/KEAP1/Nrf2 qui conduit à la ferroptose. Les effets anti-tumoraux de l'inhibition de PCSK9 ont été validés *in vivo*. J'ai aussi surexprimé Mep1a pour mimer le profil des tumeurs, ce qui induit une augmentation de protéines interagissant avec PCSK9 et un changement du métabolisme lipidique *in vitro* et *in vivo*. En conclusion mes résultats soulignent le rôle clé de PCSK9 dans le métabolisme lipidique et montre pour la première fois son implication dans le maintien de l'homéostasie redox cellulaire et la ferroptose. Ils ouvrent la voie au repositionnement de médicaments anti-PCSK9 pour traiter les cancers du foie avec un bénéfice thérapeutique réel.

Mots-clés : PCSK9, ferroptose, méprine, carcinome hépatocellulaire, hépatoblastome, métabolisme lipidique

Rewiring lipid metabolism by targeting PCSK9 as a new therapeutic approach in liver cancers

Dysregulation of energetic metabolism including increased lipid biosynthesis is an emerging feature of many cancers, including adult (hepatocellular carcinoma or HCC) and pediatric (hepatoblastoma or HBL) liver cancers. HCC is the 3rd leading cause of cancer death worldwide. HBL is a rare embryonic cancer with increasing incidence. The analysis of available transcriptomic data shows a deregulation of the expression of genes involved in the absorption and metabolism of lipids in both cancers. Thus, PCSK9, a pro-protein convertase that negatively regulates LDLR, and HMGCR, an enzyme of the *de novo* cholesterol synthesis pathway, are overexpressed. The role of PCSK9 in liver cancer is not well understood. Therefore, the objective of my project is to remodel lipid metabolism around PCSK9 using available drugs and overexpressing an enzyme involved in PCSK9 maturation e.g. meprin alpha (Mep1a) which expression is increased in liver cancers. My results show that pharmacological or genetic targeting of PCSK9 decreases cell growth and migration and increases O₂ consumption. Genetic deletion of PCSK9 increases intracellular levels of some lipids and disrupts the p62/KEAP1/Nrf2 axis that leads to ferroptosis. The anti-tumor effects of PCSK9 inhibition were validated *in vivo*. I also overexpressed Mep1a to mimic liver tumor profile and found an induction in PCSK9 interacting proteins, and a change in lipid metabolism *in vitro* and *in vivo*. In conclusion, my results highlight the key role of PCSK9 in lipid metabolism and show for the first time its involvement in the maintaining of cellular redox homeostasis and in ferroptosis. They open the way to the repositioning of anti-PCSK9 drugs to treat liver cancers with a real therapeutic benefit.

Keywords: PCSK9, ferroptosis, meprin, hepatocellular carcinoma, hepatoblastoma, lipid metabolism

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