OSTEOPONTIN:
A REGULATOR OF LIVER LIPID METABOLISM

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PhD Thesis 2016

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ABBREVIATIONS

AA	Amino acid
Aadac	Arylacetamide deacetylase
Abcb4/MDR2	Multidrug resistance 2
Abcb11/BSEP	Bile salt export pump
Abcc2/MRP2	Canalicular multispecific organic anion transporter 1
Abcc3/MRP3	Canalicular multispecific organic anion transporter 2
Abcg5	ATP-binding cassette, sub-family G, member 5
Abcg8	ATP-binding cassette, sub-family G, member 8
AC	Acylcarnitine
ACAT	Acyl-CoA:cholesterol aciltransferase
ACLY	ATP citrate lyase
ACC	Acetyl-CoA carboxylase
FPLC	Fast protein liquid chromatography
ACS	Acyl-CoA synthetase
Actb	Actin beta
Alb	Albumine
ALT	Alanine aminotransferace
AMPK	5'-AMP-activated protein kinase
ANOVA	Analysis of variance
Asp	Aspartate
AST	Aspartate aminotransferace
ATP	Adenosine-5'-triphosphate
BA	Bile acid
BCA	Bicinchoninic Acid
BMI	Body mass index
BS	Bile salt
BSA	Bovine serum albumin

CA	Cholic acid
Ccna2	Cyclin A2
Ccnd1	Cyclin D1
CDCA	Chenodeoxycholic acid
CDP	Cytidine-5'-diphophate
CDP-cho	CDP-choline
CDP-eth	CDP-ethanolamine
CEH	Cholesteryl ester hydrolase
Cer	Ceramide
Ces1d	Carboxylesterase 1D
Chka	Choline kinase alpha
Chkb	Choline kinase beta
CMH	Ceramide monohexoxyl
CHOL	Cholesterol
CK	Cytokine
CL	Cardiolipin
CM	Chylomicron
CMr	Chylomicron remnant
CoA	Coenzyme A
Col1a1	Collagen, type I, alpha 1
CV	Central vein
Cyp7a1/CYP7A1	Cholesterol 7-alpha-hydroxylase
DAB	3,3'-Diaminobenzidine
DCA	Deoxycholic acid
DEPC	Diethylpirocarbonate
DG	Diacylglyceride
Dgat/DGAT	Acyl-CoA:diacylglycerol acyltransferase
DMEM	Dubbelco's modified Eagle's medium
DPX	Di-n-butyl phthalate in xylene mounting media
dH$_2$O	Distilled water

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dNTP	Deoxynucleotide-5'-triphosphate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELOVL	Elongase
ESI	Electrospray inosization
ER	Endoplasmic reticulum
Etnk2/EK2	Ethanolamine kinase 2
ETA-1	Early T lymphocyte activation
FA	Fatty acid
FAA	Fatty acid amide
FABP	FA binding protein
FATP	FA transport protein
FBS	Fetal bovine serum
FC	Free cholesterol
FAS	Fatty acid synthase
Fru-6-P	Fructose-6-phosphate
FXR	Farnesoid X receptor
Gapdh/GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GA-3-P	Glyceraldehyde-3-phosphate
GLU	Glucose
Glu-6-P	Glucose-6-phosphate
GPL	Glycerophospholipid
GUDCA	Glycoursodeoxycholic acid
G6PD	Glucose-6-phosphate dehydrogenase

HDL	High-density lipoprotein
HBV	Hepatitis B virus
HCC	Hepatocellular carcinome
HCV	Hepatitis C virus
HDCA	Hyodeoxycholic acid
HIV	Human immunodeficiency virus
HFD	High fat diet
HMG-CoA	Hydroxy-3-methyl-glutaryl-CoA
HMGCR	HMG-CoA reductase
Hnf4a/HNF4α	Hepatocyte nuclear factor 4 α
HOMA-IR	Homeostasis model assessment of insulin resistance
HPBCD	Hydroxypropyl beta-cyclodextrin
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
HSC	Hepatic stellate cell
H&E	Hematoxilin and eosin
IDL	Intermediate-density lipoprotein
IFC	Integrated Fluidic Circuits
IL	Interleukin
IOD	Integrated optic density
iOPN	Intracellular OPN
IP$_3$	Inositol-1,4,5-trisphosphate
IR	Insuline resistance
KC	Kupffer cell
KH	Krebs-Henseleit perfusion medium
LCA	Lithocholic acid
LCFA	Long-chain fatty acid
LC/MS	Liquid chromatography/tandem mass spectrometry
LD	Lipid droplet

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	LDL receptor
Lipa/LAL	Lisosomal acid lipase
Lipc/HL	Hepatic lipase
Lipe/HSL	Hormone-sensitive lipase
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCFA	Medium-chain fatty acid
ME	Malic enzyme
MRM	Multiple reaction monitoring
MTTP	Microsomal triacylglyceride transfer protein
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dicucleotide oxydized
NADH	Nicotinamide adenine dicucleotide reduced
NADP	Nicotinamide adenine dicucleotide phosphate oxydized
NADPH	Nicotinamide adenine dicucleotide phosphate reduced
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NL	Normal liver
NPC1L1	Niemann-Pick C1-like 1 protein
Nr0b2/SHP	Small heterodimer partner
Nr5a2/LRH-1	Liver receptor homolog-1
OA	Oleic acid
OAA	Oxalacetate
OATP	Solute carrier organic anion transporter family

OPN	Osteopontin
OPN-KO	Osteopontin knock out
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphohydrolase
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
Pcna	Proliferating cell nuclear antigen
Pcyt1a/CTα	Choline phosphate cytidylyltransferase A
Pcyt2/ET	Ethanolamine-phosphate cytidylyltransferase
PE	Phosphatidylethanolamine
Pemt/PEMT	Phosphatidylethanolamine N-methyltransferase
PH	Partial hepatectomy
PLA2	Phospholipase A2
PLD	Phospholipase D
Plin2/ADRP	Perilipin 2/Adipophilin
PMME	3-phosphoethanolamine-N-methil
Pnpla2 /ATGL	Adipose triacylglyceride lipase
PNPLA3	Adiponutrin/patatin-like phospholipase domain containing 3
Ppia	Peptidylprolyl isomerase A (cyclophilin A)
PPP	Pentose phosphate pathway
PT	Portal triad
PUFA	Polyunsaturated fatty acid
PVDF	Immobilon-P transfer membranes
RER	Rough endocytoplasmic reticulum
RGD	Arginine-glycine-aspartate motif
rOPN	Recombinant osteopontin
ROS	Reactive oxygen species
RT-qPCR	Real time-quantitative polymerase chain reaction
SCFA	Short-chain fatty acid
SAMe	S-adenosylmethionine

SCD-1	Stearyl-CoA desaturase 1
SDS	Sodic dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SER	Smooth endoplasmic reticulum
SFA	Saturated fatty acid
SM	Sphingomyelin
sOPN	Secreted osteopontin
Slc10a1/NTCP	Na^+-dependent taurocholic cotransporting polypeptide
SPP1	Secreted phosphoprotein 1
SREBP	Sterol regulatory element binding protein
TAE	Tris-acetate buffer with EDTA
TBS	Tris-buffered saline
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetrametiletilendiamina
TFA	Trans fatty acid
TG	Triacylglyceride
TGF- β	Transforming growth factor beta
TGH	Triacylglycerol hydrolase
THCA	Taurohyocholic acid
THDCA	Taurohyodeoxycholic acid
TLC	Thin layer chromatography
TLCA	Taurolithocholic acid
TMC	Tauromuricholic acid
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TOF-MS	Time-of-flight mass spectrometry

TUDCA	Tauroursodeoxycholic acid
TαMCA	Tauro-alpha-muricholic acid
TβMCA	Tauro-beta-muricholic acid
T2DM	Type 2 diabetes mellitus
UDCA	Ursodeoxycholic acid
UPLC	Ultra performance liquid chromatography
VLCFA	Very-long-chain fatty acid
VLDL	Very-low-density lipoprotein
VLDLr	VLDL remnant
Vldlr/VLDLR	VLDL receptor
WT	Wild type
αMCA	Alpha-muricholic acid
βMCA	Beta-muricholic acid

A. RESUMEN/SUMMARY

RESUMEN/SUMMARY

1. Introducción y Objetivos

La enfermedad del hígado graso no alcohólica (EGHNA) es una de las principales enfermedades hepáticas crónicas en los países desarrollados. Está considerada como la manifestación hepática del síndrome metabólico, e incluye un amplio espectro de condiciones que van desde el hígado graso no alcohólico (HGNA) a la esteatohepatitis no alcohólica, acompañada en algunos casos de fibrosis que puede progresar a cirrosis, carcinoma hepatocelular y a estadíos terminales de la enfermedad hepática, siendo el transplante hepático la única solución en estos casos. La desregulación metabólica es una característica distintiva asociada al desarrollo y progresión de EHGNA, en la cual se produce un desequilibrio entre incorporación de lípidos (entrada al hepatocito y síntesis *de novo*) y su utilización (catabolismo y secreción).

El hígado es capaz de regenerarse tras daño hepático o resección para recuperar su correcta funcionalidad. La regeneración hepática implica una profunda remodelación metabólica con el principal objetivo de disponer de energía y precursores metabólicos esenciales para recuperar con éxito las funciones hepáticas.

La osteopontina (OPN) es una citoquina multifuncional que se expresa en el hígado y está relacionada con desordenes metabólicos. En obesidad, su sobreexpresión hepática se correlaciona con el contenido de triacilglicérido (TG). Su deficiencia protege frente a la hepatoesteatosis inducidas por obesidad y a la fibrosis hepática y atenúa la producción de glucosa por el hígado. Además, se ha observado que la neutralización de OPN circulante evitala fibrogénesis hepática en ratones. Los estudios realizados hasta el momento para analizar el papel de OPN en los desórdenes metabólicos se han realizado en modelos de obesidad. En obesidad los cambios metabolicos que ocurren en el hígado están asociados a un aumento en la lipólisis del tejido adiposo promovido por la resistencia a insulina, lo que da lugar a un aumento de la liberación de ácidos grasos al plasma, los cuales serán almacenados, entre otros, en el hígado. Por ello resulta difícil discernir si la desregulación metabólica del hígado se debe a un efecto directo de OPN o es consecuencia del metabolismo alterado en el tejido adiposo, donde OPN también se sobreexpresa. Así,

teniendo en cuenta que OPN está vinculada a diferentes desórdenes hepáticos caracterizados por una desregulación del metabolismo, los principales **objetivos** en este estudio son: 1) identificar el papel que desempeña OPN como regulador directo del metabolismo hepático; 2) investigar si OPN está involucrada en la modulación del metabolismo lipídico durante la regeneración hepática; 3) validar la OPN sérica como un biomarcador temprano de la disfunción metabólica del hígado en pacientes no obesos con HGNA, en los cuales no es evidente la desregulación metabólica del tejido adiposo.

2. Procedimentos experimentales

Se analizaron los flujos metabólicos y las actividades enzimáticas implicadas en el metabolismo lipídico, la expresión de diversas proteínas y el contenido lipídico en hepatocitos primarios e hígados de ratones OPN-KO y sus respectivos controles silvestres (WT), así como de ratones WT a los cuales se administró rOPN (osteopontina recombinante). Se analizó el lipidoma y el metaboloma hepático de los ratones OPN-KO y sus correspondientes WT. Además, se realizó una hepatectomía parcial del 70%, una de las técnicas más reconocidas y estandarizadas para el estudio de la regeneración hepática, en ratones OPN-KO, ratones OPN-KO tratados con atorvastatina y sus WT. Por último, se analizaron las biopsias hepáticas de pacientes no obesos con HGNA o con hígado normal, recogidas durante colecistectomías laparoscópicas programadas y sus respectivos sueros.

3. Resultados

Los resultados muestran que OPN regula la interconexión entre el metabolismo hepático de colesterol y el de la fosfatidilcolina (PC), siendo la conversión de colesterol en ácidos biliares el proceso metabólico clave. La OPN extracelular provoca la disminución de los niveles de expresión de la proteína colesterol 7-alfa-hidroxilasa (CYP7A1) y, en coherencia, la carencia de OPN aumenta la expresión de CYP7A1. A su vez la carencia de OPN incrementa la colesterogénesis en hepatocitos primarios; sin embargo, no altera los niveles hepáticos de colesterol, probablemente por el incremento de la conversión de colesterol en ácidos biliares. Estos cambios están asociados a una mayor expresión génica de Abcb4 (MDR2), responsable de la secreción biliar de PC, lo que sugiere un aumento de la secreción

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canalicular de PC a la bilis en los ratones deficientes en OPN. El hecho de que el contenido hepático de las especies de PC más abundantes en la bilis sea inferior en ratones OPN-KO apoya esta propuesta, y es coherente con la disminución del contenido hepático y de ciertas especies de ácidos biliares. Esta adaptación podría ser la causante del aumento en la expresión de genes relacionados con la vía de síntesis de CDP-colina ya que es esta vía la que genera mayoritariamente las especies de PC más representadas en la bilis; en cambio no se han detectado diferencias en la actividad fosfatidiletanolamina n-metiltransferasa (PEMT) debida a la deficiencia en OPN. El descenso en los niveles hepáticos de PC dirigido por la falta en OPN se revierte tras la adición de OPN al medio de cultivo de hepatocitos primarios deficientes en OPN. Además, la administración *in vivo* de rOPN incrementa los valores de PC hepáticos en ratones WT.

El aumento de la colesterogénesis promovido por la mayor síntesis de ácidos biliares en ratones OPN-KO está asociado a la disminución de la lipogénesis *de novo*. Ambos procesos metabólicos comparten una gran demanda de acetil-CoA como precursor, así como del poder reductor; la colesterogénesis es una vía de muy alto coste energético lo que podría dar lugar a la pronunciada disminución del contenido relativo de NADH hepático debida a la deficiencia en OPN. Los análisis metabolómicos muestran que en el hígado de los ratones OPN-KO los niveles relativos de algunos intermediarios de las vías de las pentosas fosfato y la glicólisis son inferiores a los de ratones WT. Estas modificaciones no se asocian a alteraciones en la entrada de glucosa en los hepatocitos ni en la transformación de piruvato a lactato. Tampoco existen cambios en las actividades de las enzimas glucosa-6-fosfato dehidrogenasa ni enzima málico. Además, la OPN extracelular tiene un efecto directo sobre la lipogenesis *de novo* ya que activa la acetil-CoA carboxilasa, enzima limitante en este proceso. En concordancia la carencia hepática en OPN está asociada a la menor activación de este enzima. A pesar de que OPN regula la lipogénesis *de novo*, el contenido hepático de TG no está alterado, probablemente como consecuencia de una mayor disponibilidad de ácidos grasos procedentes de las lipoproteínas plasmáticas. Se ha observado que la carencia en OPN está asociada a la sobreexpresión de la proteína receptora de lipoproteínas de muy baja densidad (VLDLR) y un aumento en las actividades de las enzimas lisosomales triacilglicérido hidrolasa (TGH) y colesteril ester hidrolasa (CEH), lo que indica una mayor actividad de la vía endocítica de lipoproteínas. Con el fin de comprobar si la modulación de la utilización hepática del colesterol por OPN tiene como consecuencia alteraciones en el

metabolismo de glicerolípidos se administró atorvastatina a ratones OPN-KO. Los resultados muestran que la inhibición de la colesterogénesis *in vivo* restaura los niveles hepáticos de PC.

Dado que la administración *in vivo* de OPN es capaz de modificar el metabolismo lipídico hepático y que se conoce que durante la regeneración hepática aumentan los niveles de OPN y existe una importante remodelación lipídica, propusimos la hipótesis de que OPN podría estar involucrada en la remodelación del metabolismo lipídico que ocurre durante la regeneración hepática después de hepatectomía parcial. Los resultados muestran que durante la regeneración hepática la deficiencia de OPN no afecta a los cambios que ocurren en el contenido hepático de TG. La esteatosis microvesicular transitoria, característica de la regeneración trashepatectomía parcial, requerida para que la regeneración tenga lugar con normalidad, no se ve alterada por la carencia de OPN. Los bajos niveles de PC en el hígado quiescente de ratones OPN-KO se mantienen 24 h después de hepatectomía parcial. Además, la deficiencia en OPN también evita el incremento que se produce en el contenido hepático de PE 24 h despues de la hepatectomía en los ratones WT. Un análisis más preciso del lipidoma hepático revela que la remodelación que ocurre en los ratones WT 24 h después de la hepatectomía parcial conduce a un perfil lipidómico similar al del hígado quiescente deficiente en OPN, que presenta cambios de menor magnitud que el del WT durante la regeneración hepática. La remodelación lipidómica 24 horas después de la hepatectomía parcial en los ratones WT está asociada al incremento de la actividad de las lipasas lisosomales TGH y CEH. Sin embargo, eso no ocurre en los animales deficientes en OPN, en coherencia con la menor modulación del lipidoma hepático durante este periodo de regeneración. La modulación del lipidoma hepático como consecuencia de la inhibición de la síntesis *de novo* de colesterol en el hígado de los ratones OPN-KO conduce a la normalización del contenido de PC. Con el objetivo de definir el papel de la regulación de la interconexión entre el metabolismo de colesterol y PC en animales carentes en OPN durante la regeneración hepática, se realizó la hepatectomía parcial a un grupo de ratones OPN-KO tratados con atorvastatina. Los resultados muestran que la remodelación lipídica se asocia a una respuesta más temprana en la regeneración hepática.

Hasta el momento no existen marcadores séricos importantes de desregulación metabólica en las primeras fases de HGNA y la mayoría de investigaciones se centran en pacientes no obesos. Teniendo en cuenta que los resultados obtenidos muestran que OPN

regula directamente el metabolismo lipídico hepático y que en el HGNA, se produce una fuerte desregulación del metabolismo hepático, propusimos que OPN podría ser un biomarcador sérico temprano de la desregulación metabólica en los pacientes no obesos con HGNA, en los que no existe una desregulación aparente del metabolismo en el tejido adiposo ni inflamación.

Los resultados de los análisis realizados muestran que los niveles séricos de OPN están incrementados en pacientes con HGNA en los que la conversión de colesterol en ácidos biliares está disminuida. Además, los niveles séricos de OPN en pacientes con HGNA se correlacionan con la concentración hepática de PC y colesterol.

4. Conclusiones

1- Ostepontina (OPN) regula la interconexión entre el metabolismo hepático de colesterol y el de fosfatidilcolina (PC). El proceso metabólico clave es la conversión de colesterol en ácidos biliares.

La OPN extracelular provoca la disminución de la expresión de la colesterol 7-alfa-hidroxilasa (CYP7A1). La carencia de OPN no modifica la concentración hepática de colesterol cuando CYP7A1 está sobreexpresada, a pesar del aumento de la colesterogénesis en hepatocitos.

La OPN extracelular aumenta la concentración hepática de PC mientras que su deficiencia está asociada a una disminución de los niveles hepáticos de PC, principalmente de las especies que son secretadas a la bilis. La inhibición de la síntesis *de novo* de colesterol restablece los niveles hepáticos de PC en los ratones deficientes en OPN.

El incremento en el uso de sustratos para la síntesis *de novo* de colesterol en los hepatocitos deficientes en OPN está asociado a una disminución de la lipogénesis *de novo* y a un menor contenido hepático de los intermediaros de las vías glicolítica y de las pentosas fosfato y del poder reductor, lo que contribuye a la disminución en la concentración de PC.

A pesar de que OPN regula la lipogénesis *de novo*, el contenido hepático de TG no está alterado, probablemente como consecuencia de un aumento en la disponibilidad de ácidos grasos vinculado a una mayor actividad endocítica .

2- La remodelación que ocurre en el lipidoma hepático 24 h después de hepatectomía parcial en los ratones WT conduce a un perfil similar al del lipidoma del hígado quiescente en los ratones OPN-KO, el cual presenta cambios de menor magnitud que el del WT durante la regeneración hepática.

3- En los pacientes no obesos con hígado graso no alcohólico, en los cuales no existe una desregulación metabólica del tejido adiposo y la conversión de colesterol a ácidos biliares está disminuida, la OPN sérica se correlaciona con las concentraciones hepáticas de PC y colesterol y podría ser un biomarcador sérico temprano de la desregulación metabólica.

B. INTRODUCTION

1. The liver

The liver is the largest solid organ of the body that extends across the entire abdominal cavity just inferior to the diaphragm. The anatomical location is of course linked to its function. It is also the largest visceral organ in humans, its weight representing between 2% to 5% of the total adult human body weight. One of the most notable characteristics is the central role in whole body metabolic integration. Besides, it has the ability to regenerate different section of itself in the event of damage.

1.1 Structure and cell types

The human liver is formed by 4 different lobes: right, left, quadrate and caudate or Spiegel lobe. Each lobe is divided into small lobules, structural units of the liver, which are connected by blood vessels and fibrotic tracts. The lobules are built around a central ramification of the hepatic vein. The edges formed by the join of some lobules, known as the portal triads, are composed of a branch of the portal vein, another from the hepatic artery, a biliar conduct and a lymphatic vessel. The branches of the portal vein and the hepatic artery are connected with the central vein by sinusoids and between them by hepatocytes row, known as Remak trabecule or cordon, which is separated from the blood flux by a monolayer of endothelial cells and by the space of Disse. The functional and microcirculatory unit of the liver is the hepatic acinus, a parenchymal space covered by two central veins. It is a structure with a central axis, named Herring channel, which is formed by the portal triad. The hepatic acinus is divided into 3 regions, depending on the distance from the axis, named periportal, intermediate and perivenous[1].

The liver is composed of several cell populations. These cells are in and around the hepatic sinusoids and are divided into two principal groups: **parenchymal cells** or hepatocytes and **non-parenchymal or sinusoidal cells** such as endothelial cells, kupffer cells, stellate cells, lymphoid cells or Pit cells and the biliar epithelium[2].
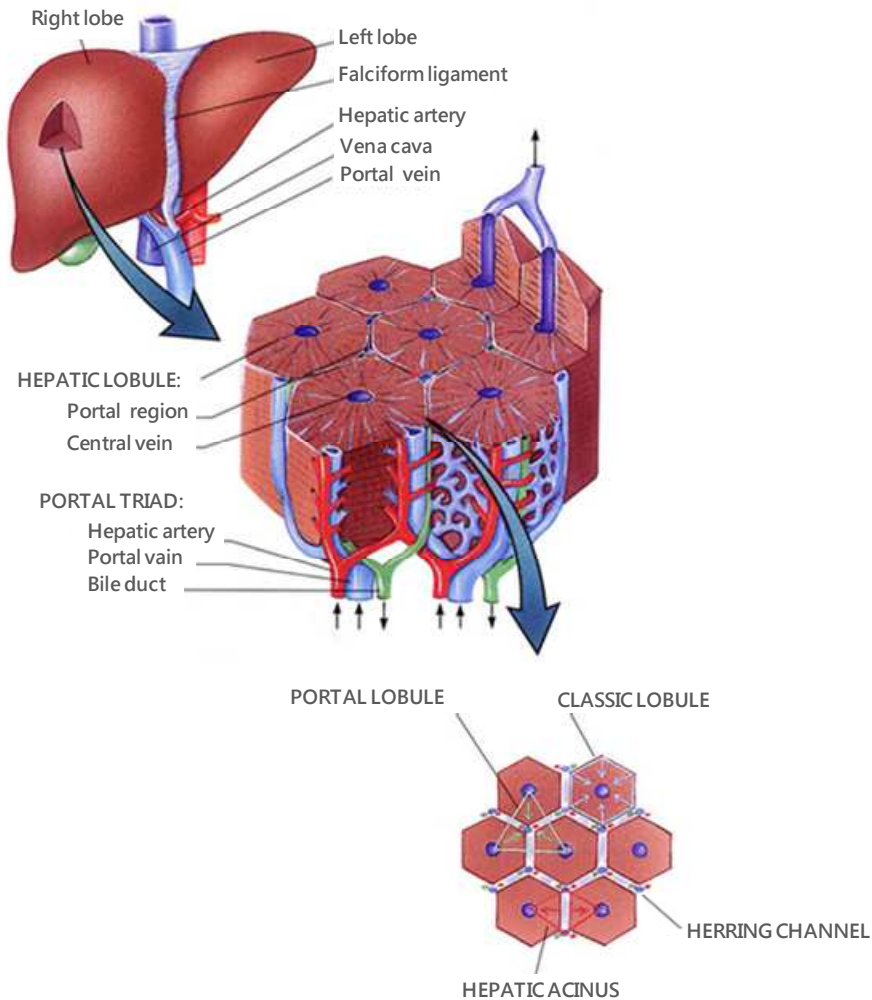
Figure B.1. Schematic image where different structural and functional liver units are represented: the lobule and the acinus. The image has been modified from http://medcell.med.yale.edu/.

Endothelial cells are the first barrier between blood and hepatocytes, being their main function to filtrate fluid, solutes and particle, which facilitates the exchange between the lumen of the sinusoid and the space of Disse easier. These sessile cells also participate in other processes like coagulation, fibrinolysis, inflammation, immune response, regulation of the blood pressure, angiogenesis and lipoprotein metabolism, among others.

Kupffer cells (KC) are a macrophage resident population that has the ability to move between the sinusoidal endothelium and the space of Disse. These phagocytic cells can

produce cytokines (CK) that play a crucial role in the innate immune response and that can modulate hepatocyte and endothelial cell metabolism.

Hepatic stellate cells (HSC) also known as Ito cells or fat-storing cells, are located in the perisinusoidal space of Disse sharing their extensions with endothelial cells. They play a crucial role in the hepatic fibrogenesis. Usually, they are the main hepatic source of extracellular matrix (ECM) components, control the microvascular tone and are the main site for vitamin A storage.

Pit cells are lymphoid cells of big size with natural killer activity. They are localized in the sinusoidal endothelial layer and they are characterized by being a liver-associated population of large granular lymphocytes. They have the capacity to kill tumor cells and they probably also play a role in the antiviral defense of the liver. In addition, pit cells may have a growth-regulatory function of the liver.
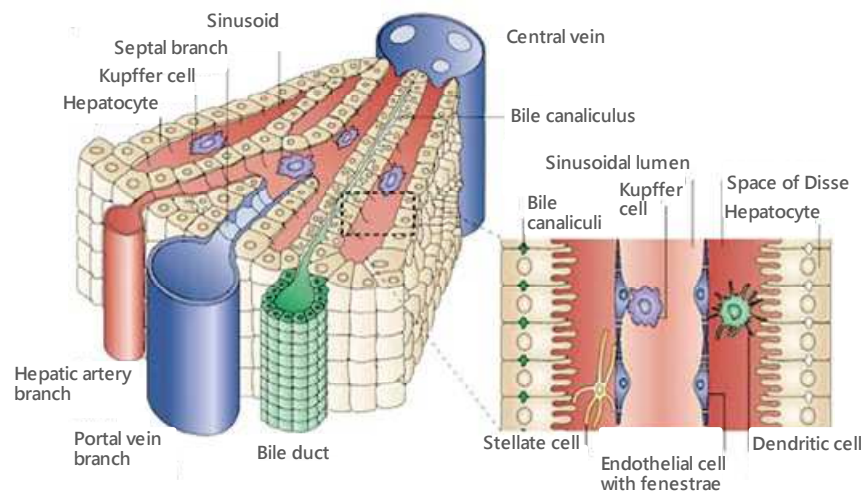
Figure B.2. Three-dimensional structure of hepatic acinus and the liver cellular composition[3].

The most abundant cell type in the liver by mass and number are **hepatocytes** (Figure B.3); human liver is made of around 80 x 10^9 hepatocytes. The hepatocyte is a complex cell type which has to fulfill several complex functions at the same time and these are accomplished by means of very effectively functioning structures and organelles. The hepatocyte is a poliedric epithelial cell, mono, bi or even multinucleate (40-50% of the whole hepatocytes) and has a big amount of organelles in the cytoplasm, which contributes to

become one of the most metabolically active cell type of the body. The hepatocyte is a polarized cell and it possesses three different specialized membrane domains. The basolateral or sinusoidal domain, is the one characterized by having microvilli that increased the contact surface with the space of Disse, making easier the exchange of substances between the hepatocyte and blood, for example the secretion of lipids into very-low-density lipoproteins (VLDL). In the canalicular or apical domain, also called the biliary pole of the hepatocyte, the apical bile acid (BA) transporters, organic ion transporters and P-glycoproteins are located, being in charge of the primary ATP dependent transport of organic components. The third domain is the lateral domain, in which cellular adhesion processes develop by tight junctions, gap junctions and desmosomes.
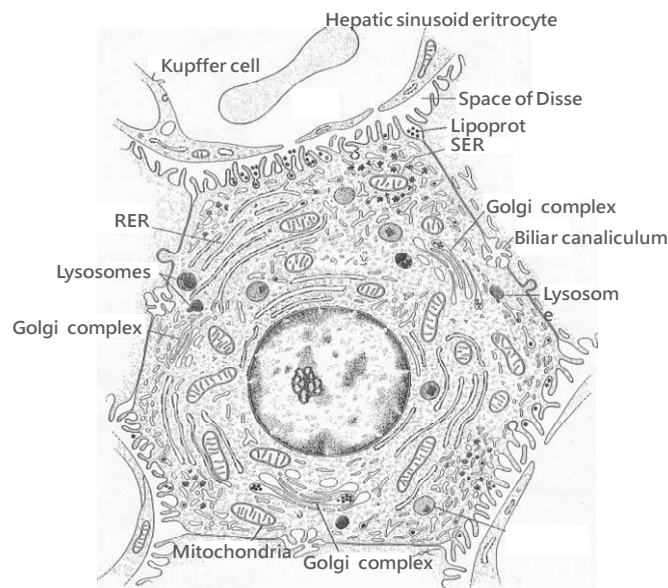
Figure B.3. Scheme of a hepatocyte. Modified image from http://es.slideshare.net/mdmunozc01/orgnulos-membranosos-2011. Rough endoplasmic reticulum (RER); smooth endoplasmic reticulum (SER).

1.2 Metabolic center

The liver has a central role maintaining the entire body metabolism homeostasis. Different metabolic processes, such as processing of absorbed nutrients and xenobiotics, BA synthesis and bile formation, maintenance of lipids, glucose, amino acids, ammonia and bicarbonate homeostasis in the organism, and synthesis of most of the plasma proteins are performed by the liver. In addition, the liver is also in charge of the storage and processing

of signal molecules and hormones, and immune processes involved in the whole body acute phase reaction.

1.2.1 Glycerolipid metabolism

1.2.1.1 Fatty acid sources

Fatty acids (FA) are very important substrates for oxidation and production of cellular energy as well as precursor molecules for all lipid classes, including those that form biological membranes. They also modify and regulate the properties of many proteins through direct covalent linkage, and perform important roles as signaling molecules in metabolic regulation. They are components of glycerolipids, such as triacylglycerides (TG) and glycerophospholipids (GPL).

Depending on the presence of double bonds, FAs are classified into saturated FAs (SFA), monounsaturated FAs (MUFA) and polyunsaturated FAs (PUFA). According to the position of the first double bond from the methyl end of the FA molecule, PUFAs are described as *n-3*, *n-6* or *n-9*. In addition, they can also differ depending on the chain length being classified in short-, medium-, long- and very-long-chain FAs. Short-chain FAs (SCFAs) consist of chain with less than 6 carbons, medium-chain FAs (MCFAs) of between 6-12 carbons, long-chain FAs (LCFAs) of between 12-20 carbons, and very-long-chain FAs (VLCFA) of more than 20 carbons.

In the liver there are three major primary sources of FAs: 1- FAs incorporated into lipoproteins (VLDL remnants (VLDLr) and QM remnants (QMr)), 2- circulating FA-albumin complex, which are released from extrahepatic tissues and 3- *de novo* lipid synthesis carried out in the liver. In addition, different species of FAs can be generated in liver by chain elongation and desaturation of exogenous and endogenous FAs.

Diet is a fundamental lipid source in the body and QMr are responsible for providing dietary FAs to the liver. The quantity and quality of dietary FAs are known to have an impact on the composition of tissue lipids both in rodents[4] and humans[5]. Some reports have demonstrated that not all dietary FAs will be a source for the same glycerolipids; each FA type could have preferences. For example, dietary n-3 PUFAs are preferentially incorporated into PLs compared to TGs[6] and among PLs, Belogun *et al.* have demonstrated that they are

incorporated into phosphatidylcholine (PC) and lysophosphatidylcholine (LPC). Additionally, dietary n-3 PUFA are able to remodelate the fatty acyl moieties of plasma and liver PC, LPC, and cholesteryl ester (CE)[4].

As mentioned, circulating FAs coming from extrahepatic tissues are also a source of hepatic FA. The uptake of FAs into the hepatic cells is mainly driven through passive diffusion or facilitated by different transporters, such as CD36, FA transport proteins (FATP) or FA binding proteins (FABP).

The FA composition of intracellular glycerolipids not only depends on diet and extrahepatic sources but also depends on the action of other enzymatic systems responsible for synthesizing, desaturating and elongating FAs. Regarding FAs *de novo* synthesis, it is performed mainly in the liver starting from small intermediates that come mainly from the degradation of carbohydrates and amino acids that finally produce acetyl-coenzyme A (CoA), the starter molecule in *de novo* synthesis. In a healthy liver, *de novo* synthesis is connected to carbohydrate metabolism. Therefore, glycolysis converts glucose to pyruvate, which is the main source of acetyl-CoA. Acetyl-CoA is transported to the cytosol as citrate. ATP citrate lyase (ACLY) then converts citrate back to acetyl-CoA and oxalacetate (OAA), and the cytosolic malate dehydrogenase (MDH) reduces that OAA to malate, which is oxidized to piruvate by the malic enzyme (ME) in a reaction that is associated to the production of reduced NADPH. In the FA *de novo* synthesis, the catalytic components required for the entire pathway are integrated into two principal enzymatic systems, acetyl-CoA carboxylase (ACC) and FA synthase (FAS). ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Acetyl-CoA and malonyl-CoA are then used as the substrates for the production of palmitate (16:0) by the multifunctional FAS. FAS uses NADPH, produced mainly by ME and pentose phosphate pathway (PPP). Subsequently, elongase and desaturase enzymes can act on *de novo* synthesized 16:0 to generate 16:1, 18:0, or 18:1 FAs. In the liver, stearoyl-CoA desaturase 1 (SCD1) is the primary desaturase responsible for the generation of C16:1 and C18:1 from SFAs, whereas LCFA elongase 5 (ELOVL5) seems to be the primary hepatic elongase for generating C18:0 and C18:1. Additional elongases and desaturases also contribute to the elongation and desaturation of longer PUFAs (for examples, C18:2 and C18:3). *De novo* synthesized FAs need to be activated by long chain acyl-CoA synthetases (ACSL) to be incorporated into TG and PLs, in which ACSL3 and ACSL5 play a crucial function in the liver.

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1.2.1.2 Phosphatidic acid synthesis

Phosphatidic acid (PA) is the simplest membrane phosphoglyceride and also a central intermediate for the synthesis of membrane and storage glycerolipids. The metabolism of PA occupies a key position in glycerolipid metabolism and membrane biogenesis. Additionally, PA has emerged as a new class of lipid mediator. It is involved in various cellular processes: signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement[7, 8].
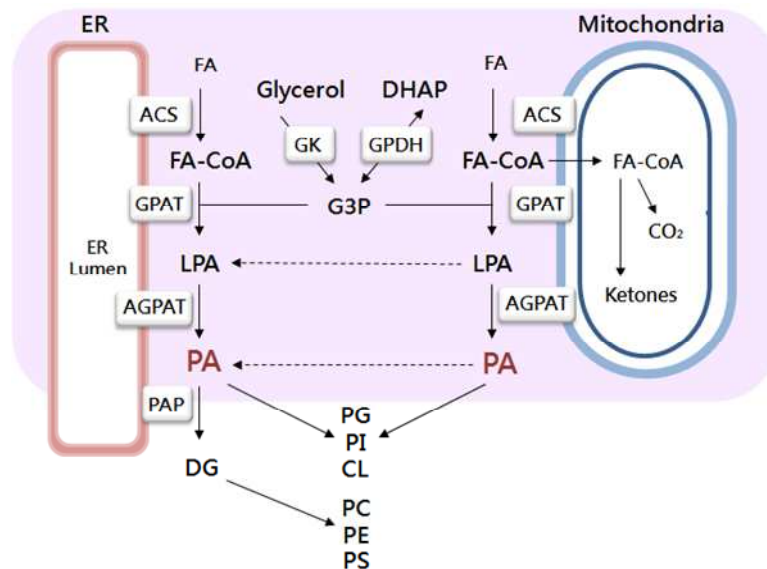
Figure B.4. Phospatidic acid biosynthetic pathway. The pink area emphasizes the biosynthetic pathway of PA as a product of *de novo* synthesis of FAs. It has also been represented the glycerolipids synthesized form PA, which is the precursor molecule. ACS, Acyl-CoA synthetase; AGPAT, acylglycerol-phosphate acyltransferase; CL, cardiolipin; DG, diacylglyceride; DHAP, dihydroxyacetone-phosphate; DHAP-AT, DHAP acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; G3P, glycerol-3-phosphate; GK, glycerol kinase; GPAT, glycerol 3-phosphate acyltransferase; GPDH, glycerol-3-phosphate dehydrogenase; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLC, phospholipase C; PS, phosphatidylserine. Image adapted from Coleman R.A., *et al.*[9].

De novo synthesis of FAs is an intrahepatic source of PA and it is produced and metabolized by several enzymatic reactions, including a synthetase and different acyltransferases, as shown in Figure B.4.

1.2.1.3 Triacylglyceride metabolism

The TGs are insoluble neutral lipids. They are formed by a glycerol backbone and 3 molecules of FAs attached by ester bonds. So the physical and chemical properties of TGs differ based on the nature of their FAs. TGs are the most abundant acylglycerides in the organism and serve important functions in the liver; in fact, a good regulation of TG metabolism is essential to maintain the proper metabolic homeostasis of the organism.

The TG content of hepatocytes is regulated in processes such as the hepatic lipid input, FA and lipoprotein uptake, FA synthesis and esterification, and hepatic lipid output in which FA oxidation and VLDL-TG export are involved.

Acyl-CoA:diacylglycerol acyltransferase (DGAT) is the enzyme that catalyzes the final and specific step in TG synthesis. In the liver there are two isoforms of DGAT enzymes, DGAT1 and DGAT2, associated to ER membranes. Accordingly to some research groups, each of them are even though involved in specific roles. Both enzymes catalyze the same reactions, but some groups have suggested that DGAT1 seems to play a role in VLDL-TG secretion[10, 11], whereas DGAT2 could be implicated in the regulation of liver cytosolic TG content and pools[12]. Another protein involved in cellular TG homeostasis is the lipid droplet (LD) associated protein adipophilin (ADRP or Plin2). It has been demonstrated that the overexpression of this protein increases cellular TG content, whereas a decrease of ADRP increases the sorting of FAs to β-oxidation[13].

Concerning the lipid export from liver, TG, together with cholesterol and PC are secreted in VLDL and transported to peripheral tissues. In hepatocytes, microsomal TG transfer protein (MTTP), together with an adequate supply of lipids are needed for the proper assembly of apolipoprotein (apo) B containing VLDLs. In the bloodstream, the VLDLs diminish in size and are enriched in cholesterol and apoE, forming the VLDLr or intermediate-density lipoprotein (IDL). These IDLs can follow two different ways: a low percentage of these lipoproteins are recognized by apoE and apoB/E hepatic receptors being endocyted, and the rest goes on with the plasma metabolization until they are transformed into low-density lipoprotein (LDL). Therefore, the VLDLs are the precursors of IDLs and LDLs.

Another important function of TG is to generate precursors for PL biosynthesis because TG stores can be partially hydrolyzed to form diacylglycerides (DG), which are precursors of

the major phospholipids (PL) PC, phosphatidylethanolamine (PE), and phosphatidylserine (PS) (Figure B.4). DGs can also be phosphorylated to form PA, needed to generate phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL), (reviewed by Coleman *et al.*)[9].

TG is subjected to continuous hydrolysis and re-esterifications. In the liver, hydrolysis of TG provides products, such as FA and DG for β-oxidation and signaling, as well as substrates for the assembly of VLDL-TG. In the liver there are different proteins involved in TG hydrolysis such as adiponutrin/patatin-like phospholipase domain containing 3 (PNPLA3), some members of the carboxylesterase family (mouse Ces3/TGH, and its human ortholog CES1/TGH; mouse Ces1/Es-x), arylacetamide deacetylase (AADAC), hepatic lipase (HL or Lipc), lysosomal acid lipase (LAL/Lipa) (reviewed by Quiroga *et al.*)[14] and adipose tissue TG lipase (ATGL or Pnpla2), which is expressed in the liver and plays an important role in the liver TG homeostasis[15]. There are also evidences that show the importance that hormone sensitive lipase (HSL/Lipe) has in the liver and not only in TG metabolism but also in cholesterol metabolism[16, 17]. Most of these lipolytic enzymes are glycoproteins associated with the ER. Each of them is supposed to have its own affinity to different TG types, depending on their characteristics (still to be concreted) and different proposed functions in the liver, such as, VLDL assembly, LDs maturation and lipolysis, among others[18]. Re-esterification to give TG provides the cells the availability of regulating the substrates for energy utilization, of regulating the lipid signaling molecules and in the same way the TG storage in LDs, which is a protective mechanism to avoid toxicity of FA and DG.

1.2.1.4 Glycerophospholipid metabolism

In the last decade the role of PLs has gained attention because of their role in the maintenance of liver metabolism homeostasis. PLs are a heterogeneous group of molecules which main function is to form cellular membranes. PLs also function as precursors of second messengers such as DG and inositol-1,4,5-trisphosphate (IP$_3$). A third and usually overlooked function is to provide energy in the form of fatty acyl components[19].

The PLs found in most of the cell membranes are basically GPL. The most abundant GPL types are PC, PE, PS, PI and CL. The GPL molecules contain an apolar, consisting of

diacyl moiety, and a polar portion constituted by a phosphate and a substituted alcohol. The nature of the acyl groups and the type of alcohol group decide the classification, properties, and biological functions of the GPLs. GPL synthesis takes place in the ER and in the inner membrane of the mitochondria in almost all the cellular types.
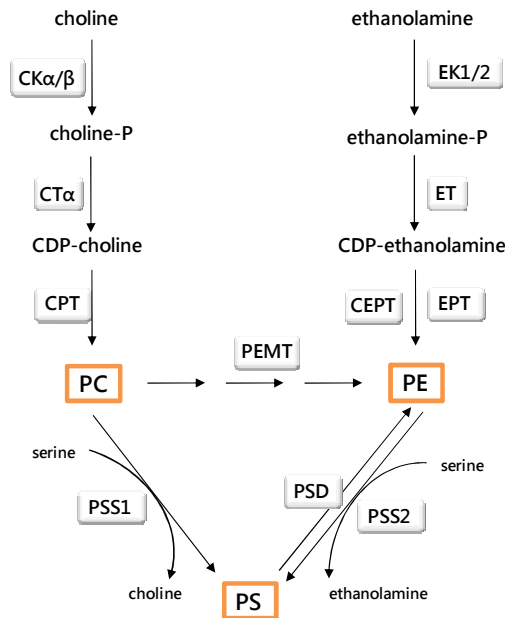
Figure B.5. Liver phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine biosynthetic pathways. CEPT, choline/ethanolamine phosphotransferase; CK, choline k inase; CPT, CDP-choline:l,2-diacylglycerol cholinephosphotransferase; CT, CTP:phosphocholine cytidylyltransferase; EK, ethanolamine kinase; EPT, CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase; ET, CTP:phosphoethanolamine cytidylyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PS, phosphatidylserine; PSD, phosphatidylserine decarboxylase; PSS1, phosphatidylcholine synthase-1; PSS2, phosphatidylserine synthase-2.

There are two different strategies for GPL synthesis and both of them are PA derived. Trough one of them the obtained DG is the direct precursor of PC, PE and PS, whereas in the other one PA is directly converted into cytidine diphophate diacylglycerol (CDP-DG), which will be use to produce PI, PG, and CL.

PC is the most abundant GPL in the liver and can be synthesized by two different pathways: *de novo* or CDP-choline pathway (Figure B.5), which is also known as Kennedy pathway, and phosphatidylethanolamine methyltransferase (PEMT) pathway, which is mainly restricted to liver cells. Evidences shows that ~30% of PC synthesized in the liver is via the

PEMT reaction[20] whereas most of the remaining biosynthetic-derived FA in liver originates via *de novo* pathway. The PC species from these two pathways reveal distinct PC profiles[21], suggesting that a profound distinction of PC profiles may contribute to the different functions of these two pathways in the liver[22].

Nevertheless, PLs also undergo remodeling processes, fatty acyl moieties in the glycerol backbone are replaced by other FAs to form "mature" GPLs and deacylation and reacylation of PLs are involved in the PL remodeling.

1.2.2 Cholesterol and bile acid metabolism

All the nucleated animal cells have the capacity to synthesize cholesterol, but this process is specially intense in liver, suprarenal cortex, skin and intestine. Cholesterol is a crucial and critical molecule in animal for maintaining cell homeostasis.

The liver plays a central role in the synthesis, redistribution, and regulation of whole body cholesterol. In the liver, there are two types of cholesterol, the metabolically active form, which is the free cholesterol (FC), and the inert form, cholesteryl ester (CE), that may be stored, hydrolyzed, or secreted as a lipoprotein component.

The two sources of cholesterol in the organism are the diet and *de novo* synthesis. In humans *de novo* synthesis is the main source, while in rodents is mainly absorbed from the diet.

De novo synthesis or mevalonate pathway starts from its precursor unit acetyl-CoA via a complex metabolic pathway divided in three consecutively phases. These three phases are the formation of mevalonate from acetyl-CoA, the conversion of mevalonate in squalene and finally, the transformation into cholesterol. *De novo* synthesis pathway is compartmentalized such that intermediates cycle between the peroxisome, cytosol and the ER (reviewed by Faust *et al.*)[23].

The first committed step in sterol synthesis, the NADPH-dependent reduction of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to mevalonate, is catalyzed at the ER membrane by HMG-CoA reductase (HMGCR), the rate limiting enzyme of the cholesterol biosynthetic pathway. This enzyme, which is the most important regulation point of cholesterol biosynthesis but not the unique, is regulated through different mechanisms.

Among them, phosphorylation and dephosphorylation mechanisms controlled by glucagon and adrenalin, or insulin, can inhibit or activate, respectively, the enzyme activity.

Cellular cholesterol synthesis, uptake and processing reactions are regulated in a complex manner by two main nuclear receptor systems, sterol regulatory element binding proteins (SREBP, mainly type 2)[24] and liver X receptors (LXRs). Simply, SREBP activation increases the transcription of gene products that function to increase cellular cholesterol levels, such as HMCGR and LDL receptor (LDLR) (Reviewed by Goldstein *et al.*)[25], whereas LXR activation facilitates reverse cholesterol transport; that is, processes leading to cholesterol removal from peripheral cells and increased biliary sterol secretion, by the transcriptional regulation of several members of the ABC transporter family of proteins, such as, ABCG1, ABCG5/G8 or ABCA1 (Reviewed by Tontonoz et al.)[26].

High cellular concentrations of cholesterol are cytotoxic so there are different mechanisms to avoid cytotoxicity. FC content in the liver is maintained by the equilibrium of input pathways, in which cholesterol synthesis and lipoprotein uptake are involved; and output pathways, which are the formation of BAs, biliary secretion and secretion into lipoproteins. Any excess of FC is compensated by reversible conversion of FC to CE, which esterification process is favorable because the resultant is an inert product highly hydrophobic that can be store in the cytosol inside LDs. The esterification is catalyzed by the enzyme acyl-CoA:cholesterol aciltransferase (ACAT), that has 2 isoforms, ACAT1 and ACAT2, which are present mainly in the liver. The CEs, thus, formed may also constitute part of the secreted neutral lipid core of VLDL[27]. Cytosolic CEs are in a dynamic state, undergoing a constant cycle of hydrolysis and re-esterification[28] and the hydrolysis of CEs is thought to occur catalyzed by cholesteryl ester hydrolase (CEH) enzymes. CEH activity has been reported in the lysosomal, cytosolic and microsomal fractions of the liver.

In hepatocytes cholesterol can also be oxidized to form BAs. The metabolism of cholesterol to BAs represents the major pathway for its elimination from the body, accounting for approximately half of the daily excretion. Bile salts (BS), together with cholesterol and PCs, are transported from the liver across the canalicular membrane of the hepatocytes into the bile and stored in the gallbladder.

BAs repress transcription of the hepatic microsomal cholesterol 7-alpha-hydroxylase (CYP7A1) through farnesoid X receptor (FXR)-mediated induction of small heterodimer

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partner (SHP) protein, which is recruited to the CYP7A1 promoter through interactions with liver receptor homolog-1 (LRH-1), a nuclear receptor activated by PLs, together with hepatocyte nuclear factor 4 α (HNF4α), which is another nuclear receptor, transcriptionally activating CYP7A1[29-31]. Likewise, it has been demonstrated that LRH-1 and HNF4α cooperate in maintaining basal CYP7A1 expression[31]. Therefore, both of them are implicated in BA homeostasis and BA biosynthesis.

After each meal, gallbladder BSs are released into the intestinal tract, efficiently reabsorbed (~95% of BSs) in the ileum, and transported back to the liver via portal blood. In the enterohepatic circulation, a minor fraction (less than 3–5%) of the secreted BAs escapes intestinal reabsorption via fecal excretion and needs to be replaced by *de novo* synthesis[32]. BA synthesis involves 17 different steps divided into two major pathways: the classical and the alternative pathway. The mentioned CYP7A1 is the first rate-limiting step of the classical pathway and of vital importance because it has been demonstrated that in a CYP7A1 deficient mice model the BA pool size is reduced by 75% without any compensation by other BA biosynthetic enzyme[33, 34]. The immediate products of these pathways are referred to as primary BAs. The chemical diversity of the BA pool is further expanded by the actions of anaerobic bacteria in the gut, which convert this primary BAs into dozens of secondary BAs[35]. In humans, the most abundant primary BAs are conjugates of chenodeoxycholic acid (CDCA) and cholic acid (CA)[36, 37], whereas in rodents, conjugated of CA and β-muricholic acid (βMCA) predominate[38, 39]. Tauromuricholic acid (TMC) and taurocholic acid (TCA) are the two most abundant BA species in murine bile. In the intestine conjugated BAs are deconjugated and then dehydroxylated at the 7-α-position to form the secondary BAs.

Concerning BSs flux in the liver, the major BS transporters are the hepatic Na$^+$-dependent taurocholic cotransporting polypeptide (NTCP;Slc10a1), which is a basolateral transporter and the BS export pump (BSEP; Abcb11), which is responsible for the 80% of canalicular BS efflux[40].
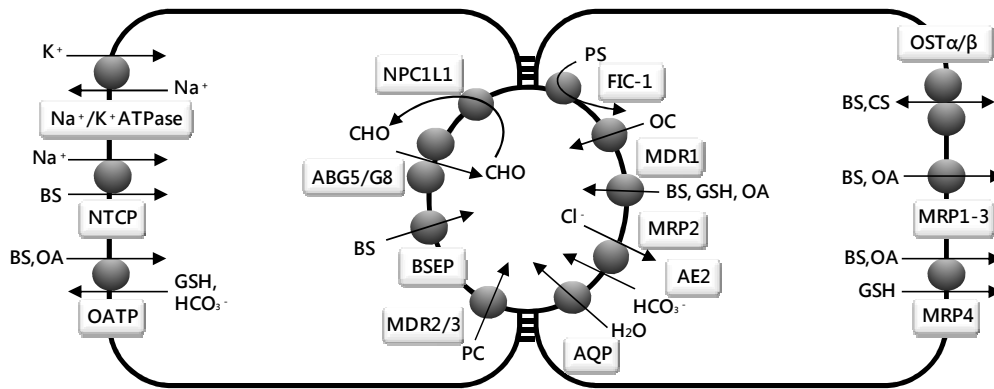
Figure B.6. Hepatobiliar transporters. ABCG5/8, ATP-binding cassette G5 and G8 heterodimers; AE2, Cl⁻/HCO3⁻ anion exchanger 2; AQP, aquaporin; BS, bile salt; BSEP, bile salt export pump; CHO, cholesterol; Cl⁻, chloride anion; FIC-1, PL-transporting ATPase; GSH, glutathione; HCO3⁻, bicarbonate; MDR1, multidrug resistance protein 1; MDR2/3, multidrug resistance protein 2/3; MRP1, multidrug resistance-associated protein 1; MRP2, canalicular multispecific organic anion transporter 1; MRP3, canalicular multispecific organic anion transporter 2; MRP4, multidrug resistance-associated protein 4; NPC1L1, Niemann-Pick C1-like 1 protein; NTCP, sodium/bile acid cotransporter; OA, organic anion; OATP, solute carrier organic anion transporter family; OC, organic cation; OSTα/β, organic solute transporter subunit α/β; PC, phospatidylcholine; PS, phospatidylserine.

Thus, once the BSs have been secreted to the canaliculum, PC and cholesterol would be released from the outer hemimembrane of the canaliculus membrane[41]. A PL flippase so-called multidrug-resistance transporter (MDR3 in humans/ MDR2 in rodents; ABCB4/Abcb4) is the responsible for PLs translocation, specially PC[42], from the inner hemimembrane to the outside. For the cholesterol secretion there are two half-transporters ABCG5 and ABCG8 (heterodimers)[43] which export sitosterol and cholesterol. Moreover, some findings suggest that, in addition to its role in enterocytes, Niemann-Pick C1-like 1 protein (NPC1L1) also functions in hepatocytes to regulate biliary cholesterol concentration[44]. BSs have the capacity to allow the solubilization of cholesterol in bile and other lipophilic compounds, therefore, the formation of micelles acts to maintain cholesterol in solution while at the same time lowers the free (intermicelle) BS concentrations. An adequate bile composition is fundamental, not only to avoid bile stones formation but also to avoid damage on hepatocytes and cholangiocytes.

1.3 Liver disease and disrupted metabolism

There are many kinds of liver diseases, some of them related with viruses (hepatitis B or C), others can be the result of drugs consumption, poisons or drinking too much alcohol, but in the last decade the prevalence of those related with metabolic disorders, such as obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM) is increasing.

1.3.1 Natural history and pathogenesis of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is one of the most common cause of chronic liver disease and impaired liver functions that affects up to 30% of the USA population. It refers to a spectrum of conditions ranging from non-alcoholic fatty liver (NAFL), characterized by simple steatosis without inflammation, to non-alcoholic steatohepatitis (NASH) which can progress to liver cirrhosis and hepatocellular carcinoma (HCC) (Figure B.7). When NAFLD progresses to this late stage, liver transplantation is the only option for treatment because until now there are none specific treatments for the disease[45-48]. NAFLD is associated with obesity, IR, and T2DM that are also connected with disrupted metabolism in the liver[49, 50], which together with dyslipidemia and hypertension, characterize the metabolic syndrome. In fact, around 65-85% of obese patients suffer from NAFLD. However, NAFLD could also develop in non-obese patients[51].

The pathogenesis of NASH is still unclear. A traditional point of view postulates the "double hit" hypothesis which theorized that a "first hit", determined by metabolic factors (obesity, T2DM, dyslipidemia) will lead to hepatic steatosis, which will sensitize the liver to subsequent "second hits", such as oxidative stress, ER stress and proinflammatory CKs, that cause hepatocellular injury and liver inflammation, promoting NASH progression. However, nowadays, there are growing evidences suggesting that simple steatosis and NASH may actually be 2 separate diseases. In this "multi-parallel hit" model[52], the accumulation of "lipotoxic/pro-inflammatory" lipid species interacts with proinflammatory factors to yield NASH since the beginning, while in the other cases the liver develops steatosis and remains free from inflammatory and fibrotic changes[53]. Therefore, there are still contraries in the pathogenesis of NASH that should be clarified.
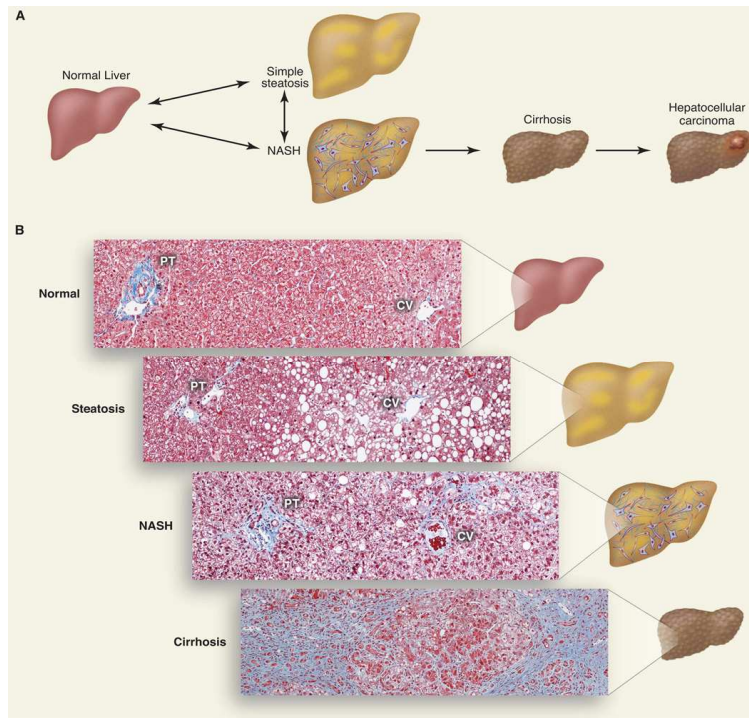
Figure B.7. The disease spectrum of NAFLD. (**A**) Schematic of progression of NAFLD. The accumulation of TG within lipid droplets in hepatocytes causes steatosis. Steatosis associated with inflammation, cell death, and fibrosis is referred to as NASH, which can progress to cirrhosis. Individuals with cirrhosis have an increased risk of hepatocellular carcinoma. (**B**) Histological sections illustrating normal liver, steatosis, NASH, and cirrhosis. Collagen fibers are stained blue with Masson's trichrome stain. The portal triad (PT), which consists of the hepatic artery, portal vein, and bile duct, and the central vein (CV) are shown[50].

NASH is characterized by inflammation and fibrosis mainly induced by toxicity[54, 55] and the release of a variety of CKs, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1 and transforming growth factor β (TGF-β). These CKs are involved in the recruitment of circulating macrophages into the liver and the activation of KCs and HSCs, both of which factors contribute to the progression of NAFLD[56].

1.3.2 Metabolic disruption in NAFLD progression

In NAFLD the normal equilibrium in hepatocyte lipid metabolism is disrupted. In response to different factors such as high serum insulin and glucose, transcription factors SREBPs and ChREBP are activated and induce the expression of genes involved in the synthesis of FAs and cholesterol in the liver, lipids that in excess will cause toxicity.

1.3.2.1 Fatty acid accumulation in liver

FAs are involved in different metabolic pathways, therefore, chronically elevated FAs can disturb metabolic homeostasis in liver leading to development of NAFLD. Hepatic fat accumulation has been strongly associated with IR, as mentioned above, but steatosis can also develop in the absence of IR[57].
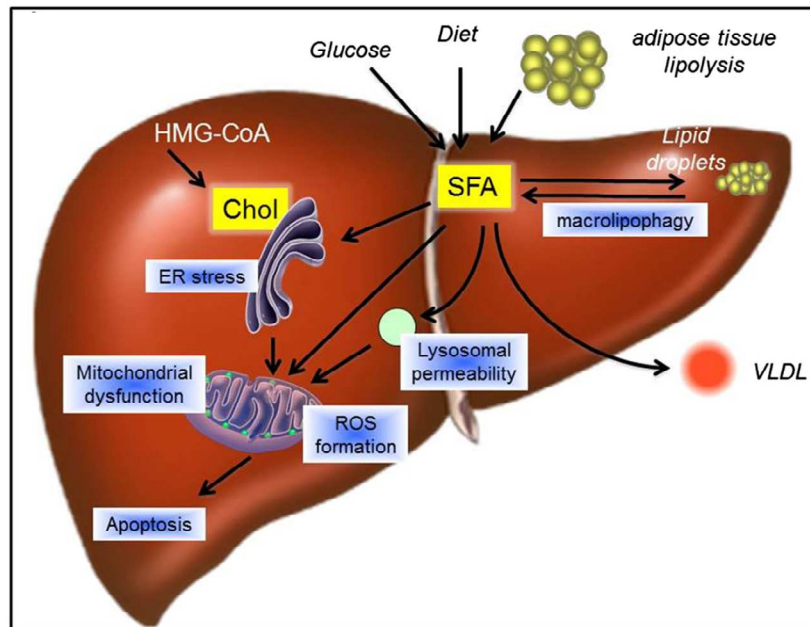
Figure B.8. Lipotoxicity in NASH. Flux and metabolic fate utilization of free FAs that cause injury to the liver. Chol, cholesterol; reactive oxygen species (ROS); saturated fatty acid (SFA); very-low-density lipoprotein (VLDL)[61].

It has been suggested that some types of FAs, such as SFAs cause lipotoxicity, which could be the main cause for NAFLD progression[53, 58-60] (Figure B.8). Lipotoxic injury occurs in the setting of excess FA traffic rather than due to simple TG accumulation. The current theory of lipotoxicity focuses in the damage induced because of the increase in the flux of FAs within hepatocytes exceeding the hepatic capacity to oxidize, store and export FAs as TGs. The net result could be the disruption of macrolipophagy, induction of ER stress and of lysosomal permeabilization, the mitochondrial dysfunction and the generation of toxic lipid metabolites, which predisposes to overproduction of reactive oxygen species (ROS), (reviewed by Sanyal *et al.*[61] and Bechmann *et al.* [60]).

Therefore, one of the main metabolic disruption, which is involved in NAFLD development and progression, is FA accumulation inside de liver; likewise, different studies have been performed to demonstrate which could be the main mechanisms involved. The excess flux of FAs within the liver could be due to a disruption in the major primary sources of FAs due to, just in part, an increase in different FA transporters, such as, FATP2/5, CD36, or VLDL receptor (VLDLR), which are closely implicated in the FA uptake[62-67]. This disruption is also related to alterations in the FA *de novo* synthesis with subsequent changes in some elongases and desaturases, such as, ELOVL6 and SCD1, respectively, that will lead to an increase in the proportion of those FA species which are toxic for the liver and important determinants for NAFLD progression[68-71]. As mentioned before, it has been suggested that this increase in FA flux within the liver will also lead to disrupt macrolipophagy and FA catabolism[60, 72-74]. External factors like the diet will be another very important source of FAs that should be taken into account. It has been demonstrated that high amounts of ingested carbohydrates, preferentially fructose as major stimulus for hepatic *de novo* lipogenesis, and more likely to directly contribute to NAFLD than dietary fat intake[75, 76]. In addition, a high fat diet (HFD) with excessive ingestion of FAs like SFAs and trans-FAs (TFAs) also contribute to the metabolism disruption and therefore to the development of NAFLD. In fact, it has been demonstrated that n-6 PUFAs have the capability of producing proinflammatory eicosanoids, which are harmful. These oxidized PUFAs can cause lipid peroxidation and alteration of PLs composition, resulting in changes in cell membrane fluidity[77]. A high ratio of n-6:n-3 PUFAs in hepatocytes and circulation has also been associated with severity of NAFLD[77].

1.3.2.1 Role of cholesterol and bile acids

Emerging experimental and human evidences have linked altered hepatic cholesterol homeostasis and FC accumulation to pathogenesis of NASH. It has been suggested that FC, rather than TG and FA accumulation, sensitized the liver to some concrete inflammatory CKs (TNF and Fas)-induced murine NASH development[78]. In addition, in NAFLD patients has been proposed that the more fat the liver contains, the higher is the synthesis of cholesterol and the lower is the absorption[79].

In NAFLD patients, the progression of NASH and fibrosis paralleled hepatic FC accumulation. FC accumulation injures hepatocytes directly, by disrupting mitochondrial and

ER membrane integrity, triggering mitochondrial oxidative injury and ER stress, and by promoting generation of toxic oxysterols. Hepatic cholesterol has been suggested to be increased in NASH patients, paralleling both the extent of hepatic FC accumulation and the severity of liver histology, which derives from enhanced SREBP-2 gene transcription[80, 81]. However, the FC increase is not connected with the corresponding increment in CE, suggesting the deregulation of hydrolysis/esterification cycle[80-83].

Cholesterol can be connected with BA through the action of CYP7A1 in the liver. High levels of BAs would increase the risk of hepatotoxicity, causing oxidative stress, inflammation, necrosis, and eventually fibrosis and cirrhosis[84, 85]. During NASH progression, plasma concentration of BAs is increased in patients, concomitant with a remodeling of BA species, including those more hydrophobic and cytotoxic secondary species[86-89]. It has been described that some BA species are increased in liver of NASH patients, which will contribute to the disease progression[86, 90, 91]. Besides, hydrophobic species and their glyco- and tauro-conjugates are highly cytotoxic, and their accumulation in the liver can induce mitochondrial dysfunction, oxidative stress, or apoptosis[92, 93]. In NAFLD there is also a disruption of hepatic BA metabolism and transporters[88, 94, 95]. The major hepatic BA homeostasis regulator FXR is involved in NAFLD progression, in fact, FXR activity is decreased and inversely related to the severity of liver histology[95-97].

2. Liver regeneration after partial hepatectomy

The liver has the capacity to regenerate after various types of injuries. The proliferation of all existing cell types ensures the replenishment of liver and bile duct mass and architecture[98-100].

There are different approaches to study liver regeneration in animal models. Nowadays one of the most studied model for liver regeneration is partial hepatectomy (PH), because it gives the possibility to study cell, tissue and organ regeneration. It has been established that the regenerative response in liver is proportional to the mass of lost tissue for resections that remove up to 70% (2/3) of the liver.

2.1 Phases and extracellular signals

Liver regeneration after partial hepatectomy (PH) has been studied extensively since the 19th century, establishing the long-standing model that hepatocytes, which account for most of the liver weight, proliferate to recover the original mass of the liver but also the rest of existing mature cellular populations that compose the intact organ.
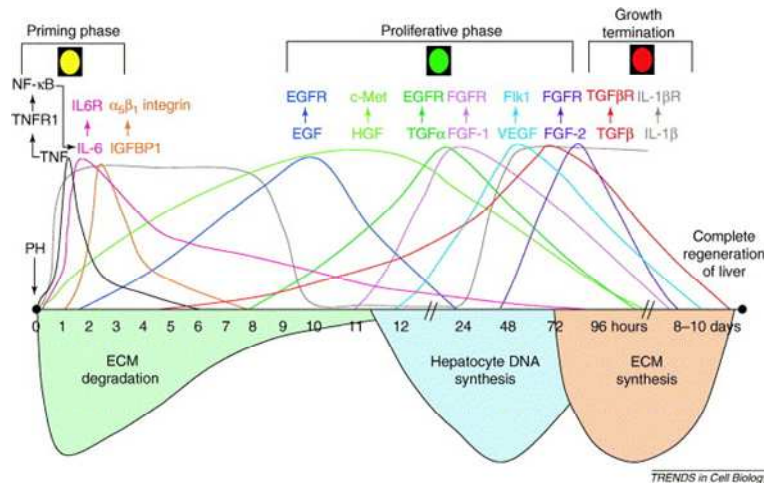

Figure B.9. Schematic of the 'start and stop' signals during liver regeneration in mice. Hepatocyte proliferation during liver regeneration involves initial cytokine induction of the G0–G1 transition (yellow light for caution), followed by growth-factor-induced progression through the G1 restriction point (green light for 'go'). Hepatocyte proliferation is halted by inhibitory factors TGFβ and IL-1β (red light for 'stop'). Factors such as VEGF and FGF might induce angiogenesis once proliferation has occurred. ECM degradation (green shaded area) occurs in the first few hours before hepatocyte DNA synthesis and division (blue shaded area), and hepatic ECM is re-established (orange shaded area) once hepatocytes have divided. Extracellular matrix (ECM), endothelial growth factor (EGF), EGF receptor (EGF), HGF receptor (c-Met), fibroblast growth factor (FGF), VEGF receptor (Flk1), hepatocyte growth factor (HGF), insulin-like growth factor-binding protein 1 (IGFBP1), interleukin (IL), nuclear factor kappa B (NFKB), partial hepatectomy (PH), tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF)[101].

In contrast to cells from proliferative tissues, hepatocytes are cells resting in G_0 phase (quiescent). When regeneration starts, normally quiescent liver cells go through a series of well-orchestrated steps within distinct and recognizable stages. The first one is the priming stage, which in rodents takes place during more or less the first 4 hours after PH in which the progression from G_0 to G_1 occurs and in which initial CK induction happens. This phase initiate in response to an increase in CKs such as TNFα, which binds its type I receptor (TNRF1) on KCs, leading to the activation of nuclear factor kappa B (NFKB)[102, 103]. In addition, C3a, C5a, and MyD88 also can activate NFKB as well as TNFα [104, 105]. The

induction of NFκB in KCs leads to the expression of IL-6 and also of TNFα. Subsequently, IL-6 and TNFα are released and bind to their receptors on hepatocytes activating different signaling pathways that will allow the initial phase progression. The insulin-like growth factor binding protein-1 (IGFBP-1) has also an important role in this initial phase and is rapidly induced, concretely 1 h, after PH[106]. However, IL-6 stimulation in the first stage of liver regeneration can supersede the effect of insulin and stimulate IGFBP-1 expression[107]. IGFBPs can bind to the fibronectin receptor (α5β1 integrin) suggesting that IGFBP-1 may support liver regeneration at least in part via its effect on signaling pathways related to the phase progression[108].

After cell cycle entry, the primed hepatocytes progress through the cell cycle in response to growth factors. Hepatocyte growth factor (HGF), mainly synthesized by HSCs, is considered the central stimulatory signal for the hepatocyte cell cycle progression[99]. The binding to the corresponding receptors promotes a signaling cascade that drives transition from G_1 to S phase. DNA replication starts approximately 34 h and reaches a maximum at 44 h after PH in mice[109, 110]. Concerning non-parenchymal cells, the proliferative phase starts shortly after (24 h) hepatocyte proliferation and enter into DNA synthesis (reviewed by Michalopoulos *et al.*)[99]. There are evidences that demonstrate that normal entrance of hepatocytes into the replicative process does not occur in a synchronized way[111-113], suggesting that a proliferation wave first affects the areas of the lobules surrounding the portal triads (periportal hepatocytes) and then moves towards the perivenous zone of the liver acini.

DNA synthesis is mostly complete by 72 hours post-hepatectomy, when the last and third phase starts. This phase is known as the remodeling phase in which liver recovers its architecture due to the activity of the endothelial cells, the biliary epithelial cells and the synthesis of the extracellular matrix. Finally, there is a growth termination phase by which quiescent and inhibitory factors like TGFβ produced by HSCs, and activin A, produced by hepatocytes, inhibit proliferation processes.

2.2 Lipid changes during liver regeneration

Essential for the successful initiation and completion of liver regeneration is that the remaining cells within the liver acquire sufficient energy substrate and metabolic precursors

to support the metabolic demands of rapid proliferation, which involves an immense metabolic remodeling.

During liver regeneration, plasma TG and cholesterol levels significantly decrease, whereas there is a dramatic increase of lipids in the liver. During the early stages of liver regeneration, hepatocytes accumulate a significant amount of lipids in LDs, which are mainly composed of TGs[114, 115]. A study based on imaging mass spectrometry (IMS) revealed the existence of characteristic changes and a dynamic distribution of multiple TG and PL species during the G_1 phase of the regenerating liver (24 h post-PH)[116].
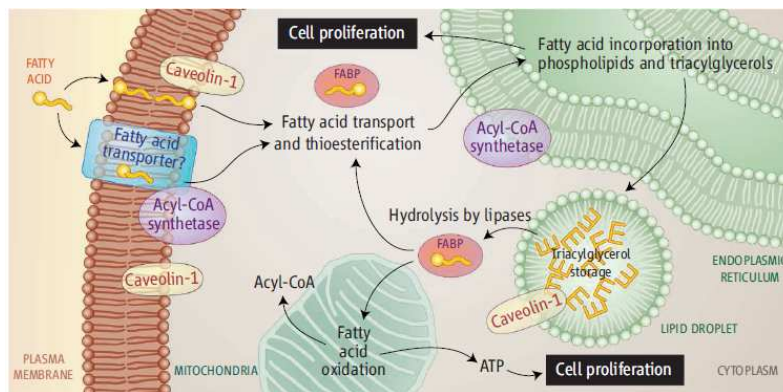
Figure B.10. Triglyceride metabolism during liver regeneration. FABP, FA binding protein[117].

It has long been recognized that the regenerating liver generates signals that couple FA release from the adipose tissue to increased hepatic FA uptake, which in turn promotes hepatic lipogenesis[118]. In fact, hepatocellular fat accumulation in G_1 phase, concretely between 12 and 24 h after PH, is specifically regulated and may be essential for normal liver regeneration[119]. TG accumulation in large LDs is transient with a peak in the G_1 phase[115]. There are some evidences suggesting that this transient steatosis is likely due to both, an increase of lipogenic activity and altered lipid transport balance into and out of the hepatocyte[115, 116, 118]. Likewise, during the first hours of the G_1 phase there is also an increase in the TG hydrolysis by cytosolic lipases that releases FAs for mitochondrial β-oxidation, which is coupled to the production of energy (ATP). Additionally, fatty acids and partially hydrolyzed lipids are ready substrates for the biosynthesis of membrane phospholipids that are required to support rapid cell division[117].

Concerning cholesterol metabolism, its content increases after PH and after cell division begins[120, 121], which is mainly stored as CE in LDs. During the entire proliferation phase, starting from 12 h and until 72 h after PH, when preexisting cholesterol has become insufficient to meet the cellular demand, an increase in HMGCR occurs with a subsequent increase in *de novo* synthesis[120]. In spite of this increase, LXR activity is diminished during liver regeneration which could be crucial to guarantee the intracellular cholesterol levels of regenerating hepatocytes[121]. Cholesterol accumulation could be also due to the lipid uptake, because there is an overexpression of some lipid receptors, such as LDLR and FABP4, during the first 24 h post-PH[116, 122]. LDLR has been shown to be an important gene not interfering with LDs formation but being associated with the hepatic lipidome remodeling during PH[123]. Therefore, cholesterol is essential to perform an adequate liver regeneration which has led to suggest that cholesterol acts as a regulator of cell cycle progression[124, 125].

BA metabolism is also modified in liver regeneration with clear variations depending on the regeneration phase. Shortly after partial resection, BA concentrations fluctuate in the remaining liver and in serum, so it is essential that BAs return back to the remnant liver[126-128], suggesting that BA flux is necessary for initiation of normal liver regeneration. In this situation, the biliary concentration of BAs and the bile flow normalized to the remaining liver tissue are increased. Therefore, in the first hours of the priming phase, biliary lipid secretion is also profoundly modified and increased[129]. In a later phase (24 h post-PH), it has been described a transient increase in BA bile output, together with a markedly hepatic BA decreased until the liver is completely restored[130], which is accompanied by a sharp repression of Cyp7a1 gene expression[131]. In fact, this decreased on BA synthesis is known to be beneficial for liver regeneration progression because it is important to control the hepatic BA levels for liver protection. During DNA replication, bile formation is also reduced returning to normal levels when the liver is almost restored[132]. All this metabolic changes occur together with adaptive changes of the gene expression of the BA basolateral transporters NTCP and OATPs and of the canalicular transporters BSEP and MRP2[133, 134], which might account for the decreased amounts of BAs inside the liver, along G_1 and S phase, until 48 h after PH.

3. Osteopontin

3.1 Structure, localization and function

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), early T lymphocyte activation (ETA-1) or bone sialoprotein-1, was originally isolated from the mineralized matrix of bovine bone[135]. This glycoprotein has a multidomain structure and different functional characteristics of a matricellular protein or of a proinflammatory soluble CK[136-138].

OPN is typically a monomer of approximately 300 amino acids and with a molecular weight ranging from 40-80 kDa due to varied post-translational modifications, such as phosphorylation, glycosylation, sulfation or enzymatic cleavage and as a result of alternative splicing and translation (reviewed by Gimba *et al.*)[139].

Concerning liver OPN variants, OPN can remain intracellular (iOPN) or secreted (sOPN); hence, allowing autocrine and paracrine signaling[140, 141].
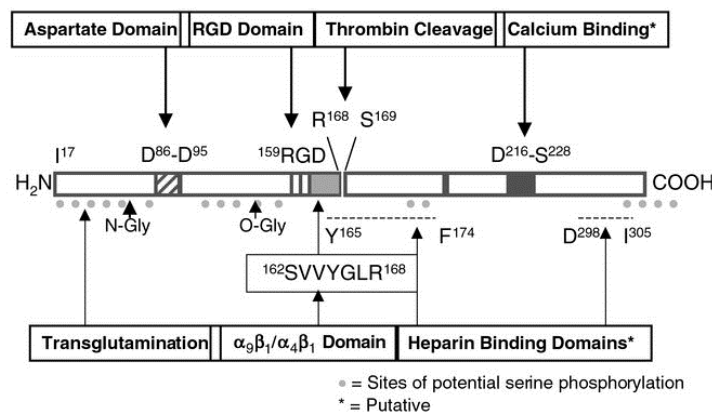
Figure B.11. The structure of osteopontin. Indicated sites of O-glycosylation and phosphorylation are intended to be representative; both vary (phosphorylation in particular) with the source of the protein. Numbering of the amino acids is based on the human protein, and the signal sequence is not illustrated[152].

Once OPN is secreted, it is proteolytically cleaved[142, 143] (Figure B.11), mainly by thrombin, which cleaves OPN into two fragments of equivalent size to display integrin and CD44 binding domains[144]. The arginine-glycine-aspartate (RGD)-motif engages a subset of cell surface integrins ($\alpha v/\beta 3$, $\alpha v/\beta 1$ and $\alpha v/\beta 5$, and $\alpha 8/\beta 1$)[145, 146]; the cryptic SVVYGLR

(SLAYGLR in mouse OPN) containing domain that interacts with other integrins (α9/β1, α4/β1 and α4/β7)[147-149], and the ELVTDFTDLPAT domain has been reported to bind to integrin α4/β1[150]. The CD44-binding site has been mapped to the N-terminal and the C-terminal portion of OPN[151]. These receptors directly or indirectly activate cellular signaling pathways, allowing OPN to mediate cell-matrix, and possibly cell-cell, interactions.

OPN is one of the most abundant non-collagenous, non-specific proteins in bone but it can also be expressed and located in a variety of tissues, including the liver and the adipose tissue, and besides blood in biological fluids such as milk or urine[149]. Concretely in the liver, OPN is expressed in parenchimal and non parenchimal cells including biliary epithelial cells[128, 153-156].

Concerning the functions of OPN, it is mainly involved in cell migration and adhesion, tissue remodeling and calcification, and inflammatory processes including macrophage activation[152, 157]. In addition, it has been shown that OPN expression is increased in tumorigenesis, angiogenesis and in response to cellular stress and injury[158-161].

3.2 Liver disease and osteopontin

It has been described that OPN is associated with different liver disorders[160, 162]. Within the liver, increased OPN expression has been mainly found in hepatocytes and inflammatory cells[163]. Notably, OPN is markedly upregulated in the liver in obesity, and hepatic OPN levels correlate with liver TG levels[164, 165].

In addition, different studies in mice models and in humans suggest a role for OPN protein in the pathogenesis and progression of NAFLD[166, 167] and in liver fibrosis[168]. Moreover, it has been demonstrated that OPN deficiency, avoids the disease progression protecting against HFD-induced liver injury and against obesity-induced hepatic steatosis[163, 165, 169].

OPN has also been connected to the progression to HCC. OPN levels are increase in patients with HCC[170] and OPN is also overexpressed in HCC tissues of different etiologies[171]. Recently, OPN has been implicated in the metastasis of HCC promoted by HSCs[172, 173]. In addition, it has been demonstrated that there is a relation between the

expression profile of OPN isoforms and HCC; OPN splice variants differentially couple to signaling pathways to modulate the migratory property of HCC cells[174].

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