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Targeting metabolism for resolving Non-Alcoholic Steatohepatitis

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TABLE OF CONTENTS

RESUMEN

RESUMEN

La enfermedad crónica del hígado comprende un amplio grupo de patologías hepáticas de diferente etiología caracterizadas por una lenta progresión de la enfermedad. A su vez, puede dar lugar al desarrollo de etapas tardías de la enfermedad tales como la cirrosis o el carcinoma hepatocelular (CHC) [1–3], dos de las patologías con mayor mortalidad en EEUU y Europa. Entre sus causas más frecuentes se encuentra la enfermedad del hígado graso no alcohólica (EHGNA) [2–4].

La EHGNA es un término empleado para designar un amplio espectro de patologías que comprenden la esteatosis, la esteatohepatitis no alcohólica (EHNA) y la cirrosis. La esteatosis se caracteriza por una acumulación de lípidos intrahepática y, aunque sea una patología de carácter benigno, puede dar lugar entre el 10 y 30% de los casos a inflamación, muerte celular y fibrosis. Esta patología recibe el nombre de EHNA y, aunque es de carácter reversible, un desarrollo de fibrosis crónica puede progresar a cirrosis en aproximadamente el 20% de los casos [1]. Actualmente la hipótesis más aceptada a la hora de explicar la progresión de la enfermedad es la implicación de los dos *hit,* donde un primer *hit* induce una acumulación de lípidos en el hígado que puede desembocar en complicaciones derivadas que contribuyen a su progresión desde esteatosis a EHNA. Estas complicaciones o segundos *hits* consisten la acumulación de especies lipídicas tóxicas, disfunción mitocondrial e incrementos en el estrés de retículo endoplasmático (ERE) y oxidativo [5]. Finalmente, el riesgo de desarrollar carcinoma hepatocelular (HCC) aumenta en gran medida en presencia de estas patologías [6].

A su vez la EHGNA está asociada con otras comorbilidades con una gran prevalencia; tales como la obesidad, diabetes de tipo 2, resistencia a insulina, dislipidemia y enfermedades cardiovasculares [3,7–12]. Su incidencia se ha estimado entre 20 y 30% de la población mundial, manifestándose particularmente en los países occidentales. Además, el aumento de la tasa de obesidad y los hábitos de vida diarios están favoreciendo la expansión de la EHGNA, convirtiéndola en un problema de salud global [10,13]. A día de hoy las principales terapias de EHGNA se centran en reducir la acumulación de lípidos y sus complicaciones derivadas: reducir la inflamación y daño hepáticos, mejorar la resistencia a la insulina y revertir la fibrosis [14]. *Elafibranor* y *liraglutide,* con resultados prometedores en fase IIb, y el ácido obeticólico, con resultados positivos en fase III [15], son las terapias en fase más avanzadas de ensayo clínico.

La desregulación del metabolismo en la patofisiología hepática es el proyecto general que se desarrolla en *Liver Disease Laboratory*, el grupo de investigación donde se ha realizado esta tesis. Entre estas alteraciones nos hemos centrado en el metabolismo del nitrógeno, y especialmente la glutamina como principal vía anabólica del amonio, y la homeostasis del magnesio (Mg^{2+}) en la enfermedad hepática EHNA y su progresión a estadios más avanzados, que serán los dos objetivos fundamentales de esta tesis doctoral.

En relación al metabolismo de nitrógeno, la hiperamonemia se caracteriza por un aumento de los niveles de amonio en sangre. Esta condición está causada por una desregulación en el metabolismo del amonio y se ha relacionado con el desarrollo de enfermedades hepáticas [16]. El hígado es el principal órgano responsable de la homeostasia de amonio. Los enzimas del ciclo de la urea y glutamina sintetasa, situados en las zonas periportal y perivenosa respectivamente, son los encargados de conjugar el amonio produciendo urea y glutamina [17,18]. Por otro lado, la glutaminasa (GLS) situada principalmente en la zona periportal, cataliza la degradación de glutamina (Gln) produciendo amonio y glutamato (Glu). Además del hígado, el intestino también contribuye a la producción de amonio. Alteraciones de la microbiota intestinal se han relacionado con el desarrollo de enfermedades hepáticas como la EHNA o cirrosis, donde se ha observado un aumento de la proporción de bacterias responsables del metabolismo de la urea [19,20]. No obstante, se ha descrito que la mayor producción de amonio en pacientes con cirrosis proviene de la deaminación de la Gln, catalizada por la glutaminasa [21].

Como anteriormente se ha mencionado, la GLS es el enzima responsable del catabolismo de la glutamina (Gln) a glutamato y amonio. La Gln se ha descrito como un regulador del estado de oxido-reducción de la célula [22], de modo que alteraciones en su metabolismo podrían estar relacionadas con las desregulaciones metabólicas observadas en la EHNA, donde estado hipermetabólico desencadena los segundos *hits* mencionados anteriormente [5]. En relación a lo mencionado, el análisis metabolómico realizado en el suero de una amplia cohorte de pacientes muestra un descenso del ratio Gln/Glu en aquellos pacientes de EHNA comparado con individuos sanos, sugiriendo una alteración en el catabolismo de la glutamina con GLS como principal enzima afectado. Además se ha descrito previamente que la isoforma de alta afinidad (GLS1) se induce respecto a la isoforma GLS2 en carcinoma hepatocelular (CHC) y otros tipos de cáncer [23–25] donde, frente a un aumento de la actividad del ciclo de Krebs para mantener el balance energético y proliferativo [26,27], la Gln sirve como sustrato energético y fuente de carbono [28–31].

También se ha descrito una inducción de GLS1 en cirrosis [26] con un importante papel en la activación de células hepáticas estelares [32] promoviendo el desarrollo fibrótico. En base lo expuesto anteriormente, nuestro primer objetivo ha sido analizar la implicación del metabolismo de glutamina, y su regulación mediada por GLS1, en la EHNA.

Por otro lado, una mayor ingesta de Mg^{2+} se ha asociado con una menor mortalidad debido a complicaciones hepáticas [33]. El magnesio es el catión divalente más abundante en la célula y actúa como cofactor de reacciones enzimáticas en las que se metaboliza ATP, de modo que es esencial para la correcta actividad de enzimas relacionadas con el metabolismo energético y de ácidos nucleicos [34]. Las alteraciones metabólicas características de la EHNA sugieren también posibles perturbaciones en los niveles de Mg^{2+} en el hígado. Aunque no se hayan realizado estudios previos donde se relacionen alteraciones en la homeostasia del catión con el desarrollo de EHGNA, sí se ha caracterizado la relación entre la hipomagnesemia y varias comorbilidades como la resistencia a insulina, complicaciones cardiovasculares y, sobre todo, obesidad [35–38]. Interesantemente, la determinación de Mg^{2+} sérico en una cohorte de pacientes muestra un aumento en aquellos diagnosticados de EHNA, sugiriendo una posible contribución de la homeostasia de Mg^{2+} en el desarrollo de la patología.

La homeostasia del magnesio viene determinada por su flujo a través de las distintas membranas celulares [39,40]. Sus propiedades físico-químicas, presentando un tamaño radial 400 veces mayor en su forma hidratada respecto a la libre, implican que sean necesarios transportadores para su flujo a través de dichas membranas [41]. Los transportadores descritos hasta la fecha son la ciclina M (CNNM), el transportador de magnesio 1 (MagT1), la familia de transportadores 41 de soluto (SLC41) o el receptor transitorio de potencial (TRPM) [42–46]. Sin embargo, la regulación de estos transportadores concretamente en el hígado, así como su contribución en la modulación de la homeostasis de magnesio, es un tema que todavía por elucidar. La CNNM se ha descrito previamente como parte del interactoma de las fosfatasas de hígado regenerante (PRL) [47], un factor pro-oncogénico descrito en varios tipos de cáncer entre los que se encuentra el CHC [48–50]. Esto sugiere una posible contribución de las proteínas de la familia CNNM en la enfermedad hepática, por lo que en el presente trabajo nos hemos centrado en determinar la contribución de CNNM al desarrollo de la EHNA, su papel en la homeostasis de Mg^{2+} hepática y los efectos de su modulación en la enfermedad.

El objetivo de esta tesis, en resumen, es determinar la contribución de GLS1 y CNNM en el metabolismo de la glutamina y la homeostasia del magnesio respectivamente, así como su papel en el desarrollo y progresión de la EHNA. Para ello se han analizado muestras de suero y tejido de pacientes sanos y diagnosticados de esteatosis y EHNA. También se han empleado modelos animales de la enfermedad, basados en la alimentación de ratones con distintas dietas: una dieta deficiente en colina con 0.1% de metionina (0.1%MCDD) y una rica en grasas deficiente en colina (CD-HFD). El primer modelo 0.1%MCDD desarrolla EHNA debido a una disrupción en la síntesis de lipoproteínas de muy baja densidad (VLDL), de modo que un defecto en el exporte de lípidos induce su acumulación en el hígado con las consecuentes alteraciones metabólicas [51]. Cabe mencionar que la disrupción de la síntesis de VLDL también es característica de los ratones deficientes del gen *metionina adenosiltransferasa 1a (Mat1a)*, los cuales desarrollan EHNA con un patrón metabólico de subtipo M similar a más de la mitad de los pacientes [52]. El segundo modelo CD-HFD desarrolla la EHNA con un patrón similar al de los humanos con un característico aumento de peso, desarrollando también resistencia a insulina y demás comorbilidades [53]. En ambos modelos los ratones de han alimentado con 0.1%MCDD o CD-HFD durante dos y tres semanas, respectivamente, cuando la patología ha sido inducida. Se ha realizado un silenciamiento especifico en el hígado inyectando por la vena de la cola un siRNA específico contra *Cnnm4* (si*Cnnm4*), *Gls1* (si*Gls1*) o inespecífico (siCtrl). El tratamiento se ha realizado mediante dos pinchazos semanales en el caso de si*Gls1* y un pinchazo semanal en si*Cnnm4*, sacrificando los ratones 0.1%MCDD y CD-HFD a un tiempo final de 4 y 6 semanas respectivamente. Los resultados obtenidos en los ensayos clínicos *in vivo* se han reforzado con estudios *in vitro* en hepatocitos primarios estimulados con un medio deficiente en metionina y colina (MCD) o ácido oleico (OA) y células humanas THLE2.

Centrándonos en el metabolismo de la glutamina, en el presente trabajo se ha caracterizado una sobre-expresión de GLS1, el principal enzima implicada en su catabolismo, en muestras de pacientes con EHNA, además de en los modelos animales mencionados anteriormente, donde se ha observado un aumento progresivo de GLS1 asociado a los diferentes estadios de la enfermedad. Además, en los estudios preclínicos de 0.1%MCDD y CD-HFD se ha observado que el aumento de lípidos inducido por la dieta es revertido con el tratamiento de si*Gls1*. En concordancia, la acumulación de

lípidos inducida en hepatocitos primarios al tratarlos con MCD y OA también es revertida al silenciar el enzima, demostrando su contribución en el desarrollo de la enfermedad.

Como se ha mencionado anteriormente, los ratones alimentados con 0.1%MCDD desarrollan EHNA a causa de un exporte de VLDL defectuoso [51]. Esto se debe a que la privación de colina induce un descenso en los niveles hepáticos de fosfolípidos (fosfatidilcolina en particular), moléculas esenciales durante la formación de VLDL [54]. Además, el déficit de metionina reduce los niveles de s-adenosilmetionina (SAMe) en el hepatocito, sustrato para la síntesis de fosfatidilcolina [55]. Sin embargo, el tratamiento de hepatocitos primarios con siRNA contra *Gls1* restaura los niveles de fosfolípidos reducidos al estimular las células con MCD. Además, la inhibición del exporte mediante lomitapide, un inhibidor de la proteína transferente de triglicéridos microsomales (MTP), revierte la reducción de lípidos inducida al silenciar *Gls1* en dichas células. Por otra parte, el tratamiento con si*Gls1* a ratones alimentados con 0.1%MCDD aumenta los niveles de fosfatidilcolina y fosfatidilserina en el hígado. En la determinación bioquímica de la composición de VLDL, aisladas de ratones tratados con P407 para inhibir su captación por parte de la lipoprotein lipasa (LPL) [56], se observa también una restauración de fosfatidilcolina y fosfatidiletanolamina en las VLDL, junto con un aumento de los niveles de triglicéridos y colesterol libre. En resumen, la resolución de la esteatosis se debe a una restauración en la formación de VLDL debido a un aumento de los niveles de fosfolípidos en el hígado.

El silenciamiento específico de *Gls1* también ha demostrado disminuir el estrés oxidativo tanto en ensayos *in vitro* como *in vivo*. Se ha caracterizado que el efecto observado al silenciar el enzima se debe a un descenso de la actividad oxidativa por parte del hepatocito, el cual presenta una menor actividad de oxidación de ácidos grasos, ciclo de Krebs y cadena transportadora de electrones. Al encontrarse reducida la actividad de dichas rutas metabólicas, la producción de ROS es menor, por lo que la síntesis de glutatión reducido [57], inducida en ambos modelos de EHNA, disminuye a causa del tratamiento con si*Gls1*.

En la ruta de transsulfuración la cisteína se conjuga con glutamato y glicina para dar lugar a glutatión reducido [57]. La cisteína proviene de la conjugación de la serina con la homocisteína, la cual puede actuar como sustrato para síntesis de cisteína o ser remetilada en el ciclo de la metionina, cuya disminución de actividad se ha descrito ampliamente en la EHNA [58,59]. En los ratones alimentados con 0.1%MCDD y tratados con si*Gls1* se

ha observado un descenso de la expresión a nivel de mRNA en los enzimas relacionados con la vía de transsulfuración, así como una inducción de los enzimas implicados en el ciclo de la metionina y los folatos. No obstante, también se ha observado un aumento de la expresión de aquellos enzimas relacionados con la síntesis de fosfatidilcolina y fosfatidilserina. De este modo, los resultados señalan que la disminución de los enzimas implicados en la ruta de transsulfuración conllevan una mayor disponibilidad de serina, la cual promueve la expresión de enzimas implicados en la síntesis de fosfolípidos. Un mayor contenido de fosfatidilcolina y fosfatidilserina en el hígado restaura los niveles de fosfolípidos, triglicéridos y colesterol libre secretados en forma de VLDL, promoviendo la resolución de la esteatosis.

En resumen, el presente trabajo demuestra la contribución de GLS1 en el desarrollo de NASH. El tratamiento mediante siRNA específico ha demostrado ser una terapia potencial efectiva, la cual reduce la actividad oxidativa reduciendo la producción de ROS y restaurando el ensamblaje de VLDL promoviendo el exporte de lípidos.

En relación a la contribución de CNNM4 en el desarrollo de la EHNA, se ha caracterizado una alteración en los niveles de mRNA de *Cnnm1* y *Cnnm4* en pacientes. Sin embargo, la contribución de CNNM4 en el desarrollo de la enfermedad se ha demostrado mediante un cribado *in vitro* en hepatocitos primarios estimulados con MCD y silenciando cada isoforma por separado, observando una reversión de la acumulación de lípidos solamente al tratarlos con si*Cnnm4*. También se ha caracterizado una sobre-expresión a nivel de mRNA y proteína en los modelos animales 0.1%MCDD y CD-HFD, y a nivel de proteína en una cohorte de tejidos de pacientes humanos de EHNA. Mediante dos ensayos pre-clínicos, siguiendo el procedimiento mencionado anteriormente, se ha demostrado la contribución de CNNM4 en el desarrollo de la EHNA, observando que la acumulación de lípidos y desarrollo de fibrosis inducidos al alimentar a los ratones con 0.1%MCDD y CD-HFD, durante 4 y 6 semanas respectivamente, se reducen al silenciar CNNM4.

Anteriormente se ha mencionado que, aunque CNNM4 se ha descrito como un regulador de la homeostasia de magnesio [46], su rol en el hígado todavía está por elucidar. Para ello se han realizado varios estudios *in vitro* en hepatocitos primarios, donde el magnesio de distintos orgánulos se ha marcado específicamente mediante dos sondas específicas denominadas Mag-S-AM, la cual se une con alta afinidad al Mg^{2+} presente en todos los compartimentos celulares [60], y Mag-S-TPP-AM, en la que una modificación mediante un grupo fosfonio le permite unirse únicamente al catión presente en la matriz

mitocondrial. Basándonos en la intensidad de fluorescencia emitida por la sonda unida y libre a distintas longitudes de onda, se han determinado la cantidad de magnesio dentro del hepatocito. Mediante este estudio se ha demostrado el papel de CNNM4 como un extrusor de Mg^{2+} en el hígado, observando una acumulación del catión al silenciar la proteína y una disminución de Mg^{2+} cuando CNNM4 se sobre-expresa, tanto por un estímulo con MCD o mediante un vector de expresión.

El estrés oxidativo y el estrés del retículo endoplasmático (ERE) se han relacionado estrechamente con el desarrollo de la EHNA [5,61]. El retículo endoplasmático actúa como reservorio de calcio (Ca^{2+}) gracias al transporte activo mediado por ATPasas [62]. Sin embargo, mediante una sonda específica de Ca^{2+} FURA-2 [63] y el método Grynkiewicz [64], se ha observado un descenso de la capacidad de liberación por parte del retículo en condiciones de MCD y una restauración al tratar los hepatocitos con si*Cnnm4*. Esto sugiere que el silenciamiento de *Cnnm4* implica una reducción del ERE, lo que se ha confirmado determinando la expresión de distintos marcadores en hígados de los ratones de ensayos pre-clínicos, observando una reversión al tratar los ratones con si*Cnnm4*. También se han determinado los niveles de ROS mitocondrial *in vitro* y el estrés oxidativo en los ensayos pre-clínicos, observando que la inducción al desarrollar EHNA se revierte con la terapia de siRNA. En resumen, los resultados señalan que el silenciamiento de *Cnnm4* reduce los niveles de ERE y estrés oxidativo en el hígado.

En el retículo endoplasmático se encuentra la proteína transferente de triglicéridos microsomales (MTP), la cual cataliza la formación de pre-VLDL y la maduración a VLDL [54,65]. La MTP es un heterodímero de dos subunidades: una subunidad M participa en la transferencia de lípidos durante la formación de la partícula y la subunidad P cataliza la formación de puentes disulfuro [66]. Sin embargo, la subunidad P también tiene una función co-chaperona [67,68], lo que sugiere que una situación de ERE pueda afectar a la actividad del heterodímero MTP. Al determinar la actividad de la proteína se observó tanto en los modelos *in vitro* como *in vivo* que la terapia con si*Cnnm4* induce un aumento de la actividad MTP. Este resultado concuerda con el aumento de expresión de apolipoproteína B100 (apoB100), observado al determinar la cantidad de la proteína en los sueros de ratones alimentados con 0.1%MCDD y CD-HFD y tratados con el siRNA, ya que la proteína se co-traduce simultáneamente con la formación de la VLDL catalizada por la MTP. Además, el análisis bioquímico de las VLDL determina una restauración parcial del contenido lipídico de las mismas al tratar los ratones con si*Cnnm4*, junto con

una tasa de secreción ligeramente mayor. La inhibición de la MTP con lomitapide [69]en hepatocitos primarios tratados con MCD y/o si*Cnnm4* confirma que la reducción de los lípidos observada al silenciar *Cnnm4* está siendo mediada por un aumento del exporte de VLDL, en este caso inducido por una mayor actividad de la MTP.

Se ha determinado que el tratamiento de los ratones alimentados con 0.1%MCDD con si*Cnnm4* induce una restauración del Mg^{2+} en las VLDL secretadas. Como se ha mencionado anteriormente, alteraciones en los niveles de Mg^{2+} se han relacionado con comorbilidades de la EHNA como la obesidad por lo que alteraciones en la composición de las VLDL secretadas podrían tener un papel en tejidos periféricos. Se ha determinado la actividad oxidativa de ácidos grasos del tejido adiposo, observando una disminución de la actividad oxidativa al desarrollar EHNA y una restauración al silenciar el enzima, posiblemente debido a un aumento de la expresión de distintos enzimas implicados en el proceso. Esto sugiere que la acumulación de Mg^{2+} hepática al silenciar *Cnnm4* conlleva un aumento del catión en las VLDL secretadas, lo que supone un efecto en la actividad oxidativa del tejido adiposo. Los resultados se han corroborado con cultivos de adipocitos primarios estimulados con medio condicionado de hepatocitos o Mg^{2+}. Se abre, por tanto, una nueva perspectiva en el desarrollo de terapias basadas en la modulación del contenido de Mg^{2+} en el tejido mediante las CNNM, no solo de la EHNA sino de sus comorbilidades.

Se ha demostrado la contribución de CNNM4 en el desarrollo de la EHNA. Su inhibición supone una acumulación de los niveles de Mg^{2+} en el hígado y la resolución de la patología, posiblemente debido a un menor ERE y una mayor actividad de la MTP, catalizando el exporte de lípidos en forma de VLDL.

En resumen, el presente trabajo demuestra el papel de GLS1 y CNNM4 en el desarrollo de la EHNA. Ambas dianas se sobre-expresan cuando se desarrolla la patología en pacientes y modelos animales de ratón. Los ensayos pre-clínicos tratando los modelos con siRNA demuestran una reducción de esteatosis y estrés oxidativo y un aumento del exporte de lípidos en forma de VLDL.

Conclusiones

En base a los resultados obtenidos e integrados en esta tesis doctoral, hemos concluido:

1) El enzima Glutaminasa 1 está sobre-expresado en la EHNA, tanto en muestras clínicas como en modelos pre-clínicos de la enfermedad
    a. En el suero de pacientes de EHNA hay ratio glutamina/glutamato reducido
    b. Hay un cambio de la isoforma 2 de la glutaminasa a la isoforma 1
    c. La producción de amonio es amortiguada por una mayor expresión de glutamina sintetasa
2) El silenciamiento dirigido de la Glutaminasa 1 mejora la EHNA *in vitro* e *in vivo*
    a. Una menor expresión del enzima reduce el flujo oxidativo en el hígado, como consecuencia de una actividad reducida en las siguientes vías:
        i. La oxidación de ácidos grasos está inhibida
        ii. El ciclo de Krebs y la fosforilación oxidativa se encuentran reducidos
    b. La reducción en el flujo oxidativo reduce la producción de especies reactivas de oxígeno
    c. La síntesis de glutatión se reduce, conllevando una mayor disponibilidad de serina en el hígado que promueve la síntesis de fosfolípidos
    d. El contenido de las lipoproteínas de muy baja densidad (VLDL) se encuentra enriquecido, promoviendo el exporte de lípidos y reduciendo el contenido de lípidos hepático

3) La Ciclina M4 está sobre-expresada en pacientes de EHNA y modelos pre-clínicos de la enfermedad
    a. La proteína actúa como un extrusor de magnesio en el hepatocito
4) El silenciamiento dirigido de la Ciclina M4 mejora la EHNA, tanto *in vitro* como *in vivo*, reduciendo la acumulación de lípidos en el hígado
    a. En modelos pre-clínicos de la EHNA el desarrollo de fibrosis está revertido
    b. La depleción de Ciclina M4 induce una acumulación de magnesio en el hepatocito
    c. El estrés oxidativo y de retículo endoplasmático se reducen tanto en modelos *in vitro* como *in vivo* de la patología

d. La actividad de la proteína transferente de triglicéridos microsomal está aumentada *in vitro* e *in vivo*, promoviendo la secreción de lipoproteínas de muy baja densidad (VLDL)
e. Las alteraciones de magnesio en las lipoproteínas de muy baja densidad secretadas podrían modular la actividad oxidativa del tejido adiposo blanco

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ABBREVIATIONS

0.1%MCDD	=	0.1% methionine and choline-deficient diet
2-APB	=	2-Aminoethoxydiphenyl borate
5-MTHF	=	5-methyltetrahydrofolate
ABC	=	Adenosine triphosphate-binding cassette
ACAD	=	Acyl-CoA dehydrogenase
ACC	=	Acetyl-coenzyme-A carboxylase
ACDP	=	Ancient conserved domain protein
Acetyl-CoA	=	Acetyl-coenzyme-A
ALT	=	Alanine aminotransferase
AMP	=	Adenosine monophosphate
ANOVA	=	Analysis of variance
AFT	=	Activating transcription factor
AOX	=	Aldehyde oxidase 1
ARF-1	=	ADP-rybosylation factor-1
ASM	=	Acid soluble metabolites
ATP	=	Adenosine triphosphate
AMPK	=	AMP-dependent protein kinase
APO	=	Apolipoprotein
ARG	=	L-arginine
ARP	=	Actin related protein
αSMA	=	Alpha-smooth muscle actin
AST	=	Aspartate aminotransferase
BCLC	=	Barcelona Clinic Liver Cancer
BDL	=	Bile duct ligation
BEGM	=	Bronchial epithelial growth medium
BHMT	=	Betaine homocysteine methyltransferase
BiP	=	Binding protein
BMI	=	Body mass index
BSA	=	Bovine serum albumin
BPTES	=	Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide
Ca^{2+}	=	Calcium
CaBP2	=	Calcium-binding protein
CBS	=	Cysthationine-β-synthase
CCL	=	Chemokine c-c ligand
CCl_4	=	Carbon tetrachloride
CCR	=	Chemokine ligand receptor
CDAA	=	Choline-deficient L-amino acid-defined diet
CD-HFD	=	Choline-deficient high-fat diet
CDP	=	Cytidyl diphosphate
CDR	=	Cirrhosis dysbiosis ratio

CE	=	Cholesteryl ester
CE-TP	=	Cholesteryl ester transfer protein
CEPT	=	CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase
CHAPS	=	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ChEBP	=	Carbohydrate response element-binding protein
CHK	=	Choline kinase
CHOL	=	Choline
CHOP	=	C/EBP homologous protein
CHPT	=	CD-choline:1,2-diaclyglycerol cholinephosphotransferase
CLD	=	Chronic liver disease
CM	=	Chylomicrons
CNNM	=	Cyclin M
CTH	=	Cystathionine gamma-lyase
COP	=	Coaptomere
CP	=	Carbamoyl-phosphate
CPT	=	Carnitine palmitoyltransferase
CPS	=	Carbamoyl-phosphate-synthase
CTLA-4	=	Cytotoxic T-lymphocyte-associated protein 4
CDP	=	Cytidylphosphate
CVD	=	Cardiovascular diseases
CXCL	=	Cytokine c-x-c ligands
CXCR	=	Cytokine c-x-c receptor
CYP2E1	=	Cytochrome P450 2E1
DGAT	=	Diacyglycerol acyltransferase
DHE	=	Dihydroethidium
DIAMOND	=	Diet-induced animal model of non-alcoholic fatty liver disease
DMEM	=	Dulbecco's Modified Essential Medium
DNL	=	*De novo* lipogenesis
DPP4	=	Dipeptidyl peptidase-4
DR5	=	Death receptor 5
DTT	=	Dithiothreitol
ECAR	=	Extracellular acidification rate
ECM	=	Extracellular matrix
EDTA	=	Ethylenediamine tetraacetic acid
EGF	=	Endothelial growth factor
EGFR	=	Endothelial growth factor receptor
EGTA	=	Egtazic acid
eIF2α	=	Eukaryotic translation initiation factor 2 alpha
EMT	=	Epithelial-mesenchymal transition
ER	=	Endoplasmic reticulum
ERK	=	Extracellular-regulated kinases
ETC	=	Electron transport chain

ETN	=	Ethanolamine
ETNK	=	Ethanolamine kinase
FA	=	Fatty acid
FASL	=	FAS ligand
FAS / FASN	=	Fatty acid synthetase
FAO	=	Fatty acid oxidation
FBS	=	Fetal bovine serum
FFA	=	Free fatty acid
FGF	=	Fibroblast growth factor
FGFR	=	Fibroblast growth factor receptor
FXR	=	Farnesoid X receptor
GAB	=	Glutaminase B
GAC	=	Glutaminase C
GDH	=	Glutamate dehydrogenase
GCL	=	Glutamate cysteine ligase
GLN	=	L-glutamine
GLP-1	=	Glucagon-like peptide 1
GLS	=	Glutaminase
GLU	=	L-glutamate
GNMT	=	Glycine N-methyltransferase
GRP	=	Glucose regulatory protein
GSH	=	Reduced glutathione
GS	=	Glutamine synthetase
GSS	=	Glutathione synthase
GSSG	=	Oxidized glutathione
HCC	=	Hepatocellular carcinoma
Hcy	=	Homocysteine
HCD	=	High-cholesterol diet
HDL	=	High-density lipoproteins
HE	=	Hepatic encephalopathy
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	=	High-fat diet
HFS-WD	=	High-fat high-sugar western diet
HS	=	*Homo sapiens*
HSC	=	Hepatic stellate cells
HSP	=	Heat-shock protein
IDL	=	Intermediate-density lipoproteins
IGF	=	Insulin-like growth factor
IGFR	=	Insulin-like growth factor receptor
IRE1α	=	Inositol-requiring enzyme 1 alpha
KC	=	Kupffer cell
KGA	=	Kidney-type glutaminase

LCAT	=	Lecithin: cholesterol acyltransferase
LDL	=	Low-density lipoprotein
LDL-R	=	Low-density lipoprotein receptor
LGA	=	Liver-type glutaminase
LOXL2	=	Lysyl oxidase-like 2
LPL	=	Lipoprotein lipase
LPP	=	Lipoprotein particles
LPS	=	Lipopolysaccharaide
LRP	=	LDLR-related protein
MagT1	=	Magnesium transporter 1
MAPK	=	Mitogen-activated protein kinases
MAT	=	Methionine adenosyltransferase
MCDHFD	=	Methionine and choline-deficient high-fat diet
MEM	=	Minimal essential medium
Mg^{2+}	=	Magnesium
MDR2	=	Multi-drug resistance 2 protein
MCP-1	=	Macrophage chemoattractant protein-1
Met	=	L-Methionine
MGAT	=	Monoacylglycerol acyltransferase
MM	=	*Mus muscuulus*
MMP	=	Matrix metalloproteinase
mRNA	=	Messenger RNA
mTOR	=	Mammalian target of rapamycin
MTP	=	Microsomal triglyceride transfer protein
MS	=	Methionine synthase
MTHFS	=	Methyltetrahydrofolate synthetase
MTHFR	=	Methyltetrahydrofolate r
NAFL	=	Non-Alcoholic Fatty liver
NAFLD	=	Non-Alcoholic Fatty Liver Disease
NASH	=	Non-Alcoholic Steatohepatitis
NEFA	=	Non-esterified fatty acids
NFκB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4^+	=	Ammonium cation
NK	=	Natural killer
NPC1L1	=	Niemann-Pick C1-like 1
NRF2	=	NFE2-related factor 2
O.C.T.	=	Optimal cutting temperature (compound)
OCA	=	Obeticholic acid
OCR	=	Oxygen consumption rate
OP	=	L-ornithine phenylacetate
Orn	=	L-ornithine
OTC	=	Ornithine-transcarbamylase

OXPHOS	=	Oxidative phosphorilation
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PCYT1	=	CTP:phosphocholine cytidyltransferase
PD-1	=	Programmed cell death
PDGF	=	Platelet-derived growth factor
PDGFR	=	Platelet-derived growth factor receptor
PDI	=	Protein disulphide isomerase
PEMT	=	Phosphatidylethanolamine N-methyltransferase
PERK	=	PRK-like endoplasmic reticulum kinase
PI3K	=	Phosphatidylinositol 3-kinase
PISD	=	Phosphatidylserine decarboxylase
PKA	=	Protein kinase A
PL	=	Phospholipid
PPAR	=	Peroxisome proliferator-activated receptor
PRL	=	Phosphatase of regenerating liver
Ptd-Cho	=	Phosphatidylcholine
Ptd-Etn	=	Phosphatidylethanolamine
Ptd-Ser	=	Phosphatidylserine
PTDSS	=	Ptd-Ser synthase
PTEN	=	Phosphatase and tensin homolog
PSG	=	Penicillin streptomycin gentamicin
Q	=	Ubiquinone
rCM	=	Remnant chylomicrons
RIPA buffer	=	Radio immunoprecipitation assay
RPM	=	Revolutions per minute
RPMI	=	Roswell Park Memorial Institute (culture medium)
ROS	=	Reactive oxygen species
RT	=	Room temperature
SAH	=	S-adenosylhomocysteine
SAHH	=	S-adenosylhomocysteine hydrolase
SAMe	=	S-adenosylmethionine
SCD1	=	Stearoyl-coenzyme-A desaturase 1
SDS	=	Sodium dodecyl sulfate
SER	=	L-Serine
siRNA	=	Small interfering RNA
SIRT	=	Selective internal radiation therapy
SLC41	=	Solute carrier family 41
SOCS	=	Suppressor of cytokine signaling
SOD	=	Superoxide dismutase
SR-B1	=	Class B scavenger receptor B1
SREBP-1c	=	Sterol regulatory element-binding protein-1 isoform c

T2DM	=	Type 2 diabetes mellitus
TACE	=	Transarterial chemoembolization
TBS	=	Tris-buffered saline
TCA	=	Tricarboxylic acid
TF	=	Transcription factor
TG	=	Triglyceride / triacylglyceride
TGFβ	=	Transforming growth factor β
THF	=	Tetrahydrofolate
TIMP	=	Tissue inhibitors of metalloproteinases
TLC	=	Thin layer chromatography
TKR	=	Tyrosine kinases receptor
TNF	=	Tumor necrosis factor
TRAIL	=	TNF-related apoptosis-inducing ligand
TRPM	=	Transient potential receptor melastatin
TRL	=	Toll-like receptor
UPR	=	Unfolded protein response
VEGF	=	Vascular endothelial growth factor
VLDL	=	Very-low-density lipoprotein
VTC	=	Vesicular tubular cluster
XBP1	=	X-box binding protein 1

1. Summary

1. SUMMARY

Non-Alcoholic Fatty Liver Disease (NAFLD) encompasses a group of pathologies that range from steatosis to non-alcoholic steatohepatitis (NASH) and ends up at cirrhosis. NAFLD also increases the risk of developing hepatocellular carcinoma (HCC) which, together with cirrhosis, cause two million deaths each year. Current therapies are focused on ameliorating the pathology at the earliest stages, reducing lipid accumulation (1st hit) and/or derived complications (2nd hit). However, the lack of effective diagnose methods and therapies make NAFLD a difficult condition to manage, becoming a global health problem with around a 25% of prevalence which is expected to grow within next years.

The main aim of our group, the Liver Disease Laboratory, is to elucidate the mechanisms underlying NAFLD. In the present thesis we have particularly focused on erly stages, basing on perturbations reported to occur in the pathology such as the ones related to nitrogen metabolism and magnesium (Mg^{2+}) homeostasis.

Ammonia implication has been widely reported in fibrosis, with glutaminase (GLS) as the main source in the organism and a down-regulation of urea cycle. We have dedicated our effort to analyze the contribution of the two isoforms GLS1 and GLS2 to the pathology. Gln/Glu metabolites are altered in NASH, showing a higher rate of Gln catabolism in serum of these patients. Importantly, Gln regulates intracellular redox balance as it can replenish the TCA cycle for producing ATP and carbon metabolites, so that alterations in Gln catabolism could trigger metabolic alterations during NASH. Moreover, the high-affinity isoform GLS1 has been reported to overexpress in late stages of NAFLD such as cirrhosis and HCC. Thus, GLS1-mediated Gln catabolism could contribute to the development of the pathology.

Perturbations in Mg^{2+} homeostasis have been related to NAFLD comorbidities such as obesity, cardiovascular diseases or diabetes. Indeed, Mg^{2+} supplementation has proved to reduce mortality caused by liver diseases, whereas the determination of the cation in serum from a cohort of patients showed an upregulation in the NASH-diagnosed group. Because of Mg^{2+} physicochemical properties, transporters are required for its flux across cell membranes. However, little research has been performed about them, and almost none about their role in liver. In this context, we have focused on cyclin M (CNNM) family, reported to modulate Mg^{2+} homeostasis and interact with phosphatases of regenerating liver (PRL), a pro-oncogenic protein described in liver cancer.

Two pre-clinical animal models of NASH have been used. On one hand, mice fed a 0.1%methionine and choline-deficient diet (0.1%MCDD) develop NASH by a disrupted very-low-density lipoprotein (VLDL) assembly. It must be mentioned that disrupted VLDL synthesis also occurs in mice lacking *Mat1a*, described to develop NASH with a metabolic fingerprint similar to humans. On the other hand, mice fed a choline-deficient high-fat diet (CD-HFD) develop NASH with a similar pattern to humans. Results have been reinforced by *in vitro* studies in primary hepatocytes and human THLE2 cell lines.

Herein, we demonstrate GLS1 contribution to NASH, as the enzyme is overexpressed in clinical NASH and *in vivo* animal models. Liver-specific *Gls1* silencing reverts the pathology by reducing intrahepatic lipid accumulation and inflammation. In this work we have characterized that reduction of Gln catabolism leads to a decreased oxidative activity and, as a consequence, a reduction of oxidative stress. Meanwhile, glutathione synthesis is reduced so that serine availability is higher, which does not enter the transsulfuration pathway and acts as substrate for phospholipids (PLs) synthesis. PLs are essential for VLDL assembly, so that NASH resolution under *Gls1* silencing might be due through a decrease in oxidative stress and a restoration of lipid export in form of VLDL.

Otherwise, clinical and pre-clinical studies have characterized a CNNM4 overexpression in NASH. An *in vitro* screening points out is contribution over the other CNNM isoforms, whereas pre-clinical *Cnnm4* silencing has proven to revert the pathology reducing lipid accumulation, inflammation and fibrosis. We also demonstrate the role of CNNM4 as a Mg^{2+} extruder in the liver and, although intrahepatic Mg^{2+} content has been reported to reduce lipid content in the cell, we have proven the prevalence of CNNM4 expression over magnesium supplementation. Regarding the mechanism by which Mg^{2+} accumulation induced by targeting CNNM4 resolves NASH, the reduction of endoplasmic reticulum stress (ERS) might lead to the observed increase of microsomal triglyceride transfer protein (MTP) activity, the protein responsible of VLDL assembly and export. Therefore, Mg^{2+}-induced ERS reduction may activate MTP to promote VLDL assembly and export.

In conclusion, our results point out CNNM4 and GLS1 contribution to NASH development as they are overexpressed in the pathology. Additionally, liver-specific silencing ameliorates NASH by promoting lipid export and reducing oxidative stress. Moreover, Mg^{2+} modulation opens a new field of study as it might also have an effect at other peripheral organs such as pancreas, adipose tissue or cardiovascular system.

2. INTRODUCTION

2. INTRODUCTION

2.1 CHRONIC LIVER DISEASE

Chronic liver disease (CLD) comprehends a broad group of hepatic pathologies from different etiology, and characterized by a slow progression that normally lasts longer than 6 months (up to 20-40 years) and which can lead to the development of late stages: cirrhosis and hepatocellular carcinoma (HCC) (Mishra and Younossi 2012; Riley and Bhatti 2001; Vernon, Baranova, and Younossi 2011). CLD is one of the leading mortality cause in USA and Europe and it can be caused by different pathologies such as viral infections of hepatitis B and C, toxins, alcohol and drug abuse, autoimmune liver diseases, hereditary diseases or Non-Alcoholic Fatty Liver Disease (NAFLD) (Riley and Bhatti 2001; Vernon et al. 2011; Younossi et al. 2016).

2.1.1 Non-Alcoholic Fatty Liver Disease (NAFLD)

Non-Alcoholic Fatty Liver Disease, NAFLD, has emerged as one of the most frequent causes of CLD in our society (Vernon et al. 2011) and it has become a major health problem in the world (Bellentani et al. 2010; Loomba and Sanyal 2013). NAFLD manifests particularly in Western countries, with an incidence of between 20 and 30% in general population. It consists on a clinical syndrome that includes a wide spectrum of hepatic disorders that go from a simple lipid accumulation in the hepatocyte (steatosis or non-alcoholic fatty liver, NAFL) to non-alcoholic steatohepatitis (NASH) characterized by inflammation, hepatocellular ballooning and fibrosis. If prolonged fibrosis occurs, NASH can lead to cirrhosis, with an elevated risk of developing HCC.

NAFLD is often associated to other pathologies such as obesity, type 2 diabetes (T2DM), insulin resistance, dyslipidemia, hypertension and cardiovascular risk (Adams et al. 2005; Bertot and Adams 2016; Lindor and Gores 2003; Loomba and Sanyal 2013; Noureddin and Rinella 2015; Teli et al. 1995; Vernon et al. 2011). All of them are considered risk factor for the development of metabolic syndrome (Siegel and Zhu 2009). NAFLD prevalence, aforementioned to be around 20-30% in population from western countries, raises up to 30-50% in diabetic patients and even to 80-90% in obese people, turning almost universal when combining both factors. In case of children, the prevalence of NAFLD has unfortunately risen up to 40-70% from 3-10% (Bellentani et al. 2010). Indeed, the increase of NAFLD prevalence is expected to rise up in near future as patients

with metabolism syndrome are increased, becoming a global health problem (Loomba and Sanyal 2013; Mishra and Younossi 2012).

As far as the progression of the pathology, it requires a series of steps usually named as the "two-hit hypothesis" (Day 2006; Sanyal 2005) (Fig. 2.1). The initial "hit" in the liver is given to the accumulation of fatty acids (FAs) derived from the adipose tissue lipolysis or their synthesis through an increased *de novo* lipogenesis (DNL), pathology known as steatosis or NAFL. For the progression of the disease researchers have proposed several possible "second hits", being the most commonly accepted: the activation of endoplasmic reticulum (ER) stress and the increase of oxidative stress by reactive oxygen species (ROS) overproduction, followed by decreased antioxidant capacity (Day 2006; Sanyal 2005). Mitochondrial functionality gets altered as cause and consequence of oxidative stress (Day 2006; Kershaw and Flier 2004), followed by hepatocyte apoptosis, contributing to the development of hepatitis and fibrosis (Berson et al. 1998; Sanyal 2005).

NAFLD is frequently considered a benign condition and NAFL has a good prognosis, being commonly reversible by changing underlying causes of the disease as the lifestyle (Mishra and Younossi 2012). However, about a 10-30% of NAFL patients progress to NASH, with a 20% of probabilities to develop cirrhosis within the next 10 years (Farrell and Larter 2006; Harrison, Torgerson, and Hayashi 2003; Marrero et al. 2002) and, finally, liver failure and HCC (4-27%) (Takuma and Nouso 2010).

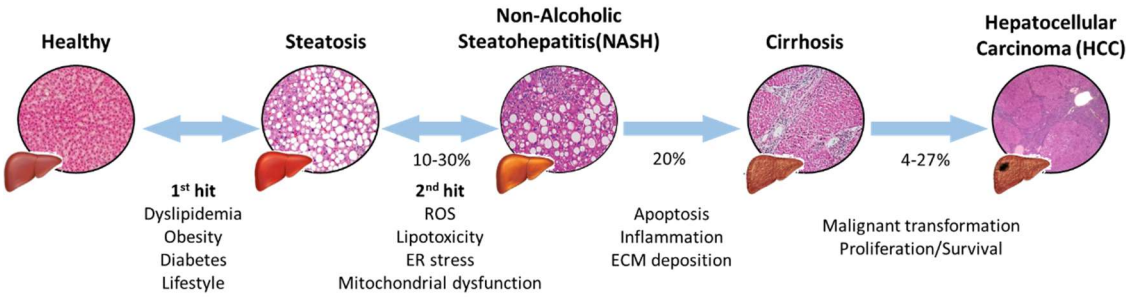


Figure 2.1. Two-hit hypothesis of liver disease progression: from steatosis to HCC. Steatosis develops as a consequence of an increased lipid accumulation in the liver due to different causes (1st hit). Reactive oxygen species (ROS), lipotoxicity, endoplasmic reticulum (ER) stress and mitochondrial dysfunction lead to NASH (2nd hit) in 10-30% patients. Sustained damage results in fibrotic response and cirrhosis in 20% of patients. Finally, the 4-27% of chronic patients can develop HCC, the most common manifestation of liver cancer

Although the "two-hit" hypothesis refers to the progression of chronic liver disease in each pathology, it is evident that there are multiple factors converging and contributing synergistically to NAFLD progression ("multiple-hit" hypothesis). In the next sections the mechanisms implicated in the initiation of the disease (alterations in lipid homeostasis, "first hits") and the progression of the disease (ROS production, ER stress and mitochondrial dysfunction, "second hits") required for the development of NAFLD will be described.

2.1.1.1 First hit: Alterations in lipid homeostasis

As aforementioned, steatosis or NAFL characterizes by an increased lipid accumulation in the liver. This is the result of an imbalance between the processes involved in the increase of lipids (FA uptake and DNL) and the ones involved in their decrease (very-low density lipoprotein export and β-oxidation) (Fig. 2.2).

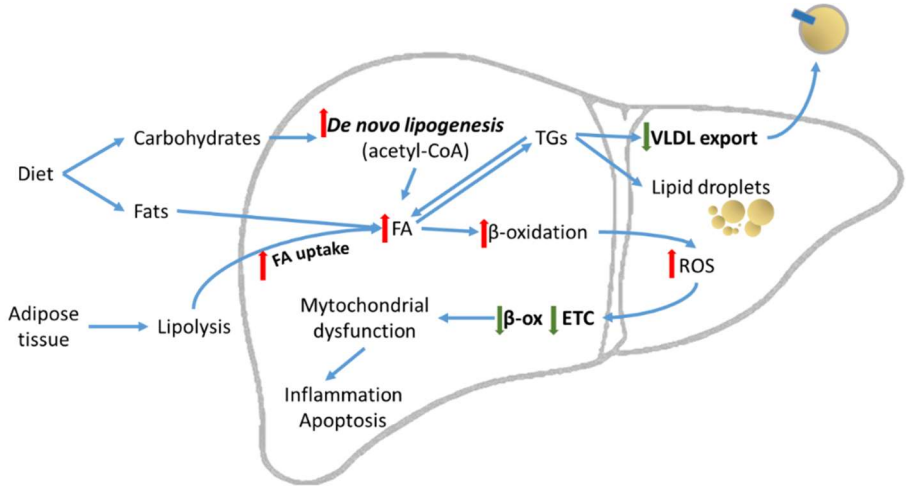


Figure 2.2 The main pathways implicated in triglyceride (TG) accumulation during chronic liver disease progression. NAFLD is characterized by a TG accumulation in lipid droplets. It can be due to an increased FA uptake from the diet or the adipose tissue, an enhanced hepatic *de novo* lipogenesis, a decreased β-oxidation or an impairment in VLDL secretion. Lipid accumulation can increase ROS levels predisposing the liver to a mitochondrial dysfunction, which later promotes inflammation and apoptosis in the hepatocytes, leading to the progression of the disease from steatosis to NASH.

2.1.1.1.1 Increased fatty acid uptake and *de novo* lipogenesis

Intrahepatic FA content can increase because of an excess of free fatty acids (FFAs) supply from the white adipose tissue, the major source of FAs in the body. Under specific circumstances, triglyceride (TG) contained in the adipose tissue are hydrolyzed releasing FFAs delivered directly to the liver instead of been uptake by other tissues. This process has been described to be increased in NAFLD (Fabbrini, Sullivan, and Klein 2010).

De novo lipogenesis (DNL) is a process mainly regulated by two enzymes that constitute an important source of FAs in the liver: acetyl-coenzyme A (acetyl-CoA) carboxylase (ACC) and fatty acid synthetase (FASN). Although DNL contribution to hepatic TG content has been estimated to be less than 5%, it has been described to be increased in NAFLD patients to 15-23%, even during post-prandial stages (Diraison, Moulin, and Beylot 2011; Donnelly et al. 2005; Lambert et al. 2014). In this context, the incoming nutrients from the diet contribute relevantly to the lipogenesis in liver, as not only FAs but also carbohydrates constitute important sources of FAs to the global liver pool.

The process is tightly regulated by molecular mechanisms that implicate the enzymes involved in the conversion of acetyl-CoA to palmitate and TG (Fig. 2.3): ACC1/2, FASN, stearoyl-CoA desaturase 1 (SCD1) and diacylglycerol acyltransferase (DGAT) 1/2. These enzymes are transcriptionally regulated by several transcription factors (TFs), particularly de sterol regulatory element-binding protein-1 isoform c (SREBP-1c) (Shimano et al. 1997; Shimomura et al. 1999), stimulated by insulin and the carbohydrate response element-binding protein (ChrEBP) (Yamashita et al. 2001), stimulated by glucose. Both TFs and ACC1/2 can be also inhibited through phosphorylation by the AMP-dependent protein kinase (AMPK) (Viollet et al. 2009).

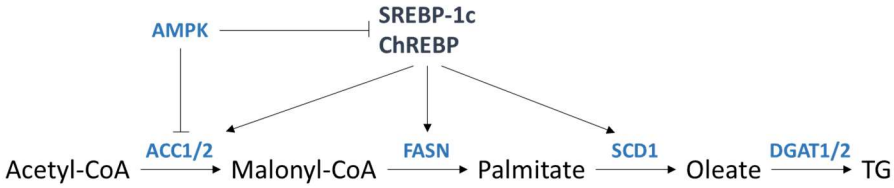


Figure 2.3. *De novo* lipogenesis. The principal steps implicated in TG synthesis from acetyl-CoA. This process is frequently augmented in NAFLD and controlled by different TFs that regulated the expression of different enzymes implicated in determined steps of lipogenesis.

2.1.1.1.2 Disrupted VLDL secretion

Exceeding FAs in the liver are conjugated into TGs, which can be stored in the hepatocyte or secreted into very low-density lipoproteins (VLDL) to the circulation for their delivery to peripheral tissues. VLDL, which will be described further in more detail (Chapter 2.2.3), are macromolecular complexes mainly formed by TGs and cholesteryl esters (CE) surrounded by phospholipids and unesterified cholesterol, all stabilized by a molecule of apolipoprotein B100 (ApoB100). An increased production of VLDL is a common feature in NAFLD but it cannot compensate the increased TG synthesis produced in the liver if

steatosis occurs (Fabbrini et al. 2010; Kawano and Cohen 2013). Moreover, it has been reported that oxidative stress and ER stress, characteristics of NASH, contribute to the degradation of apoB100 by protesomal and non-proteasomal mechanisms, impairing TG secretion from the liver and contributing to fatty liver (Ota, Gayet, and Henry N. Ginsberg 2008; Pan et al. 2004).

2.1.1.1.2.1 Methionine metabolism

Disrupted VLDL secretion was characterized by Cano and colleagues in mice lacking the *methionine adenosyltransferase 1A* gene (*Mat1a*$^{-/-}$) (Cano et al. 2011). Those *Mat1a*$^{-/-}$ mice have been reported to develop NASH with a similar metabolic fingerprint to subtype M, characteristic of more than the half of NASH patients (Alonso et al. 2017), pointing out the relevance of the integrity of VLDL assembly and export for maintenance of lipid homeostasis. MAT1A is the enzyme responsible for the metabolism of the essential amino acid methionine (Met) into S-adenosylmethionine (SAMe) (Cantoni and Durell 1957), the most important biological methyl donor in the cell. Although SAMe can be produced in almost every cell, the liver is the responsible of 50% Met metabolism and 85% SAMe methylation reactions (Finkelstein 1990; Mato et al. 2002; Mudd and Poole 1975). Additionally, SAMe is involved in polyamines synthesis and transsulfuration pathway to generate glutathione (Lieber and Packer 2002; Lu 2000; Mato et al. 1997). In this section the methionine cycle as well as its deregulation is described.

Several researchers work link the development of NASH and its chronic progression in NAFLD with alterations in Met metabolism. Best *et al.* demonstrated that diet deficient in methyl groups such as Met, choline and folate led to the development of steatosis and a progression to NASH, fibrosis and HCC if prolonged in time (Best, Hershey, and Huntsman 1932). Moreover, in human patients Kinsell demonstrated a defective Met clearance from plasma in a cirrhotic state (Kinsell et al. 1948; Kinsell and Harper 1947). Since then, study of the link between NAFLD and Met metabolism has reported a dysregulation as one of the causes of the development and progression of the disease.

a) Methionine cycle and complementary pathways

Met and SAMe levels are controlled by a group of enzymes present in the methionine cycle (Fig. 2.4). As mentioned previously, SAMe is synthetized from Met by the MAT enzyme. MAT is codified by two genes located in different chromosomes (MAT1A and MAT2A) in different liver scenarios while a third isoform (MAT2B) is expressed in regenerating liver (Halim et al. 1999). SAMe can then be demethylated into S-adenosylhomocysteine (SAH) by different methyltransferases, being glycine N-methyltransferase (GNMT) the most important for SAMe/SAH ratio. In fact, this ratio is considered as an indicator of the cell's methylation capacity (Finkelstein 2007). SAH is hydrolyzed by S-adenosylhomocysteine hydrolase (SAHH) to prevent its accumulation and generates homocysteine (Hcy) and adenine. Hcy can be remethylated or enter transsulfuration pathways depending on SAMe levels, which inhibits enzymes involved in remethylation pathway and stimulates transsulfuration ones (Mato et al. 1997).

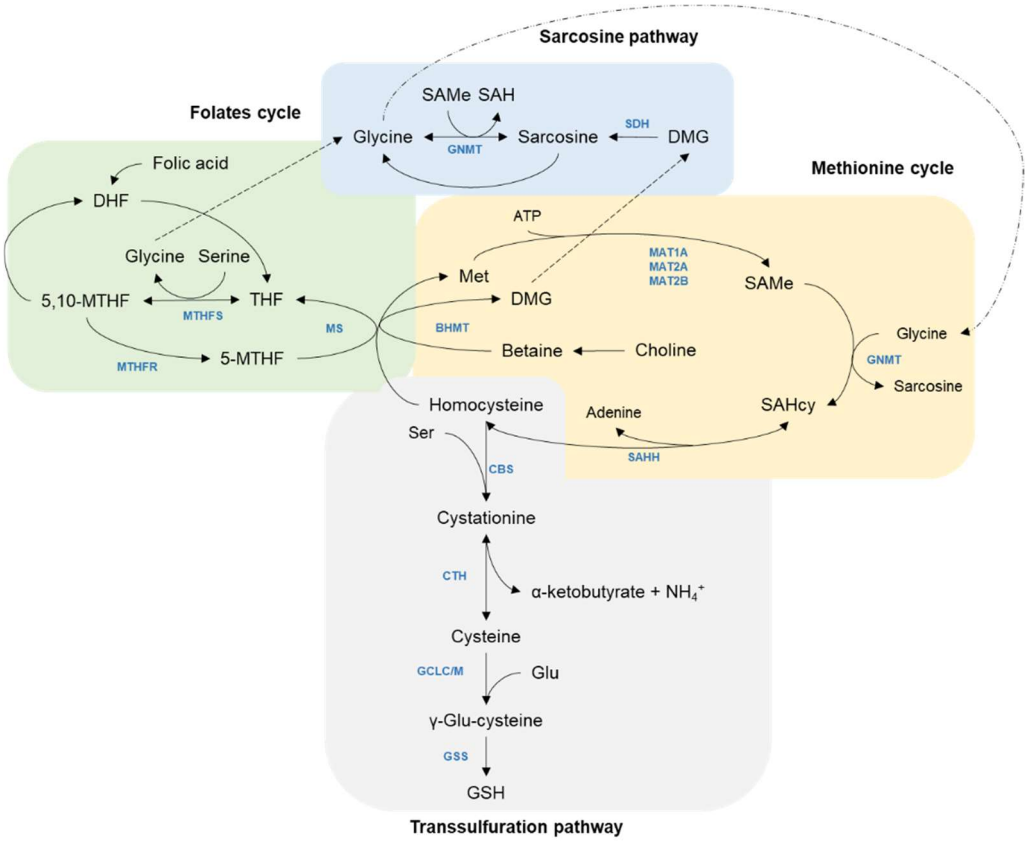


Figure 2.4. The methionine cycle is linked to folates cycle, sarcosine pathway and transsulfuration pathway. (Met = methionine; MAT = methionine S-adenosyltransferase; SAMe = S-adenosylmethionine; GNMT = glycine N-methyltransferase; SAHcy = S-adenosylhomocysteine; SAHH = S-adenosylhomocysteine hydrolase; CBS = cystathionine beta synthase; CTH = cystathionine gamma lyase; GCLC/M = glutamate-cysteine ligase, catalytic/modulator subunit; BHMT = betaine homocysteine methyltransferase; MS = methionine synthase; 5-MTHF = 5-methyltetrahydrofolate; THF = tetrahydrofolate; MTHFS = 5,10-methyltetrahydrofolate synthase; 5,10-dimethyltetrahydrofolate; DHF = dihydrofolate; DMG = dimethylglycine; SDH = sarcosine dehydrogenase).

In the transsulfuration pathway, important in liver because its high activity, Hcy is used as a substrate of cystathionine-β-synthase (CBS) generating cysteine and, finally, glutathione by the action of glutamate cysteine ligase (GCL) and glutathione synthase (GSS) (Lu 1999, 2009) (Fig. 2.4). Alternatively, HCy can be remethylated generating Met through a pathway directed by two different enzymes: betaine homocysteine methyltransferase (BHMT) and methionine synthase (MS). BHMT is a liver and renal specific enzyme that converts Hcy into Met using betaine as co-substrate, while MS is coupled to folate cycle requiring normal levels of vitamin B12 and folates. MS uses 5-methyltetrahydrofolate (5-MTHF) as methyl donor for Hcy and generating tetrahydrofolate (THF), converted to 5,10-MTHF by the enzyme 5,10-methyltetrahydrofolate synthetase (MTHFS) and regenerated to 5-MTHF by methyltetrahydrofolate reductase (MTHFR). (Fig. 2.4).

Moreover, SAMe can be catabolized by glycine-N-methyltransferase (GNMT). This enzyme represents about 1-3% of total cytosolic protein content in the hepatocyte so that it can be considered as a marker of an adult and differentiated liver (Luka, Mudd, and Wagner 2009). GNMT uses glycine as substrate and generates sarcosine, regulating SAMe levels an maintaining SAMe/SAHcy ratio constant. Sarcosine can be used to regenerate glycine and 5,10-MTHF so that GNMT is linked to folate cycle.

b) Methionine metabolism in liver disease

Met and SAMe levels and the intermediate metabolites in the pathways described above (Fig 2.4) are altered in liver disease, where low SAMe levels are frequently found as a consequence of a low expression of MAT1/3 enzymes (Avila et al. 2000). On the other hand, GNMT expression has been reported to be downregulated in liver disease, generating an excess of SAMe that leads to an aberrant methylation pattern (Avila et al. 2000; Heady and Kerr 1975; Liao et al. 2012; Mudd et al. 2001).

These data indicate the necessity of regulating SAMe content in the liver, since an impairment leads to liver injury. For this, the knockout animal models *Mat1a*$^{-/-}$ (cited above) and *Gnmt*$^{-/-}$ that develop NAFLD and HCC, have been generated (Lu et al. 2001; Luka et al. 2006). The mechanism of developing the disease of both NAFLD animal models will be described deeply in Chapter 2.1.1.

2.1.1.2 Second hit: Mechanisms underlying the progression to NASH

In the beginning of the Chapter 2.1.1 steatosis or NAFL has been mentioned to be a rather benign condition, but which can progress to NASH when a damaging situation results and a second "hit" occurs. In the next section there will be deeply discussed the mechanisms that lead the progression of the disease, being the presence of ROS the primary event and most important one. Some other factors such as lipotoxicity, ER stress and mitochondrial dysfunction also contribute to the progression.

2.1.1.2.1 Reactive oxygen species overproduction

ROS are chemically reactive compounds that normally generate in the cell as consequence of oxygen metabolism. They have an important role in cell signaling and mediate in many reactions in the cell affecting DNA, lipids and proteins (Freeman and Crapo 1982). Among ROS components there are hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH^-) and one-atom oxygen (O^-) (Thannickal and Fanburg 2010). Under normal conditions, the oxidant machinery, composed by superoxide dismutase (SOD), catalases and reduced glutathione (GSH), buffers ROS production for its clearance. However, when the production of ROS exceeds the capacity of its detoxification, a situation of oxidative stress becomes cytotoxic in the cell.

Cellular ROS can come from different cell compartments: mitochondria, ER and peroxisomes (Sanyal 2005). Mitochondria has the highest contribution of ROS production. In this organelle, fatty acid oxidation (FAO) and tricarboxylic acid (TCA) cycle processes take place, linked to the electron transport chain (ETC) to produce adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS). During OXPHOS, ETC transports electrons (e^-) and protons (H^+) through its different components, each one with higher reduction capacity than the previous one, and reducing O_2 to water in the final step. It is estimated that about 1-2% of the transferred e^- can leak the ETC leading to ROS production (Boveris and Chance 1973). An excessive e^- flow through the ETC due to an increased mitochondrial TCA and FAO during NAFLD initial stages. Meanwhile, the increase in ROS production may induce tumor necrosis factor (TNF) signaling, which enhances lipid peroxidation resulting, then, in an overproduction of e^-, mitochondrial dysfunction and a ROS excessive production (Nassir and Ibdah 2014; Pessayre et al. 2002) (Chapter 2.1.1.2.4).

In the pathogenesis of NAFLD, ROS overload has been also described to occur from alternative sources. Firstly, because of an increased cytochrome P450 2E1 (CYP2E1) expression, a ROS producing enzyme located in the ER and mitochondria (Zangar, Davydov, and Verma 2004). Secondly, an increased peroxisomal FAO with the subsequent production of H_2O_2 (Begriche et al. 2006).

2.1.1.2.2 Lipotoxicity

Lipotoxicity has been considered an important contributor to NASH development during the recent years (Cusi 2012; Neuschwander-Tetri 2010). Despite the TG accumulation is the first step produced in NAFLD, most of the recent studies indicate that this condition itself is not toxic in the liver (McClain, Barve, and Deaciuc 2007). The toxicity in the liver of FAs, instead of their amount, is determined by their relative amount: monounsaturated FAs do not induce toxicity whereas saturated FAs do (Alkhouri, Dixon, and Feldstein 2009; Listenberger et al. 2003). On the other hand, several studies have characterized the lipoapoptotic role of FFA and other bioactive lipids such as ceramides in hepatocytes mediated by death receptors such as FAS ligand (FASL), TNF-related apoptosis-inducing ligand (TRAIL) and death receptor 5 (DR5), which leads to the initiation of an extrinsic apoptotic pathway (Feldstein, Canbay, Guicciardi, et al. 2003; Lang et al. 2007; Malhi et al. 2007). The upregulation of cited death receptors is another important feature in liver from NASH patients, especially in contributing to fibrosis development (Alkhouri et al. 2009; Feldstein, Canbay, Angulo, et al. 2003).

2.1.1.2.3 Endoplasmic reticulum stress

In recent years, ER stress has been proposed as an important mechanism implicated in the development and progression of NASH (Malhi and Kaufman 2011; Özcan et al. 2004; Puri et al. 2008). Accumulation of very long chain fatty acids caused by lipid excess leads to ER stress and, simultaneously, this contributes to liver damage and NASH progression. Added to lipotoxicity, ER stress-related signaling is linked to insulin resistance, inflammation and hepatocyte death.

ER contains the highest Ca^{2+} inside the cell due to the active transport by Ca^{2+} ATPases, which have been widely reported to play a key role in Ca^{2+} active import to the lumen of the ER (Meldolesi and Pozzan 1998). The lumen is an oxidative environment, where proper proteins fold to be secreted or displayed on the cell surface. Ca^{2+}-dependent molecular chaperones, such as GRP78, GRP94 and calreticulin, stabilize folding

intermediates whereas an aberrant Ca^{2+} homeostasis triggers protein unfolding (Orrenius, Zhivotovsky, and Nicotera 2003; Rizzuto, Duchen, and Pozzan 2004; Schroder and Kaufman 2005). Then, an initial intent of the unfolded protein response (UPR) happens to adapt to the changing environment, re-establishing a native ER functionality. If not, ER stress occurs.

ER stress was first described in *in vivo* mouse models of NAFLD (Özcan et al. 2004; Rahman et al. 2007) and later characterized in NAFLD and NASH human patients (Gregor et al. 2009; Puri et al. 2008). During steatosis, a first ER stress response is implicated in an increased insulin resistance and DNL which, added to the fact that VLDL secretion is impaired, contribute to lipid accumulation in the liver (Dara, Ji, and Kaplowitz 2011; Zhang et al. 2014). During the progression of NASH, ER stress is strongly associated to inflammation by different mechanisms such as ROS production, activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), JNK pathway and ChREBP signaling. It is also associated to contribute to liver injury by promoting hepatocyte apoptosis, mainly through CHOP induction and JNK/TRAF signaling (Dara et al. 2011; Zhang et al. 2014).

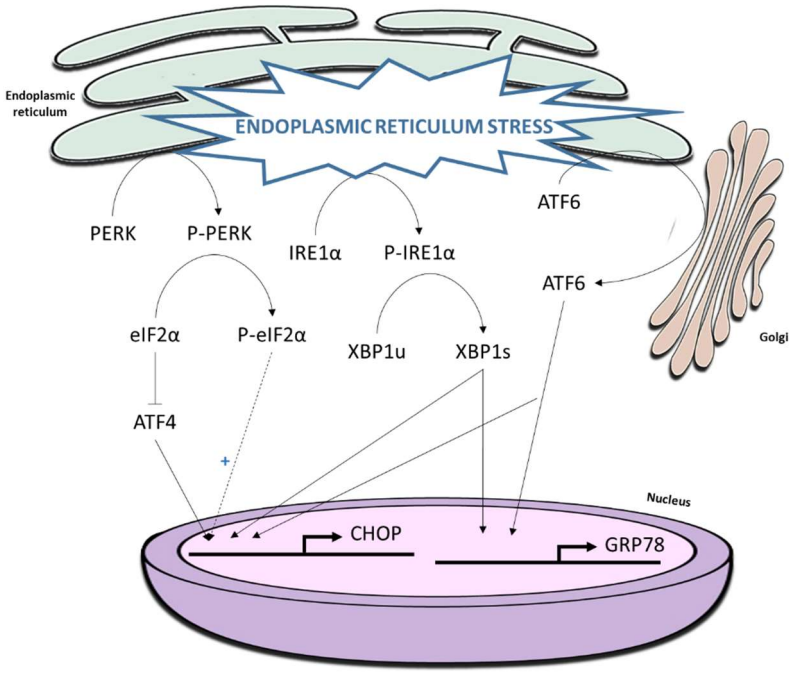


Figure 2.5. Endoplasmic reticulum stress (ERS) signaling pathways. The signaling pathway starts in the lumen of ER and translocates the signal to different proteins. PERK gets activated by phosphorylation and inhibits then eIF2α by phosphorylation, promoting ATF4 translocation into the nucleus. IRE1α activates phosphorylation and promotes XBP1 mRNA splicing from XBP1u to XBP1s isoform, translocating into the nucleus. ERS also promotes ATF6 translocation into the Golgi, finally acting as a transcription factor in the nucleus. In the nucleus, CHOP expression is promoted by ATF4, XBP1s and ATF6 while GRP78 expression is only promoted by XBP1s and ATG6. (PERK = PRK-like endoplasmic reticulum kinase; IRE1α = inositol-requiring enzyme 1α; ATF4/6 = activating transcription factor 4/6; eIF2α = eukaryotic translation initiation factor 2α; XBP1 = x-box binding protein 1; CHOP = C/EBP homologous protein; BiP/GRP78 = binding immunoglobulin protein/GRP78).

Regarding the mechanism of action by which ER stress response exerts its function, the signaling begins with the activation of inositol-requiring enzyme 1α (IRE1α) and PRK-like endoplasmic reticulum kinase (PERK), catalyzing their phosphorylation, or activating transcription factor 6 (ATF6) activation, promoting its relocation to Golgi (Xu, Bailly-Maitre, and Reed 2005). IRE1α signaling begins when the protein is phosphorylated and takes place through the splicing of x-box binding protein 1 (XBP1) mRNA from the XBP1u to the XBP1s isoform, leading to transcription functions in metabolism, apoptosis and vesicular trafficking. Meanwhile, PERK pathway stimulates the phosphorylation of NFE2-related factor 2 (NRF2) and eukaryotic translation initiation factor 2α (eIF2α). EIF2α inhibits activating transcription factor 4 (ATF4) when its dephosphorylated and the reaction catalyzed by P-PERK inhibits eIF2α promoting ATF4 mRNA translation (Scheuner et al. 2001). Finally, ATF4, ATF6 and XBP1s translocate to the nucleus and promote the transcription of C/EBP homologous protein (CHOP) and GRP78 (Fig. 2.5).

2.1.1.2.4 Mitochondrial dysfunction in NASH

Mitochondria is the main source of energy in the hepatocyte and most part of the cells. It is the responsible of generating energy as ATP and reduction power as $NADH+H^+$ and $FADH_2$ through the oxidation of nutrients by three converging pathways: β-oxidation and ketogenesis, TCA cycle and ETC (Fig. 2.5). During the development of NAFLD, lipid accumulation leads to metabolic adaptations with the aim of counteracting it.

During the last years, several works have pointed a mitochondrial disease as the cause of NAFLD and NASH progression, although there is not clear whether mitochondrial dysfunction is the cause or the consequence of the disease (Begriche et al. 2013; Nassir and Ibdah 2014). Mitochondrial dysfunction in NASH refers to a group of common events such as impairment in the ETC complexes activity and the reduction in OXPHOS and ATP production. Firstly, the presence of mitochondrial abnormalities was observed in NASH patients (Caldwell et al. 1999; Sanyal et al. 2001) but there are some controversies related to animal models in the way that mitochondrial dysfunctions affect NASH.

As long as concerns FAO, it has been described to be increased, decreased or unchanged in different NAFLD animal models (Begriche et al. 2006). However, PPARα reduced expression seems to be a common event in NAFLD and such reduction correlates with

NASH progression. Moreover, several proteins involves in mitochondrial biogenesis and ETC have been described to be downregulated (Aharoni-Simon et al. 2011). There also have been described several alterations in ETC complexes activity, having found to be reduced in human patients and NASH murine models (Garcia-Ruiz, Kaplowitz, and Fernandez-Checa 2013). These studies highlight the importance of the ETC in mitochondrial dysfunction during NASH and link it with the progressive decrease in energy status and ATP levels during the disease (Cortez-Pinto et al. 1999; Serviddio 2008; Szendroedi et al. 2009) (Fig. 2.6).

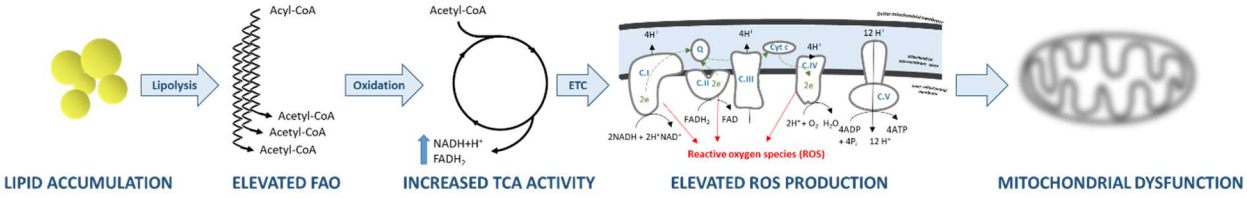


Figure 2.6. An excessive lipid accumulation leads to a final mitochondrial dysfunction. An accumulation of lipid causes an elevated fatty acid oxidation (FAO) activity. As FAO and tricarboxylic acid (TCA) cycle are coupled pathways, an increased TCA takes place with a subsequent reduced coenzyme ($NADH+H^+$ and $FADH_2$) production. A higher production than electron transport chain (ETC) can afford leads to a dysregulated electron flux through mitochondrial membrane so that reactive oxygen species (ROS) production is increased. Therefore, mitochondrial dysfunction takes place.

Mitochondria, as the most important metabolic organelle, increases its FAO activity during the initial steps of NAFLD. However, a ROS production may be increased consequently and ending up in mitochondrial dysfunction and ETC deficiency, contributing to inflammation and fibrosis and, therefore, to the development of NASH. Herein, a brief introduction of the main metabolic functions of the mitochondria in the liver and their deregulation during NAFLD will be presented.

2.1.1.2.4.1 Fatty acid β-oxidation and ketogenesis

FFAs are catabolized through mitochondrial FAO in a series of steps that produce energy in form of adenosine triphosphate ATP and ketone bodies. Depending on the metabolic state, dietary lipids can be stored as TGs in the adipose tissue or directly oxidized. During certain conditions such as fasting, TGs stored in the adipose tissue are mobilized to the liver and metabolized to FAs for energy production. FAs are then activated into acyl-CoA and translocated by carnitine palmitoyltransferases (CPT) into mitochondria, where they undergo cycles of four sequential reactions until it is converted into acetyl-CoA (or acetyl-CoA and propionyl-CoA in case of impair FAs). At this point, acetyl-CoA can either enter the TCA cycle for ATP production of be used as ketogenic substrate in extrahepatic tissues (Begriche et al. 2013). On the other hand, during β-oxidation NADH and $FADH_2$

are produced to directly link the FAO with mitochondrial ETC (Chapter 2.1.1.2.4.3) (Fig. 2.6, Fig. 2.7).

As summarized in Fig. 2.6, different mechanisms regulate β-oxidation. First, by regulating the entry of activated FA into mitochondria through CPT modulation. The isoform 1 of CPT is negatively regulated by malonyl-CoA produced from acetyl-CoA during DNL (Fabbrini et al. 2010) in a way that FAO is regulated by lipogenesis by inhibiting the entrance of FAs into the mitochondria. On another hand, CPT1 is positively regulated by peroxisome proliferator-activated receptor alpha (PPARα) by the promotion of malonyl-CoA decarboxylase (Lee et al. 2004). PPARα is also implicated in the regulation and transcription of most of FAO-implicated enzymes, being a key regulator of mitochondrial β-oxidation (Mandard, Müller, and Kersten 2004; Mello, Materozzi, and Galli 2016; Rakhshandehroo et al. 2010) . Finally, FAO is also regulated by AMPK phosphorylation, inactivating DNL and increasing β-oxidation directly binding and activating PPARα (Viollet et al. 2009). Meanwhile, FAO connection to other mitochondrial functions such as the TCA cycle ETC modulates the production of reduced coenzymes in the mitochondria ($NADH+H^+$ and $FADH_2$) and the energy production as ATP by oxidative phosphorylation OXPHOS.

However, the importance of the FAO contribution to the development of NAFLD is not well described. There are opposite works where it is reported to be downregulated and upregulated (Fabbrini et al. 2010; Sanyal et al. 2001). In this context, β-oxidation has been proposed to be increased during the initiation of NAFLD development trying to compensate the lipid accumulation that occurs in the liver. Nevertheless, during the progression of the disease, and due to the increased β-oxidation, a mitochondrial failure occurs affecting the β-oxidation capacity of the cell. In this case, despite the opposite ideas of the works cited before, decreased mitochondrial function is considered a common event in NAFLD as mitochondrial abnormalities in structure and function frequently observed in NAFLD (Berson et al. 1998; Caldwell et al. 1999; Sanyal et al. 2001).

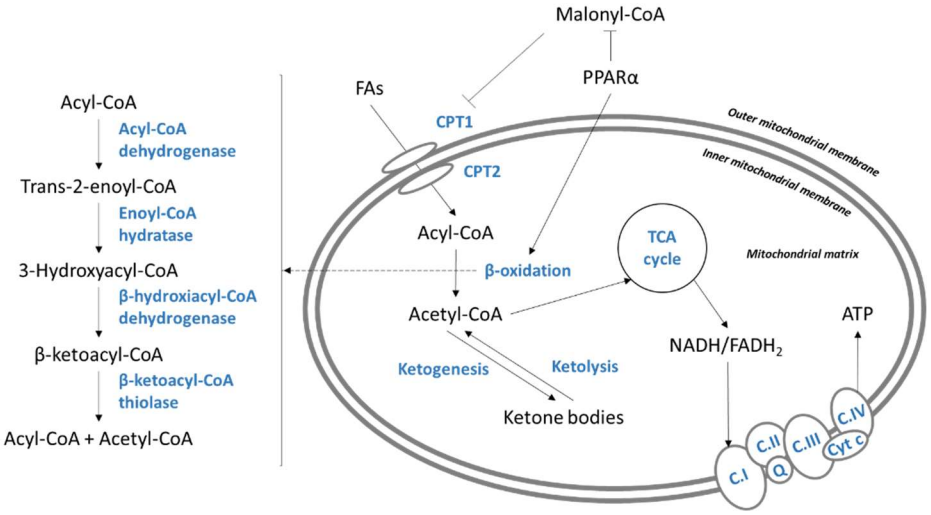


Figure 2.7. Mitochondrial fatty acid β-oxidation. Fatty acids (FAs) are converted into acetyl-CoA through β-oxidation. This process requires the internalization of FAs into the mitochondria, being firstly converted to acyl-CoA. Once inside, β-oxidation consists on a series of sequential oxidation reactions with acetyl-CoA as final product. Finally, acetyl-CoA can be fully oxidized in the TCA cycle to produce energy and reduction power or it can be used as a substrate for ketone bodies production in a process known as ketogenesis.

2.1.1.2.4.2 Tricarboxylic acid cycle

The tricarboxylic acid cycle is the central pathway of metabolism that links carbohydrate, lipid and protein metabolism through acetyl-CoA. This process consists of 8 sequential oxidative steps which convert acetyl-CoA into CO_2 to produce ATP and reductive power in form of $NADH+H^+$ and $FADH_2$. The reduced coenzymes can then enter the OXPHOS process in the ETC contributing to the electrochemical force employed by the complex V ATPase for ATP production.

2.1.1.2.4.3 Electron transport chain

It has been already mentioned that FAO and TCA converge in this pathway by the reduction power obtained. Reduced coenzymes $NADH+H^+$ and $FADH_2$ enter the ETC to be re-oxidized during OXPHOS reaction where a series of steps, controlled by the different components of the ETC, end with the complex V-catalysed ATP production. The ETC is composed by the complexes I, II, III, IV and V (also known as ATPase or ATP synthase) (Logan 2007). (Fig. 2.8)

- Complex I: NADH-ubiquinone oxidorreductase is the complex that oxidized NADH to NAD^+ transferring two electrons to the ubiquinone (Q). Four H^+ simultaneously translocate to the mitochondrial intermembrane space to generate a proton gradient.
- Complex II: Succinate dehydrogenase or SDH complex is a complex involved in the ETC and TCA. In ETC, $FADH_2$ is oxidized to FAD, delivering two extra electrons to Q and with no proton pumping to the intermembrane space.

- Complex III: Ubiquinone-cytocrome-c oxidorreductase complex. It is involved in the reduction of cytochrome c oxidizing the ubiquinol to ubiquinone and contributing to H^+ gradient by the release of four more H^+ to the intermembrane space.
- Complex IV: Cytochrome c oxidase complex is linked to complex III and participates in electron transference from C.III to oxygen, producing water and pumping four H^+ to the intermembrane space.
- Complex V/ATPase/ATP synthase: This complex finally coupled the ETC to OXPHOS by using the proton gradient created across the ETC for generating ATP. This complex redrives the previously pumped H^+ into the matrix and uses the produced electrochemical energy created by the gradient to phosphorylate ADP producing ATP.

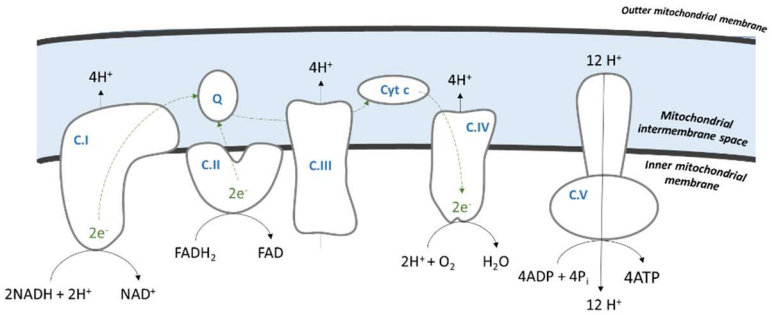


Figure 2.8 Electron transport chain (ETC). The ETC is composed by five complexes that transfer electrons from $FADH_2$ and $NADH+H^+$ to the oxygen, which is finally reduced to water. During the process, pumped H^+ from the mitochondrial matrix to the mitochondrial intermembrane space create an electrochemical gradient. Finally, the complex V or adenosine triphosphate (ATP) synthase employs the energy obtained by the formed gradient.

2.1.1.3 Animal models of NAFLD

NASH can be induced in mice when feeding with a certain diet. For the study of the disease, one of the most frequent are the following: high-fat diet (HFD), *ob/ob* mice, *db/db* mice, 0.1% methionine and choline-deficient diet (0.1%MCDD), methionine and choline-deficient high-fat diet (MCDHFD), high-cholesterol diet (HCD), *foz/foz* mice, choline-deficient high-fat diet (CD-HFD), choline-deficient L-amino acid-defined diet (CDAA), CDAA + carbon tetrachloride (CDAA+CCl_4), hepatocyte-specific *phosphatase and tensin homolog* (*Pten)*-deficient mice. In Table 2.1, adapted from Lau *et al.* (Lau, Zhang, and Yu 2017), a brief description of each diet is included and, above, the most extended ones are described deeper. Remarkably, a genetic animal model known as diet-induced animal model of non-alcoholic fatty liver disease (DIAMOND) has been recently developed and starting to be commercialized (Asgharpour et al. 2016; Luo et al. 2013).

Table 2.1. Most extended animal models for the study of NAFLD. (Lau et al. 2017) (NASH = non-alcoholic steatohepatitis; HCC = hepatocellular carcinoma; SC = standard chow; HFD = high-fat diet; 0.1%MCDD = 0.1%methionine and choline-deficient diet; HCD = high-cholesterol diet; CD-HFD = choline-deficient high-fat diet; CDAA = choline-deficient amino acid-defined diet; CCl_4 = carbon tetrachloride; Fa = fat; C = carbohydrate; Pr = protein; Su = sucrose; Ch = cholesterol; Cho = choline; Chl = cholate; Glu = L-glutamic acid; Asp = L-aspartic acid; Arg = L-arginine, Leu = L-leucine).

Model	Summary of composition	Obesity	Steatosis	NASH	Fibrosis	HCC
SC diet	20%Fa, 60% Ch, 20% Pr	No	No	No	No	No
HFD	71%Fa, 11%Ch, 18%Pr	Yes	Yes	Mild	Yes	No
ob/ob	NA	Yes	Yes	No	No	No
db/db	NA	Yes	Yes	No	No	No
0.1%MCDD	10%Fa, 40%Su, 0%Cho	No	Yes	Yes	Yes	No
MCDHFD	45%Fa, 35%Ch, 20%Pr, 0%Cho	No	Yes	Yes	Yes	Yes
HCD	1%Cho with 15%Fa or 0.5%Chl	Yes	Yes	Yes	Yes	No
foz/foz	NA	Yes	Yes	Yes	Yes	No
CD-HFD	45%Fa, 35%Ch, 20%Pr	Yes	Yes	Yes	Yes	Yes
CDAA	28.9kcal/g Glu, 15.8kca/g Asp, 12.7kcal/g Arg, 10.5kcal/g Leu, 0% Cho	No	Yes	Yes	Yes	Yes
CDAA+CCl_4	28.9kcal/g Glu, 15.8kca/g Asp, 12.7kcal/g Arg, 10.5kcal/g Leu, 0% Cho	Yes	Yes	Yes	Yes	Yes
Hepatocyte-specific *Pten*-deficient mice	NA	No	Yes	Yes	Yes	Yes
***Mat1a*$^{-/-}$ mice**	NA	No	Yes	Yes	Yes	Yes
DIAMOND	42%Fa, 42g/L Glu, 0.1%Chl	Yes	Yes	Yes	Yes	Yes

The 0.1%MCDD is a modification of the methionine and choline-deficient diet, whereas methionine content is increased mice fed this diet do not loose so much weight. This diet is characterized by a deficiency of methionine and choline in the diet, two essential precursors of SAMe and phosphatidylcholine (Ptd-Chol) respectively. Therefore, important processes in the cell such as VLDL formation, DNA methylation and antioxidant machinery are compromised. 0.1%MCDD induces steatosis, inflammation, cell death, transaminases increase and fibrosis in a short period of time. 0.1%MCDD leads to mitochondrial dysfunctions with an increased oxidative stress. Moreover, this NAFLD model is mainly useful to study the disease specifically in the liver, without other tissue implications and with some differences from patients such as the fact that mice do not gain weight and do not develop insulin resistance.

In case of HFD, NAFLD is induced by a lipid-enriched diet where most of the nutrients (70%) are derived from dietary fats. This diet induces steatosis, oxidative stress, inflammation and, differently to 0.1%MCDD, insulin resistance. Therefore, this diet is closely related to metabolic syndrome and often employed as insulin resistance model (Kasumov et al. 2010; Kowalski et al. 2013). On the other hand, in some cases HFD-fed mice do not develop a high NAS score so this fact highly depends on the mice strain. On the other hand, high-fructose consisting diets

CD-HFD combines the two NASH-inducing principles of the previous diets: disrupted VLDL export and low SAMe levels, consequence of choline deficiency; and an increased fat uptake as a consequence of feeding mice with high-fat containing diet. CD-HFD mice develop steatosis, fibrosis and inflammation. Previous reports have shown that this model develops NASH in a similar pattern to that observed in humans, showing hepatic ballooning and fibrosis with concomitant obesity as well as dyslipidemia and insulin resistance (Wolf et al. 2014).

Finally, DIAMOND animal model has been reported to be the closest one to human evolution of NAFLD with development of obesity, insulin resistance, dyslipidemia and NAFLD. It is based on feeding with a high-fat high-sugar western diet (HFS-WD) mice with a genetic background obtained from a cross between two mouse strains (129S1/SvlmJ and C57BI/6J). DIAMOND mice develop NASH within approximately 22

weeks of HFS-WD, advanced fibrosis with approximately 38 weeks and HCC in approximately 45 weeks (Asgharpour et al. 2016).

2.1.1.4 Therapies for NAFLD

Despite affecting to the 25% of the population and representing the most common state of CLD, until date there is no treatment effective approved for NAFLD. Although changing unhealthy lifestyle has been the most frequent recommendation to NAFLD patients, (Chalasani et al. 2012; Palmer and Schaffner 1990) the long-term hard compliance of the patient has made pharmacological approaches to emerge in order to reduce liver inflammation and injury, overcome insulin resistance and target the development of fibrosis (Ratziu, Goodman, and Sanyal 2015). One of the most relevant pharmacological approaches are: PPAR agonists, FXR-bile acid axis modulators, lipid-altering agents, insulin sensitizers, hepatoprotective agents, gut-liver axis modulators and anti-fibrotic therapies. Herein, there is a brief description of each therapy (Fig. 2.9).

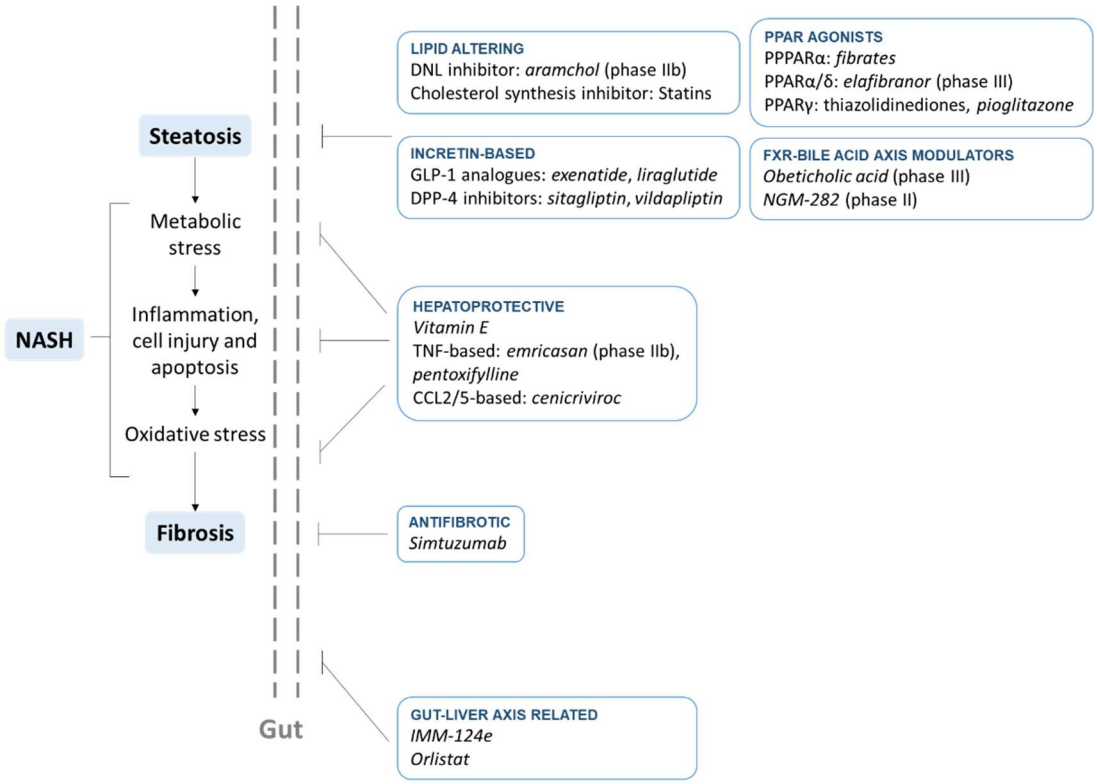


Figure 2.9. Existing therapies for NAFLD. Current therapies have a multifocal approach on targeting different hallmarks of the disease. Therapies targeting steatosis are developed to reduce lipid content (steatosis) with different mechanism of action: targeting lipid metabolism-related enzymes, through PPAR- or incretin-like therapies and modulating the FXR-bile acid axis. Other approaches are focused on protecting hepatocyte from metabolic stress, inflammation, injury or apoptosis and oxidative stress through hepatoprotective agents; or reducing fibrosis development. Gut-liver axis is another approach for resolving NAFLD. (NASH = non-alcoholic steatohepatitis, DNL = *de novo* lipogenesis; GLP-1 = glucagon-like peptide 1; DPP-4 = dipeptidyl peptidase-4; TNF = tumor necrosis factor; CCL2/5 = chemokine ligand 2/5; PPAR = peroxisome proliferator-activated receptor).

2.1.1.4.1 PPAR agonists

Peroxisome proliferator-activated receptors (PPAR) are a group of nuclear factors expressed in liver, adipose tissue, heart, skeletal muscle and kidney. They regulate β-oxidation, lipid transport and gluconeogenesis and have 3 receptors (α, β/δ and γ) that differ by tissue distribution (Brown and Plutzky 2007; Poulsen, Siersbaek, and Mandrup 2012).

PPARα agonists such as fibrates are extensively used for treating hypertriglyceridemia but without an effect in NAFLD (Musso et al. 2010). PPARδ agonists have an additional effect, due to the presence of the δ receptor in macrophage and kuppfer cell (KC), inhibiting KC activation and stimulating β-oxidation. However, a PPARβ/δ agonist named GW501516 has been withdrawn in clinical trials despite of its promisory results (Riserus et al. 2008). Remarkably, elafibranor consists on a dual PPARα/δ agonist and it has been proved to be effective in insulin sensitivity improvement and resolve NASH in phase IIb (Cariou et al. 2013). Phase III clinical trial is currently in the recruitment phase.

PPARγ agonists have been used in diabetes in the form of thiazolidinediones and shown to be effective for NASH treatment (Belfort et al. 2006; Sanyal et al. 2010). Pioglitazone has been evaluated together with vitamin E, obtaining an improvement in NASH histology, reduction in serum aminotransferases as well as hepatic steatosis and lobular inflammation. However, pioglitazone secondary effects may cause heart failure so its use for NASH is limited (Lincoff et al. 2007; Sanyal et al. 2010).

2.1.1.4.2 FXR-bile acid axis modulators

The bile acid intracellular farnesoid X receptor (FXR) inhibits bile acid synthesis and decreases hepatic gluconeogenesis, lipogenesis and steatosis (Porez et al. 2012) so that this approach has been studied for NAFLD therapies.

On one hand, obeticholic (OCA) has shown promising results. OCA is a synthetic bile acid and FXR agonist which has been evaluated in NASH non-cirrhotic patients with a significant histological improvement and a fibrosis reduction. However, pruritus was noted to develop as adverse effect so medication was stopped in some cases and it raises concerns about the need of closer monitoring for cardiovascular risk (Neuschwander-Tetri et al. 2015). Nevertheless, OCA treatment has very recently achieved promissory results in a Phase III clinical trials in reversion of fibrosis and key components of NASH disease (Younossi et al. 2019).

On another hand, fibroblast growth factor-19 (FGF-19) present an alternative FXR-bile acid axis modulator. This hormone is regulated via FXR activation and binds to FGF receptor 4 (FGFR4)/β-klotho receptor in the hepatocyte suppressing gluconeogenesis and promoting glycogen synthesis (Kir et al. 2011). The possible risk of developing cancer-promoting adverse effects has led to the development of non-tumorigenic variants such as NGM-282, currently on phase II (Luo et al. 2014).

2.1.1.4.3 Lipid-altering agents

In Chapter 2.1.1.1 it has been well described the alterations in lipid homeostasis that take place in NAFLD development. As expected, modulators in several pathways involved have been studied as a feasible approach.

In this context, stearoyl-CoA desaturase 1 (SCD1) has been studied as obese subjects present an elevated activity in case of developing NASH, whereas its inhibition has been reported to improve insulin sensitivity and decrease liver steatosis (Issandou et al. 2009; Ntambi 1995; Walle et al. 2016). Aramchol is an inhibitor whose NASH-reducing effects have been reported and it is currently being evaluated in a phase IIb trial (Iruarrizaga-lejarreta et al. 2017; Safadi et al. 2014).

Another agent such as statins, HMG-CoA reductase (HMGCR) inhibitors, has been used in prevention of cardiovascular diseases (CVD). Dyslipidemia, a common feature of metabolic syndrome and NAFLD, places patients in an increased risk for CVD and NAFLD alone has been also reported to be another risk factor (Katsiki, Mikhailidis, and Mantzoros 2016; Srikanth and Deedwania 2016; Stone et al. 2014). Nevertheless, some research has pointed out statins to be underused for NAFLD, even though they are considered as safe at moderated doses. In fact, a small prospective study showed NASH resolution (Blais et al. 2016; Pastori et al. 2015).

2.1.1.4.4 Incretin-based therapies

Incretins are gut-derived hormones secreted at low basal levels in the fasting state, and that rapidly increase after feeding. They have an effect in glucose uptake, inhibiting hepatic and promoting peripheral tissue uptake, reducing glycaemia. Therefore, incretin-based therapies have emerged to be adequate for diabetes and NAFLD treatment.

Probably the therapies related to glucagon-like peptide 1 (GLP-1) are the most popular ones under study. GLP-1 is secreted in the distal ileum and proximal colon that acts at

two levels: stimulating pancreatic β-cell proliferation and insulin synthesis and interacting with receptors in gastrointestinal tract, lung, kidney and central nervous system (Drucker and Nauck 2006). GLP-1 has metabolic functions that include gastric emptying delay, appetite suppression, enhanced liver glucose uptake, insulin secretion and glucagon release inhibition (Abu-Hamdah et al. 2009; Drucker 2006).

As GLP-1 undergoes rapid degradation by dipeptidyl peptidase 4 (DPP-4), produced GLP-1 receptor agonists need to resist the immediate cleavage (Drucker and Nauck 2006). Exenatide and liraglutide, already approved for T2DM treatment, have shown to improve NASH decreasing alanine transferase (ALT) levels and hepatic fat and fibrosis. Additionally, associated weight loss make those therapies attractive in NASH patients with metabolic syndrome (Armstrong et al. 2013; Carbone, Angus, and Yeomans 2016). Another approach consists on DPP-4 inhibitors such as sitagliptin and vildapliptin, prolonging the action of GLP-1 (Rotman and Sanyal 2017).

2.1.1.4.5 Hepatoprotective agents

Another approach for resolving NAFLD consists on the resolution of characteristic hallmarks such as inflammation, cell injury or apoptosis or oxidative stress. In this context, therapies targeting pro-inflammatory agents or proteins have been taken into consideration.

At present, vitamin E is considered as the first treatment when diet and lifestyle changes are not enough. Its antioxidant effects have been proven by to ameliorate NASH both in diabetic and non-diabetic patients (Kowdley et al. 2015; Sanyal et al. 2004). Nevertheless, several studies have pointed out secondary effects of a chronic consumption in the development of prostate cancer and hemorrhagic stroke so these need to be considered when treating NASH patient with vitamin E supplementation (Klein et al. 2011; Schurks et al. 2010).

TNF has been aforementioned (Chapter 2.1.1.2) as a central molecule in signaling pathways that lead to hepatocyte cell injury or apoptosis. Emricasan, an inhibitor of pan-caspase-mediated pathway, has been reported to lower ALT levels, particularly in HCV and NASH (Pockros et al. 2007), so its efficacy is currently being evaluated in a phase IIb trial with NASH and fibrotic patients. Pentoxifylline (PTX) is a TNF inhibitor that can modulate the functions of other pro-inflammatory cytokines (D'Hellencourt et al. 1996; Genoves et al. 2014) and has been proved to cause an histologic improvement in

NASH with a possible lipid peroxidation and a subsequent ROS reduction (Zein et al. 2012).

Finally, other pro-inflammatory cytokines released by the hepatocyte such as chemokine ligand 2 or 5 (CCL2 or CCL5) have also been a hepatoprotective approach. Cenicriviroc, an antagonist of CCL2/5 specific receptor CCR2/5 (Tacke 2018), is currently under phase IIb study for NASH patients.

2.1.1.4.6 Gut-liver axis related therapies

NAFLD and NASH has been widely linked to a disturbed gut-liver barrier integrity (Chapter 2.2.1.1.1). a and their products (specially lipopolysaccharide or endotoxin) can escape causing a massive inflammatory hepatic response so that therapies preventing this phenomenon are currently being evaluated (Compare et al. 2012). IMM-124e is an IgG-enhanced-derived colostrum which has been shown favorable results in preliminary clinical studies, especially in glycaemia and lipid profile (Mizrahi et al. 2012) and orlistat, an FDA-approved lipase inhibitor currently used for obesity, seems to improve liver enzyme levels and liver content (Zelber-Sagi et al. 2006).

2.1.1.4.7 Antifibrotic therapies

Another group of therapies has focused on the detention or reversion of fibrosis development, correlating with NAFLD patient mortality. Therefore, reversion of this hallmark implies an improvement of the condition (Bonis, Friedman, and Kaplan 2001; Ekstedt et al. 2015). A reduction of lysyl oxidase-like 2 (LOXL2) through a monoclonal antibody, named simtuzumab, has been reported to reduce collagen formation in patients and it is currently being evaluated in a phase II trial with non-cirrhotic and cirrhotic patients (Barry-Hamilton et al. 2010).

2.1.2 Liver fibrosis and cirrhosis

As previously mentioned, NAFLD is a progressive disease where a rather benign and reversible condition such as steatosis can evolve to more complicated pathologies. Around 20% of NASH patients have been estimated to progress to irreversible fibrosis/cirrhosis (Figure 2.1).

Liver fibrosis is characterized by an excessive extracellular matrix (ECM) deposition in the liver because of a chronic liver damage together with a sustained wound healing response. The accumulation of ECM proteins alters the normal hepatic architecture,

converting parenchyma into fibrotic scared tissue and generating hepatocyte regeneration nodules that finally lead to cirrhosis. A chronic exposure to a damaging agent (such as drugs, infections or particularly metabolic diseases) is the main cause of fibrosis progression which, although it can evolve rapidly in some cases, the chronic exposure leads to a slow progression that takes over years and finally ends up into cirrhosis. (Friedman 2003, 2007). Cirrhosis is considered as an end-stage of liver disease characterized by alterations in liver parenchyma, nodule formation and hepatic dysfunction. It is normally accompanied by a decreased intrahepatic blood low, resulting in portal hypertension (Bataller and Brenner 2005; Detlef Schuppan and Nezam H. Afdhal 2008; Friedman 2003).

In a healthy state, liver architecture consists on a sinusoid surrounded by hepatocytes lined over a membrane of permeable connective tissue known as Space of Disse. In such space there are inactivated hepatic stellate cells (HSCs), while liver inflammatory macrophages, known as Kupffer cells (KCs), are placed in the sinusoid. If liver reaches a fibrotic state, apoptotic hepatocytes activate KCs releasing inflammatory cytokines that activate HSCs, which contribute majorly to fibrosis by the secretion of large amount of ECM that fills the space of Disse and remodels the sinusoid. Therefore, damaged and dead hepatocytes are replaced by fibrotic scar tissue. The sinusoid remodeling also leads to its capillarization and alterations in hepatic vascularization and portal hypertension. Alterations in hepatic blood flow are considered the main causes that lead to fibrosis- and cirrhosis-derived complications that correlate with liver dysfunction: ascites, renal failure, encephalopathy and varicelar bleeding (Bataller and Brenner 2005; Beers et al. 2003; Detlef Schuppan and Nezam H. Afdhal 2008) (Figure 2.10).

As aforementioned fibrosis can progress to cirrhosis chronically, and without symptoms in many cases. Compensated cirrhosis, named when liver presents normal or not decreased hepatic function, often leads to a progression to decompensated cirrhosis. Decompensated cirrhosis is characterized by the rapid development of the different complications associated to hypertension and liver dysfunction, which can evolve even more rapidly to HCC development and are associated to poorer survival rates. Related to cirrhosis diagnose, liver biopsy is still the most reliable technique able to identify the underlying mechanism the disease, more accurately and setting the grade of cirrhosis progression. However, it is a very invasive method that cannot be completely reliable. Between alternative diagnostic methods employed, serum biomarkers and transient

elastography (Fibroscan) are used. However, these methods present the inconvenience that they cannot identify the etiology of the disease (Castéra et al. 2005; Detlef Schuppan and Nezam H. Afdhal 2008; Pinzani, Rombouts, and Colagrande 2005; Ziol et al. 2005).

Historically, liver fibrosis has been considered an irreversible disease with scar formation as a unidirectional pathway. However, several researches have recently pointed out the possibility of reverting fibrosis. Although the most effective therapy fibrosis is to eliminate the causing agent (Chapter 2.1.1.3 and Chapter 2.1.2.3), several therapeutic approaches are starting to be implanted as they have proven to improve the pathology in fibrotic patients (Bataller and Brenner 2005; Benyon and Iredale 2000; Friedman 2007). Necessarily, the development of a suitable therapy for fibrosis and, especially cirrhosis, passes through characterizing the main cellular mechanism underlying the progression of the disease.

2.1.2.1 Cell population contribution and fibrogenesis

As mentioned before, a complex interplay between different hepatic cell populations takes place during the development of fibrosis (Figure 2.10). In this section there is presented the major contribution of each hepatic cells to the disease.

2.1.2.1.1 Hepatocytes

Hepatocytes are the predominant cells in the liver in terms of volume and function. They contribute majorly to the initiation of the fibrogenic response. Many damaging and cytotoxic agents target the hepatocytes promoting their injury, therefore releasing ROS and cytokines including inflammatory mediators (interleukins, TNF) that stimulate KCs and fibrogenic agents (transforming growth factor β, TGFβ) that activate HSCs. If the injury persists, hepatocytes turn into apoptosis and release apoptotic bodies that are phagocyted by KCs and HSCs, activating them and inducing cytokines production (TNF, TRAIL, FASL and TGFβ). Thus, inflammatory and fibrogenic processes initiate in the liver and apoptotic signaling increases in the hepatocytes (Canbay, Feldstein, et al. 2003; Canbay, Friedman, and Gores 2004; Higuchi and Gores 2003; Savill and Fadok 2000).

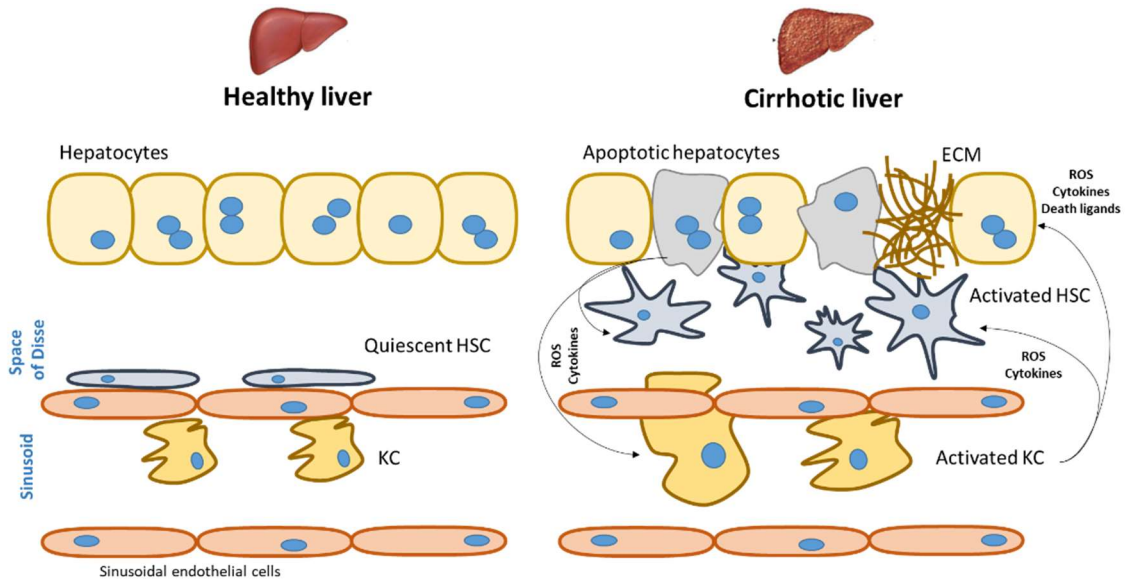


Figure 2.10 Liver structure and cell populations in healthy and fibrotic state. Healthy liver (left) is composed by hepatocytes lined in the space of Disse and surrounding the sinusoid. In the space of Disse there can be also found quiescent hepatic stellate cells (HSCs). In the healthy liver sinusoid there are also Kupffer cells (KCs). Upon liver injury, liver fibrosis occurs (right). Hepatocyte starts becoming apoptotic and releasing cytokines that activate KCs and HSCs. HSCs, when activated, produce proteins from extracellular matrix (ECM) in order to replace the dead hepatocyte and repair the tissue. Sustained liver damage perpetuates the interplay between the three cell types (hepatocyte, HSCs and KCs) leading to ECM deposition and parenchymal architecture disruption.

2.1.2.1.2 Kupffer cells and immune system

Kupffer cells (KCs) are macrophages that reside in liver located in the sinusoid. They have a high endocytic and phagocytic capacity (including endotoxins, pathogens and apoptotic bodies). KCs are in contact with gut-derived and bacterial products that can induce their activity. If liver damage occurs, KCs secrete molecules and cytokines (ROS, NOS, TNF…) that mediate the inflammatory response in the liver and regulate the immune system via antigen presentation. KCs can also secrete death ligands, such as TRAIL and FAS, which enhance hepatocyte apoptosis. Therefore, KCs activation leads to liver inflammation, hepatocyte apoptosis and HSC activation (Canbay, Feldstein, et al. 2003; Gressner et al. 1993).

In addition, more cells from the immune system participate in fibrogenesis. During hepatic inflammation, innate immune cells (monocytes, neutrophils, dendritic cells and natural killer (NK) cells) and adaptive immune cells (T and B cells) are recruited to play different roles in the inflammatory response and fibrogenic development and resolution (Maher 2001; Winau et al. 2007; Xu, Zhang, and Wang 2012)

2.1.2.1.3 Hepatic stellate cells

Hepatic stellate cells (HSC) are the main fibrosis contributors. They produce ECM independently of the etiology and amplify the fibrogenic response (Bataller and Brenner 2005; Higuchi and Gores 2003; Mederacke et al. 2013). They normally reside, if liver is healthy, in the space of Disse in contact with the hepatocyte. Upon liver injury, HSCs get activated and differentiated into myofibroblast-like cells characterized by proliferation, contraction and inflammatory and fibrogenic capacity. Once activated, HSCs migrate across the liver, accumulating in damaged sites and replacing injured or dead hepatocytes while secreting ECM. Their contribution to fibrosis is defined in three sequential steps: *initiation, perpetuation* and *resolution* (Fig. 2.11).

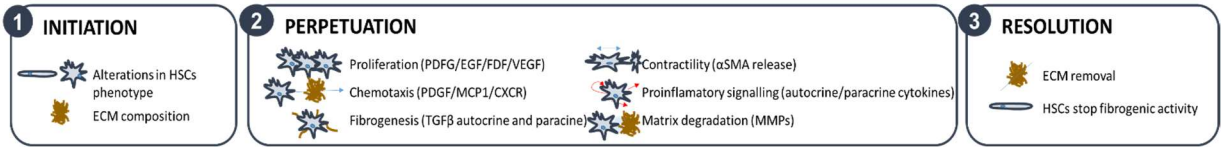


Figure 2.11 Stages of hepatic stellate cells (HSC) activation. HSC activation process initiates with alterations in their phenotype and extra-cellular matrix (ECM) composition. Perpetuation stage involves several changes that include HSC proliferation, chemotaxis, fibrogenesis, contractility, proinflammatory signaling and matrix degradation. Activation ends in a resolution step that involves ECM removal and the stop of fibrogenic activity. (HSC = hepatic stellate cells; ECM = extracellular matrix; PDGF = platelet-derived growth factor; EGF = endothelial growth factor; FGF =fibroblast growth factor; VEGF = vascular endothelial growth factor; MCP-1 = macrophage chemoattractant protein-1; CXCR = cytokine c-x-c receptor; TGFβ = transforming growth factor; αSMA = α-smooth muscle actin; MMP = matrix metalloproteinases).

2.1.2.1.3.1 Initiation

This phase consists on early and rapid alterations in HSCs phenotype and the ECM composition. On one hand, HSCs become activated rapidly due to the action of ROS and cytokines mainly derived from injured hepatocytes, KCs and cholangiocytes. The most important HSC-activating cytokines are TGFβ, platelet-derived growth factor (PDGF) and endothelial growth factor (EGF). Apart from cytokines, HSCs can be activated by engulfing apoptotic bodies derived from hepatocytes and DNA from dead cells, activating and proliferating. (Canbay, Taimr, et al. 2003; Jiang et al. 2009).

On another hand, changes in the ECM occur by altering collagen composition (from collagen IV as the major component to I and III) and changes in membrane receptors (such as integrins) that lead HSCs to migrate across the matrix (Shafiei and Rockey 2006; Yang et al. 2003; Zhang et al. 2006). In addition, the actin cytoskeleton promotes migration and contraction (Choi et al. 2006; Yee 1998) and matrix metalloproteases get activated releasing growth factors that increase fibrogenic signaling (Schuppan et al. 2001) (Fig. 2.11).

2.1.2.1.3.2 Perpetuation

Activated HSCs respond to cytokines and growth factors enhancing their fibrogenic capability through the maintenance and regulation of their proliferation, chemotaxis, fibrogenesis, contractility, proinflammatory signaling and matrix degradation (Fig. 2.11):

- Proliferation: By paracrine and autocrine mechanisms, HSCs induce their own proliferation through the action of PDGF, the most potent mitogen described for this cell type and mitogen-activated protein kinases/extracellular-regulated kinases (MAPK/ERK) (Pinzani et al. 1994, 2005). PDGF activates phosphatidylinositol 3-kinase (PI3K) and MAPK/ERK pathways other mitogens such as EGF, vascular endothelial growth factor (VEGF) and FGF reinforce its proliferative effect (Friedman 2008b; Yoshiji et al. 2003; Yu et al. 2003).
- Chemotaxis: As mentioned before, HSCs are able to migrate across the ECM. Such action is driven by chemoattractant (Ikeda et al. 1999) such as PDGF, macrophage chemoattractant protein-1 (MCP-1) and cytokine c-x-c receptor (CXCR) ligands (Bonacchi et al. 2001; Das et al. 2010; Gong et al. 2017). HSCs migration is inhibited by high levels of adenosine at the injury place, regulating their fixation and fibro genesis in the right site (Hashmi et al. 2007).
- Fibrogenesis: The main function of activated HSCs is to produce ECM. This process is mainly regulated by a TGFβ autocrine and paracrine signaling and mediated by Smad2/3 receptors that activate target gene expression associated to transcription factors and coactivators. It is also regulated by Smad7, which inhibits its signaling (Breitkopf et al. 2006; Inagaki and Okazaki 2007)
- Contractility: Because of phenotype change, HSCs presents characteristics of smooth-muscle-like cells. By the expression of α-smooth-muscle actin (αSMA) and myosin filaments, their contractile activity of HSCs gets increased (Rockey et al. 1992; Saab et al. 2002). This alteration in contractility is one of the main causes of hepatic portal hypertension.
- Proinflammatory signaling: The contribution of activated HSCs to inflammation is mediated by the release of cytokines such as cytokine c-c ligands (CCL), chemokine c-x-c ligands (CXCLs) MCP-1, cytokine c-c receptors (CCR) and TNF. On one hand, cytokines can activate HSCs. On another hand, they can activate hepatocytes and other immune cells. Related to immune cells, HSCs can interact with them modulating their response through antigen presentation (Bomble et al. 2010; Friedman 2008a).

Thus, HSCs can amplify and establish a positive loop of inflammatory signaling contributing to liver fibrosis.

- Matrix degradation: During fibrogenesis HSCs play a role in matrix remodeling by the release of metalloproteinases (MMPs), such as MMP-2 and MMP-9, that degrade collagen IV specifically leading to disruption of the basal membrane in the liver (Giannandrea and Parks 2014). HSCs also release tissue inhibitors of metalloproteinases (TIMP) importantly implicated in collagen I/III degradation during advance fibrosis (Arthur, Mann, and Iredale 1998; Fowell et al. 2011).. Regarding to TIMPs and MMPs, their targeting is an attractive approach to revert liver fibrosis.

2.1.2.1.3.3 Resolution

During this process, the excessive ECM deposited is removed and liver recovers its normal architecture and function. This process requires that HSCs stop their fibrogenic activity by becoming senescent, inactive or apoptotic (Tacke and Trautwein 2015). There are common events frequently found such as decreased TIMP production (allowing an increase in ECM degradation and collagenase activity) (Brew and Nagase 2010) and changes in the immune system, mainly mediated by NKs, that lead to HSCs apoptosis (Fasbender et al. 2016) (Fig. 2.11).

2.1.2.1.4 Non-hepatic stellate cells

Despite HSCs have been identified as major contributors to fibrosis independently of its etiology (Mederacke et al. 2013), animal models have allowed to identify other important contributors to the processes mentioned previously. Other myofibroblast sources that have been identified are portal fibroblast (Dranoff and Wells 2010; Iwaisako, Brenner, and Kisseleva 2012), bone marrow-derived mesenchymal cells (Kemp, Hows, and Donaldson 2005) and cells undergoing epithelial-mesenchymal transition (EMT) (Xia et al. 2006).

2.1.2.2 Animal models of fibrosis

Along fibrosis research animal models have been studied for understanding the pathology. Despite of the existence of several animal models of liver fibrosis, all of them present different characteristics that contribute unequally to the disease such as genetic background, contribution of the immune system or differential gene expression among other. Some models widely used are based on chemical toxins (carbon tetrachloride,

CCl_4), surgical procedures (bile duct ligation, BDL), diet models (0.1%MCDD in its latest stages, Chapter 2.1.1.2) or genetic background (*Mdr2*$^{-/-}$ and *Gnmt*$^{-/-}$).

Carbon tetrachloride is a chemically induced model of fibrosis where the toxin is administered intraperitoneal and then transformed into CCl_3^- by CYP2E1 in the liver. This compound leads to an acute phase of hepatocyte death, necrosis, inflammation and fibrogenesis activation. Previous works have been characterized even the development of HCC if sustained administration (Scholten et al. 2015).

Bile duct ligation (BDL) is a surgical procedure consisting on the ligation of the bile duct, which leads to obstructive cholestasis through bile acid accumulation in the liver. The excess of bile acid promotes hepatocyte apoptosis, inflammation and fibrogenesis. This model is characterized by the implication of portal myofibroblasts in the fibrogenic response, the proliferation of cholangiocytes and the presence of intrahepatic bile ducts. Such characteristics make BDL is considered an excellent model to study biliary cirrhosis. However, the invasiveness and difficulty as well as the highly associated mortality are disadvantages to consider of this model (Fernandez-Ramos et al. 2018)

Genetic models (*Mdr2*$^{-/-}$ and *Gnmt*$^{-/-}$) are based on mice that lack a certain protein. The multi-drug resistance 2 (MDR2) protein is responsible for the secretion of phospholipid into the bile acid so mice defective of this protein develop spontaneously biliary fibrosis and HCC (Fernandez-Ramos et al. 2018). *Gnmt*-defective mice develop chronic liver disease under all its stages, progressing from fibrosis to cirrhosis and finally developing HCC. The chronic excess of SAMe alters the immune system during NASH and fibrosis, overactivating NK/NKT cells and promoting TRAIL-induced apoptosis in hepatocytes (Fernández-Álvarez et al. 2015; Gomez-Santos et al. 2012).

2.1.2.3 Liver fibrosis therapies

Until date there is no effective and standard treatment for liver fibrosis. The current treatment implicates the removal or the causative agents that cause the disease, such as the blocking and reversion by antiviral treatments. Some of the target of research in fibrosis treatment include: anti-inflammatory drugs to avoid inflammation contribution to the progression of the disease, targeted therapies against HSCs to inactivate or induce apoptosis in them, antioxidants to protect hepatocytes from ROS-induced damage, synthetic transcription factors (PPARs and FXR) and the use of non-toxic

ursedeoxycholic bile acid for bile acid-induced fibrosis (Bataller and Brenner 2005; Trautwein et al. 2015).

However, considering the high prevalence of NASH-derived fibrotic patients there are still some major challenges for developing new therapeutic approaches: better characterization of the disease, non-invasive markers and continued studies to evaluate the progression in treated patients. For this reason, many research outcomes, particularly this work, have focused on stopping NASH progression in order to avoid the development of fibrosis and cirrhosis (Chapter 2.1.1.4).

2.1.3 Hepatocellular carcinoma

2.1.3.1 Epidemiology and etiology

Liver cancer is the fifth most common cancer type in the world and the second cause of cancer-related death. HCC is the most frequent presentation of liver cancer (70-85%) over other types (cholangiocarcinoma, hemangiosarcoma and hepatoblastoma) (Alexander et al. 2013; Suriawinata and Thung 2002)). HCC etiology is heterogeneous and multifactorial whereas the major risk of its development are chronic hepatitis B/C, alcoholism, aflatoxin B1 and NAFLD (see next Chapter) (McGlynn and London 2011; Mittal and El-Serag 2013).

The absence of symptomatology in HCC early stages makes it to be diagnosed at late stages, already as multifocal and alongside a cirrhotic surrounding environment. Such fact leads to several difficulties during HCC treatment and a poor prognosis cancer (Attwa and El-Etreby 2015; El-Serag et al. 2008; Llovet and Bruix 2003). When diagnosis very few patients are suitable for therapeutic intervention, such as transplantation or tumor resection, so survival rates of HCC patients are poor, between 6-20 months after diagnosis. Moreover, tumor recurrence after intervention is frequent and it may be enhanced by different signaling pathways that converge and contribute to the malignant transformation of the HCC, reducing the efficacy of conventional systemic therapies (Stoot et al. 2010). Therefore, more research is required particularly in molecular pathways that drive HCC for developing suitable therapies.

2.1.3.1.1 NAFLD-derived HCC

As aforementioned, around 4-27% of cirrhotic patients develop HCC (Figure 2.1). Indeed, NAFLD prevalence has increased during last years. Its expected future spreading is positioning NAFLD-associated HCC as one of the second leading causes of HCC and the most increasing one (Khan et al. 2015; Michelotti, Machado, and Diehl 2013; Wong, Cheung, and Ahmed 2014). Many risk factors mentioned before for NAFLD development (metabolic syndrome, dyslipidemia or diabetes) are also risk factors for HCC, and they are almost presented at least in one form in NAFLD-derived HCC (Michelotti et al. 2013; Welzel et al. 2011). Another important feature of NAFLD-derived HCC is the possibility of cirrhosis absence (Alexander et al. 2013; Ertle et al. 2011; Guzman et al. 2008). Improvement in HCC understanding and diagnosing, particularly in NAFLD-derived type, is mandatory for treating this disease.

2.1.3.2 Molecular pathways in HCC

One of the principal characteristics of HCC is its heterogeneity, which leads to difficulties for treatment and a poor prognosis. There are many different molecular signaling pathways activated at the same time and contributing to the development of cancer. Such pathways are implicated in the regulation of cell growth and proliferation, differentiation, angiogenesis, inflammation and apoptosis:

- Tyrosine kinases receptor (TKRs) pathway: It includes a group of receptors whose activation involves different growth and migration pathways such as Ras/MAPK and PI3K/Akt, frequently overactivated in early HCCs and almost all advanced ones (Bhat, Sonenberg, and Gores 2013; Muntane et al. 2013). Ras/MAPK is activated by different TKRs such as insulin-like growth factor receptor (IGFR) or the receptors EGFR, PDGFR and FGFR leading to the activation of transcription factors and proliferation genes. PI3K/Akt is activated by IGFR1 and inactivated by PTEN, frequently downregulated in HCC, while other TKRs are activated by mammalian target of rapamycin (mTOR).
- VEGF angiogenic pathway: HCC is highly vascularized tumor. However, it is characterized by the presence of hypoxic regions that induce a pro-angiogenic response to generate new vessels from the surrounding parenchyma into the tumor. Such angiogenic response is mainly mediated by the overexpression of VEGF (Cao et al. 2015; Zhang et al. 2012)

- JAK/STAT pathway: This pathway is frequently overexpressed in HCC and promotes the transcription of genes involved in proliferation, migration and differentiation. JAK/STAT is autoregulated in a negative feedback loop in which its activation induced the transcription of suppressor of cytokine signaling (SOCS), that binds to JAK inhibiting the pathway. A frequent overexpression of JAK/STAT in HCC is associated to high methylation of SOCS promoter that prevents the negative regulation (Calvisi et al. 2006).
- Epigenetics: This term defines a variety of mechanisms that control gene expression without affecting genome: DNA methylation, histone acetylation/methylation, miRNAs, transcription factors and chromatin remodeling. They contribute to HCC development by enhancing pro-oncogenic gene expression or downregulating tumor suppressor genes.
- WNT/β-catenin: Due to the frequency of aberrant Wnt mutations, β-catenin has been widely described as a tumor promoter. The canonical WNT/β-catenin pathway regulates embryogenesis, including hepatobiliary development, maturation and zonation. In adult liver the pathway is mostly inactive but it is re-activated during cell renewal and/or regeneration, as well as certain pathologies. Particularly in HCC, WNT/β-catenin is frequently hyperactivated promoting tumor growth and dissemination (Khalaf et al. 2018; Perugorria et al. 2019). Interestingly, Kim *et al.*, have recently described the promotion of growth factor signaling as a key function of β-catenin. The accumulation in the nucleus is restricted to late stages of HCC, whereas until then β-catenin is complexed with cadherin family members driving tumor cell survival by enhancing the signaling of growth factor receptors such as EGFR (Kim et al. 2019).
- TGFβ: This factor plays a dual role in HCC development acting as tumor suppressor during HCC initiation and implicated in invasiveness, angiogenesis and metastasis in advanced HCC (Breitkopf et al. 2006). TGFβ seems to switch from early to late phases of cancer development so that TGFβ-targeting therapies need to understand the crosstalk between this factor and other signaling pathways (Arrese et al. 2018). Particularly, this factor has been reported to interact with a TGFβR-1 promoting Smad phosphorylation and promoting the expression of protumoral genes such as MMPs, C-MYC, PI3K/Akt, ERK1/2 or P38 among other (Liu, Chen, and Zeng 2018).

2.1.3.3 Clinical management for HCC

The complexity if the management of HCC calls for a multidisciplinary approach and specialized nursing. In order to estimate survival, a staging system is required to quantify the tumor burden and liver dysfunction. For this, the Barcelona Clinic Liver Cancer (BCLC) distinguishes among a very early stage (BCLC 0), an early stage (BCLC A), an intermediate (BCLC B), an advanced stage (BCLC C) and a terminal stage (BCLC D) (Llovet, Bru, and Bruix 1999). In this Chapter the most frequent therapies, considering the phase on the tumor, will be described.

2.1.3.3.1 Surgical therapies

Resection is ideal for patients with a solitary tumor at an early stage (BCLC 0 or A), regardless the tumor size and in whom liver function is preserved and there is no significant portal hypertension (Roayaie et al. 2015). These patients have a survival above 60% at 5 years with low postoperative mortality (<3%). However, 70% of such patients have tumor recurrence at 5 years and no adjuvant therapies have proved to reduced it (Bruix et al. 2015).

Liver transplantation, which cures the disease, can be performed in patients with a limited burden and who are not candidates for resection. The Milan criteria for liver transplantation are the benchmark (Mazzaferro et al. 1996). Transplantation with tumors that meet the criteria is associated with 60-80% survival at 5 years and 50% at 10 years with a recurrence lower than 15% (Mazzaferro et al. 2018). While patients are on the waiting list, or if they exceed the Milan criteria, they receive neoadjuvant treatments such as ablation or transarterial therapies (Llovet et al. 2002).

2.1.3.3.2 Tumor ablation

Ablation is recommended for BCLC 0/A patients that are not candidates for surgery (EASL 2018; Marrero et al. 2018). The main method consists on an image-guided percutaneous radiofrequency ablation that causes tumor necrosis. If comparing with resection, this method has fewer complications but provides worse local control for larger tumors. Other ablative options include microwave, cryoablation and ethanol injection.

2.1.3.3.3 Transarterial therapies

This therapy is considered for intermediate-stage patients (BCLC B). The main treatment consists on transarterial chemoembolization (TACE), a transarterial infusion of a cytotoxic agents followed immediately by embolization of tumor-feeding blood vessels.

By this, adjacent nontumoral liver tissue is generally protected because its blood supply comes mainly from the portal vein (Llovet and Bruix 2003). Median survival from this treatment ranges from 26 to 40 months (Burrel et al. 2012; Kudo et al. 2014) and it is not improved when combining the therapy with sorafenib or brivanib (Kudo et al. 2014; Meyer et al. 2017).

Another transarterial treatment consists on selective internal radiation therapy (SIRT) and it is bases on the infusion of microspheres with yttrium-90, whose radiation has antitumoral properties. Differently as TACE, it does not include a macroembolic step (Salem et al. 2016). Phase III clinical trials to evaluate SIRT in BCLC C showed no improvement if combining with sorafenib (Chow et al. 2018; Vilgrain et al. 2017).

2.1.3.3.4 Systemic therapies

These therapies are recommended for late-stage patients (BCLC B and C). Related to them, sorafenib was the first systemic drug approved by the FDA and it is the standard of care for frontline therapy (Villanueva 2019). Sorafenib is an inhibitor of the serine-threonine kinases Raf-1 and B-Raf as well as TKR activity of VEGFR and PDGFβ. Most agents and other treatment approaches have failed to improve on or parallel the efficacy of this treatment: erlotinib, brivanib, sunitinib, linifanib, everolimus, peggylated arginine deiminase, SIRT, TACE, doxorubicin and FOLFOX (Villanueva 2019).

In recent years, a significant progress has been achieved in testing new therapies. Regorafenib is another inhibitor of multiple kinases that increased survival or patients during treatment with sorafenib, decreasing death risk by 37% (Bruix et al. 2017). Indeed, regorafenib became the first drug approved by the FDA for second-line treatment. Other second-line treatments are cabozantinib and ramucirumab (Villanueva 2019).

Finally, immune-based therapies for HCC are emerging. The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor tremelimumab has been tested in a small phase 2 clinical trial (Sangro et al. 2013). Moreover, non-responders to sorafenib have been treated with nivolumab, a programmed cell death (PD-1) immune checkpoint inhibitor, achieving a response that prompted FDA approval under the accelerated program (El-Khoueiry et al. 2017). Another PD-1 inhibitor such as pembrolizumab showed a similar response (Zhu et al. 2018). The combination of targeted therapies with PD-1 inhibitors has been tested and ongoing phase 3 trials will establish these therapies role in the clinical management of HCC.

2.2 METABOLISC ALTERATIONS IN NASH

As mentioned previously in chapter 2.1.1.2, lipid accumulation in NAFLD results from a metabolic imbalance that can include: increased FA uptake or DNL, a disrupted β-oxidation or derangements in VLDL assembly and/or secretion. It also has been pointed out previously mitochondrial dysfunction as the cause of NAFLD and NASH progression (Begriche et al. 2013; Nassir and Ibdah 2014). In this chapter, the importance of non-lipid-related metabolism, particularly nitrogen (ammonium/ammonia and glutamine) (Chapter 2.2.1) and magnesium homeostasis (Chapter 2.2.2) will be described. Coupled to this, in Chapter 2.2.3 the process of VLDL assembly and export, as well as their circulation along the organism, will be described.

2.2.1 Nitrogen metabolism

Related to mitochondrial dysfunction (Chapter 2.1.1.2.4) and its causes, many studies have pointed out a possible affection in carbamoyl-phosphate-synthase (CPS) and ornithine-transcarbamylase (OTC) function (Begriche et al. 2013). Both enzymes are placed in the mitochondria and play a role in the urea cycle which, together with glutamine synthetase (GS, described below), participate in ammonia clearance in the liver (Haussinger 1983; Meijer et al. 1985). Remarkably, patients with urea cycle disorders have been characterized to have a hundred-fold increased risk of developing HCC compared to normal subjects (Seminara et al. 2010; Wilson et al. 2012)

Ammonia is one of the main products of nitrogen metabolism and is normally converted by hepatic urea cycle to be secreted by kidneys. Hyperammonemia is a metabolic condition characterized by elevated levels of ammonia, and it has been reported to be a common event in both acute and CLD (Munoz and Maddrey 1988). Indeed, this condition has been previously associated with increased Child-Pugh grade of liver cirrhosis (Khan, Ayub, and Khan 2016) so that ammonia-lowering therapies have been studied finding improvement in chronic liver disease outcomes (Ghabril et al. 2013; Kristiansen et al. 2014; Wright et al. 2012). It has been also reported that lowering ammonia concentrations in cirrhotic patients restores skeletal muscle proteostasis and restores cirrhosis-derived sarcopenia (Kumar et al. 2016). In this context, some therapeutic approaches have been developed to rewire nitrogen metabolism such as L-ornithine phenylacetate (OP), which has been applied to the treatment of hyperammonemia and hepatic encephalopathy (HE, deeper described in Chapter 2.2.1.1.1) (Jalan et al. 2007).

Focusing on NASH development and its progression to cirrhosis, the relevance of the accumulation of hepatic ammonia in NAFLD has not been elucidated yet. Hepatic TG accumulation is known to inhibit ureagenesis, increasing ammonia concentrations around perivenous hepatocytes (Zhu et al. 2000). Therefore, lipid accumulation and mitochondrial dysfunction during NASH would lead to hyperammonemia in pre-cirrhotic stages. Hyperammonemia has been also associated with alterations in several genes such as the Toll-like receptor (TRL) pathway and an increased hepatocyte apoptosis (Jia et al. 2014), while the scavenging of ammonia has been recently reported to prevent the progression of fibrosis (De Chiara et al. 2019).

2.2.1.1 Ammonia metabolism in liver

Ammonia exists in the organism as ammonium ion (NH_4^+) at physiological pH. It is produced mainly by amino acid deamination, from biogenic amines, amino groups of nitrogenous bases and in the intestine by intestinal bacterial flora through the action of urease (described below). Ammonia is also the product of glutaminase (GLS)-mediated catabolism of glutamine. Glutamate dehydrogenase (GDH) participates in both ammonia scavenging and generation as it catalyzes the reversible glutamate synthesis and degradation (Spanaki and Plaitakis 2012).

On one hand, GLS and CPS are mainly located in periportal hepatocytes as urea cycle takes place in these cells. On the other hand, glutamine synthetase (GS, Chapter 2.2.1.2) is located in perivenous hepatocytes so direct ammonium scavenging process is realized. (Fig. 2.12) (Haussinger 1987). Glutamine is transported across the plasma membrane by a specific transporter called system N (Kilberg, Handlogten, and Christensen 1980). As observed in Fig. 2.12, periportal glutamine catabolism and perivenous resynthesis imply opposite glutamine variations across plasma membrane of the hepatocytes from the two different compartments. Haussinger *et al.,* reported this process to be consequence of a concentration gradient between the compartments and the plasma. By this, glutamine cycling allows the liver to adjust the hepatic ammonium flux into urea or glutamine (Gln) according to requirements of acid-base homeostasis guarantying a well-balanced pH regulation (Haussinger 1987).

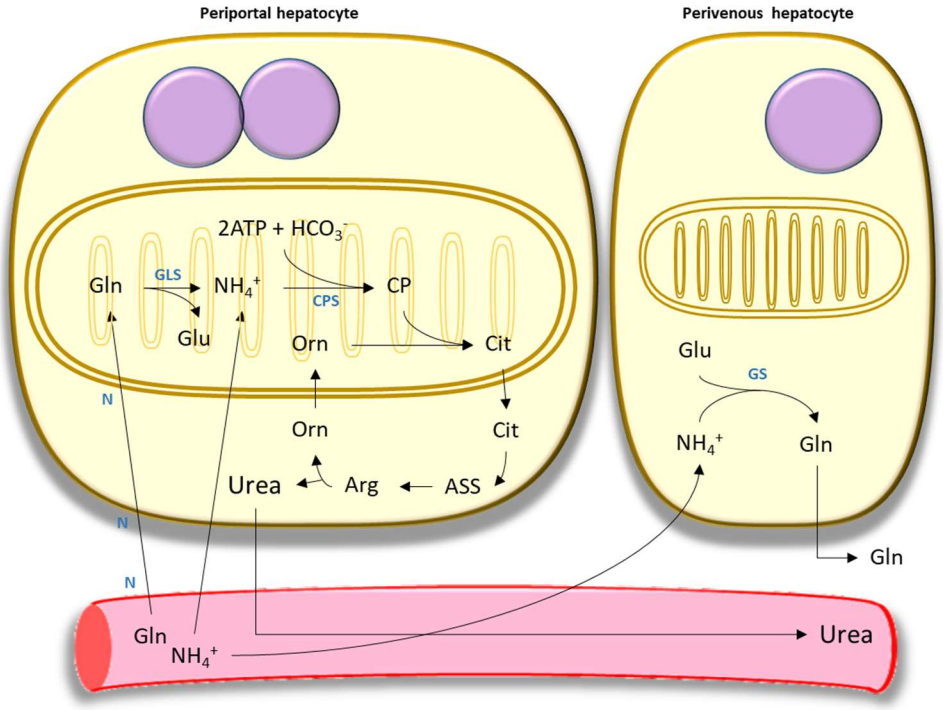


Figure 2.12. Periportal glutamine (Gln) breakdown to glutamate (Glu) and ammonium (NH_4^+) by ammonium-activated glutaminase (GLS) promotes NH_4^+ flux into the urea cycle. In the cycle NH_4^+ is converted into carbamoyl-phosphate (CP) by carbamoyl-phosphate synthetase (CPS), then metabolized into citruline (Cit), argininosuccinate (ASS) and arginine (Arg). Finally, arginine is metabolized into ornithine (Orn), which re-enters the cycle, and urea, which is delivered to the plasma for its secretion. Perivenous ammonium scavenging through glutamine synthesis is catalysed by glutamine synthetase (GS).

2.2.1.1.1 Gut: liver: brain axis

Ammonia homeostasis is clinically relevant for maintenance of the other organs. In this context, several research works have focused on elucidating the contribution of liver-mediated ammonia alterations in gut microbiome (microbiota) and brain, as well as the interconnection between these organs. In the physiological state, Gln (described deeper in next Chapter) is a crucial energy source for gut, where the amino acid is converted by GLS releasing ammonia. As cited above, ammonia is mainly scavenged in the liver periportal hepatocytes by the urea cycle, whereas remnant ammonia is used as substrate for Gln synthesis by GS from perivenous hepatocytes. Under healthy conditions, urea enters then the systemic venous circulation and is excreted by kidney (Olde Damink et al. 2003). When liver fails to scavenge ammonia, other organs are forced to adapt to an ammonia excess mainly synthesizing Gln as detoxification, However, it does not contribute to net nitrogen removal as Gln only acts as a non-toxic nitrogen carrier (Olde Damink, Jalan, and Dejong 2009). Ammonia also induces oxidative stress by generating free radicals and leads to the nitrotyrosination of brain proteins (Oja, Saransaari, and Korpi 2017; Rose 2012).

In brain, astrocytes are the main contributors of Gln synthesis. The ammonia accumulation and its subsequent metabolism into Gln lead to a cascade of neurochemical events that lead to a brain dysfunction named hepatic encephalopathy (HE) (Butterworth 2002; Haussinger et al. 2000). Inflammation also promotes the development of HE in patients with cirrhosis and pro-inflammatory cytokines modulate ammonia effect (Seyan, Hughes, and Shawcross 2010). Indeed, there are evidences for a role of neuroinflammation in liver failure, activating microglia and increasing the synthesis of pro-inflammatory cytokines, monocytes recruitment and blood-brain barrier permeability alterations (Butterworth 2013). Nevertheless, the exact mechanism by which inflammation causes HE is not clear yet. Related to mechanisms that underlie its development, Zhang and collaborators found alterations in microbiota in cirrhotic patients who had developed HE compared to the ones who had not (Zhang et al. 2013). Bajaj also characterized alterations in fecal flora, poor cognition, endotoxemia and inflammation in patients with HE (Bajaj et al. 2012), pointing out the existing inter-connection among brain, liver and gut.

Gut and liver have a pivotal role in the absorption and metabolism of several compounds. Liver receives around a 70% of its food supply from the gut, and abnormalities in liver such as alterations in bile acid metabolism or cirrhosis development lead to changes in microbiota (Rai, Saraswat, and Dhiman 2015). As cited previously, ammonia can be produced by bacteria through the action of urease, additionally to GLS and GDH contribution, which catabolizes urea into CO_2 and NH_3 (Collins and D'Orazio 1993). Gut microbiota, defined as the population of microorganisms that inhabit this organ (specially bacteria), is unique for each individual and it can be modulated by several factors as it uses ingested dietary and host-derived components to generate energy for their own cellular processes and growth, being also able to produce metabolites (Mancini et al. 2018).

Alterations in the integrity of gut microbiota have been linked to NASH development. Particularly, Zhu and co-workers identified an increase of alcohol-produced bacteria in NASH microbiomes as well as an elevated ethanol concentration in NASH (Zhu et al. 2013). Moreover, alterations in microbiota have been correlated with the development of cirrhosis. The cirrhosis dysbiosis ratio (CDR) describes the alterations in microbiota that accompany cirrhosis progression (Bajaj et al. 2014). Indeed, the severity of liver pathologies affect the composition of the microbiota where it leads to a release of higher

endotoxin levels and endotoxin-mediated inflammation derived from LPS (Bajaj et al. 2014). In a healthy liver stage, a balance gut microbiota is accompanied by gastro-intestinal homeostasis and intestinal integrity. Furthermore, under CLD conditions dysbiotic microbiota increases gastric pH and decreases bile acid synthesis and intestinal motility. Such imbalance in microbiota leads at the meantime to alterations in brain blood-barrier, astrocyte swelling, neurotransmission and oxidative stress (Mancini et al. 2018).

Taking into consideration the link between brain and gut, therapies targeting urease producing bacteria have been investigated to ameliorate HE (Collins and D'Orazio 1993; Hansen and Vilstrup 1985). Successful modulation of microbiota leads to HE amelioration: prebiotics, probiotics, synbiotics (pre- combined with pro-biotics) are used to decrease bacterial urease activity and reduce ammonia absorption as they decrease pH, endotoxemia, inflammation and toxin intake (Rai et al. 2015). Lactulose is also a standard therapy to decrease ammonia production and absorption (Clausen and Mortensen 1997) whereas rifaximin, a synthetic antibiotic, also modulates microbiota for treating HE (Garcovich et al. 2012). Remarkably, the main source of ammonia production in patients with cirrhosis is the GLS-mediated Gln deamination (Romero-Gómez et al. 2009).

2.2.1.2 Glutamine

L-glutamine (Gln) is a neutral L-α-amino acid containing 5 carbons. Its molecular weight is 146.15 kDa and its elemental composition comprises carbon (41.09%), hydrogen (6.9%), oxygen 32.84% and nitrogen (19.17%). Gln has 2 nitrogen groups, α-amino and the easily-hydrolysable side-chain amide group. (Fig. 2.13A)

Gln is the most abundant amino acid in the organism and it is a fundamental metabolic intermediate, the main donor of ammonia groups (NH_3) and pH homeostasis maintenance (Cruzat et al. 2014; Curi et al. 2005, 2016). Gln is also a proteinogenic amino acid as it accounts for 5-6% of incorporated amino acids into proteins (Roth 2008). About 80% of Gln in the organism is contained in skeletal muscle, where its concentration is even 15-30 times higher than in plasma (Horvath et al. 1996; Scheppach et al. 1994). In plasma Gln concentration varies between around 500 to 800 μM, which represents about 20% of total amino acids pool in the blood (Roth 2008). Particularly in liver, Gln presents from 40 to 60% of total pool (Cruzat, Macedo Rogero, et al. 2018; Labow, Souba, and Abcouwer 2001).

Gln concentration and availability in the organism depends on the balance between its synthesis/uptake and catabolism/release. Tissues such as lungs, liver, brain, skeletal muscles and adipose tissue have organ-tissue specific Gln synthesis, while primarily consuming tissues such as intestinal mucosa, leucocytes and renal tubule cells are high in Gln-catabolizing enzymes. Meanwhile, Gln metabolism is modulated by many factors such as glucocorticoids, thyroid hormones, growth hormone and insulin (Cruzat, Macedo Rogero, et al. 2018; Cruzat, Rogero, et al. 2018).

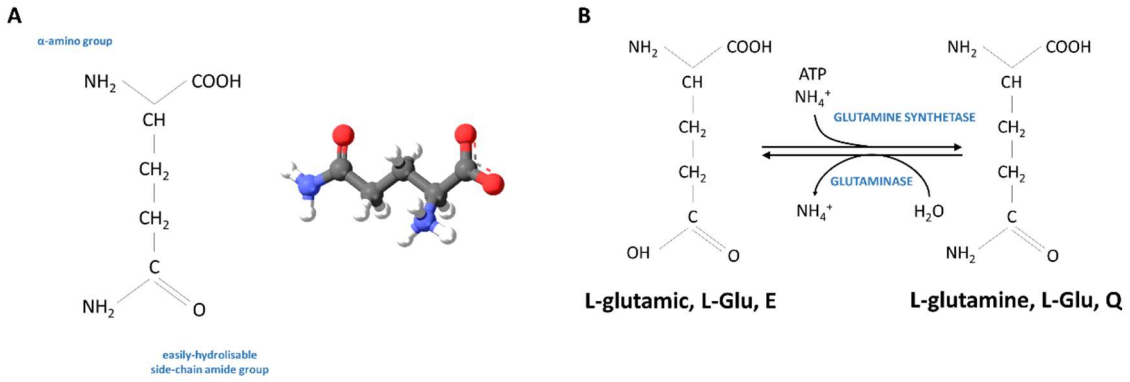


Figure 2.13. Glutamine structure and metabolism. A. Glutamine structure is composed by an α-carbon bound to a α-amino, an α-carboxyl groups and a three-carbon side chain with an amide group. **B.** L-glutamine is synthetized from L-glutamate and ammonium (NH_4^+) through the ATP-dependent glutamine synthetase. L-glutamine catabolism into L-glutamic with NH_4^+ is mediated by glutaminase.

2.2.1.3 Gln metabolism in liver

It has been previously mentioned that liver is the main metabolic organ and Gln is an important precursor for metabolites, so that the amino acid is essential for energy metabolism and hepatocyte proliferator in the liver. Gln is a key precursor for gluconeogenesis under starvation conditions to maintain glucose homeostasis in blood. Additionally, liver regulates blood pH and detoxification of ammonium via the urea cycle as it converts Gln into glutamate (Chapter 2.2.1.1) (Haussinger and Schliess 2007). Ammonia is delivered to the liver for ATP-dependent CPS synthesis, which regulates glutamine flux. Activated HSC has been also reported to be dependent on Gln conversion into α-ketoglutarate and non-essential amino acids to proliferate as a Gln reduction caused an impaired HSC activation (Li et al. 2017). Moreover HSC have been also characterized to use Gln for proline synthesis, a key component of collagen and ECM formation (Li et al. 2017).

Gln concentration in the organism depends on the balance between its synthesis and catabolism. The two main intracellular enzymes of Gln metabolism are GS (EC 6.3.1.2),

responsible of its synthesis and mainly placed in perivenous hepatocytes, and GLS (EC 3.5.1.2), responsible of its catabolism and mainly placed in periportal hepatocytes (Figure 2.12, Figure 2.13).

2.2.1.3.1 Glutamine synthetase (GS)

GS catalyzes the reaction that synthetizes Gln from NH_4^+ and Glu consuming ATP (Fig. 2.12, Fig. 2.13B) (Krebs 1935). The enzyme is primarily found in the cytosol as it produces Gln for synthetizing cytoplasmic proteins and nucleotides. (Curi et al. 2016). GS activity depends on Glu availability which, in turn, is synthetized from 2-oxoglutarate and NH_4^+ through the action of glutamate dehydrogenase (GDH) or from the catabolism of other amino acids such as branched-chain ones (mainly leucine) (Holecek 2018; Tan, Sim, and Long 2017).

Related to GS distribution in the liver, the enzyme is located in the perivenous region acting as a high-affinity scavenger that has escaped periportal detoxification to urea synthesis (Figure 2.12). When ammonia is produced it is delivered via blood stream to such perivenous hepatocytes and used for glutamine synthesis. Therefore, GS acts as a glutamine scavenger for an efficient ammonia detoxification (Haussinger 1983, 1990) as it has been proved through inhibition of GS by methionine sulfoximide or destruction of perivenous cells by carbon tetrachloride (CCl_4) treatment (Haussinger 1983).

2.2.1.3.2 Glutaminase (GLS)

GLS catabolizes Gln releasing Glu and NH_4^+ (Fig. 2.12, Fig. 2.13B) (Neu, Shenoy, and Chakrabarti 1996) and is its placed in the mitochondrial matrix. GLS placement inside the mitochondria allows to produce Glu for entering the TCA as 2-oxoglutarate for obtaining energy or metabolic intermediates (Curi et al. 2016), combining with bicarbonate to form carbamoyl-phosphate (CP) (Meijer et al. 1985) or being used as a precursor for glutathione synthesis (Lu 1999).

In a healthy liver, glutaminase is found in periportal hepatocytes together with CPS and requires ammonia as an essential activator (Fig. 2.12) (Haussinger, Weiss, and Sies 1975; Joseph and McGivan 1978). CPS remains inactive in the absence of NH_4^+ so that GLS acts as an amplification system for ammonia inside the mitochondria, being determinant for urea cycle flux (Meijer et al. 1985).

There are two different phosphate-activated glutaminase isoforms, glutaminase 2 (GLS2) and glutaminase 1 (GLS1). The *GLS2* gene is located on chromosome 12 and encodes two splice variant, highly expressed in normal adult liver (Mates et al. 2013) with a low activity and allosteric regulation: liver-type glutaminase (LGA, short transcript isoform) and glutaminase B (GAB, long transcript isoform). Likewise, the *GLS1* gene is located in chromosome 2 and encodes two splice variants, mainly expressed in kidney under normal conditions, with a high activity and low Km: kidney-type glutaminase (KGA, long transcript isoform) and glutaminase C (GAC, short transcript isoform).

2.2.1.3.2.1 Glutaminase 1 in cancer

Tumor cells are major Gln consumers and compete with healthy ones for this amino acid (Eagle 1955; Medina et al. 1992). Particularly, hepatoma cells consume Gln from 5 to 10 times higher than non-malignant hepatocytes (Bode et al. 1995) As a consequence, a Gln depletion takes place in the non-tumor environment and it correlates with tumor growth. Therefore, Gln metabolism has been studied during last years in order to find suitable cancer therapies (Souba and Sc 1993).

A metabolic switch from the GLS2 to the GLS1 isoform has been described to occur in many cancer cell types such as colorectal cancer (Daemen et al. 2018; Xiang et al. 2015, 2019). This occurs due to a metabolic reprogramming in order to its maintain homeostasis in a poorly-vascularized and nutrient-deprived environment (Nagarajan, Malvi, and Wajapeyee 2016; Pavlova and Thompson 2016). Particularly, the Warburg Effect occurs in many cases so anaerobic glycolysis takes place despite of oxygen presence (Pavlova and Thompson 2016; Warburg 1956) whereas there is characteristic a DNL increase accompanied by a reduced FAO in cancer cells (Long et al. 2018). As a consequence, the reduced acetyl-CoA flux coming from FAO must be compensated by an exogenous supply of Gln, which serves as an important source of reduced nitrogen for biosynthetic reactions and a source of carbon for TCA cycle replenishment that pairs the glycolytic flux (Altman, Stine, and Dang 2016; DeBerardinis and Chandel 2016; Jiang et al. 2016; Pavlova and Thompson 2016). Particularly in liver cells, it have been also described the reprogramming switch from GLS2 to GLS1 isoform in cancer, as well as an increased TCA activity that allows them to maintain energy balance and their proliferative state (Yu et al. 2015; Yuneva et al. 2012).

Indeed, targeting Gln entry into the TCA cycle is being evaluated in clinical trials for treatment of certain malignancies (Bromley-Dulfano et al. 2013; Garber 2016; Gross et

al. 2014; Robinson et al. 2007). Several compounds have been deported to inhibit GLS1 by different mechanism and, as a consequence, reduce tumor growth: UPGL00004, 968, Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), CB-839 or telaglenastata, thiourea derivatives (THDP17), ebselen and 6-diazo-5-oxo-L-norleucine (Díaz-Herrero et al. 2014; Gross et al. 2014; Huang et al. 2018; Sappington et al. 2016; Thomas et al. 2013; Yuan et al. 2016).

2.2.1.3.2.2 Glutaminase 1 in cirrhosis and previous stages of NAFLD

The high-affinity isoform GLS1 is not overexpressed only in HCC but also in previous stages. Yu and collaborators also characterized GLS1 increase in cirrhotic patients (Yu et al. 2015). Remarkably, in their study they show an increase of the enzyme as pathology progresses to HCC. More recently, GLS1 induction has been shown in fibrotic livers whereas inhibition of the enzyme blocked the activation of HSCs, characterized to contribute to fibrosis development (Du et al. 2018).

Despite of characterizing its overexpression in cirrhosis and HCC, the relevance of GLS1 expression in the development of NASH is poorly understood. The treatment of phenylbutirate, an ammonia scavenger, has proven to reduce the palmitate-mediated induction of triglyceride levels by decreasing endoplasmic reticulum stress (Rahman et al. 2009). In agreement with this, the hepatic metabolism of Gln has been previously reported to be implicated in regulating cellular redox balance in the pathophysiology of numerous diseases, suggesting a possible role of GLS1 in the development of NASH (Alberghina and Gaglio 2014; Faubert et al. 2013). Therefore, one of the main objectives of this thesis (Chapter 3) is to characterize GLS1 expression and its contribution to the development of NASH.

2.2.2 Magnesium homeostasis

Magnesium (Mg^{2+}) intake has been also related to liver diseases-derived mortality (Wu et al. 2017). This cation is the most abundant divalent one in the cell and the fourth most abundant element in the body. Mg^{2+} is required for the correct activity of many enzymes related to energy and involving nucleic acids metabolism (Baaij et al., 2015). Mg^{2+} transport rate across cell membranes varies: it is higher in heart, liver and kidney and lower in skeletal muscle, red cells and brain (Swaminathan 2003). The cation is found in a free form in cells and can potentially function as secondary messenger similarly to Ca^{2+} signaling (Baaij et al. 2015). However, this function in intracellular signaling is poorly understood. A Mg^{2+} deficiency has been related to the triggering of inflammatory response, mitochondrial dysfunction and decrease of the antioxidant capacity; all of them hallmarks of NASH. Indeed, previous studies have suggested a link between Mg^{2+} deficiency and lipid metabolism (Lal et al. 2003; Rayssiguier 1984)

In this chapter, the role of Mg^{2+} in cell homeostasis and its efflux through its specific transporters will be deeply described.

2.2.2.1 Role of magnesium in the organism

Intracellular Mg^{2+} concentrations range from 5 to 20 mM. From this, 1-5% is ionized and the remainder is bound to proteins, negatively charged molecules and ATP. Extracellular levels of the cation only account for around 1% of the total (Aikawa 1981; Swaminathan 2003) and it is primarily found in serum and red blood cells (Touyz 2004). Serum Mg^{2+} can be categorized into three fractions: free, bound to protein or complexed with anions. Among the three, the ionized free form has the greatest biological activity (Touyz 2004)

Mg^{2+} is primarily found in the cell, where plays a role as a counter ion for energy-rich ATP an nucleic acids. It acts as a cofactor in more than 300 enzymatic reactions (Saris et al. 2000; Swaminathan 2003), especially in stabilizing ATP-generating reactions through formation an ATP-Mg or GTP-Mg complex. Thus, these complexes are required universally for glucose utilization, lipogenesis, protein synthesis, nucleic acids, coenzymes or methylation among other (Aikawa 1981). More functions are detailed herein (Swaminathan 2003):

- Enzyme substrate (ATP-Mg or GTP-Mg): Kinases B (hexokinase, creatine kinase, protein kinases), ATPases or GTPases ($Na^{+}K^{+}$-ATPase, Ca^{2+}-ATPase) and cyclases (adenylate cyclase and guanylate cyclase).

- Direct enzyme activation: Phosphofructokinase, creatine kinase, 5-phosphoribosyl-pyrophosphate synthetase, adenylate cyclase and Na^+K^+-ATPase.
- Membrane function: cell adhesion and transmembrane electrolyte flux.
- Calcium antagonist: muscle contraction and relaxation, neurotransmitter release and action potential conduction in nodal tissues.
- Structural function: proteins, polyribosomes, nucleic acids, multiple enzyme complexes and mitochondria.

2.2.2.2 Magnesium transport across biological membranes

The characterization of all molecules involved in the transport of magnesium requires further investigation. To a large extent, analysis and measurement of the fluctuations of magnesium has been limited by its intracellular abundance, which apparently appears sufficient to develop its role as cofactor. However, during last 25 years several reports have pointed out Mg^{2+} fluctuations due to various stimuli with a subsequent physiological role (Romani and Maguire 2002). Regulation of cytosolic Mg^{2+} concentration involves a delicate balance between ion influx, efflux buffering and compartmentalization within organelles. The study of Mg^{2+} compartmentalization and trafficking, involved in both physiologic and pathological processes, demands sensors with controllable localization for the measurement of the organelle-specific levels with subcellular resolution.

Magnesium is unique among divalent cations, as it has the smallest ionic radius and the largest hydrated one. When hydrated the cation has a 400 times larger radius than the unbound form, thus requiring the action of specialized proteins known as Mg^{2+} transporters to be shuffled into and out of cells (Jahnen-dechent and Ketteler 2012). In the last decade, several proteins have been identified as regulators of Mg^{2+} homeostasis in vertebrates: ancient conserved domain protein/cyclin M 1-4 (ACDP1-4/CNNM1-4), magnesium transporter 1 (MagT1), MRS2, solute carrier family 41 (SLC41) and transient potential receptor melastatin 6 or 7 (TRPM6 or TRPM7).

MRS2 was the first mitochondrial transporter described in human (Zsurka, Gregan, and Schweyen 2001), while other transporters were discovered as they were upregulated due to hypomagnesemia conditions (Goytain and Quamme 2005) (CNNM2, SLC41 and MagT1). TRPM6 was discovered also in hypomagnesemia patients and, together with TRPM7, it has been reported to be involved in regulating Mg^{2+} homeostasis in mammals

(Schlingmann et al. 2002). Herein, a brief description of the aforementioned transporters has been included.

2.2.2.2.1 Cyclin M/Ancient conserved domain protein

Regarding the new outcomes about magnesium transporters, the cyclin M (CNNM), also known as ACDP, has emerged as a key regulator of Mg^{2+} homeostasis. CNNMs are membrane proteins, encoded by four genes: *CNNM1*, *CNNM2*, *CNNM3* and *CNNM4*. All the isoforms, except CNNM1 which is mainly expressed in brain, are evolutionary expressed throughout development in all adult tissues. Although it is well established that CNNM proteins play a key role in the transport of magnesium ions through cell membranes in different organs (Funato et al. 2014), their specific role still remains under debate and, therefore, their significance as a diagnosis or treatment target. CNNM isoforms show large differences in Mg^{2+} transport activity: where CNNM4 possesses the highest activity, CNNM2 shows an intermediate one and CNNM1 and CNNM3 Mg^{2+} transport activity is the lowest (Chen et al. 2018; Hirata et al. 2014).

Remarkably, the study of CNNM in the development of pathologies has been only studied until date as taking part in the interactome with phosphatases of regenerating liver (PRL) (Yadav and Tamene 2017), a pro-oncogenic protein widely reported to play a role in cancer development (Chen et al. 2018; Gulerez et al. 2016). Although previous research has been developed about the role of pro-oncogenic PRLs in cancer (Chen et al. 2018), a new perspective has emerged by targeting CNNMs instead of PRLs to gain specificity. In this chapter each CNNM isoform will be described deeply and presented as suitable candidates for magnesium modulators.

2.2.2.2.1.1 Cyclin M1 (CNNM1)

As aforementioned, CNNM1 is mainly expressed in brain and testis (Wang et al. 2003). Until date, few research works are available about this protein, whose molecular weight has been estimated to be 115 kDa (Wang et al. 2003). Similarly to the other CNNM isoforms, CNNM1 location has been reported to be in cell membrane, where acts playing a role in magnesium transport. The most remarkable fact is that it has been described to directly interact with the PRL isoforms PRL1 and PRL2, suggesting a possible implication of the protein in several cancer development (Yadav and Tamene 2017).

2.2.2.2.1.2 Cyclin M2 (CNNM2)

This 105 kDa protein is ubiquitously expressed in adult tissues, mainly in the basolateral membrane of renal and epithelial cells. CNNM2 possess Mg^{2+} efflux activity and it has

been reported to play a key role in renal and intestinal (re)absorption of the cation (Baaij et al. 2012; Stuiver et al. 2011). Remarkably, CNNM2 exact function is still being investigated at several works point out that the protein regulates Mg^{2+} homeostasis without being a transporter (Sponder et al. 2016). Furthermore, a relationship has been established between CNNM2 mutations and hypomagnesaemia, where patients showed low Mg^{2+} levels accompanied by derived muscle weakness, tremor and headaches (Stuiver et al. 2011). A research work performed by Arjona points out the relevance of CNNM2 and magnesium homeostasis in brain development, as mutations in kidney HEK293 cells downregulate intracellular magnesium levels and mice lacking the gene suffer an impaired brain development and seizures (Arjona et al. 2014)

CNNM2 has been reported by Yadav to directly interact with PRL1 and PRL2, and indirectly with PRL3 in a CNNM-mediated way (Yadav and Tamene 2017). PRLs regulate magnesium influx binding to CNNM2, promoting tumor progression and cellular proliferation (Funato et al. 2014; Hardy et al. 2015).

2.2.2.2.1.3 Cyclin M3 (CNNM3)

CNNM3 is a 86 kDa membrane protein ubiquitously expressed in all adult tissues (Wang et al. 2003). Chen and collaborators described CNNM3 to act as a dimer for Mg^{2+} transport across cell membrane (Chen et al. 2018). The interaction between CNNM3 and PRL2 has been correlated with tumor growth, in HEK291 kidney cells, and the modulation of intracellular magnesium levels. PRL2 is overexpressed under low intracellular magnesium conditions, interacting with CNNM3 and promoting Mg^{2+} influx. However, all research has been performed in non-liver cell types so its role in liver remains understood.

2.2.2.2.1.4 Cyclin M4 (CNNM4)

CNNM4 has a 95 kDa molecular weight and shares a high homology with the isoform CNNM2 (Wang et al. 2003). Except from colon, where CNNM4 is highly expressed, CNNM4 expression is low and ubiquitous in all adult tissues. The protein has been reported to be in the basolateral membrane of colon epithelial cells, playing a key role in Mg^{2+} extrusion across the membrane for its uptake by the organism (Yamazaki et al. 2013). Mutations in CNNM4 are implicated in Jalili syndrome, characterized by the development of amelogenesis imperfecta and cone-rod dystrophy (Parry et al. 2009). Moreover, Yamazaki also has characterized a CNNM4 additional function in regulating calcium homeostasis in sperm. In a germ-cell-specific mice model deficient for CNNM4,

Ca^{2+} balance has been reported to be altered as, in sperm cells, CNNM4 is required for Mg^{2+} efflux and Ca^{2+} influx (Yamazaki et al. 2016).

CNNM4 has also been reported to interact with PRL (Yadav and Tamene 2017). Funato and co-workers have characterized an inhibitory effect of PRL in CNNM4-mediated magnesium efflux, as PRL overexpression reduces CNNM4-mediated Mg^{2+} efflux affecting energy metabolism. Such inhibition leads to a promotion of cancer malignancy by suppressing AMPK activation and activating downstream mTOR by regulating Mg^{2+} and ATP levels (Funato et al. 2014).

In summary, despite of few studies have been performed trying to determine the role of CNNM and their implication of magnesium homeostasis, none has been reported about their function in the liver so this has prompt us to hypothesize that CNNM and magnesium homeostasis is somehow affecting liver integrity and contributing to the development of NASH (Chapter 3).

2.2.2.2.2 Magnesium transporter 1

MagT1 is a selective transporter ubiquitously expressed in eukaryote cells. It was first described to be upregulated in mouse renal epithelial cells under hypomagnesemia conditions. The protein is targeted to the plasma membrane and biophysical analysis has demonstrated its voltage- and pH-dependent activity (Goytain and Quamme 2005). MagT1-deficient patients present a novel immunodeficiency as it plays a role in regulating Mg^{2+} levels (Li et al. 2011). Zhou and collaborators performed an exhaustive characterization of MagT1, confirming its location in cell surfaces. They also characterized the regulation of its expression through extracellular concentration of Mg^{2+} as MagT1 plays a role in the uptake of the cation (Zhou and Clapham 2009).

2.2.2.2.3 MRS2

The MRS2 protein is poorly expressed in the organism and mainly located in the inner mitochondrial membrane, where it plays a role in mediating Mg^{2+}-influx into the organelle (Kolisek et al. 2003). Therein, it forms a highly-conductive and selective channel that controls homeostasis (Schindl et al. 2007). Remarkably, such maintenance of stable mitochondrial Mg^{2+} levels together with MRS2 have been reported to be crucial for group II intron splicing and suppression of intron mutations, guaranteeing the correct assembly of mitochondrial membrane complexes (Gregan, Kolisek, and Schweyen 2001; Zsurka et al. 2001).

2.2.2.2.4 Solute carrier 41

These proteins belong to a family of membrane transporters, proposed as Mg^{2+} transporters due to their relation to the bacterial Mg^{2+} transporter family MgtE (reviewed by Schmitz) (Schmitz, Deason, and Perraud 2007). There are three existing solute carrier 41 (SLC41) isoforms: A1, A2 and A3. SLC41A1 and SLC41A2 share around a 70% of similarity (Wabakken et al. 2003) and contain multiple trans-membrane regions. SLC41A1 has been reported to be expressed in almost all tissues, (Schlingmann et al. 2002) specially in heart, testis and adrenal and thyroid glands. On the other hand, SLC41A2 expression is limited to immune cell lineages and SLC41A3 has been reported to be poorly and ubiquitously expressed (Runnels, Yue, and Clapham 2001).

Regarding their biological function in regulating Mg^{2+} homeostasis, SLC41A1 has been suggested to play a role in Mg^{2+} efflux acting as a Na^{+}/Mg^{2+} exchanger (Kolisek et al. 2012) while SLC41A2/3 act as putative Mg^{2+} carriers by transporting the cation with channel-like properties (Fleig, Schweigel-Röntgen, and Kolisek 2013). Interestingly, SLC41A1 is regulated by phosphorylation by the action of cAMP-dependent protein kinase A (PKA) (Fleig et al. 2013).

2.2.2.2.5 Transient receptor potential

The transient receptor potential melastatin (TRPM) family is composed by eight subclasses TRPM1-8 in which TRPM6 and TRPM7 are highly expressed in liver (Bilecik et al. 2019). TRPM6 expression has been primarily characterized in organs responsible for Mg^{2+} absorption and secretion (such as intestine and kidney) while its homologous TRPM7 has been detected in many mammalian cell types (Brandao et al. 2013).

Biophysical analysis of TRPM7 has determined its high permeability for divalent cations such as Ca^{2+}, Zi^{2+} and, particularly, Mg^{2+}. The channel is open under standard conditions and inhibited if intracellular concentration of Mg^{2+} is elevated, both in its free or ATP-conjugated form (Nadler et al. 2001). Such properties appear to be similar to TRPM6 (Voets et al. 2004), which has been additionally pointed out to play a key role for epithelial magnesium transport as a loss-of-function leads to hypomagnesemia and secondary hypocalcemia (Schlingmann et al. 2002). Nonetheless, the elegant review by Schlingmann and colleagues remarks that both proteins play a distinct role in Mg^{2+} transport but suggests more research to validate their functional relevance. Which is clear

is their role in Mg^{2+} uptake as the deficiency of these proteins is partially rescued by MagT1 (See chapter 2.2.2.2.2).

2.2.3 Lipid transport

As cited above, intrahepatic lipid accumulation is caused by an imbalance between DNL and FA uptake, on one hand, and β-oxidation and VLDL export on the other hand. In Chapter 2.1.1.1.2 the impaired VLDL secretion is already mentioned and herein a complementary description is included.

2.2.3.1 VLDL composition

VLDL are complex particles that consist of a neutral lipids core, from which most part is TG, surrounded by a monolayer of amphipathic lipids such as phospholipids and unesterified cholesterol and bound to a molecule of apoB. This apoB exists into two isoforms: apoB100, composed by 4536 amino acids, and apoB48, the N-terminal 48% of apoB100 (Chapter 2.2.3.6.2.2). The editing process that converts apoB100 mRNA to apoB48 and the expression patterns are well established: in humans apoB100 is expressed in liver during VLDL formation and apoB48 is synthesized in the intestine while in mice liver expresses both apoB100 and apoB48 (Davidson and Shelness 2000; Innerarity et al. 1996). Therefore, in mice apoB100 and apoB48 assemble in a similar way although there are slight differences in the need for another proteins (Boren, Rustaeus, and Olofsson 1994), the kinetics for intracellular turnover (Rustaeus et al. 1995) or the need for FAs to incorporate (Boren et al. 1994; Stillemark et al. 2000).

Hepatic VLDL production's purpose is to transport hepatic FA synthetized through DNL for storing in adipose tissues. Thus, they play a role in the whole-body conversion of dietary carbohydrate into TG so that it has been recently proposed that glycaemia is regulated partly by converting excessive dietary carbs into hepatic FAs (Schwarz et al. 2003).

2.2.3.2 The VLDL assembly process

The first mechanism of VLDL assembly was provided in 1976 (Alexander, Hamilton, and Havel 1976), proposing the fusion of a newly synthesized apoB with a lipid droplet produced in the smooth ER compartment of the secretory apparatus. This mechanism has been completed in more detail, reaching a new one that proposes that TG becomes associated with apoB in at least two distinct stages of the assembly process. Each step

occurs at different locations: while first step occurs in the ER the second one takes place in the Golgi (Gibbons et al. 2004; Olofsson, Stillemark-billton, and Asp 2000; Shelness and Sellers 2001).

2.2.3.2.1 Formation of pre-VLDL

In the first step, a small TG associated with an apoB molecule during its co-translational location in the membrane of the rough ER. This process is accompanied by a phospholipid (PL) shell or monolayer that encapsulates the TG. In case of apoB48-pre-VLDL in mice, this particle is actually secreted from the cells whereas apoB100-pre-VLDL is mainly retained in the cells (Boren et al. 1994). By this, a small dense apoB-containing VLDL precursor is created in the ER through the action of microsomal triglyceride transfer protein (MTP) (Gordon, Wetterau, and Gregg 1995; Rustaeus et al. 1998) (Fig 2.14A).

Regarding the regulation of this step, a co- and post-translational degradation mechanism has been proposed (Olofsson et al. 2000). An excess in apoB production followed by its degradation has been widely reported (Davidson and Shelness 2000; Olofsson, Asp, and Boren 1999) (Fig 2.14B). Such process has been characterized to be inhibited by the lipidation rate of the protein (Boren et al. 1994; Bostrom et al. 1988; Dixon, Furukawa, and Ginsberg 1991). Therefore, apoB remains associated with the translocon until it is sufficiently lipidated (Mitchell et al. 1998; Pariyarath et al. 2001) and, in this position, interacts with cytosolic chaperones such as heat-shock protein 70 (HSP70). Once ubiquitinated under lipidation absence, apoB excess is then sorted to proteasomal degradation (Fisher et al. 1997; Liao, Yeung, and Chan 1998; Yeung, Chen, and Chan 1996; Zhou et al. 1995) and generating a 70-kDa fragment (Cavallo et al. 1999).

2.2.3.2.2 Maturation of pre-VLDL

Once VLDL precursor is formed, a second stage of VLDL assembly involves the fusion of the apoB-containing precursor with a TG droplet. This process is located out from the rough ER so pre-VLDL must be transferred to the smooth membrane compartment (Stillemark et al. 2000) and it has been characterized the absence of apoB100 in the TG droplet (Hamilton et al. 1998) (Fig. 2.14C).

Although this process is not well understood yet, a dependency activity of ADP-ribosylation factor-1 (ARF-1) has been described (Asp et al. 2000) as well as the interest of the enrichment of the VLDL precursor (Raabe et al. 1999; Wang, Tran, and Yao 1999). In this step several proteins are recruited in the membrane for the cytosol for pre-VLDL

budding: clathrin and the proteins from coaptomere I (COP-I) and II (COP-II). Prevailing hypothesis points that proteins leave the rough ER via Sar1/COP-II transport vesicles (Bannykh, Nishimura, and Balch 1998). Pre-VLDL stays close to the ER and fuses forming a vesicular tubular cluster (VTC) through the action of ARF-1 and COP-I (Bannykh et al. 1998; Lavoie et al. 1999), that also promotes its fusion with *cis*-Goldi (Lippincott-Schwartz, Cole, and Donaldson 1998; Martínez-Menárguez et al. 1999; Presley et al. 1997). Similarly as the initial step, MTP is required for transference of TG from the cytosol to the lumen of the VLDL (Wang et al. 1999) (Fig. 2.14C).

It also has been described that TG formed by diacylglycerol acyltransferase (DGAT) from extracellular non-esterified FAs (NEFA) are not precursors of this secretory pool (Gibbons et al. 1992). Indeed, the secretory pool comes from hepatocellular lipolytic mobilization (Gibbons, Islam, and Pease 2000) so that they are re-esterified on the luminal side of the secretory apparatus, which requires an isozyme of DGAT distinct from that involved in extracellular FA esterification (Owen, Corstorphine, and Zammit 1997).

2.2.3.2.3 Other proteins involved in the VLDL assembly

Additionally, other proteins such as apolipoprotein E (apoE), low-density lipoprotein receptor (LDL-R) and chaperones have been suggested to play a role during VLDL formation.

- ApoE has a role in VLDL assembly and secretion as its absence has demonstrated to make VLDL poorer in TG and smaller (Mensenkamp et al. 1999, 2000). It has been also reported that apoE determines VLDL clearance and the risk of developing atherosclerosis (Knouff et al. 1999).
- LDL-R has been reported to act as a gatekeeper by preventing the release of dense apoB100-containing vesicles from the liver. By this, dense cholesterol-carrier apolipoproteins are not secreted so that atherogenic risk is reduced (Stillemark et al. 2000).
- Chaperones have been described to form a network that determines the size of the secreted particle (Linnik and Herscovitz 1998). Among them, there have been identified binding protein (BiP), calreticulin, calcium-binding protein (CaBP2), glucose regulatory protein 94 (GRP94) and protein disulphide isomerase (PDI).

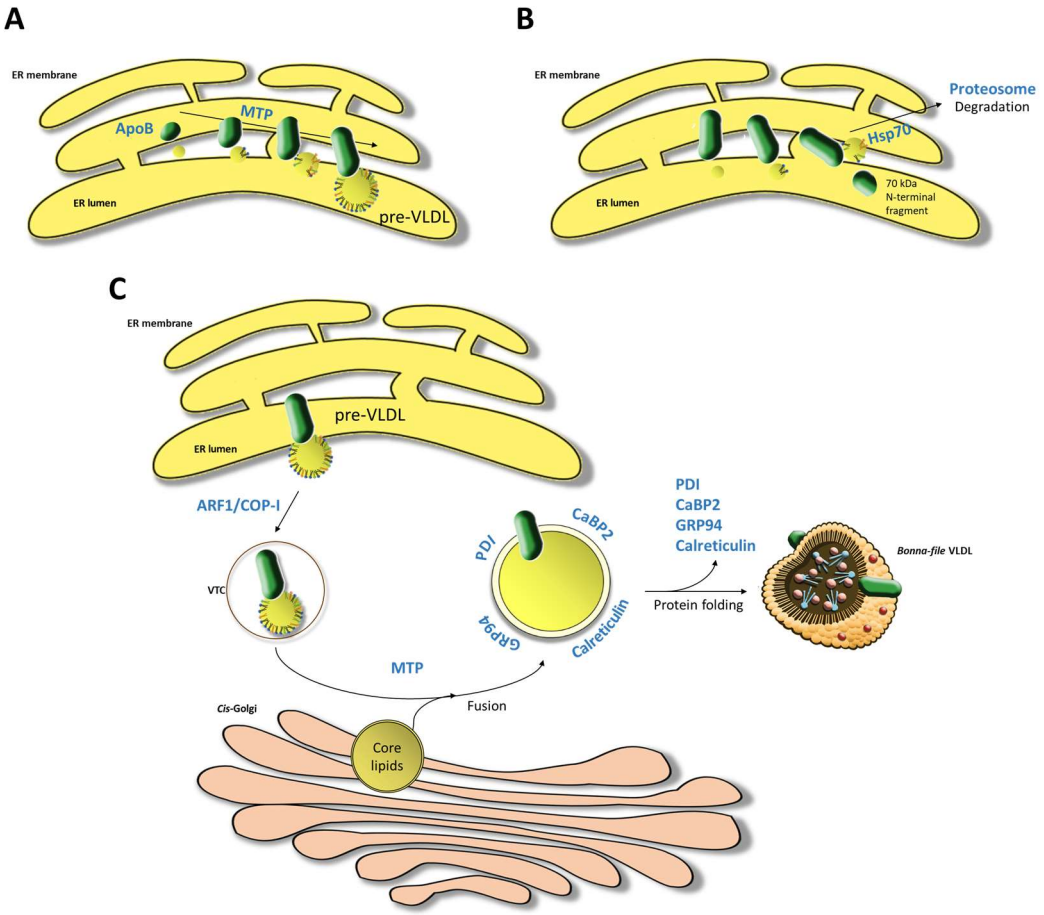


Figure 2.14. VLDL assembly occurs in two steps. A. In the first step apoB is co-translationally and post-translationally lipidated by microsomal triglyceride transfer protein (MTP) forming a nascent pre-VLDL. **B.** If a correct lipidation of apoB does not occur, nascent apoB are sorted by degradation interacting with heat shock protein 70 (Hsp70) and the proteasome. A 70 kDa N-terminal fragment of apoB is then released. **C.** If apoB is correctly lipidated, pre-VLDL exits from endoplasmic reticulum (ER) via the ARF1/COP-I complex forming vesicular tubular complexes (VTC) that reach the Golgi second-step compartment. In *cis*-Golgi, pre-VLDL is fused with lipids and proteins that stabilize the particle such as protein disulphide isomerase (PDI), GRP94, calcium binding protein 2 (CaBP2) and calreticulin. Once *bonna-file* VLDL is formed, proteins are released.

2.2.3.3 Regulation mechanisms of VLDL secretion

Glucose uptake by the hepatocyte stimulates VLDL output by enhancing TG lipolysis in a process dependent to glucose phosphorylation by glucokinase (Brown, Wiggins, and Gibbons 1999). It has been described that insulin signaling suppresses VLDL secretion by interfering with the maturation phase of VLDL assembly (Brown and Gibbons 2001; Durrington et al. 1982) without inhibiting the overall lipolytic mobilization of hepatocellular inner TG. Insulin also suppresses some factors responsible for normal transference of newly mobilized lipids into a TG-rich VLDL precursor. The absence of inner lipid mobilization effect of the hormone in liver contrasts with the inhibitory effect of the hormone in adipose tissue (Gibbons et al. 2000).

Inhibition of MTP (described deeper in Chapter 2.2.3.5) has no effect on TG lipolysis. However, MTP inhibition results in recycling of a higher amount of the mobilized pool back into the cytosol rather than into VLDL. This effect has been also associated with a delay in the removal of newly synthetized apoB and TG from ER and Golgi membranes, with a subsequent lipid accumulation in the liver (Hebbachi, Brown, and Gibbons 1999; Hebbachi and Gibbons 1999).

A dysregulation in VLDL secretion mechanisms is considered to have a major physiological impact on the regulation of plasma TG concentrations (Gibbons et al. 2002; Sparks and Sparks 1994). Moreover, a defective insulin action results in a failure of suppressing VLDL output stimulating lipid release, a characteristic commonly observed in insulin-resistant patients (Gibbons et al. 2002). VLDL purpose is to transport FAs newly synthetized in the liver from carbohydrates for their storage into the adipose tissue (Schwarz et al. 2003) so hypertriglyceridemia is associated with an excessive glucose and carbohydrate uptake by the organism.

2.2.3.4 Role of phospholipids in VLDL assembly

Phospholipids are required for lipoprotein formation and stability. Together with cholesterol, they form a monolayer that surrounds the neutral lipid core consisting on TG and CEs. The most common phospholipids are phosphatidylcholine (Ptd-Chol), phosphatidylethanolamine (Ptd-Etn) and phosphatidylserine (Ptd-Ser).

2.2.3.4.1 Phosphatidylcholine

Ptd-Chol is synthesized in by the CDP-choline pathway, also known as the Kennedy pathway (Kennedy 1957; Kennedy and Weiss 1956). Choline (Chol) can enter the cell through three different transporters: the high-, intermediate- and low- affinity (Traiffort, O'Regan, and Ruat 2013). Once inside the cell, Chol is rapidly phosphorylated by ATP to phospho-Chol via the cytosolic choline kinase (CHK) which has two distinct isoforms CHKα and CHKβ (Aoyama, Liao, and Ishidate 2004; Fagone and Jackowski 2013). The second reaction converts cytidylphosphate (CDP) and phospho-Chol into CDP-Chol via the enzyme CTP:phosphocholine cytidyltransferase (PCYT) which also has two isoforms encoded by the gene *CTα* or *CTβ*, PCYT1A and PCYT2B respectively. This reaction is considered the rate-limiting reaction for Ptd-Chol synthesis via the Kennedy pathway (Choy, Farren, and Vance 1979). Finally, CDP-choline is converted in the ER into Ptd-Chol by the CD-choline:1,2-diaclyglycerol cholinephosphotransferase (CHPT) and dual-

specificity CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) (Henneberry and McMaster 1999; Henneberry, Wistow, and McMaster 2000). (Fig 2.15).

Liver is also able to synthetize Ptd-Chol through a complementary pathway (Bremer, Figard, and Greenberg 1960; Sundler and Akesson 1975) consisting on methylation of Ptd-Etn. The enzyme phosphatidylethanolamine N-methyltransferase (PEMT) catalyzes all the three reactions (Vance 2013; Vance and Vance 1988). PEMT is a small ER membrane protein highly enriched in specialized ER-mitochondria membrane interaction sites (Vance 1990). In rodents around a 30% of Ptd-Chol has been reported to come from this alternative pathway (DeLong et al. 1999) (Fig. 2.15).

In addition to apoB, Ptd-Chol is also required for assembly and secretion of VLDLs and chylomicrons as it comprises around 60-80% of the phospholipids on the surface of apoB-containing lipoproteins (Skipski et al. 1967). Low Ptd-Chol levels or a low Ptd-Chol/Ptd-Etn ratio may lead to degradation of the nascent VLDL (Verkade et al. 1993). Indeed, choline-deficient diets are widely used employed for NASH studying as mice show a TG deposition in the liver (Chapter 2.1.1.3) (Chiba et al. 2016; Takahashi, Soejima, and Fukusato 2012). Additionally, genetic animal models with low levels of SAMe such as *Mat1a*$^{-/-}$ mice show low Ptd-Chol levels due to a reduced PEMT biosynthetic flux (Cano et al. 2011).

2.2.3.4.2 Phosphatidylethanolamine

The two major pathways for Ptd-Etn synthesis are the CDP-ethanolamine pathway in the ER, similar as the CDP-choline, and the phosphatidylserine decarboxylase (PISD) pathway in the mitochondria (Borkenhagen, Kennedy, and Fielding 1961; Percy et al. 1983; Zborowski, Dygas, and Wojtczak 1983). In the CDP-ethanolamine pathway, which is the main one in the liver, ethanolamine (Etn) is imported to the cell and phosphorylated by two cytosolic Etn-specific kinases (ETNK1 and ETNK2) abundantly expressed in liver (Gustin et al. 2008; Lykidis et al. 2001; Tian et al. 2006). Secondly, the cytosolic CTP:phosphoethanolamine cytidyltransferase (PCYT2) converts phospho-Etn into CDP-Etn (Nakashima, Hosaka, and Nikawa 1997; Poloumienko et al. 2004). This step is the rate-limiting one of the pathway (Sundler 1975; Sundler and Akesson 1975; Tijburg et al. 1987). Finally, CEPT or CDP-ethanolamine:1,2-ethanolamine phosphotransferase (Henneberry and McMaster 1999; Henneberry, Wright, and McMaster 2002) catalyzes the formation of Ptd-Etn. (Fig 2.15)

Alternatively, PISD pathway operates only on the outer aspect of mitochondrial inner membranes (Borkenhagen et al. 1961; Percy et al. 1983; Zborowski et al. 1983). This enzyme is translocated to the ER into the mitochondria ATP-dependently (Shiao, Lupo, and Vance 1995; Voelker 1989) and catalyzed the decarboxylation reaction of Ptd-Ser into Ptd-Etn, which is rapidly exported to other organelles such as ER or plasma membrane (Kainu et al. 2013; Shiao et al. 1995; Vance, Aasman, and Szarka 1991)(Fig. 2.15).

The exact role of Ptd-Etn in VLDL secretion is still poorly understood. It has been described that Ptd-Etn content of newly secreted VLDL particles and apoB-containing ones isolated from Golgi was much higher than the one of circulating VLDLs (Hamilton and Fielding 1989; Skipski et al. 1967), suggesting a possible role in VLDL assembly and/or secretion. This might also suggest that Ptd-Etn stimulates the removal of the lipoproteins from the circulation.

2.2.3.4.3 Phosphatidylserine

Oppositely as Ptd-Chol and Ptd-Etn, Ptd-Ser cannot be directly synthetized from CDP-diacylglycerol. Therefore, Ptd-Ser synthesis occurs through serine-exchange reaction through the different action of two Ptd-Ser synthases: PTDSS1 and PTDSS2 (Vance and Steenbergen 2005). The isoform 1 catalyzes the exchange reaction between Ptd-Chol and serine producing Ptd-Ser and releasing choline. The isoform 2 catalyzes the interconversion between Ptd-Etn and serine with subsequent Ptd-Ser and Etn release. Such enzymes are placed in the mitochondria-associated membrane proximal to the (Jelsema and Morre 1978; Tijburg et al. 1987; Vance and Vance 1988). (Fig. 2.15)

Although Ptd-Ser content in most biological membranes is lower than other phospholipids, it is required to maintain the structure of different membranes (Vance and Steenbergen 2005). Ptd-Etn is mainly synthetized through the CDP-Etn pathway but Ptd-Ser can act also as Ptd-Etn precursor being catabolized by PISD as mentioned above (Kainu et al. 2013; Shiao et al. 1995; Vance et al. 1991).

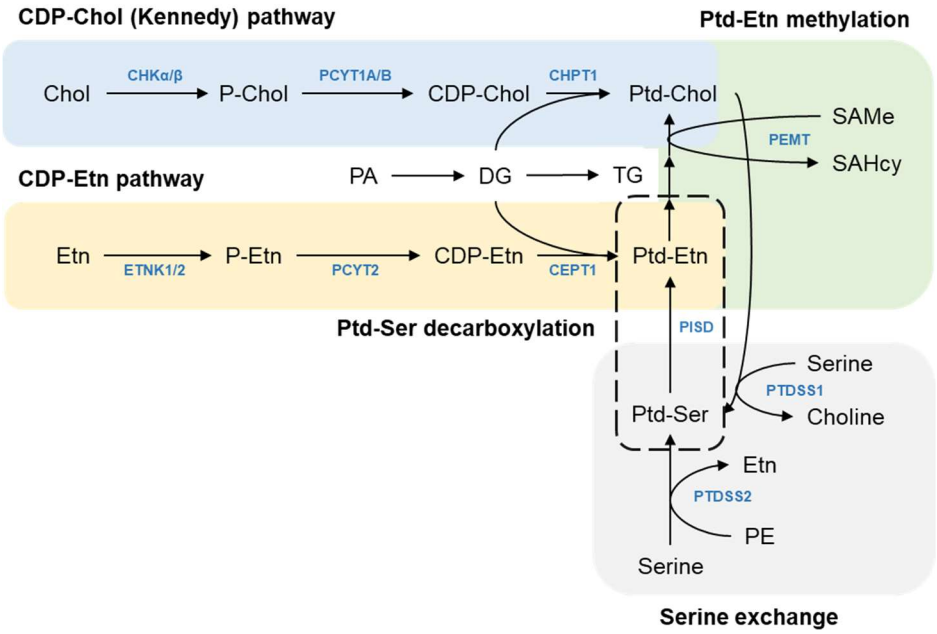


Figure 2.15. Biosynthetic pathways for phosphatidylserine (Ptd-Ser), phosphatidylethanolamine (Ptd-Etn) and phosphatidylcholine (Ptd-Chol) synthesis. Ptd-Chol can be formed from Chol via the CDP-Choline pathway, also known as the Kennedy pathway, or from PEMT which methylates Ptd-Etn using SAMe as donor**.** Similarly, Ptd-Etn can be also formed via the CDP-Etn pathway or by Ptd-Ser catabolism through PISD. Finally, Ptd-Ser can be formed by serine exchange with Ptd-Chol or Ptd-Etn mediated by PTDSS1 or PTDSS2 respectively. (Chol = choline; CHKα/β = choline kinase α/β; P-chol = phospho-choline; PCYT1A/B = CTP:phosphocholine cytidyltransferase; CDP-chol = citidyl-choline; CHPT1 = choline phosphotransferase; PEMT = phosphatidylethanolamine methyltransferase; SAMe = S-adenosylmethionine; SAHcy = S-adenosyl homocysteine; Etn = ethanolamine; ETNK1/2 = ethanolamine kinase 1/2; P-Etn = phospho-ethanolamine: PCYT2 = CTP:phosphoethanolamine cytidyltransferase; CDP-Etn = cytidine diphosphate ethanolamine; CEPT1 = CDP-choline: 1,2-diacylglycerol choline/ethanolamine phosphotransferase; PISD = phosphatidylserine decarboxylase; PTDSS1 = phosphatidylserine synthase 1; PTDSS 2 = phosphatidylserine synthase 2).

2.2.3.5 Microsomal triglyceride transfer protein

Microsomal triglyceride transfer protein (MTP/MTTP) is an essential component of the TG-droplet production machinery and locates abundantly in the ER and Golgi apparatus of the hepatocyte (Levy et al. 2002). The protein plays a key role during two phases from the first step of VLDL formation: (1) translocation of apoB to the lumen through the rough ER (Gordon et al. 1996; Rustaeus et al. 1998) and (2) after apoB transduction has been completed (Chapter 2.2.3.2). It belongs to the family of lipid transfer proteins (Mann et al. 1999; Shoulders et al. 1994). MTP facilitates the transfer of lipids, mainly TG but also cholesteryl-esters (CE) and phospholipids, to nascent apoB while it is being co-translationally translocated across the ER membrane. Kinetic studies have characterized two MTP binding sites: a fast one implicated in TG and phospholipid transfer and a slow one for only phospholipid transfer (Atzel and Wetterau 1993, 1994).

A MTP lack leads to abeta-lipoproteinemia (Sharp et al. 1993; Wetterau et al. 1992), a total loss of apoB-containing lipoproteins from plasma as the pre-VLDL particle cannot go through the second maturation step in the assembly process. This correlates with the absence of VLDL in serum of MTP-knockout mice, accompanied by lipid accumulation in the liver (Raabe et al. 1999).

2.2.3.5.1 Microsomal triglyceride transfer protein forms an heterodimer

MTP is a heterodimer of 97 kDa M subunit of 894 amino acids and a 55 kDa PDI P subunit held together by non-covalent interactions (Wetterau et al. 1990, 1991; Wetterau and Zilversmit 1985). The first MTP 3D modelling was first realized from basis of the lipovitellin structure (Read et al. 2000) and it has been completed by Biterova and colleagues (Biterova et al. 2019). They have reported an interaction with a protein disulphide isomerase (PDI) in an MTPα-PDIβ heterodimer complex with a lipid-binding cavity.

- The P subunit is known to facilitate disulphide bond formation during biosynthesis of nascent proteins even if the heterodimer has been disrupted (Garcia et al. 1992; Wetterau et al. 1990, 1991). However, the P subunit lacks lipid transfer activity by itself (Hussain et al. 2012). PDI has been described by Wettereau to maintain MTP retention in the ER (Wetterau et al. 1990, 1991) and it have been also reported to act as a co-chaperone (Pandhare and Deshpande 2004; Wang et al. 1999). Disruption of

the MTP-PDI heterodimer results in the aggregation of the M subunit and the loss of the lipid transfer activity (Wetterau et al. 1991).

- The M subunit belongs to a family of large lipid transfer proteins (Sellers et al. 2005; Smolenaars et al. 2007) and share sequence homology to apoB. MTP N-terminal β-barrel domain has been suggested to bind with the N-terminus of apoB (Bradbury et al. 1999; M. M. Hussain et al. 2003; M. Hussain, Shi, and Dreizen 2003; Mann et al. 1999), while C-terminal β-sheet binds to lipids and exerts the transfer activity of MTP (Read et al. 2000).

2.2.3.6 Lipoproteins composition and transport

Lipoprotein particles (LPPs) are biochemical structures formed by proteins (apolipoproteins) that bind lipids forming a hydrophobic core for their transport. LPPs are synthesized in the rough ER of hepatocytes where, as mentioned in chapter 2.2.3.2, the initial assembly of apoB-containing particles occur. In the present chapter, each lipoprotein particle and their respective apoproteins will be described, as well as their transport along the organism.

2.2.3.6.1 Lipoprotein particles

LPPs are classified by the composition of their apoproteins in their outer membrane and the lipids placed inside. As mentioned, lipids are assembled into a hydrophobic core that allows their transport across the organism and that determines the density of the particle. Based on these, LPP main groups are classified as chylomicrons (CMs), remnant chylomicrons (rCMs) very-low-density lipoproteins (VLDL), intermediate-density liproteins (IDL) low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Density and diameter determine the group of each LPP (Table 2.2).

Table 2.2. Physical properties and lipid compositions of lipoprotein classes. (CM = chylomicron; VLDL = very-low-density lipoprotein; LDL = low-density lipoprotein; HDL = high-density lipoprotein; TG = triacylglycerol; CE = cholesteryl ester; FC = free cholesterol)

	CM	VLDL	LDL	HDL
Density (g/mL)	<0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (Å)	6000-2000	600	250	120-70
Total lipid (wt%)	99	91	80	44
TG	85	55	10	6
CE	3	18	50	40
FC	2	7	11	7
Phospholipids	8	20	29	46

2.2.3.6.1.1 Chylomicrons

Chylomicrons (CMs) are large TG-rich particles generated in the intestine and involver in the transport of dietary fat to peripheral tissues and liver. They are formed by apolipoproteins A1, A2, A4, A5, B48, C2, C3, E. ApoB48 is the core structural protein, and there is only a molecule per lipoprotein.

CMs, whose size varies depending of CM fat content, are synthesized in the ER of enterocytes by the action MTP and monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase 2 (DAGT2), which catalyze the formation of DAG and TG respectively. CEs are esterified by lecithin: cholesterol acyltransferase (LCAT) and then secreted into the lymph for nutrient delivering into muscle and adipose tissue (Chung et al. 2004). The removal of TG from CMs by peripheral tissues leads to the formation of smaller rCMs, which, compared to their precursors, have a higher cholesterol content. ApoC2 from rCM acts promoting LPL activity and its transference to HDL decreasing LPL capacity

2.2.3.6.1.2 Very-low-density lipoproteins

As previously described in chapter 2.2.3.2, very-low-density lipoproteins (VLDLs) are secreted by the liver and have mainly TG and CE inside the particle. They are formed by the apolipoproteins B100 (also B48 in mice), C1, C2, C3 and E. Similarly to CMs, there is only an apoB100/48 molecule per VLDL and their size varies depending on TG content.

Once transported to the blood torrent, TG are hydrolyzed by lipoprotein lipase (LPL, described below) releasing FA. This catabolism is similar to the one from CM and both processes compete so that elevated CM levels can inhibit VLDL clearance. The removal of TG from VLDL leads to the formation of IDL particles. The liver clears around a half of IDL and hepatic lipase hydrolyzes remaining TG leading to a decrease in TG content. Exchangeable lipoproteins are transferred from IDL to other lipoproteins, leading to the formation of LDL (Dallinga-Thie et al. 2010).

2.2.3.6.1.3 Low-density lipoproteins

Derived from VLDL and IDL, they are even richer in cholesterol as the carry the most part of cholesterol in the organism. They are mainly formed by apoB100 and consist on a spectrum of particles varying in size and density. Small low-density lipoprotein (LDL) particles have been associated to hypertriglyceridemia, low HDL levels, obesity and T2DM as they are considered to be the most pro-atherogenic particles.

Small dense LDL have a decreased affinity for LDL-R, resulting in a prolonged retention time in circulation, and they enter more easily the arterial and bind intra-arterial proteoglycans with a higher affinity. Additionally, LDLs are more susceptible to oxidation, enhancing their uptake by macrophages.

2.2.3.6.1.4 High-density lipoproteins

High-density lipoproteins (HDL) are cholesterol and phospholipid-enriched particles, composed by apolipoproteins A1, A2, A4, C1, C2, C3 and E. ApoA1 is the core structural protein and each HDL can content more than one molecule. Their size is heterogeneous and can be classified based on density, size, charge or apolipoprotein composition:

- Based on density gradient ultracentrifugation: HDL2, HDL3, very-high-density HDL
- Based on nuclear magnetic resonance: large, medium, small
- Based on gradient gel electrophoresis: HDL2a, HDL2b, HDL3a, HDL3b, HDL3c
- Based on 2D gel electrophoresis: pre-β1-HDL, pre-β2-HDL, α1-HDL, α2-HDL, α3-HDL, α4-HDL
- Apolipoprotein composition: A1 particles, A1:A2 particles, A1:E particles

HDLs play an important role in transporting cholesterol from tissues to the liver, known as reverse cholesterol transport, so they are considered anti-atherogenic. They also have anti-oxidant, anti-inflammatory, anti-thrombotic and anti-apoptotic properties.

2.2.3.6.2 Apolipoproteins

Apolipoproteins favor LPPs structure and assembly, with a role in distributing lipids among tissues and sometimes act as cofactor for many lipid metabolism related enzymes (Rall, Weisgraber, and Mahley 1982). Apolipoproteins consist on a single polypeptide chain often with relatively tertiary structure that bind to non-polar lipids or recognize specific receptors. There are many isoforms (A, B, C and E) which will be described below (Table 2.3).

2.2.3.6.2.1 Apolipoprotein A

As indicated in Table 2.3, there are many isoforms of apoA that differ in their molecular weight and their biological activity: A1, A2 and A4.

- ApoA1: This apolipoprotein circulates in plasma primarily as a component of HDL and CMs (Assmann, Cullen, and Schulte 1998; Fidge 1980). A 70% of total apoA1 is synthesized in the intestine, entering circulation associated with CMs but

Table 2.3. Main apolipoproteins in the organism. (HDL = high-density lipoprotein; CM = chylomicron; ; VLDL = very-low-density lipoprotein; IDL = intermediate-density lipoprotein; LDL = low-density lipoprotein; LCAT = lecithin:cholesterol acyltransferase; TG = triacylglycerol; LDL-R = LDL receptor; LPL = lipoprotein lipase).

Apolipo-protein	MW (kDa)	Primary source	Lipoprotein association	Function
ApoA1	28.0	Liver, intestine	HDL, CM	HDL structure, LCAT activation
ApoA2	17.0	Liver	HDL, CM	HDL structure, hepatic lipase activation
ApoA4	45.0	Intestine	HDL, CM	Unknown
ApoA5	39.0	Liver	HDL, VLDL, CM	Enhances LPL-mediated TG uptake
ApoB48	241.0	Intestine	CM	CM structure
ApoB100	512.0	Intestine	IDL, LDL, VLDL	Structural protein, binds to the LDL-R
ApoC1	7.6	Liver	HDL, VLDL, CM	Activates LCAT
ApoC2	8.8	Liver	HDL, VLDL, CM	Activates LPL
ApoC3	8.8	Liver	HDL, VLDL, CM	Inhibits LPL, controls TG turnover
ApoE	34	Liver	HDL, VLDL, CM	Binds to LDL-R

rapidly transferred to HDL, and a 30% in liver, entering associated with nascent HDL with little CE (Mahley et al. 1984a). ApoA1 is a single polypeptide of 243 amino acids, with a high homology between mouse and human (Chapman 1980), that form an amphiphilic helix that has a key role in structure maintenance (Baker, Gotto, and Jackson 1975; Segrest et al. 1974) and also acts as cofactor for LCAT (Fielding, Shore, and Fielding 1972) playing a key role in reverse cholesterol transport to the liver.

- ApoA2: Mainly synthesized in the liver, apoA2 is the second most abundant component of HDL (Assmann et al. 1998; Fidge 1980). It is a dimer of two identical 77 amino acid subunits covalently bound by a disulphide bridge. Like other apolipoproteins, apoA2 binds to lipid with a high degree of ordered secondary structure that include amphiphilic regions (Sparrow and Gotto 1982). It is also known that displaces apoA1 from HDL (Lagocki and Scanu 1980) and that can also form an heterodimeric structure with apoE that does not exhibit the characteristic affinity of apoE to the receptor (Innerarity et al. 1978).

- ApoA4: This protein predominates in newly secreted CMs and it is mainly found in lipoprotein-free fraction (Fidge et al. 1980). It is formed by a single polypeptide or 371 amino acids (Weisgraber, Bersot, and Mahley 1978) synthesized both in the intestine and liver. The amphiphilic tandem has been proved to be a potent LCAT activator when this enzyme is active, while LCAT inactivity results in apoA4 redistribution into a lipoprotein-free fraction (DeLamatre et al. 1983).

2.2.3.6.2.2 Apolipoprotein B

ApoB exists in two forms: apoB100 and apoB48. As mentioned above, apoB100 is synthesized in the liver and is co-translationally bound to lipids during VLDL formation (Chapter 2.2.3.2, Fig. 2.14). Mice have been also characterized to express apoB48 in liver as part of VLDL formation (Boren et al. 1994). ApoB100 consists of 4536 amino acids while apoB48 is composed by half the length of the native apoB100. (Contois et al. 2009). ApoB100 is also present in IDL and LDL while apoB48 is synthesized by the intestine and found in CMs and their remnants (Kane 1983). The metabolism of each apoB isoform is markedly different as apoB100 is secreted as nascent VLDL which is then hydrolyzed into IDL and IDL (Elovson et al. 1981; Packard et al. 2000). A small part of VLDL and IDL are taken back by liver while almost a half of produced LDL are catabolized per day (De Castro-Orós, Pocoví, and Civeira 2010).

Importantly, there is one apoB molecule per hepatic-derived LPP as it plays a key role in VLDL formation (Pahwa and Jialal 2019). In Chapter 2.1.1.1.2 disrupted VLDL secretion was mentioned as one of NASH hallmarks that caused an alteration in lipid homeostasis. Related to this, a defective apoB100 synthesis has been linked as an important factor of steatosis development (Charlton et al. 2002; Ota, Gayet, and Henry N Ginsberg 2008) so that therapies ameliorating its synthesis present an attractive approach to ameliorate lipid accumulation in liver without a risk of increasing ROS production.

ApoB100 also acts a ligand for LDL-R-mediated clearance, which implies around 66-75% of LDL catabolism chiefly by liver (Brown and Goldstein 1983). The recognition of the molecule is the first step in the receptor-mediated LDL catabolism (Goldstein and Brown 1977). ApoB100 is a component of all atherogenic or potentially atherogenic particles so that it provides a direct measure of the number of these particles in circulation. Mutations affecting apoB results in familiar hypercholesterolemia so that therapies targeting apoB have been attempted in order to reduce cardiovascular diseases (Wilson et al. 2019).

2.2.3.6.2.3 Apolipoprotein C

Similarly to apoA, apoC is present in different isoforms that share a common redistribution among lipoprotein classes (Nestel and Fidge 1982). During fasting, apoC are mainly present in HDL, while in a fed-state they are redistributed in VLDL and CM so they seem to play a role in the equilibrium in the dynamic metabolic remodeling of lipoproteins (Hui, Innerarity, and Mahley 1984). The 3 isoform types are described herein:

- ApoC1: It consists on 57 amino acids in a single polypeptide (Shulman et al. 1975) and it has been shown to activate LCAT (Soutar et al. 1975). It participates in cholesterol esterification and transference to HDL as part of the excess surface components generates during VLDL and CM lipolysis.
- ApoC2 ApoC2 is a single 79 amino acid polypeptide and acts as a cofactor for LPL (Havel et al. 1970; LaRosa et al. 1970). A deficiency has been associated with impaired clearance of VLDL and CMs despite of a functional LPL activity (Breckenridge et al. 1978). They have been also reported to activate LCAT.
- ApoC3: The most abundant apoC (Nestel and Fidge 1982) consists on a single polypeptide of 79 residues (Brewer et al. 1974). ApoC3 modulates the uptake of TG-rich remnants by hepatic receptors (Shelburne et al. 1980; Windler, Chao, and Havel 1980) and also activates LCAT and modulates apoC2-dependent activation of LPL (Breckenridge et al. 1978).

2.2.3.6.2.4 Apolipoprotein E

ApoE is composed by 299 amino acids and constitutes chylomicrons and their remnants, VLDL and HDL (Rall et al. 1982). Synthesized with a 18 amino acid signal peptide that is co-translationally cleaved (Zannis et al. 1984), ApoE has an α-helical structure (Roth et al. 1977) with a lipid binding domain (Rall et al. 1982) that exerts several functions:

- Cholesterol transport and metabolism: ApoE-containing proteins, specifically HDL, play a major role in cholesterol transport and are increased in plasma in animals fed a high-fat or high-cholesterol diet (Mahley et al. 1984b). It is also synthesized in brain, playing a key role in cholesterol metabolism through the blood-barrier.
- Receptor-mediated uptake of specific lipoproteins: ApoB/E receptors are present in both extrahepatic and hepatic tissues. In addition, a unique receptor that interacts with apoE-containing lipoproteins has been described in liver and it would present the rCM receptor (Hui et al. 1984; Mahley et al. 1981). ApoE binding to the receptor has a

much higher affinity compared to LDL binding and it relates to the rapid plasma clearance rate of apoE-containing lipoproteins (Boyles et al. 1989) so apoE is the major determinant for receptor binding of HDL and VLDL.

- Formation of cholesterylester-rich particles: Small non-ApoE-containing HDL are increased in size by a parallel increase in CE and apoE, so that three HDL are formed: small HDL_1 (15 nm), large HDL_1 (20nm) and HDLc (25 nm) (Gordon, Innerarity, and Mahley 1983). Without apoE cholesteryl-rich particles cannot be formed.
- Heparin binding: ApoE-containing lipoproteins bind to heparin representing an important physiological mechanism for lipoprotein binding to endothelial surfaces or acting as ground substance of the arterial wall (Innerarity et al. 1978).
- Inhibition of mitogenic stimulation of lymphocyte: An immunoregulatory receptor that binds to apoE has been described in lymphocytes with a role on inhibiting early transformation events for activation of lymphocyte (Curtiss et al. 1981; Curtiss and Edgington 1976).

2.2.3.6.3 Lipoprotein transport and metabolism: receptors and transporters

LPPs circulation along the organism require the presence of several receptors and transporters that recognize them allowing their incorporation and metabolism. The LDL-R, LDLR-related protein (LRP), class B scavenger receptor B1 (SR-B1), ATP-binding cassette transporters (ABC) and Niemann-Pick C1-like 1 (NPC1L1) are the most relevant.

- LDL-R is present in liver and other tissues and recognizes apoB100 and apoE mediating in LDL, rCM and IDL uptake (Goldstein and Brown 2009). After internalization, LPP are degraded in lysosomes and cholesterol is then released. Liver LDL-R plays a role in determining plasma LDL levels and their number is regulated by cholesterol content inside the cell (Goldstein, DeBose-Boyd, and Brown 2006). When cholesterol levels are low, the transcription factor for SREBP is transported from the ER to the Golgi to cleave and activate the expression of the protein, which migrates to the nucleus and stimulates LDL-R expression.
- LRP is a member of LDL-R family expressed in multiple tissues including liver. It recognizes apoE and mediates in rCM and IDL uptake (van de Sluis, Wijers, and Herz 2017).
- SR-B1 is expressed in liver and other tissues (adrenal glands, macrophages…), mediating the selective uptake of CE from HDL (Trigatti 2017). In macrophages it facilitates cholesterol efflux from cells to HDL particles.

- ABCA1 is expressed in hepatocytes, enterocytes and macrophages mediating in cholesterol and phospholipids from the cell to HDL particles with low lipid content (pre-β-HDL) (Wang and Smith 2014).
- ABCG1 is expressed in many cell types and also mediates in cholesterol efflux to HDL particles (Baldan et al. 2006).
- ABCG5 and ABCG8 are expressed and liver and intestine forming a heterodimer. In intestine they mediate in the movement of plant sterols and cholesterol from the enterocyte into intestinal lumen decreasing their absorption. In liver, they move cholesterol and sterols into bile facilitating their excretion (Kidambi and Patel 2008).
- NPC1L1 is expressed in intestine and mediates in cholesterol and plant sterol uptakes from intestinal lumen to enterocyte (Kidambi and Patel 2008).

Once LPPs are recognized and taken or incorporated, several enzymes play a key role in metabolizing them. The lipoprotein lipase (LPL), hepatic lipase, endothelial lipase, LCAT and cholesteryl ester transfer protein (CE-TP) are the most relevant.

- LPL is synthesized in muscle, heart and adipose tissue and then secreted and attached to endothelium of the adjacent blood capillaries. It plays a role in hydrolysis of TG from CM and VLDL, releasing fatty acids to be incorporated by the cell and producing rCM and IDL. For this, apoC2 acts as a cofactor. LPL is inhibited by apoC3, apoA2 and in diabetic patients. It is activated by apoA5 and insulin (Olivecrona 2016).
- Hepatic lipase is localized in liver cells and mediates the hydrolysis of TG and phospholipids in IDL, LDL and HDL leading to smaller particles) (Kobayashi et al. 2015).
- Endothelial lipase hydrolyses only phospholipids from HDL (Yasuda, Ishida, and Rader 2010).
- LCAT is synthesized in the liver and secreted to plasma, where it catalyzes the synthesis of cholesteryl esters in HDL. This allows the transference from the surface of the HDL particle to the core, facilitating the continued uptake of free cholesterol by the particle (Ossoli et al. 2016).
- CE-TP is synthesized in liver and in plasma mediates the transfer of CE from HDL to other particles such as VLDL, CM and LDL. It also catalyzes the transference of TG from VLDL and CM to HDL. Its inhibition leads to cholesterol increase in HDL and decrease in LDL (Mabuchi, Nohara, and Inazu 2014).

3. HYPOTHESIS AND OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

Non-alcoholic fatty liver disease (NAFLD) is a broad term and encompasses a group of pathologies ranging from a simple steatosis to non-alcoholic steatohepatitis (NASH) and fibrosis. NAFLD has an estimated prevalence of around 25% worldwide, and it is expected to grow up within next years. The sedentary lifestyle and the increased consume of processed food are spreading comorbidities such as obesity, hypertension or insulin resistance. Current research is particularly focused on therapies that modulate lipid metabolism and stop the progression of the pathology at its earliest stages, when it is reversible. *Elafibranor* (PPARα/δ agonist), obeticholic acid (bile acid analogue) and *liraglutide* (GLP-1 analogue) are examples of the most advanced therapies with promising results at Phase II. However, the complexity of the pathology and the comorbidities associated make NAFLD a difficult condition to manage and treat.

Our group, the Liver Disease Laboratory, has a wide expertise in the research of the mechanisms underlying NAFLD development and progression. As it will be presented in this work, our research has been focused on metabolic perturbations that lead to NASH development and progression. We have recently set our focus on non-lipid related metabolic pathways that lead to common perturbations observed during NAFLD: nitrogen metabolism and magnesium (Mg^{2+}) homeostasis. Taking into account that metabolism is a complex network of metabolic reactions, modulating such perturbations would have effect in several NASH hallmarks such as steatosis, inflammation, fibrosis or VLDL assembly and export.

Related to nitrogen metabolism, hyperammonemia has been widely pointed out as a trigger of NAFLD development. This condition has been linked to late-state pathologies such as cirrhosis, associating it with an increased Child-Pugh grade, and HCC. Indeed, in a previous work our group we have developed a new method for ammonia determination in paraffin tissue, stablishing an ammonia score with a dependency of the stage of the disease. By this, we have characterized an ammonia excess in clinical samples and pre-clinical models of liver disease. Moreover, ammonia-lowering therapies have been related to an improvement in chronic liver disease outcomes, as the regulation of the balance between its buffering and production presents a potential approach. On one hand, ammonia is buffered in the urea cycle, already reported to be dysregulated during liver disease, and glutamine synthetase activity, which conjugates ammonia with glutamate (Glu) to form glutamine (Gln). On the other hand, ammonia is produced from Gln

catabolism by glutaminase (GLS), whose activity has been reported to be the main source of ammonia during fibrosis. Furthermore, the high-affinity isoform GLS1 has already been reported to overexpress in late stages from liver disease such as cirrhosis and hepatocelular carcinoma. Related to this, we have found a decreased Gln/Glu ratio in a metabolomics analysis of serum from a cohort of NASH patients compared to healthy ones, suggesting a perturbation in glutamine metabolism and prompting us to think about GLS1 contribution, not only in cirrhosis or cancer but also in a previous stage as NASH.

Taken into consideration previously exposed, we have hypothesized that GLS1 could trigger NASH development.

Moreover, we have focused on Mg^{2+} as another causative agent of the pathology, as alterations in its homeostasis have been linked to NAFLD comorbidities such as obesity, metabolic syndrome and insulin resistance. Taking also into account that Mg^{2+} has been reported to play a key role in all ATP-involving reactions in the organism, together with the altered energy metabolism characterized to occur during NASH, we have attempted to characterize the contribution of Mg^{2+} perturbations to the development of NASH. In a cohort of clinical samples, we found a dysregulation of the cation in serum from NASH patients compared to steatosis and healthy ones. However, little research has been realized about the modulators of Mg^{2+} homeostasis. Few works have reported several proteins involved in Mg^{2+} flux across cell membranes, whereas almost none has been elucidated about their role in the liver yet. Moreover, cyclin M (CNNM) is a Mg^{2+} modulator in the cell which has been previously described to interact with phosphatase of regenerating liver (PRL), a protein reported to be overexpressed during HCC. Therefore, CNNM could be somehow playing a role in the disease by causing intrahepatic Mg^{2+} dysregulations.

Based on previously exposed, we have hypothesized that perturbations in Mg^{2+} homeostasis, with CNNM as the main contributor, could lead to the development of NASH.

As aforementioned, metabolism must be considered as a complex network in which all the pathways are interconnected among them. Therefore, and based on previously exposed, the main objective of this thesis is to characterize the contribution of CNNM and GLS1, two non-lipid-related proteins, to the development of NASH.

Thus, the aims of this thesis are:

1. Characterize GLS1 contribution to perturbations in glutamine catabolism in NASH, studying the expression of the enzyme and determining its effect over ammonia.

2. Determine the mechanism by which GLS1 differential expression contributes to NASH development, modulating the enzyme and characterizing the effects in different hallmarks of the disease.

3. Identify the effect of CNNM in the modulation of Mg^{2+} homeostasis in NASH, characterizing the expression of the protein in the pathology and distinguishing among the four different isoforms CNNM1, CNNM2, CNNM3 and CNNM4.

4. Determine the mechanism by which the specific CNNM isoform contributes to the development of NASH and characterize the effect of its modulation in the pathology, determining if it is contributing to Mg^{2+} homeostasis.

4. EXPERIMENTAL PROCEDURES

4. EXPERIMENTAL PROCEDURES

4.1 NAFLD HUMAN SAMPLES

All the studies were performed in agreement with the Declaration of Helsinki and according to local national laws. The Human Ethics Committee of each hospital approved the study procedures and written informed consent was obtained from all patients before inclusion in the study.

Liver samples were obtained from a two independent hospitals. The cohort of NASH patients and healthy controls used to determine GLS1, GLS2 and glutamine synthetase expression (Chapter 5.1) was obtained from the Department of Gastroenterology, Azienda Ospedaliero-Universitaria & University of Modena and Reggio Emilia (UNIMORE, Modena, Italy). On the other hand, the cohort used to determine *CNNM1*, *CNNM2*, *CNNM3*, *CNNM4* and *GLS1* mRNA expression and CNNM4 protein expression in liver (Chapter 5.1 and Chapter 5.2) was obtained from a cohort of morbidity obese patients diagnosed in Marqués de Valdecilla University Hospital (MVUH, Santander, Spain) with discarded alcoholic disease and viral hepatitis infection. Finally, the serum samples from NASH patients where we measured magnesium levels were obtained from a cohort of morbidity obese patients in MVUH. The characteristic of these patients are indicated in Table 4.1, Table 4.2 and Table 4.3.

Table 4.1. Characterization of the cohort of non-alcoholic steatohepatitis (NASH) patients used for the immunohistochemical characterization of glutamine metabolism intermediates. (N = number of total patients; BMI = body mass index; NAS = NASH activity score) (Related to Figure 5.1).

	Average ± SEM
N	16
AGE (years)	42 ±12
BMI (kg/m^2)	24.5 ± 1.6
NAS score	2.7 ± 1.6

Table 4.2. Characterization of the cohort of non-alcoholic steatohepatitis (NASH) patients used for the determination of mRNA expression of cyclin M1-4 (CNNM1, CNNM2, CNNM3 and CNNM4) and the immunohistochemical characterization of CNNM4 (Related to Figure 5.1 and 5.16).

	Average ± SD
N	40
AGE (years)	44.6 ± 10.4
Gender (F/M)	25/15
Weight	135.0 ± 20.4
BMI (kg/m^2)	47.6.5 ± 5.7
LDL	99.7 ± 29.7
HDL	41.8 ± 10.0
TG	165.3 ± 89.1
Cholesterol	180.5 ± 37.5
AST	28.3 ± 14.4
ALT	34.4 ±19.1
Glucose	107.8 ± 36.0
Arterial Hypertension	46.25%
Diabetes mellitus	31.56%
Dyslipidemia	50.88%
Metabolic syndrome	60.38%
Insulin resistance	58.13%
NAS score > 5	19.5%
Ballooning	52.5%
Inflammation	65%

Table 4.3. Characterization of the cohort of non-alcoholic steatohepatitis (NASH) patients used for the determination of magnesium content in serum (Related to Figure 5.16).

	Average ± SD
N	51
AGE (years)	43.6 ± 11.35
Gender (F/M)	30/21
Weight	138.0 ± 20.36
BMI (kg/m^2)	48.0 ± 5.11
LDL	102.93 ± 30.22
HDL	40.50 ± 10.64
TG	167.27 ± 78.89
Cholesterol	181.02 ± 37.54

AST	34.43 ± 38.72
ALT	42.51 ± 49.55
Dyslipidemia	45.1%
Metabolic syndrome	54.9%
Insulin resistance	54.9%
NAS score ≥ 5	17.0%
Ballooning	41.5%
Inflammation	54.7%

4.2 ANIMAL EXPERIMENTS

All the animal experiments were conducted in accordance with the Spanish Guide for Care and use of Laboratory animals, and with the International Care and Use Committee Standards. All procedures were approved by the CIC bioGUNE's Animal Care and Use Committee and the competent authority (Diputación de Bizkaia). Mice were housed in a temperature-controlled animal facility (AAALAC-accredited) within 12-hour light/dark cycles. They were fed a standard diet (Harlan Tekland) with water *ad libitum*.

4.2.1 0.1% Methionine and Choline Deficient Diet (0.1% MCDD)

C57BL/6J wild-type mice were fed with a methionine (0.1%) and choline (0%) deficient diet for 2, 4 and 6 weeks. 2 weeks after the beginning of the diet mice were divided in two groups and subjected to an *in vivo* silencing *Cnnm4* or *Gls1* or unrelated siRNA control, receiving either 200µll of a 0.75µg/µl solution of or specific *in vivo* siRNA (Custom Ambion, USA) or control siRNA (Sigma-Aldrich, USA) using Invivofectamine ® 3.0 Reagent (Invitrogen, USA) following the manufacturer's instructions. Tail vein injection was performed twice a week until the fourth week (Figure 4.1A). At the end of the treatment animals were sacrificed and liver were split into several pieces for subsequent analysis including: RNA or protein extraction, formalin fixation for histology and immunohistochemistry or metabolic analysis. Blood for serum analysis was collected once a week during the treatment

4.2.2 Choline Deficient High Fat Diet (CD-HFD)

C57BL/6J wild-type mice were fed with a choline-deficient high-fat diet for 3 and 6 weeks. 3 weeks after the beginning of the diet mice were divided in two groups and subjected to an *in vivo* silencing *Cnnm4* and *Gls1* or unrelated siRNA control, receiving either 200µll of a 0.75µg/µl solution of or specific *in vivo* siRNA (Custom Ambion, USA)

or control siRNA (Sigma-Aldrich, USA) using Invivofectamine ® 3.0 Reagent (Invitrogen, USA) following the manufacturer's instructions. Tail vein injection was performed twice a week until the sixth week (Figure 4.1B). At the end of the treatment animals were sacrificed and liver were split into several pieces for subsequent analysis including: RNA or protein extraction, formalin fixation for histology and immunohistochemistry or metabolic analysis. Blood for serum analysis was collected once a week during the treatment.

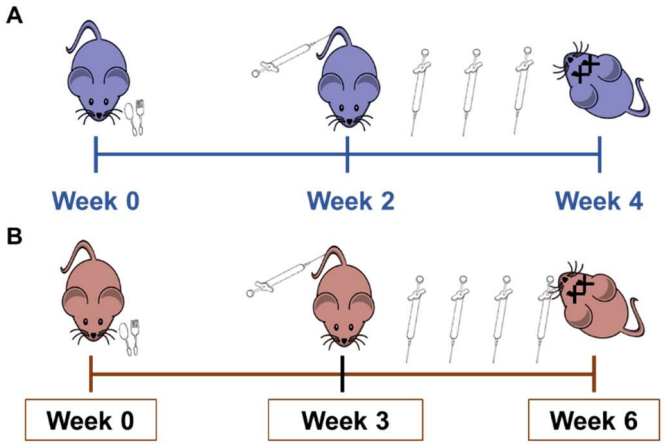


Figure 4.1. Pre-clinical study for cyclin M4 (CNNM4) and glutaminase 1 (GLS1) silencing in non-alcoholic steatohepatitis (NASH) *in vivo* animal models. A. NASH model induced by feeding mice a 0.1% methionine and choline-deficient diet during four weeks and starting treatment at the second week of the diet. **B.** NASH model induced by a choline-deficient high-fat diet (CD-HFD) during six weeks, starting the treatment at the third week of the diet.

4.3 CELL ISOLATION, CULTURE AND TREATMENTS

4.3.1 Primary and commercial cell lines

In this work primary cell cultures (adipocytes, hepatocytes, KC and HSC) and the commercial human THLE2 cell line have been used.

4.3.1.1 Primary hepatocytes isolation

Primary hepatocytes from 3-month old wild type (C57BL/6J) were isolated by perfusion with collagenase Type I (Worthington, USA). Briefly, animals were anesthetized with isoflurane (1.5% isoflurane in O_2). Then, the abdomen was opened and a catheter was inserted into the inferior vena cava. Liver was perfused with buffer A (1x PBS, 5mM EGTA, 37ºC and oxygenated) and the portal vein was cut. Next, liver was perfused with buffer B (1x PBS, 1mM $CaCl_2$ 37ºC and oxygenated) to remove EGTA, and finally perfused with buffer C (1x PBS, 2mM $CaCl_2$, 0.65 BSA, collagenase type I, 37ºC and oxygenated). After buffer C perfusion, liver was separated from the resto of the body and

placed into a petri dish with MEM (Gibco, USA). Gall bladder was carefully removed and, then, liver was mechanically disaggregated with forceps. The digested liver diluted in MEM was filtered through a sterile gauze and filtered liver cells were collected and washed three times (1x4' at 400RPM and 2x5' at 500RPM) in 10% FBS (Gibco)/1% PSG (Gibco) supplemented MEM, conserving all supernadant Kupffer and Hepatic Stellate cells isolation. After the final wash, hepatocytes contained in the pellet were resuspended in 10% FBS 1% PSG MEM for subsequently culturing.

Primary hepatocytes were seeded over previously collagen-coated culture dishes at a density of 7600 cells/mm^2 in 10% FBS/1% PSG supplemented MEM and placed in an incubator at 37ºC, 5%CO_2-95% air. After 6 hours of attachment, culture medium and unattached hepatocytes were removed with fresh 0% FBS/1% PSG MEM for the aimed treatment (Table 4.4)

4.3.1.2 Kupffer cells and hepatic stellate cells isolation

Supernatants from the hepatocyte wash were joined together and centrifuged (1350g, 10', 4°C). The pellet was resuspended in 10 ml 0% FBS/1 % PSG RPMI culture medium (Gibco) and then loaded onto a 25/50% Percoll PLUS (GE Healthcare, UK) gradient and again centrifuged (1350g, 30', 4°C) with minimum acceleration/deceleration. The nonparenchymal cells were collected with a pipette from the interface between the two density cushions of 25% and 50%. Collected cells were centrifuged again (1350g, 10', 4°C) and the resulting pellet was resuspended in 0% FBS/1% PSG RPMI. Kupffer cells were removed from the media by selective adherence, by incubating the resuspended cells on uncoated plastic culture plates for 8 min at 37°C. Afterwards the media was removed and used for primary mouse hepatic stellate cell isolation. Primary Kupffer cells were incubated in 0% FBS/1 % PSG RPMI at 37°C in a humidified atmosphere of 5% CO_2-95% air. The remaining media was centrifuged again and the resulting pellet was resuspended in 0% FBS/1 % PSG RPMI. The hepatic stellate cells were then seeded on uncoated plastic culture plates and cultured in 0% FBS/1 % PSG RPMI medium at 37°C in a humidified atmosphere of 5% CO_2-95% air.

Table 4.4. Reagents used for *in vitro* experiments. (FBS = fetal bovine serum; 2-APB = 2-aminoethyl diphenylborinate; BPTES = Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide; MTP = microsomal triglyceride transport protein; Mg = magnesium; MCD = methionine and choline-deficient; MEM = minimal essential medium; DMEM = Dulbecco's Modified Essential Medium; DMSO = dimethyl sulfoxide).

Reagent	*Dose*	*Vehicle*	*Time*	*Function*	*%FBS*	*Supplier*
2-APB	300 µM	DMSO	24h	TRPM7 inhibitor	0%	Sigma Aldrich
BPTES	10 µM	DMSO	48h	GLS1 inhibitor	0%	Sigma Aldrich
Ctrl	0	MEM/ DMEM	24/48h	Control medium	0%	Gibco
Lomitapide	600 nM	DMSO	24h/48h	MTP inhibitor	0%	Sigma Aldrich
Mg-deficient medium	0mM	DMEM	24h	Mg depletion	0%	GE HealthCare
Mg-enriched medium	5mM	MEM/ MCD	24h	Mg enrichment	0%	Sigma Aldrich
MCD		DMEM F-12	12-48h	Increase hepatocytes lipid content	0%	Gibco
Oleic acid	400 nM	MEM	6h	Increase hepatocytes lipid uptake	0%	Sigma Aldrich

4.3.1.3 Adipocytes isolation

Primary adipocytes we obtained by collagenase disaggregation from fresh adipose tissue. Briefly, the tissue was digested during 30 minutes with 0.15% collagenase type I (Sigma) in a BSA-KRB-HEPES-Glucose buffer (40%BSA, 120 mM ClNa, 5 mM ClK, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4(H_2O)_7$, 10 mM HEPES and 6 mM glucose at pH 7.4). Once incubated, digestion product was filtered through a 100 µM-diameter-pore mesh and washed three times with the BSA-KRB-HEPES-Glucose buffer.

4.3.1.4 THLE2 cells

THLE-2 cells were purchased from ATCC (ATCC® CRL-2706™). They were maintained on Bronchial Epithelial Growth Medium (BEGM™, Lonza) supplemented with BEGM Bullet Kit™ (Lonza) and 10%FBS. They were split with 0.05% trypstin-EDTA and collected in BEGM. After centrifugation at 123g during 5 minutes, supernatant was discarded and pellet resuspended.

4.3.2 Cell treatments

4.3.2.1 Primary hepatocytes

Primary hepatocytes were subjected to different treatments. Reagents, concentrations, times and culturing conditions are summarized in Table 4.4.

4.3.2.2 Primary hepatic stellate cells and kupffer cells

Hepatic stellate cells (HSC) were cultured in 0% FBS RPMI-1640 Medium (Gibco) and collected at different times after plating (0, 3, 5 and 7 days). Kupffer cells (KC) were cultured in 0% FBS MEM (Gibco) and stimulated with 200ng/ml lipopolyssacharide (LPS, Sigma) during 24 hours.

4.3.2.3 Primary adipocytes

Primary adipocytes were cultured in two different experiments. First, they were incubated during 6 h with BSA-KRB-HEPES-Glucose buffer with supplemented magnesium (5 mM) and compared to non-supplemented medium (1.2 mM). Second, they were incubated with conditioned medium obtained from primary adipocytes treated with MCD with/without specific *Cnnm4* silencing.

4.3.2.4 THLE2 cells

Upon attachment, THLE2 were transfected by overnight incubation with 100 nM CNNM4 siRNA (Ambion, USA) or an unrelated control (Ambion, USA) using dharmaFECT1 reagent (Dharmacon). Once attached, THLE2 were maintained at BEGM supplemented with MD 21793 (BEGM Bullet Kit; CC317), 5ng/mL EGF, 70 ng/mL phospho-etanoliamine and 10% FBS overnight. Next day culture media was removed and cells were incubated in 0% DMEM or 0%MCD DMEM F-12 for 12 hours. Gene knockdown was confirmed by RT- and qPCR.

4.3.3 Cell transfection

4.3.3.1 Plasmid transfection

CNNM4 plasmid was transfected into primary mouse hepatocytes using jetPRIME® (Polyplus, USA) and lipofectamine (ThermoFischer, USA) as transfection reagents following manufacturer's protocol. Transfection protocol was realized following manufacturer's instructions. Briefly, 3µg CNNM4 plasmid were mixed with 200µl jetPRIME® buffer and resuspended during 10 s. Then, 6 µl jetPRIME® transfection reagent were added and resuspended again. After a 20 minute incubation, transfection mixture was added to $0.5*10^6$ cells in a 6-well plate. Transfections were performed in cell

suspension medium and transfection mix was replaced for fresh medium 6h after transfection unless indicated. CNNM4 plasmid was kindly provided by Dr. Michel Tremblay (McGill University Goodman Cancer Research Centre, Montreal, Canada) and consists on a pDEST-26 vector with the complete cDNA of CNNM4 inserted.

4.3.3.2 Gene silencing by siRNA transfection

Cells were transfected with specific siRNAs at a final concentration of 100nM using DharmaFECT 1 reagent (Dharmacon) following manufacturer's protocol. DharmaFECT 1 and siRNA were diluted separately in 0% FBS/1% PSG MEM for 5' at RT. Dilutions were then mixed and incubated 20' at RT. siRNA transfection mixed were then added to cell suspension medium and replaced for fresh medium after 6h. siRNA transfection volumes, indicated for 6-well plates) and sequences are summarized in Table 4.5**.**

Table 4.5. siRNAs transfected with DharmaFECT 1 indicated for 6-well seeded cells.(mm = murine mouse; Hs = homo sapiens; F = forward/sense sequence; R = reverse/antisense sequence)

siRNA	*DharmaFECT 1 volume*	*siRNA (100 µM) volume*	*Sequence*	*Medium volume*
Hs CNNM4	8µl in 0.2ml	1µl in 0.2ml	F 5'- CCAUGUCGGAGAUAAUGGATT -3' R 5'- UCCAUUAUCUCCGACAUGGTG -3'	2 ml
Hs GLS1	8µl in 0.2ml	1µl in 0.2ml	F 5'- CUGAAUAUGUGCAUCGAUATT -3' R 5'- UAUCGAUGCACAUAUUCAGTT -3'	2 ml
mm Cnnm1	8µl in 0.2ml	1µl in 0.2lml	F 5'- GAUCCUGAAUGCUGUAAUATT -3' R 5'- UAUUACAGCAUUCAGGAUCCG -3'	2 ml
Mm Cnnm2	8µl in 0.2ml	1µl in 0.2ml	F 5'- CTCAATTTGCATGAAATTTAA -3' R 5'- UUAAAUUUCAUGCAAAUUGAG -3'	2 ml
Mm Cnnm3	8µl in 0.2ml	1µl in 0.2ml	F 5'- GAUGAUGAAUAUAAAGUAATT -3' R 5'- UUACUUUAUAUUCAUCAUCAG -3'	2 ml
Mm Cnnm4	8µl in 0.2ml	1µl in 0.2ml	F 5'-CACUAUUGUUCUCACCAAATT-3' R 5'- UUUGGUGAGAACAAUAGUGTT- 3'	2 ml
Mm Gls1	8µl in 0.2ml	1µl in 0.2lml	F 5'- GCAAUAGGAUAUUACUUAATT- 3' S 5'- UUAAGUAAUAUCCUAUUGCAA- 3'	2 ml

4.4 RNA ISOLATION AND cDNA EXPRESSION DETERMINATION

4.4.1 RNA isolation

Total RNA from whole liver or cultured cells was isolated using TRIzol reagent (Invitrogen) according to manufacturer's instruction. In case of cell mRNA extraction, 5 µg of Glycogen (Ambion, USA) were used in the RNA precipitation step to facilitate the

visibility of the RNA pellet. RNA concenctration was determined spectrophometrically using the Nanodrop ND-100 spectrophotometer (ThermoFisher Scientific, USA)

4.4.2 Retrotranscription

1-2 µg of isolated RNA were treated with DNAse I (Invitrogren) and used to synthesize cDNA by M-MLV reverse transcriptase in the presence of random primers and RNAseOUT (all from Invitrogen). Resulting cDNA was diluted 1/10 (1/20 if 2 µg were used) in RNAse free water (Sigma-Aldrich).

4.4.3 Real Time quantitative PCR (RT-qPCR)

qPCRs were performed using either the ViiA 7 or the QS6 Real time PCR System with SYBR Select Master Mix (Applied Biosystems, USA). 1.5 µl of cDNA were used and including the specific primers for a total reaction volume of 6.5 µl in a 384-well plate (Applied Biosystems). All reactions were performed in triplicate. PCR conditions for the primers were optimized and 40 cycles with a melting temperature of 60 ºC and 30s per step were used. Both *Homo Sapiens* and *Mus musculus* primers were designed using the Primer 3 software via the NCBI-Nucleotide webpage (http://www.ncbi.nlm.nih.gov/nucleotide/) and synthesized by Sigma Aldrich. Primer sequences are detailed in Table 4.6 and Table 4.7. After checking the specificity of the PCR products with the melting curve, data were normalized to the expression of a housekeeping gene (GAPDH, ARP).

Table 4.6. List of primers used to determine mRNA expression of *Homo Sapiens* genes.

Gene symbol	Forward sequence	Reverse sequence
ARP	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG
CNNM1	TAAGCACCTTCTACACGCGG	CAGCACCTCCTCCACAACTT
CNNM2	ATGATGATGACCTTCCCCGC	CGAGGTCGTTGTAGGGATCG
CNNM3	TCCTGTCCCGAGAAGTGGAT	CTGGTTGACACTGGGATGCT
CNNM4	GAGCTGCAACAACTCGTGTG	TCCACCTCGGTGAAGGAGAT
GLS1.1	GGAAGCCTGCAAAGTAAACCC	CCAAAGTGCAGTGCTTCATCC
GLS1.2	TGTGGTCAGATAATCCCATTTTACA	TCTATTGCCACTAAAGACATCACA

Table 4.7. List of primers used to determine mRNA expression of *Mus musculus* genes.

Gene symbol	Forward sequence	Reverse sequence
Abcd1	CCAGGGTTTCGAAGTCGTCCA	AGTGCCATCCGCTACCTAGA
Acadl	GTCCGATTGCCAGCTAATGC	CACAGGCAGAAATCGCCAAC
Acadm	TCAAGATCGCAATGGGTGCT	GCTCCACTAGCAGCTTTCCA
Aox1	TTGGCGCCTCTGATCCTAAC	AAAATGTTCCTGGCCGCCTA
Arp	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG
Cbs	CTTCAGGGACATCCCAGTGT	AGCTGCCAGGTACATCTGCT
Cept1	TCAGTGATCACGTTAGCTGTT	TCGTCATGTGAGCAACCACA
Chpt1	TGTTCCGGTTCTTGGAGTTGT-	AAGACACTGGTGCCTGCAAT
Cnnm1	CAACGAGGGTGAAGGAGACC	CGTCGAGGATCTCCGACTTG
Cnnm2	GCGAGGCTATCCTGGACTTC	TGTTGGAACGTTCTCCCTCG
Cnnm3	CTATCGTTGAGCCCGAGGAC	GGACAGCGTCCAGTTTGGTA
Cnnm4	AGGTGAACAATGAGGGCGA	CCGGGTCCGATTATCAGTGTA
Cth	GCAATGGAATTCTCGTGCCG	GCAGCCACTGCTTTTTCCAA
Cpt1	GACTCCGCTCGCTCATTCC	GAGATCGATGCCATCAGGGG
Etnk2	ATCAGTCCCAGCCTTTCTGC	GGGAATCCAACTGGGACAGG
Gclc	TGCAGGAGCAGATTGACAGG	TAGAGAAAGCAAGCGGGTGG-
Gls1.2	TGGGCAACAGTGTTAAGGGA	AAGGAATGCCTTTGATCACCT
Gls2.1	ATCTTAGCCAGGACACGCTG	AGGGGAGAAAGAGAACGACT
Gls2.3	GTGTCTGGGATCCAGGTGTG	ATATGCGATCCACATGGCCC
Gnmt	ACCAGTATGCAGATGGGGAG	CCAATTGTCAAAGGATGGCT
Gss	GACAACCCCTACCCTGTGTG	TGGAAGAGACAAGCTCCCCT
Mat1A	CCGCTATCTCCCTCTTTGCC	CCTCCCCCTACAAACCCAAC
Nrf1	CTTCATGGAGGAGCACGGAG	CGTGGAGTTGAGGATGTCCG
Nrf2	AGCCAGCTGACCTCCTTAGA	AGTGACTGACTGATGGCAG
Pcyt1a	TTTCTAGGTGCAGGGCTGTG	GGGAGAGAGGGAACAGGGAT
Pcyt2	GTGCCCACCTCTTCTCTTCC	GCAAGGCCAGTTTCCTTTGG
Pemt	CCACTGCTTCACACAGGCTA	AACCTAGGAATGCAAGGCCC
Pisd	TAAGCTGAAGCCTCAGGCAC	GGTTACGCCCTTTACCTGCT
Pparγ	GAATGCGAGTGGTCTTCCAT	TGCACTGCCTATGAGCACTT
Ptdss1	CTGCAGTTCCAGTCCCTAGC	TGGGATCAAACCACCTGACG
Ptdss2	TTCTTCCTGCGGGACATCAC	CTGCAGTTCCAGTCCCTAGC
Sahh	CACCAGATGTCCCATCGCTT	GGGAAGAGCAGAAATGGCCT
Ucp3	CTTTCTGCGTCTGGGAGCTT	CGCTTTGTTCTGTTCCAGGC

4.5 PROTEIN

4.5.1 Protein extraction and analysis

Extraction of total protein was performed as indicated. Cells were washed with cold PBS buffer and resuspended in 200 µl of RIPA lysis buffer (1.6 mM NaH_2PO_4, 8.4 mM Na_2HPO_4, 0.5% Azide, 0.1 M NaCl, 0.1% SDS, 0.1% Triton X-100, 5 mg/ml sodium deoxycholate). They were centrifuged (13000 rpm, 20' at 4 °C) and the supernatand (protein extract) was quantified for total protein content by the Bradford protein assay (Bio-Rad) and determined using a Spectramax M3 spectrophotometer (Molecular Devices, USA)

In the case of frozen liver tissue, approximately 50 µg of tissue was homogenized by using a Precellys 24 tissue homogenizer (Precellys, France) in 500 µl of buffer. In all cases, the lysates were centrifuged (13000 RPM, 20 min, 4 °C) and the supernatant (protein extract) was quantified for total protein content by the Bradford protein assay or by BCA protein assay (Pierce, USA) depending on the type of lysis buffer used and determined using a Spectramax M3 spectrophotometer.

For Caspase 3 activity assay, cells and frozen liver tissue were lysed in Caspase buffer (HEPES 10 mM pH 7.4, 0.1% CHAPS, DTT 125 mM, EDTA 2 mM). Caspase 3 buffer lysed cells were quantified by Bradford protein assay.

All lysis buffers except caspase buffer were supplemented with protease and phosphatase inhibitor cocktails (Roche, Switzerland).

4.5.2 Subcellular protein extraction

Cytosolic, membrane and nuclear fractions lysates from frozen liver tissue samples were obtained using the Subcellular Proteome Extraction Kit (Calbiochem, USA) following manufacturer's procedure. The lysates were quantified by BCA protein assay (Pierce).

Mitochondrial were isolated from frozen liver tissue samples using the Mitochondria/Cytosol Fractionation Kit (Abcam, UK) as indicated by the manufacturer. Briefly, 20 mg of frozen livers were grinded in mortar previously cooled with liquid nitrogen. Then, they were resuspended in the cytosolic buffer and mechanically homogenized in cold. Cytosol were centrifuged (13000 rpm, 10') three times. Pellets obtained from the sequential centrifugations were collected as crude mitochondria and finally mixed and lysed in the mitochondrial buffer for BCA quantification.

4.5.3 Western Blotting

Protein extracts were boiled at 95 °C for 5 min in SDS-PAGE sample buffer (250 mM Tris-HCl pH 6.8, 500 mM β-mercaptoethanol, 50% glycerol, 10% SDS and bromophenol blue). An appropriate amount of protein (between 5 and 50 µg), depending on protein abundance and antibody sensitivity, were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 3% to 15% acrylamide gels (depending on the molecular weight of the protein of interest), using a Mini-PROTEAN Electrophoresis System (Bio-Rad). Gels were transferred onto nitrocellulose membranes by electroblotting using a Mini Trans-Blot cell (Bio-Rad). Membranes were blocked with 5% nonfat milk in TBS pH 8 containing 0.1% Tween-20 (Sigma Aldrich) (TBST-0.1%), for 1 hour at RT, washed three times during 10' with TBST-0.1% and incubated overnight at 4 °C with commercial primary antibodies. Primary antibodies and their optimal incubationconditions are detailed in Table 4.8. Membranes were then washed three times during 10' with TBST-0.1% and incubated for 1 hour at RT in blocking solution containing secondary antibody conjugated to horseradish-peroxidase (HRP, Table 4.8). Immunoreactive proteins were detected by using Western Lightning Enhanced Chemiluminescence reagent (ECL, PerkinElmer, USA) and exposed to Super Rx-N X-ray films (Fuji, Japan) in a Curix 60 Developer (AGFA, Belgium).

Table 4.8. List of antibodies used for Western Blot. (BSA = 3% BSA in TBS.T 0.1%; Milk = 5% non-fat milk in TBS-T 0.1%).

Antibody	*ID*	*Supplier*	*Dilution*	*Incubation solution*
ApoB48/B100	AB742	Chemicon	1/1000	BSA
BIP/GRP78	10062019	BD	1/1000	Milk
CNNM4	14066-1-AP	Proteintech	1/1000	Milk
eIF2α	5324S	Cell Signalling	1/1000	Milk
GAPDH	AB8245	Abcam	1/10000	Milk
GLS1	N/A	AGIOS Pharmaceuticals	1/1000	Milk
GLS2	LS-C80586	LSBio	1/1000	Milk
HRP-conjugated secondary antibody to goat	A8919	Sigma Aldrich	1/1000	Milk
HRP-conjugated secondary antibody to mouse	#7076	Cell Signalling	1/1000	Milk

HRP-conjugated secondary antibody to rabbit	#7074	Cell Signalling	1/1000	Milk
Phospho-eIF2α (Ser 51)	9721	Cell Signalling	1/1000	BSA
XBP1s	MABC521	Millipore	1/1000	Milk

4.6 TISSUE STAINING ASSAYS

4.6.1 Hematoxylin and eosin

Paraffin-embedded sections (5 µm thick) of formalin-fixed liver samples were initially deparaffinised in xylene or xylene-substitute and rehydrated through graded alcohol solutions. Once hydrated, sections were subjected to a determined staining. After the deparaffinization and rehydration process, sections were subjected to conventional hematoxylin and eosin staining. Images were taken with an upright light microscope (Zeiss, Germany).

4.6.2 Sirius Red

Parafin-embedded sections (5 µm thick) of formalin-fixed liver samples were initially deparaffinised in xylene or xylene-substitute and rehydrated through graded alcohol solutions. Rehydrated sections were then stained with Sirius red solution 1 (0.01% Fast Green FCF in picric acid, Sigma Aldrich) for 15 min and then with Sirius red solution 2 (0.04% Fast Green FCF/0.1% Sirius red in picric acid, Sigma Aldrich) for another 15 min. The sections were then dehydrated directly in 100% alcohol and mounted in DPX mounting medium (Sigma Aldrich). Images were taken with an upright light microscope (Zeiss).

4.6.3 Sudan Red

Optimal cutting temperature (O.C.T)-included frozen liver samples were cut into 10 µm sections. Sections were washed in 60% isopropanol and then stained with fresh Sudan III (0.5% in isopropanol; Sigma Aldrich) solution for 30 min. Samples were then washed again in 60% isopropanol and then counterstained with eosin. The sections were then washed with distilled water and mounted in DPX mounting medium. Images were taken with an upright light microscope (Zeiss).

4.6.4 Ammonia

Paraffin-embebed sections (5 µm thick) were used for ammonia stain with Nessler´s reagent according to the following protocol. Liver sections were incubated 5 minutes with 100 mL of Nessler's reagent and washed for 10 seconds with sterile distilled. Samples were counterstained with Mayer's haematoxylin, washed with water and dehydrated briefly before clearing with histoclear. Samples were mounted with DPX permanent mounting medium. Nessler's reagent becomes darker yellow in the presence of ammonia. At higher concentrations of ammonia, a brown precipitate is formed. Images were taken with an upright light microscope (Zeiss).

4.6.5 ROS determination by DHE

O.C.T-embebed 8 µm sections were incubated with MnTBAP 150 µM 1h at RT. The samples were then incubated with dihydroethidium (DHE) 5 µM for 30 min at 37 ºC and sctions were mounted with Fluoromount-G (Southern Biotech, USA) containing 0.7 mg/l of DAPI to counterstain nuclei. Images were taken using an Axioimager D1 (Zeiss).

4.6.6 Immunohistochemistry

Paraffin-embebed sections (5 µm thick) were unmasked according to the primary antibody to be used and subjected to peroxide blocking (3% H_2O_2 in PBS, 10', RT). For stainings with mouse-hosted antibodies in mouse tissues, samples were blocked with goat anti-mouse Fab fragment (Jackson Immunoresearch, USA) (1:10, 1h, RT) and the blocked with 5% goat serum (30', RT). Then, section were incubated in a humid chamber with the primary antibody in DAKO antibody diluent (DAKO) followed by Envision anti rabbit or anti-mouse (DAKO) or ImmPRESS anti-rat (Vector, USA) HRP-conjugated secondary antibody incubation (30', RT). Unmasking and incubation conditions for each antibody are indicated in Table 4.11. Colorimetric detection was confirmed with Vector VIP chromogen (Vector) and sections were counterstained with hematoxylin. Samples were mounted using DPX mounting medium. Images were taken with an upright light microscope (Zeiss).

4.6.7 Immunofluorescence

For O.C.T-embebed 10 µm sections were incubated with primary antibody (Table 4.9) conjugated to Cy3/Cy5 and mounted with Fluoromount-G (Southern Biotech) containing 0.7 mg/l of DAPI to counterstain nuclei. Images were taken using an Axioimager D1 (Zeiss).

Table 4.9. List of antibodies used for immunohistochemistry or immunofluorescence.

Antibody	*ID*	*Supplier*	*Dilution*	*Incubation solution*	*Unmasking*
α-SMA	C6198	Sigma Aldrich	1/300	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT
CNNM4	Ab191207	Abcam	1/100	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT
GLS1	N/A	Agios Pharmaceuticals	1/100	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT
GLS2	LS-C80586	LSBio	1/100	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT
GLUL	NB110-41404	Novus Biologicals	1/100	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT
F4/80	MCA497-BB	Serotec	1/50	2% BSA in 0.01% PBS-azide	Proteinase K, 15' at RT

4.6.8 Data analysis

The average sum of intensities or stained area percentage of each sample were calculated using the FRIDA software (http://bui3.win.ad.jhu.edu/frida/, John Hopkins University).

4.7 CASPASE 3 ACTIVITY ASSAY

Caspase 3 activity was measured in cells as previously described (Beraza et al., 2007). Cells were lysed in caspase buffer and the protein content was determined by Bradford protein assay. 20 µl of 25x reaction buffer (PIPES pH 7.4 250 mM, EDTA 50 mM, 2.5% CHAPS, DTT 125 mM) were mixed with 2.5 µl of fluorogenic caspase-3 substrate (Enzo Life Sciences, USA) and with 10-50 µg of protein lysate in a total volume of 500 µl. This reaction mixture was divided into two duplicates and performed in 96 well plates. The mixture was incubated at 37°C with gentle shaking for 5 hours. Readings were taken at each hour using a Spectramax M3 spectrophotometer (excitation wavelength 390 nm, emission wavelength 510 nm). Caspase 3 activity was determined by calculating the increase in fluorescence per hour of incubation.

4.8 METABOLISM ANALYSIS

4.8.1 Liver lipid metabolism

4.8.1.1 Liver lipid quantification

30 mg of frozen liver were homogenized with 10 volumes of ice-cold PBS in a potter homogenizer. Fatty acids were measured in the homogenates using the Wako Chemicals kit (Richmond, VA) and lipids were quantified as described (Folch et al., 1957). Briefly, lipids were extracted from 1.5 mg of protein from liver homogenates. Phosphatidilcholine (PC), phosphatidylethanolamine (PE), fatty acids (FAs) and cholesterol (Ch) were separated by thin layer chromatography (TLC) and quantified as described (Ruiz and Ochoa, 1997). Triglycerides (TGs) were measured in the lipid extract with the A. Menarini Diagnostics (Italy) kit.

4.8.1.2 Lipid quantification in primary hepatocytes

Primary hepatocytes cultured in high lipid content medium (OA) or methionine/choline deficient medium (MCD) were fixed in 4% paraformaldehyde (10', RT) in PBS and incubated with BODIPY 493/503 (Molecular Probes, Invitrogen) at 1 mg/ml (1h, RT). BODIPY immunocytofluorescence images were taken using an Axioimager D1 (Zeiss) microscope. Quantification of lipid bodies was performed using Frida Software and represented as mean area per total number of cells.

4.8.1.3 Hepatic "de novo" lipogenesis

De novo lipogenesis was performed as previously described (Nassir et al., 2013), with slight modifications. Briefly, freshly isolated liver tissue slices (40 mg) were incubated in high glucose DMEM with insulin (150nm) and (H_3) Acetic acid 20 µCi/ml for 4 hours. Tissue slices were washed five time in cold PBS and homogenated in PBS. Then, lipids were extracted as previously described (Folch et al., 1957). Lipids were separated by TLC (Ruiz and Ochoa, 1997), each lipid was scraped and the radioactivity was measured in a scintillation counter.

4.8.1.4 Hepatic β-oxidation assay

Fatty acid β-oxidation measured in Figure 5.13 was assessed as described before (Gao et al., 2015a; Hirschey et al., 2010). Fresh liver slices were homogenized in a potter homogenizer in cold buffer (Tris-HCl 25mM, sucrose 500nM, EDTA Na_2 pH 7.4 1mM) and sonicated (10''). Homogenized livers were centrifuged (500g, 10', 4ºC). Protein concentration was determined and approximately 500 µg were diluted in 200 µl. Assay

was performed as follows: 400 µl of assay mixture containing 0.5 µCi/ml (1-^{14}C) was added to the samples and incubated 1h at 37ºC in Eppendorf tubes with a Whatman paper circle in the cap. The reaction was stopped by adding 400 µl of perchloric acid 3M, and NaOH 1M was added to impregnate the Whatman cap. After 2 hours, the Whatman cap was retired and the radioactivity associated was measured in a scillation counter. Eppendorf tubes were centrifuged (2100g, 10', 4ºC). 400 µl of the supernatant were collected and the radioactivity was counted in a scillation counter. The supernatant contained the acidsoluble metabolites (ASM) and the Whatman caps captured the released CO_2.

Relative FAO showed in Figure 5.26 was measured by using a commercial assay kit (Biomedical Research, Buffalo, USA). Briefly, 20 mg of liver tissue were homogenized in 750 µl cell lysis solution 1x and centrifuged at 100000 RPM during 5'. Soluble fraction was then determined and protein samples were normalized: 100 µl per well were loaded with each respective loading control and samples were incubated during 2 h at 37 ºC with 50 µl FAO assay solution. Colorimetric determination was finally determined at 550 nm.

4.8.2 Adipocyte lipid metabolism

Lypolitic activity of primary adipocytes was determined by measuring the amount of glycerol and NEFA release into extracellular medium. Determinations were realized according to commercially available kits from Sigma Aldrich and FUJIFILM Wako Diagnostics (USA), for determining glycerol and NEFA respectively.

Briefly, 6 µl sample were incubated with 200 µl reactive at 37 ºC during 5 minutes, under agitation and in light-absence conditions. Colorimetric density was determined at 540 nm wavelength and compared to a standard glycerol sample.

For NEFA determination 5 µl sample were mixed with 10 µl distilled water and incubated with 160 µl R1 reactive during 5 minutes at 37 ºC under agitation. After determining colorimetric absorbance at 550 nm wavelength, 80 µl R2 reactive were added. After another 5 minutes incubation at 37 ºC under agitation, colorimetric absorbance was determined again. NEFA concentration in the sample was determined by calculating the different between two measurements and comparing to a standard NEFA sample.

4.8.3 Seahorse analysis

Cellular metabolic profile was determined using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences, USA), providing real-time measurements of oxygen

consumption rate (OCR) and extracellular acidification rate (ECAR). For Seahorse analysis in hepatocytes 20000 primary hepatocytes were plated per well with 500 µl of assay medium prewarmed to 37°C, composed of DMEM without bicarbonate containing 1 mM sodium pyruvate, 2 mM l-glutamine, and cultured at 37°C. Measurements of oxygen consumption rate (OCR) was performed after equilibration in assay medium for 1h. After an OCR baseline measurement, sequential injections through ports in the XF Assay cartridges of pharmacologic inhibitors: Oligomycin (1mM), an inhibitor of ATP synthase, which allows a measurement of ATP-coupled oxygen consumption through oxidative phosphorylation (OXPHOS); carbonyl cyanide 4- trifluoromethoxy-phenylhydrazone (FCCP) (300 nM), an uncoupling agent that allows maximum electron transport, and therefore a measurement of maximum OXPHOS respiration capacity; and finally Rotenone (1 µM), a mitochondrial Complex I inhibitor, were performed and changes in OCR were analyzed.

4.8.4 ATP detection assay

Intracellular ATP levels was determined in liver isolated mitochondria from frozen liver tissue. For ATP measurements, ATPlite™ luminescence ATP detection kit (Perkin Elmer) was used, following manufacturer's recommendation. In brief, 50 µl of the mammalian cell lysis solution were added to 100 µl of the cell suspension and incubated on an orbital shaker (700 rpm, 5', RT). 50 µl of the substrate solution were added and incubated (700 rpm, 5', RT). Plate was adapted to the dark for 10' and the luminescence was measured in a luminometer. Obtained values were normalized to total protein concentration.

4.8.5 Complex V (ATPase) activity assay

Complex V activity was determined by using a commercial kit and following manufacturer's instructions (Cayman Chemical, USA). Briefly, two mixtures were prepared in a tube A (978 ul of Complex V Activity Assay Buffer, 20 µl Bovine Heart Mitochondria Assay Reagent, 2 µl Rotenone 1mM) and a tube B (635 µl of Complex V Assay Enzyme Mix, 20 µl of Complex V ATP Reagent and 20 µl of Complex V NADH Reagent). Mitochondria were isolated (Chapter 4.6.2) and normalized to 5 µg/µl up to a final volume of 20 µl and mixed with 50 µl of mixture from tube A and 30 µl from tube B. Absorbance was immediately measured at 340 nm during 30 minutes in 30 second intervals. Complex V activity was calculated as relative % of the rate of each sample well and the rate of vehicle control.

4.9 MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN ASSAY

Relative microsomal triglyceride transfer protein (MTP) activity was determined by using a commercially available kit (Sigma Aldrich) and following the protocol provided by manufacturer. Liver pieces were in ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.02% (w/v) sodium azide. The homogenates were subjected a 150000 g centrifugation for 10 minutes and supernatant fractions were collected. After determining protein concentration per sample, a final volume of 20 µl was incubated with 8 µl of a mixture of donor and acceptor particle (1:1) and 172 µl MTP assay buffer. Plate was sealed and incubated at 37 ºC for 3-6 hours. Fluorescence was measured at 465 nm excitation and 535 emission wavelengths.

4.10 MAGNESIUM DETERMINATION

4.10.1 Extracellular magnesium quantification

Extracellular magnesium was quantified using the QuantiCrom$_{TM}$ Magnesium Assay Kit (BioAssay Systems, USA). Briefly, 5 µl of serum or culture media were mixed with 200 µl of a 1:1 mix of Reagent A and Reagent B. After 2' incubation at RT, OD was determined at 500 nm length using a Spectramax M3 spectrophotometer (Molecular Devices, USA). Then, 10 µl of EDTA were added and OD_{500} was determined again. Magnesium concentrations were finally calculated by comparing to the OD_{500} from a standard concentration (2mg/ml).

4.10.2 Intracellular magnesium determination

Primary hepatocytes grown in glass coverslips were loaded with 2 µM Mag-S-AM (Afzal, Pitteloud, and Buccella 2014) or 1 µM Mag-S-TPP-AM (not published) in 0% FBS/1% PSG medium and incubated at 37 ºC and 5% CO_2 during 30' or 1h respectively. After removing the dye-containing medium, a 30' incubation in 0% FBS/1% PSG was performed. Coverslips were then washed in a 20mM Tris-HCl, 2.4 mM $CaCl_2$, 10mM glucose, pH 7.4 buffer and mounted on a thermostatitez perfusion chamber on a Eclipse TE 300-based microspectrofluorometer (Nikon, USA) and visualized with a 40x oil-inmersion fluorescence. Intracellular Mg^{2+} content was determined by comparing the relative fluorescence ratio between the magnesium-labelled probe, light excited at 340nm, and the not-labelled probe, excited at 380nm. The excited light ratio was determined with a Delt system (Photon Technologies International, Princeton).

4.11 INTRACELLULAR CALCIUM DETERMINATION

Similarly as the protocol for intracellular magnesium determination, primary hepatocytes grown in glass coverslips were loaded with Mag-FURA-2 (ThermoFischer, USA) diluted in 0% FBS/1% PSG medium and incubated at 37 ºC and 5% CO_2 during 30' or 1h respectively. After removing the dye-containing medium, a 30' incubation in 0% FBS/1% PSG was performed. Coverslips were then washed in a 20mM Tris-HCl, 2.4 mM $CaCl_2$, 10mM glucose, pH 7.4 buffer and mounted on a thermostatitez perfusion chamber on a Eclipse TE 300-based microspectrofluorometer (Nikon, USA) and visualized with a 40x oil-inmersio fluorescence. Intracellular Mg^{2+} levels were determines using the method described by Grynkiewicz (Grynkiewicz, Poenie, and Tsienb 1985). The 340/380 nm excited light ratio was determined with a Delta system (Photon Technologies International, Princeton) and converted into Ca2+ concentration from the standard equation:

$$[Ca^{2+}]i = \frac{(R - R_{\min})}{(R_{max} - R)} \times K_d \times Q$$

Where Kd is the Ca2+ dissociation constant of Mag-FURA-2 (0.22 mM) and Q is the ratio of the minimum/maximum fluorescence instentisy at 380 nm.

4.12 OXIDATIVE STRESS DETERMINATION

4.12.1 Lipid peroxidation assay

Lipid peroxidation was determined in frozen liver samples using the Lipid Peroxidation (MDA) Assay Kit (ab118970) (Abcam) following manufacturer's instructions.

4.12.2 Total ROS

Oxidative stress was measured using the CellROX™ Deep Red reagent (Life Technologies), following manufacturer's protocol. Primary hepatocytes were incubated with CellROX™ Deep Red reagent (5 µM, 30', 37ºC in a CO_2 incubator) diluted in 0% FBS/1% PSG MEM. Cells were washed twice with PBS, trypsinized and resuspended in 1% FBS-PBS for subsequent run on a flow cytometer. Fluorescence was detected by APC excitation laser and the average intensity of fluorescence is indicated.

4.12.3 Mitochondrial ROS

Mitochondrial ROS was measured using MitoSOX™ Red reagent (Life Technologies), following manufacturer's instructions. Briefly, primary hepatocytes and hepatoma cells were incubated with MitoSOX reagent (2.5 µM, 10', 37ºC in a CO_2 incubator) in normal

culture medium. Then, cells were washed twice with PBS and the fluorescence was measured at an excitation of 510 nm and emission of 595 nm using a spectrophotometer. Final values were normalized to total protein concentration.

4.13 STATISTICAL ANALYSIS

All the experiments were performed at least in triplicate. Data is expressed as mean ± SEM and represented as fold change vs. control mean value when indicated. Statistical significance was determined by using Prism 5 (GraphPad Software). One-way analysis of variance (ANOVA) followed by post hoc Bonferroni test was used in case 3 groups were compared or using the Student´s t-test in case 2 groups were compared. A $p<0.05$ was considered statistical different.

5. RESULTS

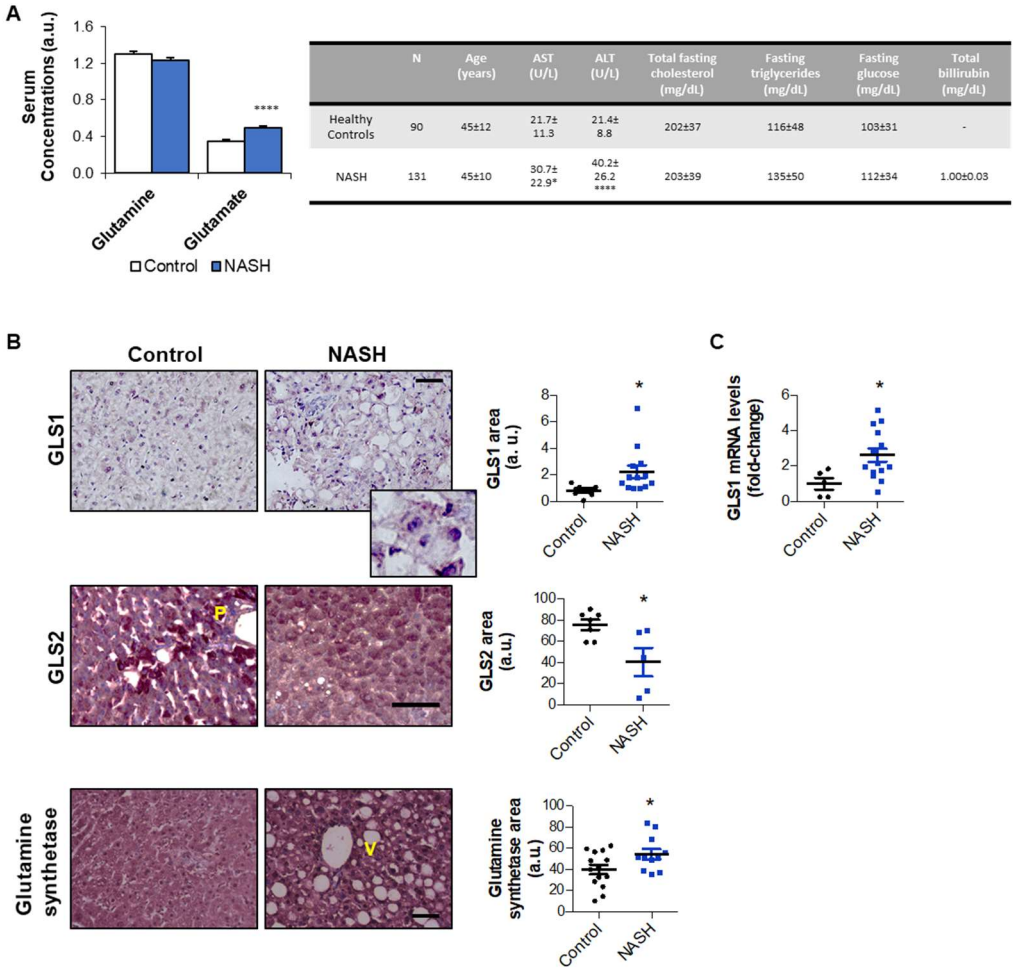
5. RESULTS

5.1. TARGETING HEPATIC GLUTAMINASE (GLS1) AMELIORATES NASH BY RESTORING VLDL TRIGLYCERIDE ASSEMBLY

5.1.1 GLS1 is overexpressed in clinical NASH

High-throughput metabolomics is a widely used method to investigate metabolic phenotypes in specific conditions. Here, we screened serum levels of glutamine and the glutaminase reaction product, glutamate, in a large cohort of patients (Barr et al. 2010). Whereas there are no significant differences in serum glutamine levels between controls (n= 90) and NASH patients (n= 131), serum glutamate levels are significantly increased in NASH patients (Fig. 5.1A), suggesting that glutamine catabolism may be aberrant in NASH. Glutaminase is the main regulator enzyme of hepatic glutamine catabolism, catalyzing the conversion of glutamine to glutamate and ammonia (the latter is excreted by the urea cycle). Whereas GLS2 is the major isoform expressed in the healthy liver, a switch from GLS2 to GLS1 occurs in liver fibrosis (Du et al. 2018), cirrhosis and liver cancer (Yu et al. 2015; Yuneva et al. 2012). Herein, a group of patients diagnosed with NASH, characterized in Table 4.1, show increased hepatic GLS1 levels relative to healthy controls. Likewise, in another cohort of patients with NASH (Table 4.2), mRNA levels of GLS1 were shown to be increased relative to healthy controls. Under these conditions, the isoform 2 of glutaminase, GLS2, usually distributed around the hepatic periportal compartment in healthy people, is decreased in the livers of NASH patients. In addition, glutamine synthetase, usually expressed in the perivenous hepatocytes of healthy livers, catalyzing the synthesis of glutamine from glutamate and elimination of residual ammonia that escapes from detoxification in the periportal hepatocytes, is induced (Fig. 5.1B,C).

Overall, we provide evidence that hepatic GLS1 expression is increased in NASH patients.



	N	Age (years)	AST (U/L)	ALT (U/L)	Total fasting cholesterol (mg/dL)	Fasting triglycerides (mg/dL)	Fasting glucose (mg/dL)	Total billirubin (mg/dL)
Healthy Controls	90	45±12	21.7± 11.3	21.4± 8.8	202±37	116±48	103±31	-
NASH	131	45±10	30.7± 22.9*	40.2± 26.2 ****	203±39	135±50	112±34	1.00±0.03

Figure 5.1. Glutaminase 1 (GLS1) is overexpressed in clinical non-alcoholic steatohepatitis (NASH). A. Serum levels of glutamine and the product of glutamine catabolism, glutamate, in a large cohort of patients diagnosed with NASH (n= 131) relative to a control group of healthy subjects (n= 90). A table showing the main serum biochemical parameters relative to these patients is shown. **B**. Liver immunohistochemical staining and respective quantification for the isoform 1 of glutaminase (GLS1), and inset zoom, the isoform 2 of glutaminase (GLS2) and glutamine synthetase in another cohort of NASH patients (n= 16) versus a control group of healthy subjects (n= 16). Scale bar corresponds to 100 µm. V-venous region; P-portal region. C. mRNA levels of GLS1 in a cohort of NASH patients against a control group of age- and body-weight matched healthy controls. Data is shown as average ± SEM and Student´s t-test was used to compare groups. *p<0.05 and ****p<0.0001 against the control group are shown (See also Table 4.1, Table 4.2).

5.1.2 GLS1 is overexpressed in mouse models of NASH

Taking into consideration the relevance of GLS1 expression in clinical NASH, we have evaluated GLS1 expression in an *in vivo* pre-clinical mouse model of NASH, the mice fed a choline and methionine deficient diet. Even though this model presents some constraints it is one of the most often used models in NASH research. Herein, the choline deficient and 0.1% methionine diet (0.1%MCDD) was used as one of the limitations of a methionine and choline deficient diet is the rapid weight loss. Adding 0.1% methionine is able to prevent this (Alberghina and Gaglio 2014). Animals fed a 0.1%MCDD rapidly accumulate hepatic fat in the form of macrovesicular steatosis and progress to

inflammation and fibrosis, hallmarks of NASH, after two and six weeks of the diet, respectively (Fig. 5.2). Under these circumstances, animals maintained on a 0.1%MCDD show a time-dependent increase in GLS1 expression, both at the protein and the mRNA level (Fig. 5.3A-D). After four-weeks of 0.1%MCDD the accumulation of GLS1 is mainly localized in the hepatocyte's mitochondria as shown by double immunofluorescence (Fig. 5.3B). On the other hand, the expression of the GLS2 isoform of glutaminase, usually distributed around the periportal regions, is reduced in steatotic livers (Fig. 5.3A,C,D). Likewise, in a NASH model of eight-month-old *Mat1a*$^{-/-}$mice (Alonso et al. 2017; Cano et al. 2011), GLS1 liver expression is induced together with glutamine synthetase, whereas GLS2 is reduced (Fig. 5.4).

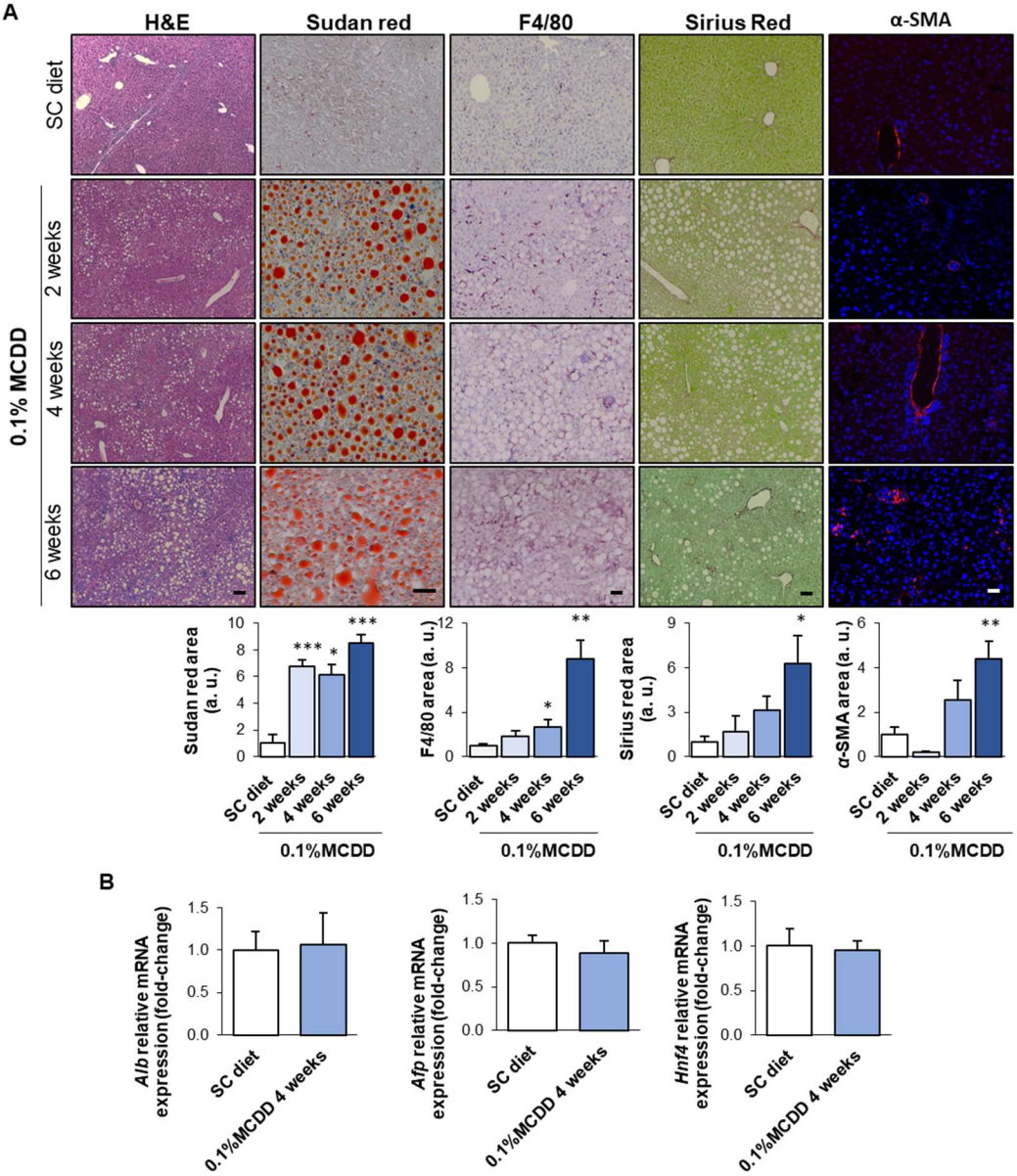


Figure 5.2. Longitudinal characterization of non-alcoholic steatohepatitis (NASH) and dedifferentiation markers in mice fed a choline deficient and 0.1%methionine diet (0.1%MCDD) (Related to Figure 5.3).

A. Representative histochemical staining and respective quantifications for hematoxylin and eosin (H&E), Sudan Red, F4/80, Sirius Red and alpha smooth muscle actin (α-SMA). Scale bar corresponds to 100 μm. At least n=5 animals were used for each experimental group. Data is shown as average ± SEM and Student´s t-test was used to compare groups of animals against standard chow diet (SC diet). *p<0.05, **p<0.01 and ***p<0.001 versus SC diet are shown; **B.** Hepatic albumin (*Alb*), α-fetoprotein (*Afp)* and hepatic nuclear factor 4 (*Hnf4*) mRNA levels in mice fed a choline deficient and 0.1% methionine diet (0.1% MCDD) for 4 weeks against a standard chow diet (SC diet). At least n=5 animals were used for each experimental group. Data is shown as average ± SEM and Student´s t-test was used to compare groups of animals.

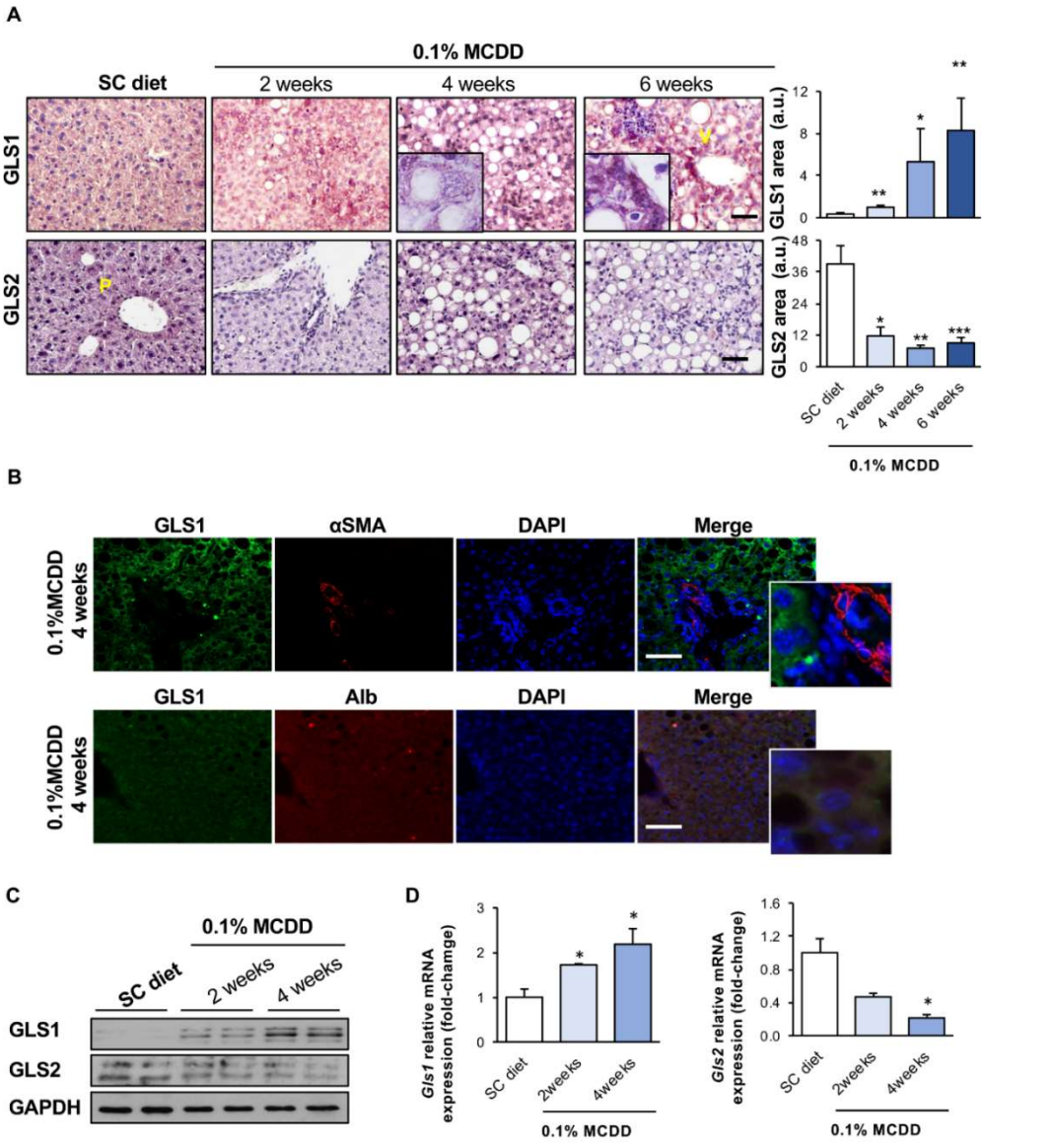


Figure 5.3. Glutaminase 1 (GLS1) is overexpressed in a mouse model of non-alcoholic steatohepatitis (NASH) of choline deficient and 0.1% methionine diet (0.1% MCDD)-fed rodents. A. Hepatic Glutaminase 1 (GLS1), with higher magnification zoom shown in inset, and Glutaminase 2 (GLS2) immunostaining and respective quantifications. Scale bar corresponds to 100 μm. V-venous region; P-portal region; **B**. Immunofluorescence double co-staining for GLS1 and albumin, a marker of hepatocytes, and alpha smooth muscle actin (αSMA), a marker of hepatic stellate cells (HSC). **C.** Hepatic GLS1 and GLS2 protein levels by Western blot analysis. Glyceraldehyde-3-phosphate (GAPDH) was used as a loading control; and **D.** Hepatic *Gls1* and *Gls2* mRNA levels in mice fed a choline deficient and 0.1% methionine diet (0.1% MCDD) against a standard chow diet (SC diet). Data is shown as average ± SEM and Student´s t-test was used to compare groups. *p<0.05, **p<0.01 and ***p<0.001 are shown versus age- and gender-matched animals maintained on SC diet (See also Figure 5.2, Figure 5.4, Figure 5.5 and Figure 5.6).

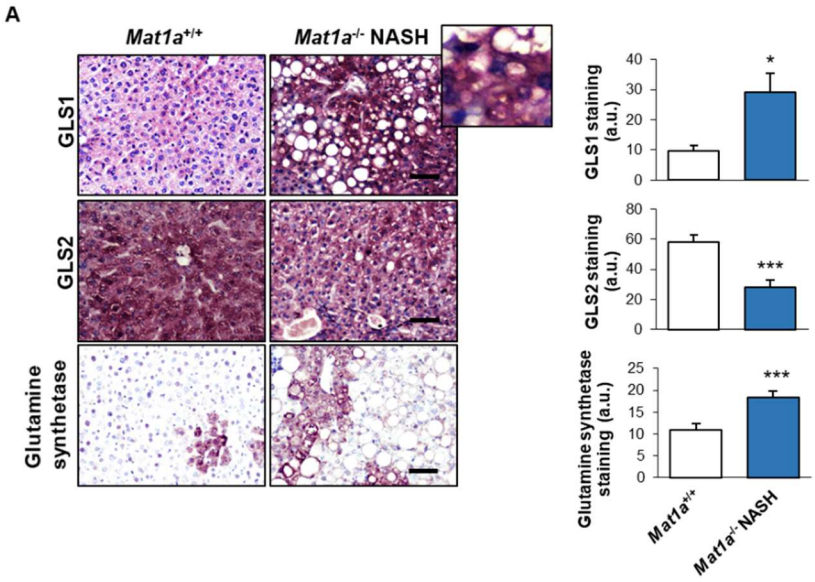


Figure 5.4. Glutaminase 1 (GLS1), Glutaminase 2 (GLS2) and Glutamine Synthetase expression in pre-clinical models of Non-Alcoholic Steatohepatitis (NASH) (Related to Figure 5.3). **A.** Hepatic GLS1, and inset zoom, GLS2 and Glutamine Synthetase immunostaining and respective quantification in eight-month old mice deficient in Methionine Adenosyltransferase 1A (*Mat1a*$^{-/-}$) relative to wild type animals (*Mat1a*$^{+/+}$). Scale bar corresponds to 100 µm. At least n=5 were used for each experimental group. Data is shown as average ± SEM and Student´s t-test was used to compare groups of animals. **p*<0.05 and ****p*<0.001 versus *Mat1a*$^{+/+}$ are shown.

In addition, we have evaluated the expression of glutamine metabolism key regulators in a mouse model of mice fed a choline-deficient, high-fat diet (CD-HFD). Previous reports have shown that this mouse model develops NASH in a similar pattern to that observed in humans, showing hepatic ballooning and fibrosis, with concomitant obesity as well as dyslipidemia and insulin resistance (Wolf et al. 2014). After six weeks of CD-HFD, we observed increased body weight in these animals relative to the standard chow (SC) diet-fed age-matched rodents (Fig. 5.5A). Hepatic triglycerides are increased, and hepatic inflammation is significant, although serum transaminases and triglycerides are not significantly altered (Fig. 5.5B-G). Finally, after six-weeks of CD-HFD, hepatic fibrosis is not significant (Fig. 5.5B). Similar to what occurs in the 0.1%MCDD-fed rodents, CD-HFD fed animals present impaired VLDL triglyceride export (Fig. 5.5H). Importantly, GLS1 levels are induced after as little as three weeks of CD-HFD (Fig. 5.5I,J).

Finally, earlier evidence have highlighted that GLS1 expression is increased with advanced tumor grade and therefore dedifferentiation (Li et al. 2018). Herein, animals fed a 0.1%MCDD during four weeks do not present alterations in dedifferentiation parameters such as albumin, alpha-fetoprotein, and the transcription factor hepatocyte nuclear factor 4 (HNF4), suggesting that GLS1 increase is specifically related to steatosis and NASH and not a dedifferentiation hallmark (Fig. 5.2B).

Overall, we provide evidence that hepatic GLS1 expression is increased in mouse models of NASH.

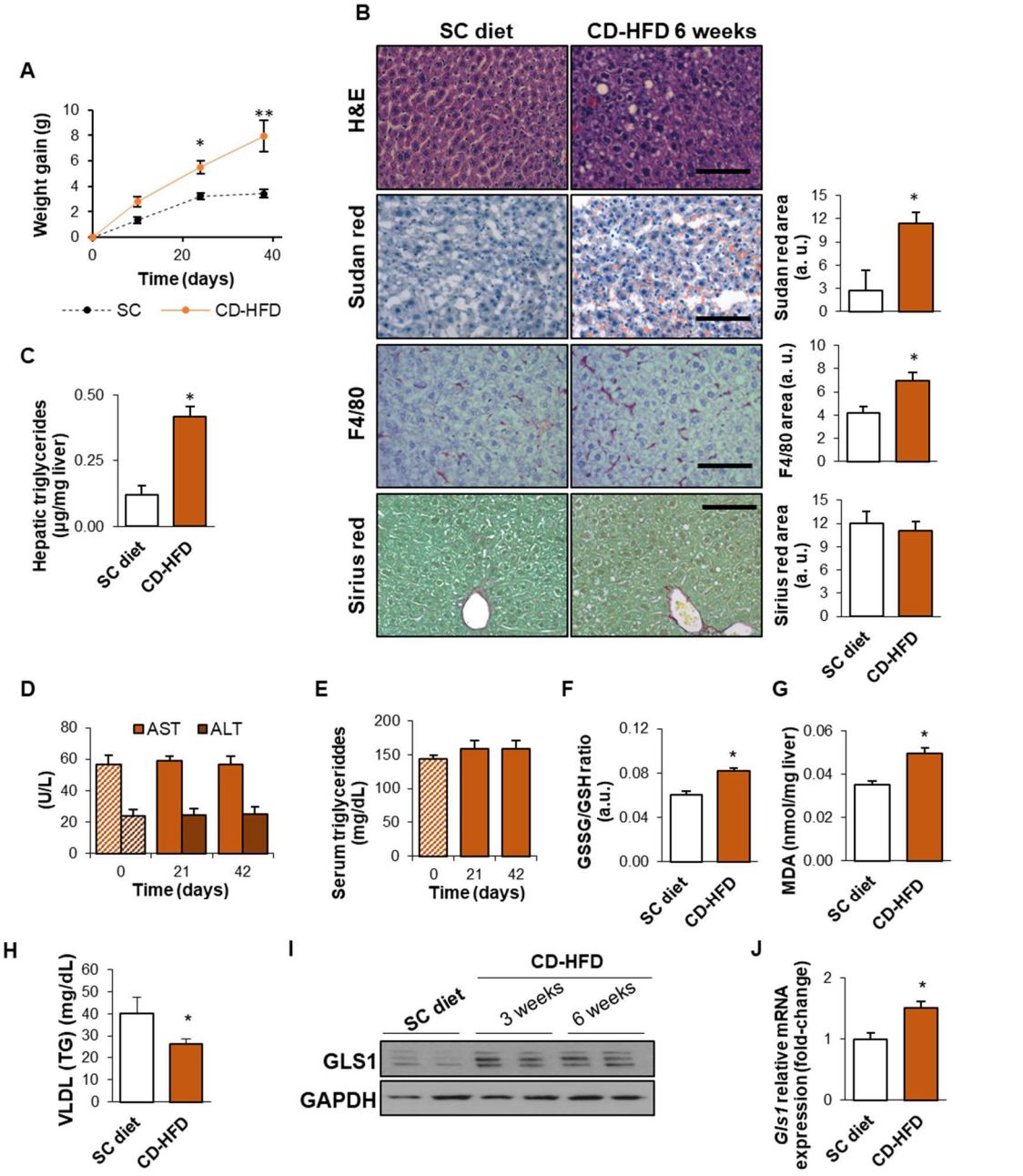


Figure 5.5. Longitudinal characterization of mice fed a choline-deficient high-fat diet (CD-HFD) (Related to Figure 5.3, Figure 5.11, Figure 5.12 and Figure 5.13)**. A.** Weight gain; **B.** Hematoxylin & eosin (H&E), Sudan Red, F4/80 and Sirius Red staining and respective quantifications. Scale bar corresponds to 100 µm; **C.** Hepatic triglycerides content; **D.** Serum transaminase levels; **E.** Serum triglycerides; **F.** Oxidized glutathione (GSSG) and reduced glutathione (GSH) ratio; **G.** Malondialdehyde (MDA) levels as a measurement of lipid peroxidation; **H.** Triglycerides content in serum very-low-density Lipoproteins (VLDL) isolated form serum obtained from the vena cava after 2-h of fasting**; I.** Representative hepatic Glutaminase 1 (GLS1) protein levels by Western Blot analysis. Glyceraldehyde-3-phosphate (GAPDH) was used as loading control; and **J.** Hepatic *Gls1* mRNA levels in mice fed a CD-HFD for six weeks compared to animals fed a control diet (SC diet). At least n=5 was used for each experimental condition. Data is shown as average ± SEM and Student's t-test was used to compare with the SC diet group. *p<0.05 and **p<0.01 is shown versus SC diet.

5.1.3 Targeting GLS1 *in vitro* resolves hepatocyte lipid accumulation

Based on recent reports suggesting the important role of GLS1 in the glutaminolysis of HSC during fibrosis progression (Du et al. 2018), particularly in animals treated with carbon tetrachloride (CCl_4), we aimed at identifying the main hepatic cells where GLS1 expression is induced in mouse models of NASH as a result of choline and methionine deprivation. Thus, after four weeks of 0.1%MCDD, a time point where inflammation is present and fibrosis is not significant (Fig. 5.2A), we perfused mouse livers to isolate hepatocytes as well as liver stroma cells, namely HSC and the liver-resident macrophages, the Kupffer cells (KC). We compared the expression of GLS1 in the different hepatic populations of 0.1%MCDD-fed rodents relative to animals fed a SC diet. We found that mRNA levels of *Gls1* are significantly increased in isolated hepatocytes from animals maintained on a 0.1%MCDD relative to the controls, whereas no changes were observed in either HSC or KC (Fig. 5.6A). Likewise, in cultured primary mouse HSC, that undergo activation *in vitro*, *Gls1* mRNA levels are increased after 7 days of culture, corresponding to an increase in the activation of HSC (Zubiete-Franco et al. 2017). On the other hand, *Gls1* mRNA levels are not significantly induced in cell cultures of primary mouse KC after stimulation with lipopolysaccharide (LPS) (Fig. 5.6.B,C). Moreover, staining of consecutive slides from liver biopsy of an animal fed a 0.1%MCDD for four weeks, shows that GLS1 staining does not overlap with F4/80 staining (a marker of KC) and fibrosis areas (Sirius red staining). On the other hand, after six weeks of 0.1%MCDD, liver biopsies show co-staining of GLS1 and Sirius red staining (Fig. 5.6D). Overall, these results show that glutamine catabolism in the hepatocyte could be relevant in our mouse model of NASH, although the relevance of other hepatic cell types for the net induction of GLS1 levels in the livers must be taken into consideration in NASH models presenting a higher degree of fibrosis.

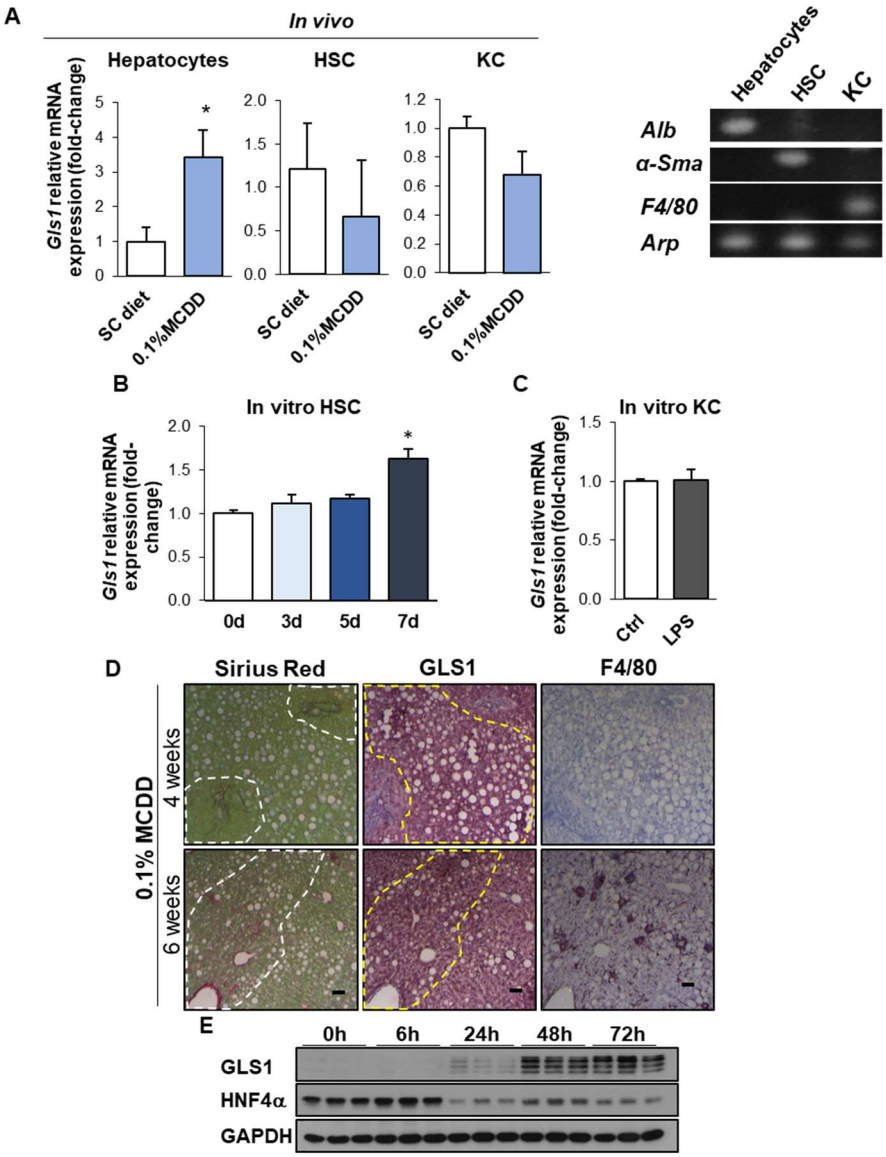


Figure 5.6. GLS1 levels in the different hepatic cell populations: hepatocytes, hepatic stellate cells (HSC) and Kupffer cells (KC). (Related to Figure 5.2, 5.3, 5.7) **A.** *Gls1* mRNA levels in primary hepatocytes, HSC and KC obtained from choline deficient and 0.1%methionine diet (0.1%MCDD)-fed mice for 4 weeks compared to standard chow fed control group (SC). Different markers of each population were analyzed by DNA agarose gel in order to confirm cell purity: albumin (*Alb*) for primary hepatocytes, α-smooth muscle actin (*α-Sma*) for HSC and *F4/80* for KC. *Arp* was used as loading control. Data is shown as average ± SEM and Student's t-test was used to compare with the SC diet group. *p<0.05 is shown versus SC diet. **B.** *Gls1* mRNA levels in *in vitro* activated primary HSC during 7 days of culture. Data is shown as average ± SEM and Student's t-test was used to compare groups. *p<0.05 is shown versus 3d. **C.** *Gls1* mRNA levels in isolated mouse KC stimulated with 200 ng/ml lipopolyssacharide (LPS). Data is shown as average ± SEM and Student's t-test was used to compare with the non-stimulated group (Ctrl). **D.** Representative consecutive slides staining for GLS1, F4/80 and Sirius red staining in liver biopsies of animals maintained for four weeks or six weeks on 0.1% MCDD (fibrosis areas with white dashed line and GLS1 with yellow dashed line). **E.** Representative hepatic Glutaminase 1 (GLS1) and Hepatic Nuclear Factor 4a (HNF4a) protein levels by Western Blot analysis. Glyceraldehyde-3-phosphate (GAPDH) was used as loading control.

In order to further assess the relevance of the high-activity glutaminase GLS1 isoform expression in the hepatocytes during NASH, primary mouse hepatocytes were isolated. Primary cultures of hepatocytes represent substantial limitations that include dedifferentiation. As expected and in agreement with previous evidence (Sahai et al.

2006), GLS1 is gradually increased during culture (Fig. 5.6E). Isolated mouse hepatocytes were maintained on a methionine- and choline- deficient (MCD) medium that has been previously shown to induce steatosis and injury in the hepatocyte cell line AML-12 (Sahai et al. 2006) as well as in primary mouse hepatocytes (Iruarrizaga-lejarreta et al. 2017). Under conditions of MCD treatment, GLS1 was inhibited in vitro by using either molecular approaches (siRNA) or the small pharmacological inhibitor BPTES (Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide). BPTES is an allosteric, time-dependent, and specific inhibitor of GLS1 that exhibits unique binding at the oligomerization interface of the glutaminase tetramer (DeLaBarre et al. 2011; Thangavelu et al. 2014; Thomas et al. 2013). *Gls1* silencing in primary hepatocytes cultured for 48 h in MCD media reduced the accumulation of triglycerides (Fig. 5.7, Fig. 5.8A). Likewise, the pharmacological inhibition of GLS1 by using BPTES, as detected by the decreased cellular glutamate/glutamine ratio, significantly reduced the accumulation of triglycerides (Fig. 5.8D-E, G). Under these conditions, cell viability, as assessed by caspase activity, was not significantly altered after *Gls1* silencing or pharmacological inhibition by using BPTES (Fig. 5.8C, F). In addition, *GLS1* silencing in a human hepatocyte cell line, the THLE-2 cells, also reduces lipid accumulation after MCD treatment (Fig. 5.8H, I). Moreover, in isolated mouse hepatocytes incubated in the presence of oleic acid, a cell model where hepatocytes accumulate lipids due to increase uptake and also inhibition of VLDL export (Nossen et al. 1986), both GLS1 silencing and pharmacological inhibition, also reduces hepatocytes lipid content (Fig. 5.9).

Finally, although primary cultures of hepatocytes represent substantial limitations that include dedifferentiation, when we silenced *Gls1* in mouse hepatocytes treated with MCD media, dedifferentiation parameters, such as albumin, alpha-fetoprotein, and HNF4, were unaltered, suggesting that the decrease in hepatocyte steatosis observed with *Gls1* silencing is independent of the dedifferentiation stage (Figure 5.8B).

Taken together, GLS1 silencing ameliorates steatosis in isolated primary mouse hepatocytes.

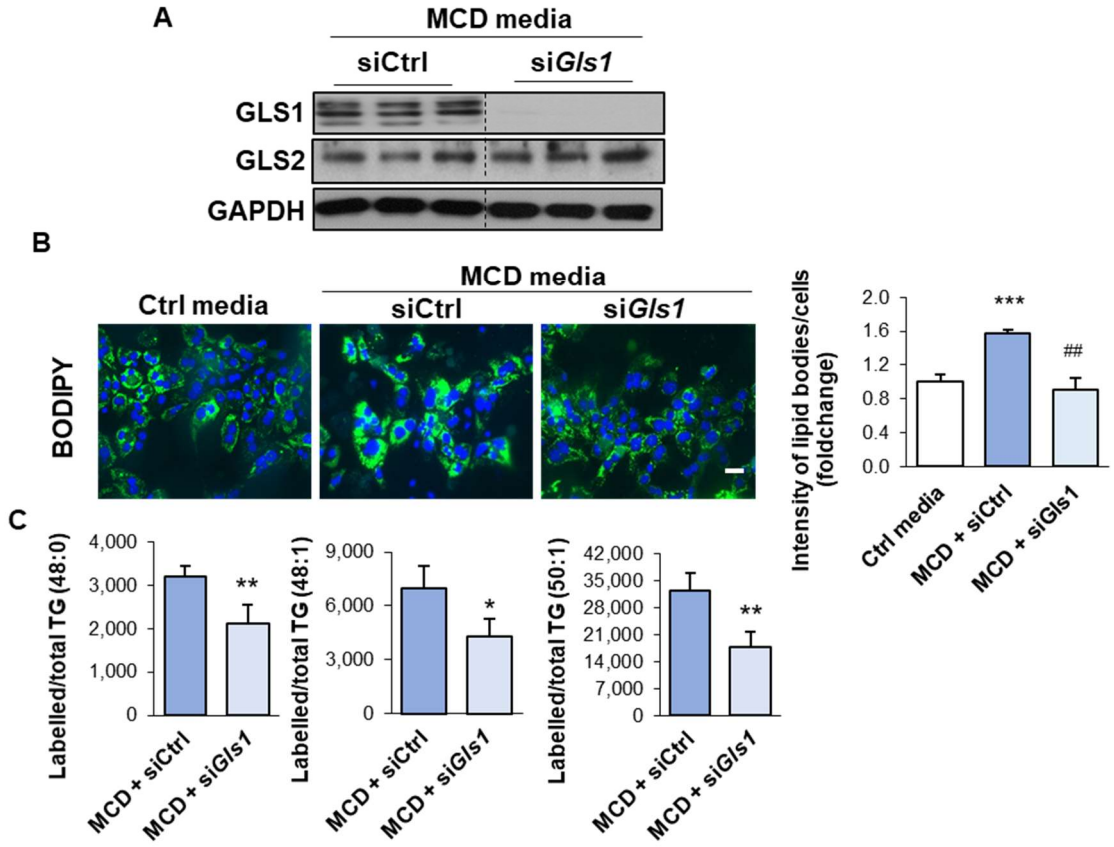


Figure 5.7. Targeting Glutaminase 1 (GLS1) in vitro reduces hepatocyte lipid content. A. Western blot analysis of total protein levels of Glutaminase 1 (GLS1) and Glutaminase 2 (GLS2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control; **B.** Representative BODIPY staining micrographs and respective quantification in mouse isolated hepatocytes treated for 48 h with a control (Ctrl) or a methionine- and choline-deficient media after treatment with siRNA against *Gls1* (MCD + si*Gls1*) or unrelated control (MCD + siCtrl). Scale bar corresponds to 100 µm. At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare between the groups. ***p<0.01 versus Ctrl and $^{\#\#}p$<0.01 versus MCD + siCtrl are shown. **C.** Mass-spectrometry analysis of different triglyceride (TG) isoforms in mouse isolated hepatocytes treated for 48 h with MCD media after treatment with siRNA against *Gls1* (si*Gls1*) or unrelated control (siCtrl). At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare between the groups. *p<0.05 and **p<0.01 versus MCD + siCtrl are shown. (See also Figure 5.6, Figure 5.8 and Figure 5.9).

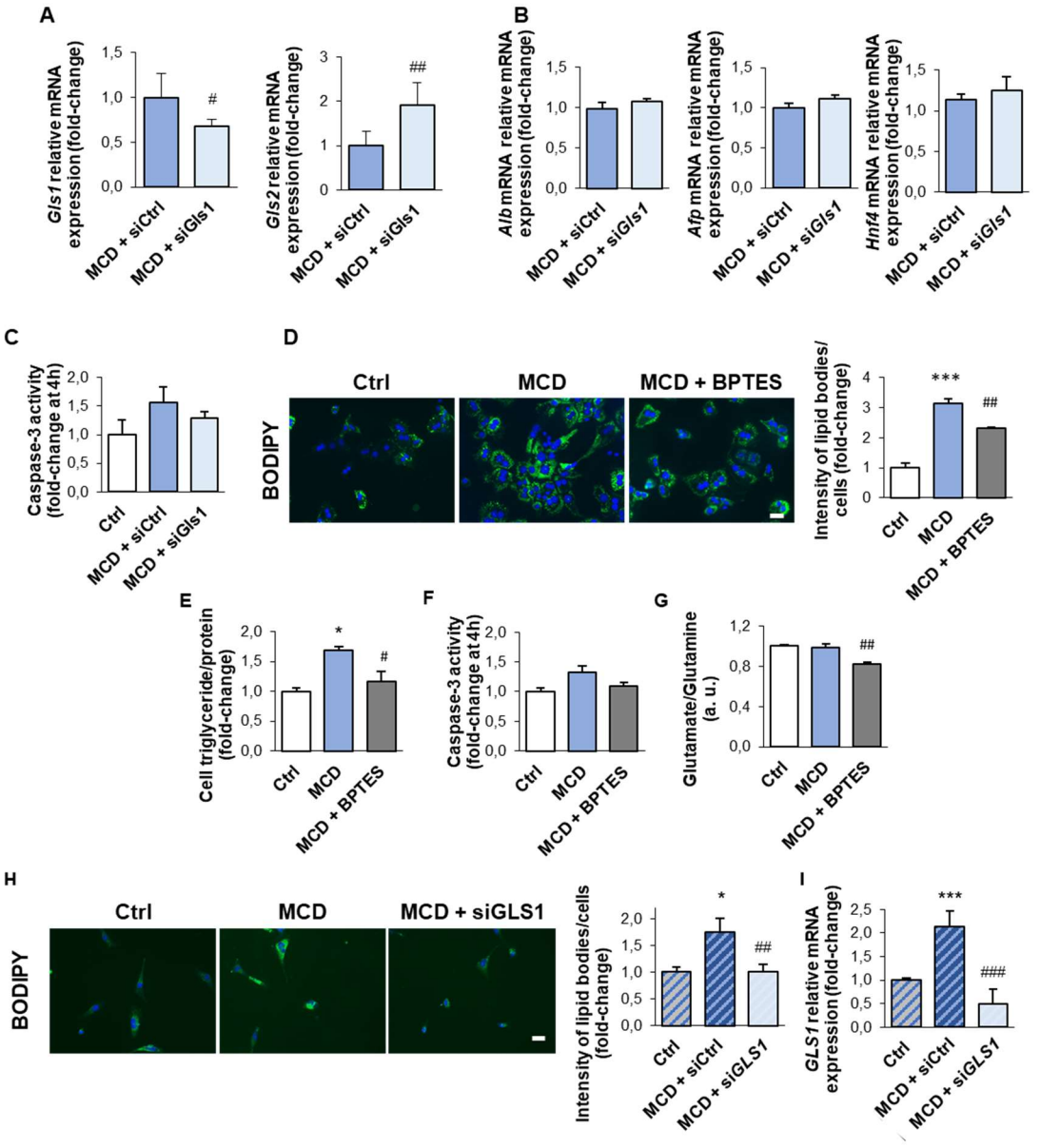


Figure 5.8. Glutaminase 1 (GLS1) inhibition in mouse and human hepatocytes reduces methionine and choline-deficient (MCD)-induced lipid accumulation without inducing cell death or dedifferentiation (Related to Figure 5.7). **A.** *Gls1* and *Gls2* mRNA levels in control medium and methionine and choline-deficient stimulated primary hepatocytes treated with siRNA against *Gls1* (si*Gls1*) or an unrelated control (siCtrl). Data is shown as average ± SEM and Student's t-test was used to compare groups. $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus MCD + siCtrl are shown. **B.** Albumin (*Alb),* α-fetoprotein *(Afp)* and hepatic nuclear factor 4 (*Hnf4)* mRNA levels in control medium and methionine and choline-deficient (MCD) stimulated primary hepatocytes treated with siRNA against *Gls1* (MCD+si*Gls1*) or an unrelated control (MCD+siCtrl). Data is shown as average ± SEM and Student's t-test was used to compare groups; **C.** Caspase activity assay in mouse isolated hepatocytes treated for 48h either with control media (Ctrl), MCD by silencing *Gls1* through siRNA (si*Gls1*) or an unrelated control (siCtrl). At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare groups. **D.** Representative BODIPY staining micrographs and respective quantification and **E.** cell triglycerides determination in mouse isolated hepatocytes treated either with control media (Ctrl), methionine and choline deficient media (MCD) and MCD media in the presence of the GLS1 pharmacological inhibitor, 10 μm BPTES (Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide) (MCD + BPTES) for 48h. Scale bar corresponds to 100 μm. At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare with the Ctrl media or the MCD group. $^{*}p<0.05$ and $^{***}p<0.001$ versus Ctrl, and $^{\#}p<0.05$ and $^{\#\#}p<0.01$ versus MCD are shown; **F.** Caspase activity assay in mouse isolated hepatocytes treated for 48h either with control media (Ctrl), MCD in the presence or absence of 10 μM BPTES (MCD + BPTES). At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare groups; **G.** Glultamine/Glutamate ratio and **H.** Representative BODIPY staining micrographs (Scale bar corresponds to 100 μm) and respective quantification and **I.** *GLS1* mRNA levels in human THLE2 cells treated either with control media (Ctrl) with or without MCD conditions during 12 h in

the presence of a siRNA against *GLS1* (si*GLS1*) or an unrelated control (siCtrl). Scale bar corresponds to 100 µm. At least triplicates were used for each experimental condition. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni's post-test was used to compare differences between groups. ***p<0.001 versus Ctrl and $^{\#\#\#}p$<0.001 versus MCD + siCtrl are shown.

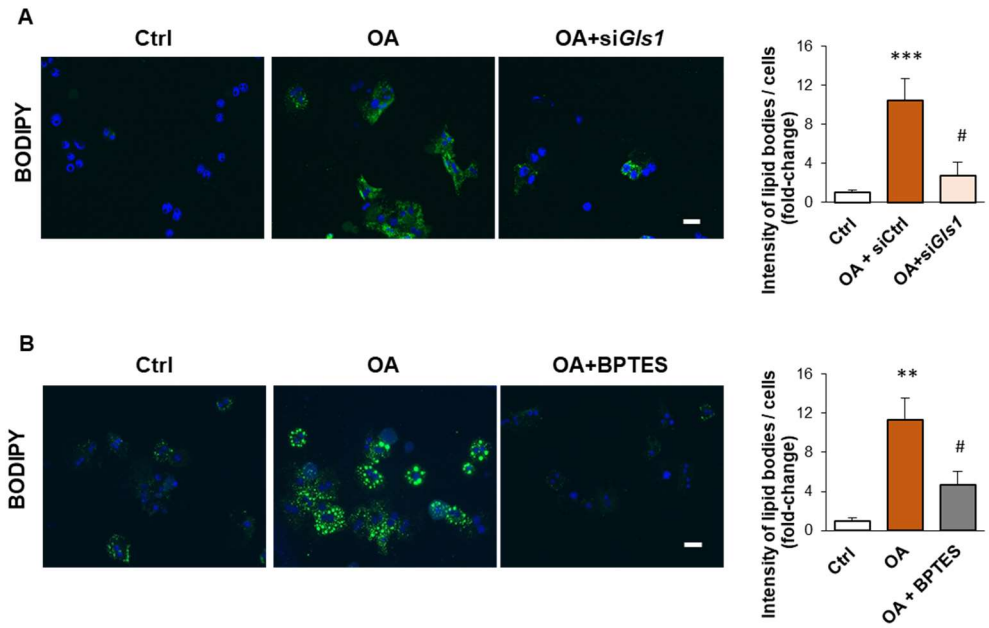


Figure 5.9. Inhibition of glutaminase 1 (GLS1) reduces oleic acid (OA)-induced lipid accumulation in primary hepatocytes. (Related to Figure 5.7) Representative BODIPY staining micrographs and respective quantification in mouse isolated hepatocytes treated either with control media (Ctrl) and 400µM oleic acid treated with **A.** a siRNA against *Gls1* (OA+si*Gls1*) or an unrelated control (OA+siCtrl); **B.** with the presence of a GLS1 pharmacological inhibitor, 10 µm BPTES (Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide) (OA+BPTES) or a vehicle (OA) for 48h. Scale bar corresponds to 100 µm. At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare with the Ctrl media or the OA group. **p<0.01 and ***p<0.001 versus Ctrl, and $^{\#}p$<0.05 versus OA are shown.

5.1.4 Targeting GLS1 *in vivo* resolves NASH

Taking into consideration that GLS1 is increased in NASH, the potential therapeutic role of silencing *Gls1 in vivo* was assessed. Thus, we have evaluated the effects of *Gls1* silencing in mice fed a 0.1%MCDD. Depriving mice of methionine and choline for four weeks caused an increase in hepatic content of fatty acids, cholesteryl esters, diglycerides, and triglycerides. As expected, the concentration of serum triglycerides and cholesterol was reduced, attributable to a phosphatidylcholine-related defect in hepatic VLDL secretion, as previously described (Rizki et al. 2006) (Table 5.1). Liver histology confirmed marked lipid accumulation after four weeks of 0.1%MCDD (Fig. 5.10A). In addition, four weeks of 0.1%MCDD lowered blood glucose (Table 5.1).

Hepatic *Gls1* was silenced in 0.1%MCDD-fed rodents by using twice-a-week tail vein injections of Invivofectamine® conjugated to either *Gls1*-specific or Control siRNA, from weeks 2 to 4 of 0.1%MCDD. As a result of *Gls1* silencing, GLS1 hepatic levels are

reduced and GLS2 expression is increased, whereas glutamine synthetase expression is not significantly altered (Fig. 5.10A-C). Of relevance, the levels of hepatic ammonia, a secondary product of the glutaminase reaction, are not significantly altered after *Gls1* silencing (Table 5.1). Under these circumstances, reduced glutaminase activity was confirmed by measuring the incorporation of ^{13}C labeling from glutamine into glutamate, confirming that glutaminase activity is, on one hand, induced in the diet and on the other there is a tendency for decreased glutaminase activity after *Gls1* silencing in vivo (Fig. 5.10D). Importantly, GLS1 specific silencing in vivo, both at the protein and the mRNA level, significantly reduced liver steatosis, measured as Sudan red staining and by biochemical assay (Fig. 5.10A, Table 5.1). Furthermore, *Gls1* silencing in vivo in 0.1%MCDD-fed rodents significantly increased hepatic phospholipid content whilst decreasing cholesteryl esters and restoring serum triglycerides levels (Table 5.1).

Table 5.1. Physical and biochemical characteristics of mice after four weeks of choline deficient with 0.1% methionine diet (0.1%MCDD) relative to standard chow (SC) diet and treatment with GLS1 silencing (Related to Figure 5.10). **p*<0.05, ***p*<0.01 and **p*<0.001 vs. SC diet is shown as well as $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 vs.0.1%MCDD + siCtrl.

	SC diet	0.1%MCDD + siCtrl	0.1%MCDD + si*Gls1*
Body weight (g)	29.8 ± 1.1	24.5 ± 0.7***	24.7 ± 1.2*,#
Liver/Body weight	0.0384 ± 0.0019	0.0360 ± 0.0008	0.0422 ± 0.0020
Serum biochemical analysis			
ALT (U/l)	47 ± 3	150 ± 13*	88 ± 19#
AST (U/l)	29 ± 10	169 ± 16***	93 ± 24*,#
Triglycerides (nmol/µl)	78 ± 8	43 ± 2****	57 ± 2**,#
Non-esterified fatty acids (nmol/µl)	0.43 ± 0.07	0.49 ± 0.08	0.40 ± 0.05
Cholesterol (mg/dl)	117 ± 7	34 ± 5****	39 ± 9****
Glucose (mg/dl)	234 ± 18	158 ± 14*	189 ± 8
Liver biochemical analysis			
Fatty acids (nmol/mg protein)	21.6 ± 1.3	60.1 ± 4.5****	49.8 ± 4.3***
Diglycerides (nmol/mg protein)	11.2 ± 0.7	29.0 ± 1.2****	30.4 ± 2.3****
Triglycerides (µg/mg liver)	8.4 ± 2.4	47.4 ± 2.5****	36.6 ± 2.3****,#
Phospholipids (nmol/mg liver)	0.53 ± 0.10	0.38 ± 0.05	0.76 ± 0.04#
Free Cholesterol (nmol/mg protein)	23.7 ± 0.9	24.4 ± 0.7	25.7 ± 0.5
Cholesteryl esters (nmol/mg protein)	10.8 ± 1.2	33.4 ± 2.0****	20.3 ± 1.2**,###
Glycogen content (a.u.)	75 ± 5	25 ± 9**	23 ± 5**
Ammonia content (fold-change)	1.0 ± 0.4	1.3 ± 0.5	**5.11** ± 0.1

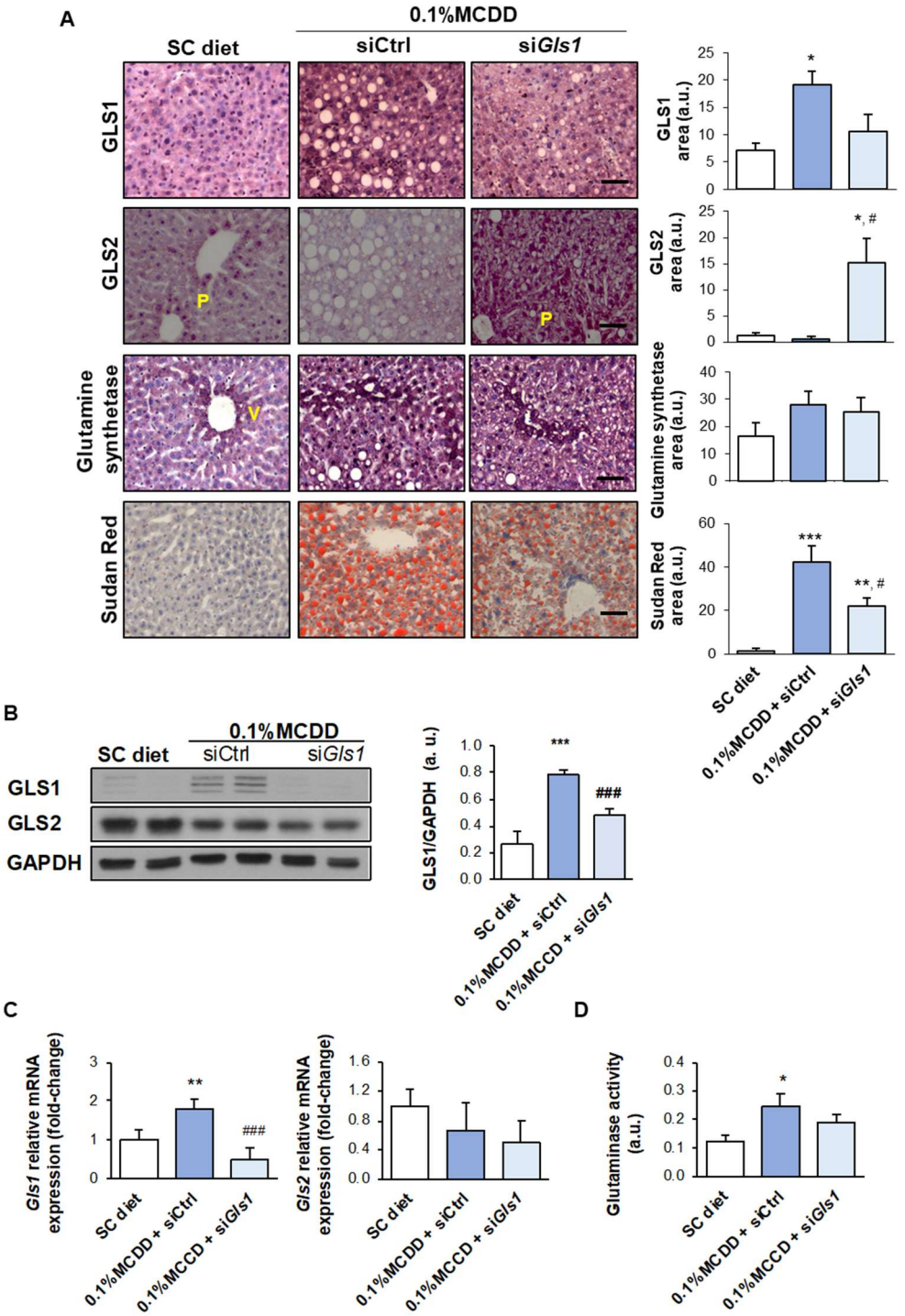


Figure 5.10. Targeting glutaminase 1 (GLS1) *in vivo* resolves the accumulation of hepatic triglycerides and non-alcoholic steatohepatitis (NASH). A. GLS1, GLS2 and glutamine synthetase levels quantified by Immunohistochemistry and representative micrographs of Sudan Red staining and respective quantification in animals maintained on a choline deficient and 0.1% methionine diet (0.1% MCDD). From weeks two to four of diet, two different experimental groups were treated either with siCtrl or si*Gls1*. Scale bar corresponds to 100 µm. At least n=5 were used for each experimental group. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. *p<0.05, **p<0.01 and ***p<0.001 versus SC diet and $^{\#}p$<0.05 versus 0.1%MCDD + siCtrl are shown. **B.** Representative Western Blot analysis and quantification of GLS1 and GLS2 levels. Glyceraldehyde-3-phosphate (GAPDH) was used as loading control **C.** mRNA levels of *Gls1* and *Gls2*. **D.** *In vivo* glutaminase activity, measured as the ratio between (U-^{13}C)glutamate and (U^{13}-C)-glutaminase, in mice fed either a standard chow diet (SC diet) or 0.1% MCDD for four weeks with or without silencing for GLS1 (0.1%MCDD + si*Gls1* or 0.1%MCDD + siCtrl). At least n=5 animals were used for each experimental group. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. *p<0.05, **p<0.01 and ***p<0.001 versus SC diet and $^{\#\#\#}$p<0.001 versus 0.1% MCDD + siCtrl are shown. (See also Table 5.1, Figure 5.11).

Finally, in animals fed a CD-HFD for six weeks, we have silenced GLS1 by using twice-a-week tail vein injections of Invivofectamine® conjugated to either GLS1-specific or control siRNA from weeks 3 to 6 of CD-HFD (Fig. 5.11A, B). In these animals, GLS1 silencing was able to significantly reduce hepatic steatosis (Fig. 5.11C) without changes to body weight and food intake (data not shown).

In summary, GLS1 inhibition ameliorates liver steatosis in pre-clinical mouse models of NASH.

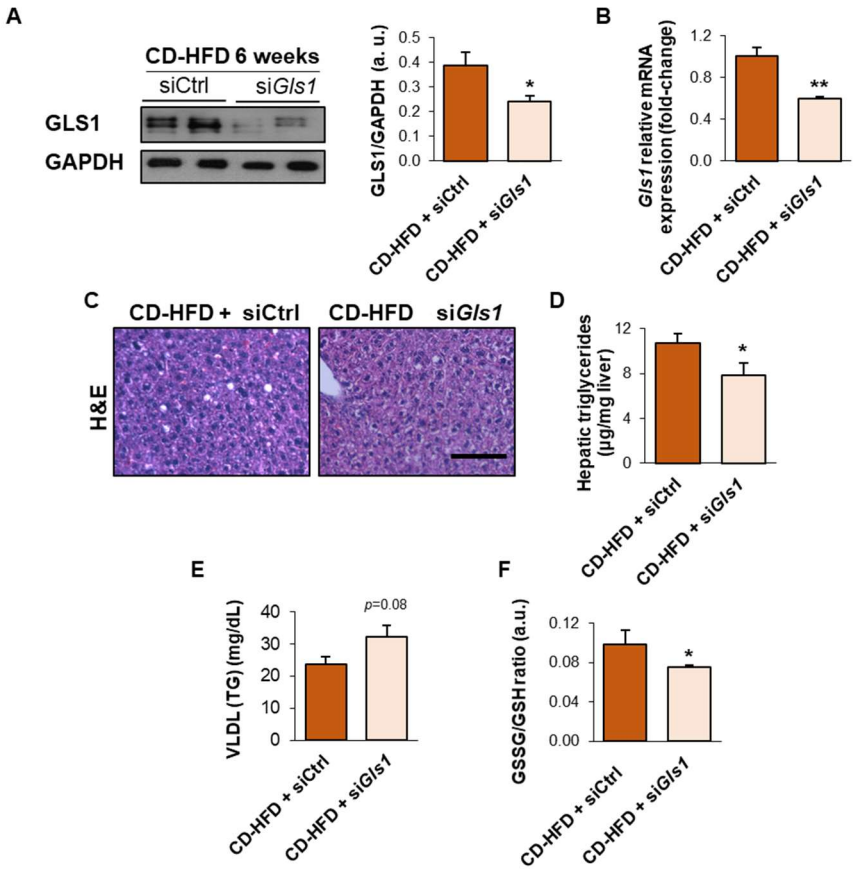


Figure 5.11. Targeting glutaminase 1 (GLS1) *in vivo* resolves the accumulation of hepatic triglycerides and inflammation induced by choline deprivation and high fat diet (Related to Figure 5.5, Figure 5.12 and Figure 5.13). A. Representative Western blot analysis and quantification of GLS1 levels. Glyceraldehyde-3-phosphate (GAPDH) was used as loading control; B. mRNA levels of *Gls1*; C. H&E staining. Scale bar corresponds to 100 µm; D. Hepatic triglycerides levels; E. Triglycerides content in serum very-low-density Lipoproteins (VLDL) isolated form serum obtained from the vena cava after 2-h of fasting; and F. Oxidized and reduced glutathione ratio (GSSG/GSH) in animals maintained on a choline deficient and high fat diet (CD-HFD) for 6 weeks. From week 3 to 6 of diet, two different experimental groups were treated either with siCtrl or si*Gls1* (CD-HFD + siCtrl or CD-HFD + si*Gls1*). At least n=5 were used for each experimental group. Data is shown as average ± SEM and Student´s t-test was used to compare between groups. *p<0.05 and **p<0.01 versus CD-HFD + siCtrl are shown.

5.1.5 Targeting GLS1 *in vitro* and *in vivo* restores VLDL export after methionine and choline deprivation

As mentioned before, phospholipids are required for correct VLDL assembly and therefore methionine and choline deprivation results in impaired VLDL export. Taking that into account, the impact of *Gls1* silencing in Ptd-Chol synthesis and VLDL export was evaluated both in vitro and in vivo. Firstly, hepatocytes grown for 48 h in complete and MCD media under conditions of *Gls1* or control silencing were incubated with (U-^{13}C)glucose for one hour. Using mass spectrometry we have measured the incorporation of the glucose tracer in intracellular Ptd-Chol species. As expected, under conditions of choline and methionine deprivation there is a tendency for reduced incorporation of carbon tracers in Ptd-Chol, reflecting impaired Ptd-Chol synthesis from glucose. On the other hand, *Gls1* silencing under conditions of methionine and choline deprivation promoted the synthesis of some Ptd-Chol species (Fig. 5.12A). Moreover, we treated isolated mouse hepatocytes with lomitapide, described previously to inhibit MTP and shown to hinder VLDL export (Sirtori et al. 2014). As expected, treatment with lomitapide caused the accumulation of cell lipids to a similar extent as in cells treated with complete and methionine- and choline-deficient media. Interestingly, under these conditions GLS1 silencing does not prevent cell lipid accumulation after lomitapide treatment, suggesting that *Gls1* silencing-induced lipid lowering may somehow be related to VLDL export (Fig. 5.12B).

In addition, hepatic phospholipid content was determined in mice maintained on a diet deprived of methionine and choline and after control or *Gls1* silencing. We observed that, as a result of in vivo *Gls1* silencing in 0.1%MCDD-fed rodents, hepatic Ptd-Cho and phosphatidylserine (Ptd-Ser) levels are augmented, whereas Ptd-Et levels remain unaltered (Fig. 5.12C). Thus, we decided to evaluate the composition of the secreted VLDL particles in mice fed a 0.1% MCDD and where we have silenced *Gls1*. For this, circulating VLDL catabolism was inhibited through the administration of poloxamer 407, a non-ionic detergent described to inhibit lipoprotein lipase (LPL) (Millar et al. 2005), an enzyme mostly abundant in tissues involved in fatty acid metabolism such as muscle and adipose tissue (Karpe et al. 1998). When we silence *GLS1* in vivo in mice fed a 0.1%MCDD, the lipid content of the secreted VLDL particles was significantly enriched in lipids, such as triglycerides, phospholipids, and

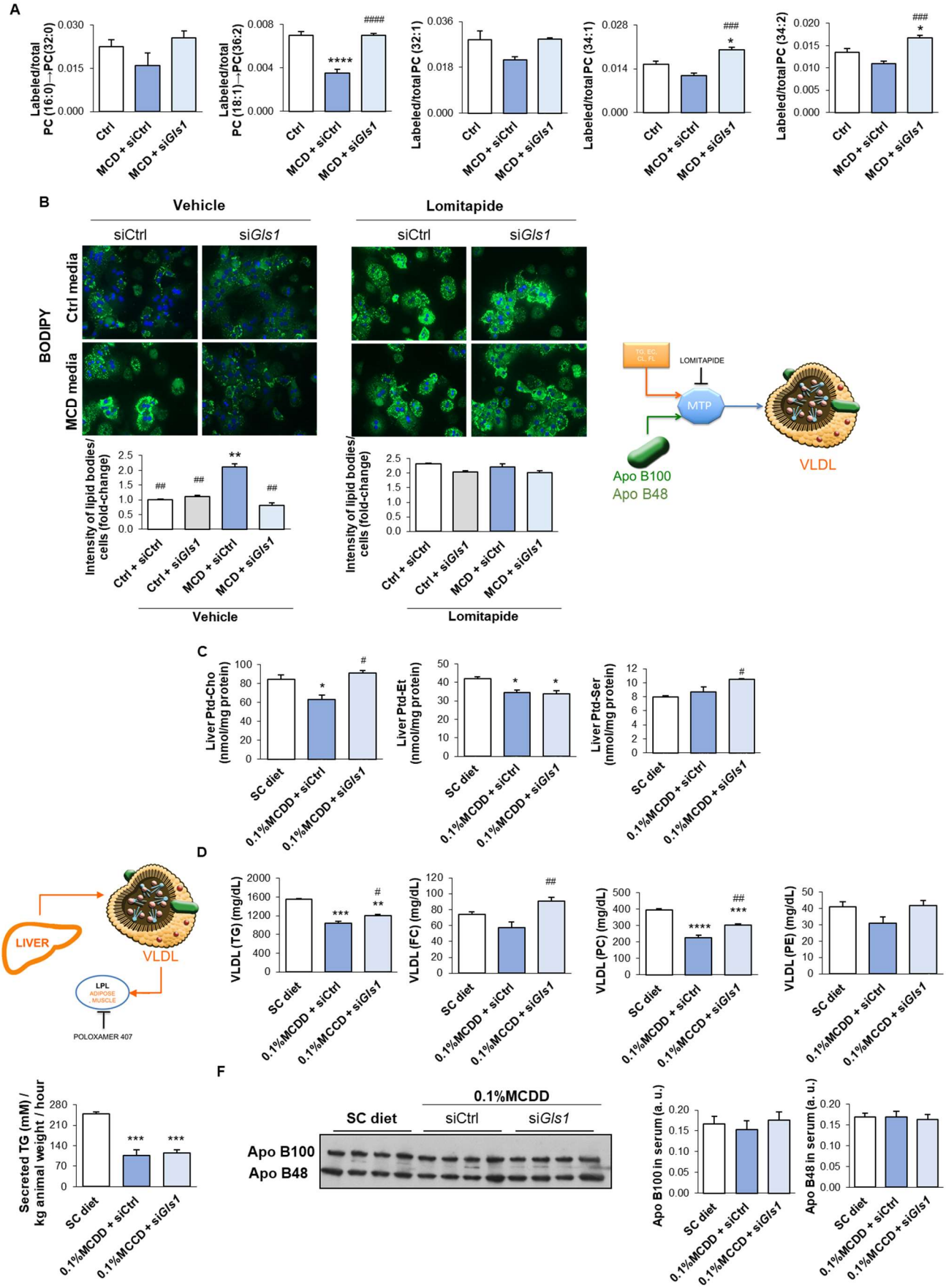

A
Labeled/total PC (16:0)→PC(32:0)
Labeled/total PC (18:1)→PC(36:2)
Labeled/total PC (32:1)
Labeled/total PC (34:1)
Labeled/total PC (34:2)
Ctrl
MCD + siCtrl
MCD + siGls1
B
Vehicle
Lomitapide
siCtrl
siGls1
BODIPY
Ctrl media
MCD media
Intensity of lipid bodies/ cells (fold-change)
Ctrl + siCtrl
Ctrl + siGls1
MCD + siCtrl
MCD + siGls1
LOMITAPIDE
MTP
Apo B100
Apo B48
VLDL
C
Liver Ptd-Cho (nmol/mg protein)
Liver Ptd-Et (nmol/mg protein)
Liver Ptd-Ser (nmol/mg protein)
SC diet
0.1%MCDD + siCtrl
0.1%MCDD + siGls1
D
LIVER
VLDL
LPL
ADIPOSE MUSCLE
POLOXAMER 407
VLDL (TG) (mg/dL)
VLDL (FC) (mg/dL)
VLDL (PC) (mg/dL)
VLDL (PE) (mg/dL)
0.1%MCCD + siCtrl
0.1%MCCD + siGls1
E
Secreted TG (mM) / kg animal weight / hour
F
0.1%MCDD
SC diet
siCtrl
siGls1
Apo B100
Apo B48
Apo B100 in serum (a. u.)
Apo B48 in serum (a. u.)

Figure 5.12. Targeting Glutaminase 1 (GLS1) in vitro and in vivo restores very-low-density lipoproteins (VLDL) triglyceride export after choline and methionine deprivation. A. Mass-Spectrometry analysis of the incorporation of ^{13}C carbons of glucose into phosphatidylcholine (Ptd-Chol) in mouse isolated hepatocytes treated with methionine- and choline-deficient media (MCD) for 24 h after overnight treatment with siRNA against *Gls1* (si*Gls1*) or unrelated control (siCtrl) and followed by 1 h incubation with 10mM U-^{13}C(glucose). At least quadruplicates were used for each experimental condition. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. *p<0.05; ****p<0.0001 versus Ctrl and $^{\#\#\#}p$<0.001; $^{\#\#\#\#}p$<0.0001 versus MCD + siCtrl are shown; **B.** Representative BODIPY staining micrographs and respective quantification in mouse isolated hepatocytes treated with control media (Ctrl) or methionine- and choline -deficient media (MCD) for 24 h after overnight treatment with siRNA against *Gls1* (si*Gls1*) or unrelated control (siCtrl). In some experimental conditions lomitapide was added at 600 nM for 24 hours. Scale bar corresponds to 100 μm. At least triplicates were used for each experimental condition. Data is shown as average ± SEM and Student´s t-test was used to compare between groups. *p<0.05 and **p<0.01 versus Ctrl + siCtrl and $^{\#\#}p$<0.01 versus MCD + siCtrl are shown. **C.** Liver phosphatidylcholine (Ptd-Cho), phosphatidylethanolamine (Ptd-Et) and phosphatidylserine (Ptd-Ser) hepatic levels; **D.** Serum very-low-density lipoprotein (VLDL) phospholipids and lipid content; **E.** Hepatic triglycerides secretion rate and **F.** Apo B100, Apo B48 and quantifications by Western blot analysis in mice fed either a standard chow (SC) diet or a diet deficient in choline with 0.1% methionine (0.1% MCDD) for four weeks. From weeks two to four of diet, two different experimental groups were treated either with siCtrl or si*Gls1*. Animals were administered poloxamer 407 (P407) and serum VLDL isolated and analyzed at six hours after P407 administration. At least n=5 were used for each experimental group. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. **p<0.01, ***p<0.001 and ****p<0.0001 versus SC diet and $^{\#}p$<0.05 and $^{\#\#}p$<0.01 versus 0.1%MCDD + siCtrl are shown (See also Figure 5.5 and Figure 5.11).

cholesterol derivatives (Fig. 5.12D). In spite of this, the number of VLDL particles, determined by the VLDL secretion rate and the molecules of apoB secreted, was not altered (Fig. 5.12E,F). Likewise, in mice fed a CD-HFD for six weeks, *Gls1*-silencing results in a tendency for increased VLDL triglyceride content in VLDL isolated from vena cava serum after 2 h of fasting (5.11E).

Overall, we provide evidence that *Gls1* silencing ameliorates liver steatosis by targeting VLDL assembly through mechanisms that have not been previously explored.

5.1.6 Targeting GLS1 *in vitro* and *in vivo* reduces oxidative stress

Oxidative stress plays a crucial role in the pathogenesis and progression of NASH. In agreement, we have observed that harvesting the primary mouse hepatocytes with media deprived of methionine and choline increases both total and mitochondrial reactive oxygen species (ROS) levels (Fig. 5.13A). Reduced glutathione (GSH) is considered to be one of the most important ROS scavengers. Importantly, the ratio between GSH and oxidized glutathione (GSSG) may be used as a marker of oxidative stress. Thus, four weeks of 0.1%MCDD is associated with augmented GSSG/GSH ratio and increased incorporation of labeled glutamine into GSH, highlighting that GSH synthesis is increased under these conditions (Fig. 5.13B). Likewise, the total levels of malondialdehyde (MDA), a marker of lipid peroxidation, were induced (Fig. 5.13C).

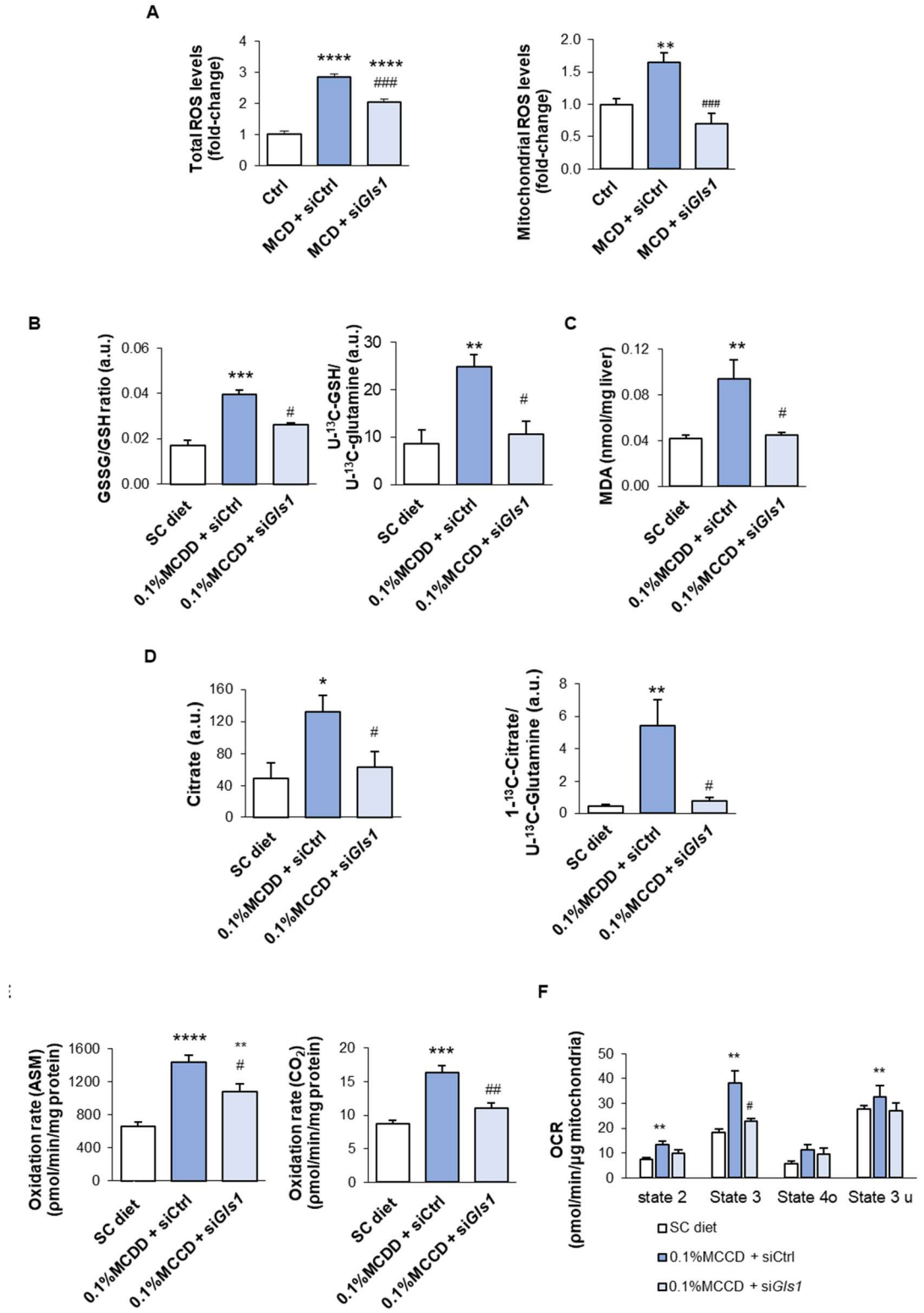


Figure 5.13. Targeting Glutaminase 1 (GLS1) in vitro and in vivo reduces oxidative stress. A. Total and mitochondrial reactive oxygen species (ROS) levels in mouse isolated hepatocytes treated with control media (Ctrl) or methionine and choline deficient media (MCD) for 48 h after overnight treatment with siRNA against *Gls1* (si*Gls1*) or unrelated control (siCtrl). At least triplicates were used for each experimental condition. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 versus Ctrl media as well as $^{\#\#\#}p$<0.001 versus MCD media + siCtrl are shown. **B.** Oxidized glutathione (GSSG) and reduced glutathione (GSH) ratio and incorporation of ^{13}C carbons from U-^{13}C(glutamine) carbons into 5-^{13}C(GSH); **C.** Malondialdehyde (MDA) levels as a measurement of lipid peroxidation; **D.** Citrate and incorporation of U-^{13}C(glutamine) carbons on 1-^{13}C(citrate) levels; **E.** Fatty acid oxidation

(FAO) rate quantified from the incorporation of ^{14}C-palmitate into CO_2 and in acid-soluble metabolites (ASM); **F.** Mitochondrial Oxygen Consumption Rate (OCR) in different states of the respiration (State 2, State 3, State 4o, State 3u) in mice fed either a standard chow diet (SC) or a diet deficient in choline with 0.1% methionine (0.1% MCDD) for four weeks. From weeks two to four of diet, two different experimental groups were treated either with siCtrl or si*Gls1*. At least n=5 were used for each experimental group. Data is shown as average ± SEM and one-way ANOVA followed by Bonferroni post-test was used to compare between multiple groups. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 versus SC diet and $^{\#}p$<0.05 and $^{\#\#}p$<0.01 versus 0.1%MCDD+siCtrl are shown (See also Figure 5.5 and Figure 5.11).

Noteworthy, *Gls1* silencing significantly reduces oxidative stress in primary hepatocytes and in the in vivo NASH models (Fig. 5.13A-C, Fig. 5.11F). Numerous causes of oxidative stress have been associated with NASH. Impaired tricarboxylic acid (TCA) cycle, FAO and oxidative phosphorylation (OXPHOS) originate ROS (Rosca et al. 2012). Here, we have measured hepatic TCA cycle activity, evaluated as the incorporation of U-^{13}C-glutamine into 1-^{13}C-citrate; hepatic FAO, measured using radioactive incorporation of labeled palmitate into CO_2 and into incompletely oxidized acid-soluble metabolites (ASM); as well as mitochondrial oxidative phosphorylation (OXPHOS) as measured by oxygen consumption rate (OCR) using a Seahorse analyzer. All the analyzed parameters were significantly higher after four weeks of 0.1%MCDD and, importantly, *Gls1* silencing during 0.1%MCDD restored TCA, β-oxidation, and OXPHOS pathway fluxes to control diet levels in association with reduced oxidative stress (Fig. 5.13D-F).

In summary, targeting hepatic GLS1 in vitro and in vivo reduces ROS levels by reducing oxidative metabolism.

5.1.7 GLS1-mediated reduction of oxidative stress is associated with restored hepatic phospholipid content

Choline is essential for the *de novo* synthesis of Ptd-Chol, the major phospholipid component of plasma lipoproteins, via the cytidine-diphosphate pathway (CDP). Decreased hepatic Ptd-Chol reduces the levels of circulating VLDL (Cole, Vance, and Vance 2012). In the liver, Ptd-Cho can be additionally synthesized from the methylation of Ptd-Et, a reaction catalyzed by the enzyme PEMT and using SAMe, an intermediate of the methionine cycle, as a methyl donor (Noga, Zhao, and Vance 2002) . The other intermediate of the methionine cycle, homocysteine, at the crossroads of the metabolic pathways, is either degraded via the transsulfuration pathway to cysteine and then GSH or is remethylated back to methionine. Indeed, depletion of SAMe and GSH are early events in the MCD model of NASH (Caballero et al. 2010) (Table 5.2, Fig. 5.13B).

In one carbon metabolism, a carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form methylene-THF (MTHF). MTHF either can be used for the synthesis of purines or reduced to methyl-THF, which can be used to methylate homocysteine to methionine. Likewise, serine, which has previously been shown to be able to transfer one-carbon units to recycle homocysteine to methionine in tumor cells (Maddocks et al. 2016), can be used in combination with homocysteine to synthesize GSH, or can be metabolized to Ptd-Ser by Ptd-ser synthase II (PTDSS2) (Kuge and Nishijima 1997). In mammals, Ptd-Ser can be further metabolized to Ptd-Et and later converted to Ptd-Chol by PEMT activity. Then, Ptd-Cho can undergo a base-exchange process with Ptd-Ser, releasing choline through the exchange with serine. Thus, even under conditions of choline deprivation, newly formed choline can be metabolized to Ptd-Cho through the CDP-choline pathway (DeLong et al. 1999; Henneberry, Wistow, and McMaster 2000).

We have previously shown that *Gls1* silencing on 0.1%MCDD-fed mice reduced oxidative stress and decreased GSH synthesis (Fig. 5B). In agreement, the expression of the genes involved in the transsulfuration pathway are reduced after *Gls1* silencing, whereas expression of enzymes involved in the folate and methionine cycles is augmented (Fig. 5.15A). Likewise, the mRNA levels of the genes involved in the CDP-choline and CDP-ethanolamine pathways and the enzymes catalyzing the base-exchange among the different phospholipids are induced after *Gls1* silencing (Fig. 5.14B). Although *Pemt* mRNA expression is not upregulated after *Gls1* silencing, the increase in the Ptd-Cho/Ptd-Et ratio observed (1.4 ± 0.3 in 0.1%MCDD + si*Gls1* *vs.* 1.0 ± 0.25 in 0.1%MCDD + siCtrl, $p<0.01$) can be indicative of increased PEMT activity that relies on the transfer of methylation units.

These results indicate that GLS1-mediated reduction of oxidative stress is associated with restored hepatic phospholipid content (Fig. 5.15).

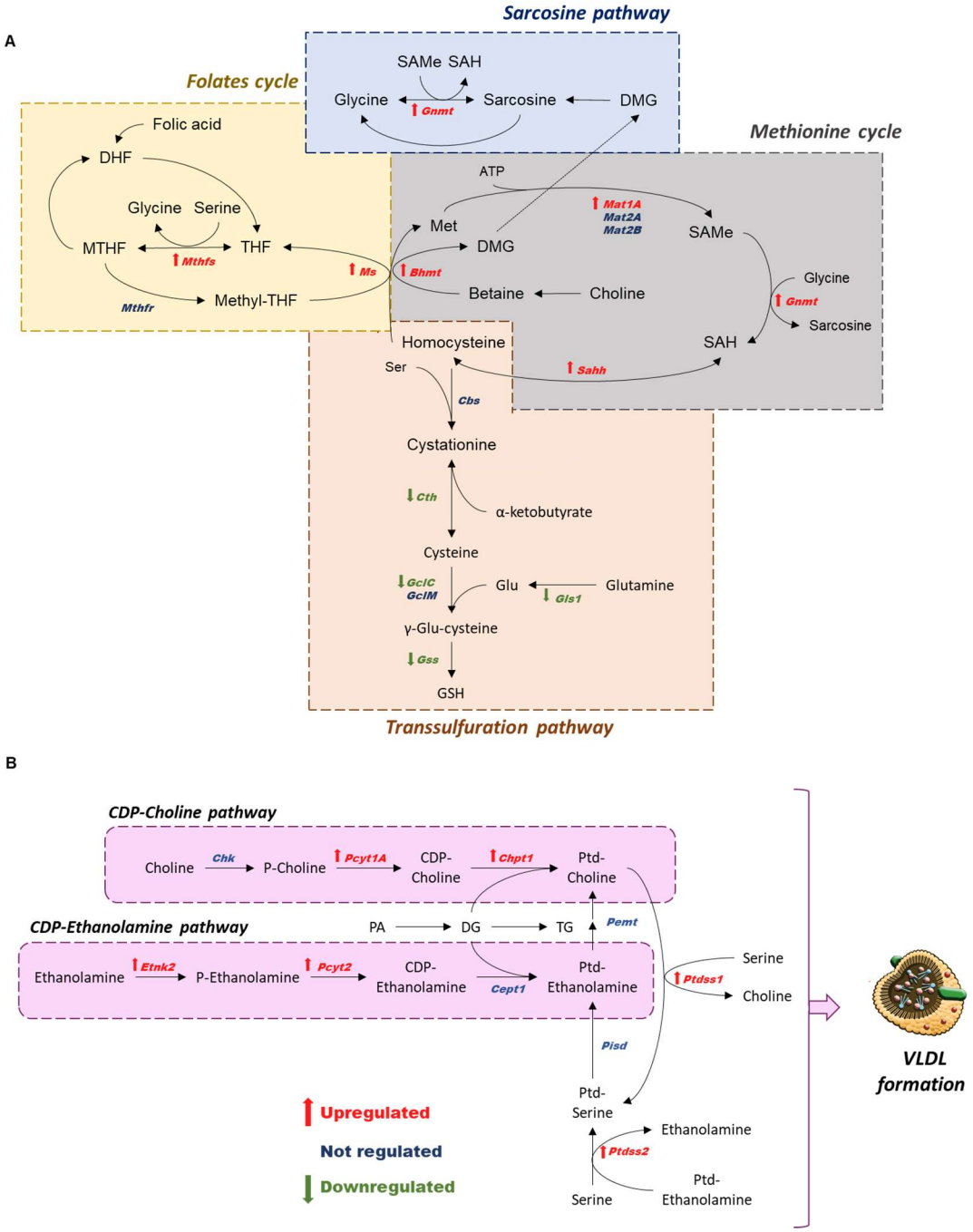


Figure 5.14. GLS1-mediated reduction of oxidative stress is associated with increased phospholipid synthesis and the activation of folate and methionine cycles. A. Differential expression of mRNA levels from genes significantly different involved either in glutathione (GSH) synthesis through the transsulfuration pathway, and the folates and methionine cycles in mice on a 0.1% MCDD and with *Gls1* silencing (si*Gls1*) versus control silencing (siCtrl) for four weeks. (*Bhmt* = betaine-homocysteine S-methyltransferase; *Cbs* = cysthationine-beta synthase; *Cth* = cysthathionine gamma-lyase; *Gclc* = glutamate-cysteine ligase, catalytic subunit; *Gclm* = glutamate-cysteine ligase, modifier subunit; *Gls1* = glutaminase 1; GNMT = glycine N-methyltransferase; GSS = glutathione synthetase; *Mat1a* = methionine adenosyltransferase 1A; *Mat2a* = methionine adenosyltransferase 2A; *Mat2b* = methionine adenosyltransferase 2B; *Ms* = methionine synthetase; *Mthfr* = methylenetetrahydrofolate reductase; *Mthfs* = synthetase; *Sahh* = S-adenosyl-homocysteinase). (DMG = dimethyglycine; MTHF = L-methylfolate; SAMe = S-adenosylmethionine; SAH = S-adenosylhomocysteine; THF = tetrahydrofolate). **B.** Differential expression of mRNA levels from genes significantly different involved in phospholipid biosynthesis in mice on a 0.1%MCDD with si*Gls1* versus siCtrl for four weeks. (*Cept1* = choline/ethanolamine phosphotransferase 1; *Chk* = choline kinase; *Chpt1* = choline phosphotransferase 1; *Etnk2* = ethanolamine kinase 2; *Pcyt1a* = phosphate cytidylyltransferase 1, choline; *Pcyt2* = phosphate cytidylyltransferase 2, ethanolamine; *Pemt* = phosphatidylethanolamine methyltransferase; *Pisd* = phosphatidylserine decarboxylase; *Ptdss1* = phosphatidylserine synthase 1; *Ptdss2* = phosphatidylserine synthase 2). At least n=5 were used for each experimental group. Student´s t-test was used to compare the two groups and significance was set to $p<0.05$ (See also Table 5.2).

Table 5.2. Methionine cycle metabolites (Related to Figure 5.14). Methionine cycle metabolites in mice fed a 0.1%-methionine and choline-deficient diet (0.1%MCDD) compared to a standard chow diet (SC diet). At least n=5 was used for each experimental condition. Data is shown as average ± SEM and Student's t-test was used to compare with the SC diet group. *p<0.05, **p<0.01, ***p<0.001 are shown versus SC diet.

Metabolite (a. u.)	SC diet	0.1%MCDD
S-adenosylmethionine	44.6±11.23	14.7±3.0**
S-adenosylhomocysteine	20.7±2.76	54.6±9,3*
Sarcosine	2.7±0.13	3.0±0.17
Choline	231.6±5.78	177.4±19.04*
Betaine	1315.0±71.49	273.2±27.35***
Dimethylglycine	2.8±0.28	0.7±0.12***

6. CONCLUSIONS

7. CONCLUSIONS

Based on presented Hypothesis and Aims in Chapter 3 and the presented results in Chapter 4, and after discussing results in Chapter 5, it can be concluded that:

1) Glutaminase 1 is overexpressed in NASH clinical and pre-clinical models
    a. NASH patients show a decreased glutamine/glutamate ratio in serum
    b. There is a switch from glutaminase 2 to the isoform 1 in clinical and pre-clinical samples
    c. Ammonia production is buffered by an increased glutamine synthetase expression
2) Targeted silencing of glutaminase 1 ameliorates NASH *in vitro* and *in vivo*
    a. The downregulation of the enzyme leads to reduced oxidative flux in the liver as consequence of a reduced activity of the following pathways:
        i. Fatty acid oxidation is inhibited
        ii. Tricarboxylic acid cycle activity and oxidative phosphorylation are reduced
    b. Decreased oxidative flux reduces reactive oxygen species production
    c. Glutathione synthesis is reduced, leading to a higher serine availability in the liver that promotes phospholipid synthesis
    d. Very-low-density lipoprotein content is enriched, promoting lipid export and reducing hepatic lipid content
3) Cyclin M4 is overexpressed in NASH clinical and pre-clinical models
    a. Cyclin M4 acts a Mg^{2+} extruder in the hepatocyte
4) Targeted silencing of Cyclin M4 ameliorates NASH both *in vitro* and *in vivo* by resolving hepatic lipid accumulation
    a. In pre-clinical NASH fibrosis development is also reverted
    b. Cyclin M4 depletion induces a Mg^{2+} accumulation in the hepatocyte
    c. Oxidative and endoplasmic reticulum stress are reduced in both *in vitro* and *in vivo* NASH models
    d. *In vitro* and *in vivo* microsomal triglyceride transfer protein activity is higher, promoting very-low-density lipoprotein secretion
    e. Mg^{2+} alterations in secreted very-low density lipoprotein might modulate white adipose tissue oxidative activity

8. BIBLIOGRAPHY

8. BIBLIOGRAPHY

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9. SUPPORT

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