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INTERCONNECTIONS AMONG INFLAMMATION, CHRONIC LIVER DISEASE AND HIV: UNDERSTANDING THE HEPATOPROTECTIVE EFFECTS OF THE ANTIRETROVIRAL DRUG RILPIVIRINE

Doctoral Thesis

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Que el trabajo titulado "Interconnections among inflammation, chronic liver disease and HIV: understanding the hepatoprotective effects of the antiretroviral drug rilpivirine", presentado por la Graduada en Farmacia **ALEKSANDRA GRUEVSKA**, ha sido realizado bajo nuestra dirección y asesoramiento.

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Lo que firmamos en Valencia, a 14 de marzo de 2022

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“Don’t be afraid of hard work. Nothing worthwhile comes easily. Don’t let others discourage you or tell you that you can’t do it”

Gertrude Elion (Nobel Laureate)

На моите родители,

за безрезервната поддршка

A Carlos,

por confiar siempre en mí

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ABBREVIATIONS & ACRONYMS

3TC	Lamivudine
ABC	Abacavir
ADIPOR1	Adiponectin Receptor 1
AIDS	Acquired Immunodeficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AMPK	5' Adenosine Monophosphate-Activated Protein Kinase
APS	Ammonium Persulfate
ART	Antiretroviral Therapy
ARV	Antiretroviral
ASK	Apoptosis Signal-Regulating Kinase
AST	Aspartate Transaminase
ATV	Atazanavir
AZT	Zidovudine
Bax	BCL2-Associated X Protein
BCA	Bicinchoninic Acid
BCL2	B-Cell Lymphoma 2
BDL	Bile Duct Ligation
BIC	Bictegravir
BM	Bone Marrow
BMI	Body Mass Index
bp	Base Pair
BSA	Bovine Serum Albumin
CAB	Cabotegravir
CAP	Controlled Attenuation Parameter
cART	Combined Antiretroviral Therapy
CCI	Charlson Comorbidity Index
CCL	CC-Chemokine Ligand
CDC	Centre For Disease Control
CDKN1A	Cyclin-Dependent Kinase Inhibitor
cDNA	Complementary DNA
CL	C-Chemokine Ligand

CLD	Chronic Liver Disease
CMV	Cytomegalovirus
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Motif Chemokine Receptors
DAMPs	Damage-Associated Molecular Pattern Molecules
DCs	Dendritic Cells
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DOR	Doravirine
DRV	Darunavir
DTG	Dolutegravir
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECACC	European Collection of Authenticated Cell Cultures
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EFV	Efavirenz
EGTA	Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ETR	Etravirine
EVG	Elvitegravir
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FDCs	Fixed-Dose Combinations

Flu	Fludarabine
FOXOs	Forkhead Box O Transcription Factors
FPV	Fosamprenavir
FTC	Emtricitabine
FTR	Fostemsavir
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GGT	Gamma-glutamyl Transferase
GO	Gene Ontology
HAART	Highly Active Antiretroviral Therapy
HBSS	Hank's Balanced Salt Solution
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HDL	High Density Lipoprotein
HFD	High Fat Diet
Hg	Hemoglobin
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
HO/BI	Homosexual/Bisexual
HRP	Horseradish Peroxidase
HSCs	Hepatic Stellate Cells
HTSX	Heterosexual
i.p.	Intraperitoneal
IDU	Injection Drug User
IFN	Interferon
IGFBP3	Insulin-Like Growth Factor Binding Protein 3
IKK	IκB Kinase
IL	Interleukin
ILCs	Innate Lymphoid Cells
INR	International Normalized Ratio
INSTIs	Integrase Strand Transfer Inhibitors
IP-10	Interferon Gamma-Induced Protein 10

IQR	Interquartile Range
IκB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor
JAK	Janus Kinase
JNK	c-Jun N-Terminal Kinase
KCs	Kupffer Cells
LAV	Lymphoadenopathy-Associated Virus
LDL	Low Density Lipoprotein
LF	Liver Fibrosis
LPS	Lipopolysaccharides
LPSc	Lipopolysaccharides cocktail
LPV	Lopinavir
LSECs	Liver Sinusoidal Endothelial Cells
MACS	Multicenter AIDS Cohort Study
MAIT	Mucosal-Associated Invariant T
MAPK	Mitogen-Activated Protein Kinases
MCD	Methionine and Choline Deficient Diet
MCP1	Monocyte Chemoattractant Protein 1
MEM	Minimum Essential Medium
miR	micro RNA
MKK	MAPK Kinase
MKKK	MAPK Kinase Kinase
MLK	Mixed Lineage Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MVC	Maraviroc
MW	Mann-Whitney
n	Number of independent experiments
NAD^+	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
ND	Normal Diet

NEAA	Non-Essential Amino Acids
NF-κB	Nuclear Factor-Kappa B
NK	Natural Killer
NLRs	NOD-Like Receptors
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NKT	Natural Killer T
NVP	Nevirapine
p.o	*per os*
PAGE	Polyacrylamide Gel Electrophoresis
PAI1	Plasminogen Activator Inhibitor 1
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PGC1α	Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1 alpha
PI3K	Phosphatidylinositol 3-Kinase
PIs	Protease Inhibitors
PLWH	People Living with HIV
PPRγ	Peroxisome Proliferator-Activated Receptor gamma
PRRs	Pattern Recognition Receptors
qRT-PCR	Quantitative-Real Time-Polymerase Chain Reaction
RAL	Raltegravir
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RPV	Rilpivirine
RT	Room Temperature

RT	Reverse Transcriptase
RTV	Ritonavir
SAPK	Stress-Activated Protein Kinases
SASP	Senescence-Associated Secretory Phenotype
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SIRT	Sirtuin
SOCS	Suppressor of Cytokine Signalling
SQV	Saquinavir
STAT	Signal Transducer and Activator of Transcription
STR	Single Tablet Regimen
T-20	Enfuvirtide
TAF	Tenofovir Alafenamide
TAK1	Transforming Growth Factor Beta-Activated Kinase 1
TBST	Tris-Buffered Saline-Tween
TC	Total Cholesterol
TDF	Tenofovir Disoproxil Fumarate
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TG	Triglycerides
TGFβ	Transforming Growth Factor Beta
TIMP	Tissue Inhibitors of Metalloproteinase
TLR	Toll Like Receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNFα	Tumour Necrosis Factor Alpha
TPV	Tipranavir
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRM	Tissue-Resident Memory
TYK2	Tyrosine Kinase 2
VEGF	Vascular Endothelial Growth Factor
Veh	Vehicle
VL	Viral Load
WB	Western Blot

WHO	World Health Organisation
αSMA	Alpha Smooth Muscle Actin
β-ME	Beta-Mercaptoethanol

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RESUMEN

INTRODUCCIÓN

Durante las últimas décadas, la utilización de la terapia antirretroviral combinada (TARc) para combatir la infección por el virus de la inmunodeficiencia humana (VIH) ha convertido esta enfermedad en una patología crónica. A pesar de que la TARc ha mejorado enormemente la calidad de vida de los pacientes, según diversos estudios epidemiológicos, estos pacientes envejecen antes y sufren de enfermedades relacionadas con la edad como cáncer, osteoporosis, enfermedades metabólicas (diabetes mellitus y enfermedad hepática) y patologías neurodegenerativas aparecen a edades más tempranas que la población no infectada. Los mecanismos responsables de este fenómeno aún no se conocen, sin embargo, los estudios describen un proceso de envejecimiento prematuro en los pacientes. Este proceso está relacionado con un estado inflamatorio persistente y senescencia, acompañado del efecto tóxico crónico tanto de la propia infección vírica como de la terapia antiviral. Así pues, el principal criterio clínico para la elección de unos fármacos antirretrovirales u otros es su seguridad en terapias administradas de por vida.

El envejecimiento está mediado por una tríada de procesos reguladores: senescencia/inflamación/hipercoagulación. Con el conocimiento disponible, está claro que la senescencia y la inflamación están estrechamente conectadas y deben verse como un proceso entrelazado. La senescencia es una detención irreversible del ciclo celular impulsada por diversos estímulos como: acortamiento de los telómeros, estrés genotóxico, estrés oxidativo, estímulos mitógenos y citoquinas inflamatorias. Las células senescentes, cuya proporción aumenta con la edad, secretan numerosos factores, entre ellos citoquinas inflamatorias, quimioquinas y sus reguladores generando así inflamación de bajo grado, considerada como el núcleo del envejecimiento y las enfermedades relacionadas con la edad. Varios estudios han demostrado la asociación de estas moléculas con numerosas patologías humanas, incluidas las enfermedades infecciosas, inflamatorias crónicas y autoinmunes, y la

formación de tumores. Además, estos mediadores están estrechamente interrelacionados. El aumento de la expresión tanto de interleucina (IL)-6 como de la quimiocina interferón-γ proteína inducible 10 (CXCL10) podría estar mediado por IL-18, mientras que factor de necrosis tumoral alfa (TNFα) e IL-6 son los principales contribuyentes a los aumentos del inhibidor del activador del plasminógeno (PAI1). PAI1, que es un objetivo transcripcional de p53, se emplea como marcador de hipercoagulabilidad, un sello distintivo de la edad avanzada y parte del vínculo bien establecido entre la inflamación y la regulación de la fibrinólisis. Otra molécula crucial en esta tríada de procesos reguladores es la proteína de unión al factor de crecimiento similar a la insulina (IGFBP3), un efector posterior de la senescencia mediada por PAI1.

La infección por VIH se ha relacionado con la inflamación crónica y la inmunosenescencia; la remodelación del sistema inmunitario que se produce progresivamente con el tiempo y se asocia con la inflamación crónica y las numerosas consecuencias clínicas del envejecimiento. En personas de edad avanzada, este proceso da como resultado un deterioro funcional de la inmunidad y una capacidad reducida de adaptación al estrés metabólico. En cuanto al VIH, la activación inmune persistente presente en la infección conduce a un posterior agotamiento de las células T. Por lo tanto, varias anomalías detectadas durante ese proceso en pacientes VIH son similares a lo que sucede durante el envejecimiento, incluida la reducción del repertorio de células T, la acumulación de expansiones oligoclonales de células efectoras/de memoria dirigidas hacia agentes infecciosos, la involución del timo y el agotamiento de las células T naïve. Los pacientes infectados por el VIH no tratados suelen presentar niveles plasmáticos elevados de moléculas inflamatorias (IL-1β, IL-6, TNFα, proteína C reactiva (PCR)), y expansión de las células T senescentes. Tras tratamiento con TARc, el estado inflamatorio general se reduce, pero el nivel de inflamación, según lo definido por IL-6, PCR y el dímero D, permanece elevado. Por último, las coinfecciones con otros patógenos son más

comunes en los pacientes con VIH y parecen tener un impacto clínico e inmunológico dañino en la patología del VIH y pueden contribuir al síndrome de "envejecimiento acelerado".

Una de las enfermedades con mayor prevalencia en pacientes con VIH es la enfermedad del hígado graso no alcohólico (EHGNA). EHGNA se ha convertido en una de las enfermedades hepáticas crónicas (EHC) más frecuente en los países desarrollados como resultado de un aumento drástico en las alteraciones metabólicas que se consideran factores de riesgo como la obesidad y la diabetes mellitus. La enfermedad se caracteriza por la acumulación de lípidos intrahepáticos (esteatosis) y puede desarrollar una etapa más agresiva, la esteatohepatitis no alcohólica (EHNA), cuyas principales características son necroinflamación, daño en los hepatocitos y fibrosis hepática. En último término, la enfermedad hepática crónica puede progresar a estadios más severos como cirrosis (en aproximadamente el 10 % de los pacientes con EHNA) y carcinoma hepatocelular (CHC). La progresión a EHNA implica un importante riesgo de mortalidad relacionada con el hígado, que ocurre en hasta un tercio de los individuos afectados por EHGNA, lo que constituye una importante carga social y económica a nivel mundial. Como las opciones de tratamiento para EHGNA, y particularmente para las formas avanzadas de esta enfermedad, son limitadas, cualquier progreso en nuestro conocimiento de este tema beneficiaría especialmente a estos pacientes. Los pacientes con VIH tienen un mayor riesgo de desarrollar EHGNA (30-40%) que la población general (15-30%), y en el caso de los pacientes coinfectados con el virus de la hepatitis C (VHC) las cifras son aún mayores 40-60%. Varios mecanismos patogénicos sugeridos pueden explicar este efecto, incluido el aumento de las comorbilidades metabólicas, el efecto hepatotóxico de la TARc de por vida y la infección crónica por VIH.

Durante la evaluación de los efectos de varios antirretrovirales sobre el desarrollo y progresión de la EHC, se descubrió que la rilpivirina (RPV) muestra acciones

antinflamatorias y antifibróticas sustanciales y, hasta ese momento imprevistas, en diferentes modelos de ratón con EHC, lo que apunta a un mecanismo hepatoprotector directo, independientemente de la etiología de la enfermedad hepática. Es importante destacar que la RPV redujo la inflamación del hígado y la progresión de la fibrosis cuando se administró en combinación con el daño, y también cuando se administró después de la consolidación de la fibrosis, lo que destaca el potencial de este compuesto no solo para aliviar sino también para revertir la fibrosis. Los estudios mecánicos revelaron un efecto proapoptótico selectivo de RPV en células estrelladas (CEH) activadas a través de la interferencia con la señalización de transductor de señal y activador de la transcripción 1 (STAT1), que no se observó en los hepatocitos. Además, se describió una interacción entre las CEH y los hepatocitos a través de la señalización de Janus quinasa activada (JAK)-STAT, que puede conducir a la regeneración del hígado: el tratamiento con RPV restableció el número de hepatocitos proliferativos mediante la activación de STAT3, restableciendo así la homeostasis del parénquima hepático. Sin embargo, esta activación fue secundaria y dependiente de la apoptosis de las CEH mediada por RPV.

Además de la implicación de la ruta de señalización de STAT, en esta tesis también se han estudiado los efectos de RPV sobre el factor de transcripción NF-κB y la cascada de proteínas activadas por mitógenos/estrés (MAPK/SAPK). El papel crucial de NF-κB en el hígado está subrayado por el hecho de que la ablación genética de los reguladores de NF-κB en modelos de ratón conduce a daño hepático espontáneo, fibrosis del hígado y CHC. En respuesta a estímulos dañinos, NF-κB media actividades tanto proinflamatorias como antiapoptóticas en los hepatocitos, para protegerlos de la muerte celular mientras se inician las respuestas inflamatorias. Esta doble función de NF-κB requiere un delicado equilibrio, ya que una activación desregulada puede provocar un aumento de la inflamación y una mayor lesión hepática y fibrogénesis posteriores. Diversos estudios han demostrado que la inhibición de NF-κB en células de Kupffer resulta en una reducción de la fibrosis.

En la misma línea, la activación de NF-κB en las CEH parece promover la fibrosis a través de múltiples mecanismos, incluidos los efectos fibrogénicos y antiapoptóticos directos, y la secreción de quimiocinas reclutadoras de macrófagos.
Las quinasas JNK y p38 se activan por estrés, clasificándose juntas como SAPK, y transducen diversas señales extracelulares que regulan la proliferación, diferenciación, apoptosis y metabolismo celular. Las SAPK activadas se detectan en el hígado de pacientes con obesidad y/o EHGNA y su activación en los hepatocitos juega un papel fundamental en la patogénesis de esta enfermedad. En las CEH, JNK participa en la inflamación y la fibrogénesis.

OBJETIVOS

Los objetivos generales de esta tesis doctoral fueron investigar las interconexiones entre la inflamación, la enfermedad hepática crónica y el VIH, así como explorar los mecanismos moleculares antiinflamatorios responsables de los efectos hepatoprotectores del fármaco antirretroviral rilpivirina.

Los objetivos específicos fueron:

1. Comparar la expresión de marcadores de inflamación y senescencia en células mononucleares de sangre periférica aisladas de pacientes infectados por el VIH bajo terapia antirretroviral combinada y población control no infectada.
2. Estudiar la correlación de estos marcadores en la cohorte mencionada de infectados por el VIH con su historia clínica, análisis bioquímicos y tratamiento farmacológico.
3. Analizar la respuesta inflamatoria en células mononucleares de sangre periférica aisladas de pacientes con enfermedad hepática crónica y tratados con rilpivirina *ex vivo*.

4. Estudiar el efecto de rilpivirina *in vivo*, centrándose en vías relacionadas con la respuesta inflamatoria.

5. Caracterizar los mecanismos moleculares implicados en los efectos antiinflamatorios inducidos por rilpivirina observados *in vivo* utilizando células hepáticas cultivadas.

METODOLOGÍA Y RESULTADOS

Para los dos estudios clínicos realizados en esta tesis, con el fin de analizar la expresión génica y proteica de diferentes marcadores inflamatorios, se aislaron células mononucleares de sangre periférica (PBMCs) de: i) pacientes infectados por el VIH y sus controles no infectados correspondientes, y ii) pacientes con EHC. Los PBMCs son células inmunitarias circulantes que incluyen linfocitos T, B y células NK en un rango de 70 a 90%, monocitos de 10 a 20% y células dendríticas que representan solo 1 a 2%. A menudo se usan en estudios de expresión génica porque se pueden recolectar fácilmente varias veces en cantidades suficientes, y se ha demostrado que sus perfiles de expresión son notablemente similares (80% de concordancia) con otros tipos de células, incluidas, entre otras, las del cerebro, colon, corazón, riñones e hígado. Los PBMCs tienen la característica única de tener niveles detectables de transcritos para una gran proporción de los genes codificados en el genoma humano. Estas células sanguíneas circulantes se encuentran con todas las células del cuerpo humano y brindan una defensa activa contra daños y cualquier cambio en el macro- y microambiente afecta la expresión génica en estas células sanguíneas. Por lo tanto, las células sanguíneas circulantes pueden proporcionar información sobre la salud o enfermedad de cualquier tejido en particular mediante el cambio del patrón de expresión de su transcriptoma. Las PMBC se han utilizado en estudios biológicos y estudios de exploración de biomarcadores y dado que contienen varios tipos importantes de células inmunitarias, pueden experimentar

reacciones inmunitarias similares a las situaciones *in vivo*, incluso en condiciones de cultivo *in vitro*.

Aparte de los PBMCs aisladas de los pacientes con VIH y EHC, en esta tesis se han empleado 3 modelos animales.

- Modelo nutricional de EHGNA, usando dieta rica en grasas (HFD)
- Dos modelos de fibrosis de hígado: CCl_4 y ligadura del conducto biliar (BDL)

Por último, también se emplearon dos líneas celulares. Como modelo de hepatocitos se usaron las células de hepatoma humano Hep3B, mientras que la línea inmortalizada LX-2, se utilizó como modelo de CEH.

Expresión de marcadores de inflamación y senescencia en pacientes con VIH y controles

En el primer estudio, evaluamos la expresión de un conjunto de 17 genes relacionados con la inflamación y la senescencia en PBMC de pacientes con VIH y controles no infectados. En pacientes con VIH, se detectó un aumento estadísticamente significativo en los genes de las moléculas inflamatorias IL-6, IL-18 y CXCL10 mientras que, curiosamente, *SERPINE1*, *IGFBP3*, *TP53* estaban regulados a la baja. Los niveles de ARN mensajero (ARNm) de STAT3 también fueron significativamente más bajos en las personas infectadas por el VIH en comparación con los controles. La disminución de la expresión génica de *TP53* y *SERPINE1* en pacientes con VIH también se corroboró a nivel proteico. En el caso de STAT3, también analizamos el nivel de proteína de su forma activa (fosforilada) y descubrimos que los pacientes con VIH no mostraban una disminución de la expresión de pSTAT3. Por último, observamos una transcripción de *SIRT1*

significativamente disminuida en pacientes con VIH, y se detectó un efecto similar a nivel proteico.

Dado que los pacientes con VIH tienen una relación CD4/CD8 alterada, analizamos si las diferentes proporciones de tipos de células dentro de los PBMCs explicaban las diferencias observadas entre los primeros genes estudiados. Con este objetivo, en primer lugar, evaluamos los niveles de expresión de *CD4* y *CD8A* y se obtuvo el resultado esperado -los pacientes con VIH mostraban una mayor expresión de *CD8A*. Las otras poblaciones celulares principales dentro de los PBMCs, es decir, monocitos y linfocitos B que tienen CD14 y CD19 como marcadores respectivamente, también se compararon y no se encontraron diferencias significativas entre los controles y los pacientes con VIH.

Correlación entre la expresión de diferentes genes en pacientes con VIH y controles no infectados

También se analizó la existencia de correlación entre las expresiones de diferentes genes en la población control y en pacientes con VIH. Los genes inflamatorios demostraron correlación entre ellos y algunos también demostraron correlación con los factores demográficos y bioquímicos. Los genes de senescencia también mostraron un alto grado de correlación y en este caso, 4 genes estaban muy relacionados: *SIRT1*, *TP53*, *IGFBP3* y *STAT3*. Es importante destacar que se detectaron varias diferencias al comparar este análisis entre pacientes y controles. *SERPINE1* no mostró en los pacientes con VIH las correlaciones observadas en la población de control, a excepción de *CDKN1A*. *SERPINE1* y *TP53* parecen estar asociados de una manera distinta. Mientras que en la población control, *SERPINE1* correlacionó negativamente con *TP53*, en pacientes infectados por el VIH esta correlación desapareció. *IGFPB3* no mostró ninguna correlación en la población de control, mientras que se correlacionó positivamente con *TP53*, *STAT3* y *SIRT1* en

pacientes con VIH. En resumen, estos resultados revelan una desregulación en los ejes p53-PAI1 y p53-IGFBP3 en los individuos infectados por el VIH.

Expresión de marcadores de inflamación y senescencia en pacientes con VIH en relación con la terapia antirretroviral

A la exposición crónica a TARc también se le ha atribuido un papel en el desarrollo de patologías relacionadas con la edad. A la luz de esto, analizamos la expresión del panel de 17 genes previamente evaluados, en relación con el tratamiento actual (recibido durante al menos 1 año). Si bien todos los pacientes tenían inhibidores de la transcriptasa inversa análogos de nucleósidos o nucleótidos (ITIAN) como base (emtricitabina/tenofovir, abacavir/lamivudina) con o sin medicamento de refuerzo (cobicistat), diferían en el medicamento adicional en su TARc y, por lo tanto, podrían agruparse en aquellos que recibieron inhibidores de la transcriptasa inversa no nucleósidos (ITINAN) (efavirenz, rilpivirina o etravirina), inhibidores de la proteasa (IP) (ritonavir, atazanavir o darunavir) e inhibidores de transferencia de cadena de la integrasa (INSTI) (raltegravir, dolutegravir o elvitegravir). No se analizaron los pacientes tratados con más de un fármaco de diferentes grupos farmacológicos. Si bien no detectamos ningún patrón particular en los genes inflamatorios (*IL6, IL18* y *CXCL10*), sí que observamos que los ITINAN fueron la clase de fármacos que provocó el mayor efecto sobre los genes relacionados con la senescencia/envejecimiento (*TP53, SERPINE1, IGFBP3*). Es de destacar que los niveles de ARNm de *SIRT1* fueron más bajos en todos los pacientes con VIH, independientemente de su TARc actual.

Tratamiento de células mononucleares de sangre periférica con RPV *ex vivo*

Otro de los objetivos fue explorar los mecanismos moleculares responsables de los efectos hepatoprotectores de RPV observados *in vivo* e investigar las interrelaciones

entre la expresión de marcadores inflamatorios específicos en pacientes con EHC. Por esta razón, reclutamos a 38 pacientes con EHC, de los cuales se aislaron PBMC y se trataron con RPV (1 y 4 μM) *ex vivo* durante 24 h. En primer lugar, estudiamos la expresión génica de las principales citocinas activadoras de las rutas STAT1 y STAT3, como IFNγ, IL-6, IL-22 e IL-10. Además, también se analizó la expresión génica de algunos de los genes diana de STAT1 como *CXCL10, CXCL9,* de su receptor *CXCR3,* además de otras quimiocinas y citocinas proinflamatorias. Los PBMCs tratadas con RPV mostraron una expresión génica incrementada de las principales citocinas activadoras de STAT3 (*IL6, IL22* e *IL10*) y disminuida en aquellas vías mediadas por STAT1 (*CXCL10* y *CXCL9*). También demostramos que la expresión de la proteína pSTAT3 aumentó, mientras que la de pSTAT1 disminuyó significativamente en PBMC tratadas con RPV *ex vivo*. Además, en el presente estudio se demostró que RPV disminuye la expresión de *CCL2*, el gen de una quimiocina inflamatoria importante que desempeña un papel fundamental en el reclutamiento de monocitos, y de *SERPINE1*, cuyas funciones ya se han comentado.

Efectos del tratamiento con RPV en las respuestas inflamatorias en un modelo de ratón de EHC

Para comprender mejor el efecto antinflamatorio que mostró RPV en el estudio *in vivo* previo realizado por nuestro grupo y en los PBMCs tratadas *ex vivo*, se realizó un análisis transcriptómico en las muestras de hígado de ratones tratados con HFD y HFD+RPV en un modelo de EHGNA. El estudio reveló la presencia de una serie de procesos biológicos regulados a la baja en el grupo de los ratones tratados con RPV que estaban asociados con la activación de linfocitos y la migración celular. Por ello se planteó la hipótesis de que las quimiocinas pueden tener un papel potencial en los mecanismos moleculares responsables de los efectos hepatoprotectores de RPV.

Efecto de RPV sobre la expresión de CXCL10 y STAT1 en Hep3B

Varios estudios sugieren que CXCL10 juega un papel importante en el desarrollo de inflamación y fibrosis hepática y que su expresión se correlaciona con la inflamación hepática y la gravedad histológica en la infección crónica por VHC. En el hígado, los hepatocitos son una fuente importante de CXCL10. En células Hep3B mostramos que el tratamiento con RPV disminuyó la expresión de CXCL10, uno de los principales genes diana de STAT1, tanto a nivel de ARNm como sus niveles extracelulares y, además, nuestros resultados señalan que la translocación de STAT1 en el núcleo también se reduce con el tratamiento con RPV en la misma línea celular. Sin embargo, después del silenciamiento transitorio de *STAT1* descubrimos un escenario mucho más complejo. Es decir, al contrario de lo esperado, las células silenciadas para *STAT1* tenían mayores niveles de ARNm de *CXCL10* y el efecto de RPV se conservó en ambos diseños experimentales, con o sin estimulación con IFNγ. Estos hallazgos sugieren que el silenciamiento de *STAT1* solo detuvo la expresión de *CXCL10* estimulada por IFNγ, mientras que otros factores transcripcionales pueden estar involucrados en la regulación de la expresión de CXCL10.

Efecto de RPV sobre la señalización de nuclear factor-kappa B (NF-κB) y la cascada de proteínas activadas por mitógenos (MAPK)

Otro factor de transcripción que podría estar implicado en la regulación de la expresión de CXCL10 es NF-κB. Estudios previos en nuestro grupo revelaron que la activación de la proteína p65 del complejo NF-κB inducida por HFD, CCl_4 y BDL disminuyó después del tratamiento con RPV *in vivo*, reduciendo así la inflamación hepática. Estos hallazgos además estaban en concordancia con el análisis transcriptómico de muestras de hígado completo del modelo de ratón con EHGNA. Para descubrir el mecanismo molecular detrás de estas observaciones, se analizó el efecto de RPV *in vitro* sobre la expresión de p65 nuclear. Se demostró que en condiciones basales hubo una tendencia decreciente en su expresión. Sin embargo,

cuando las células Hep3B se estimularon con TNFα, un inductor clásico de NF-κB, hubo una reducción modesta en la expresión nuclear de p65 con el tratamiento con RPV. Además, los efectos de RPV sobre el complejo NF-κB también se observaron en su regulador IκBα, que cambió su expresión bajo tratamiento. Se demostró que RPV aumentó los niveles de proteína IκBα, mientras que, en las mismas condiciones, la forma fosforilada de IκBα se redujo significativamente, lo que significa que el IκBα está menos degradado y es capaz de inhibir la actividad transcripcional de NF-κB. Esto está en línea con los hallazgos previos que señalan el efecto antinflamatorio de RPV.

El análisis transcriptómico del modelo de ratón con EHGNA también reveló diferencias entre los grupos HFD+Veh y HFD+RPV con respecto a la señalización de MAPK/SAPK, siendo esta otra vía que puede tener relevancia para la EHC y los efectos antinflamatorios del tratamiento con RPV observados *in vivo*. Se realizó un análisis proteico por western blot de la expresión de p38 y JNK en los hígados de los ratones en este modelo. Estos experimentos mostraron que los niveles de las formas fosforiladas de estas quinasas estaban incrementados en los grupos del HFD y que el tratamiento con RPV disminuyó significativamente su expresión proteica, lo cual confirma los resultados observados en el análisis transcriptómico. Además, también exploramos la expresión proteica de p38 y JNK en otros dos modelos murinos de daño hepático (CCl_4 y BDL) y obtuvimos resultados similares.
Con el objetivo de comprender mejor estos efectos observados *in vivo*, se realizaron estudios *in vitro* en células Hep3B y LX-2. Las células Hep3B se pretrataron durante 24 h con un cóctel de lipopolisacáridos (LPS) (LPSc) que contiene LPS, IFNγ y TNFα como estímulo proinflamatorio y se añadió RPV durante otras 24 h. Se observó que en condiciones basales no hubo cambios en los niveles de proteína p38 y JNK1/2, sin embargo, el estímulo proinflamatorio incrementó la expresión de pp38 y JNK1/2 total. RPV redujo este incremento en un modo dependiente de la

concentración en el caso de pp38 y mostró una modesta reducción en la expresión de la proteína pJNK1/2 solo con RPV 4.
Con respecto a las células LX-2, se utilizó TGFβ como estímulo profibrogénico y en co-tratamiento con RPV. Como se esperaba, TGFβ aumentó significativamente el nivel de proteína JNK fosforilada y RPV condujo a una disminución dependiente de la concentración en su expresión. La expresión de p38 fosforilada no aumentó con la estimulación de TGFβ, sin embargo, RPV disminuyó significativamente su activación en condiciones basales.

CONCLUSIONES

1. En comparación con los controles emparejados no infectados, las células mononucleares de sangre periférica de pacientes con VIH muestran una mayor expresión de los genes inflamatorios *IL6, IL18* y *CXCL10,* algunos de los cuales están relacionados con ciertos parámetros demográficos, bioquímicos e inmunológicos y el tratamiento antirretroviral combinado actual.

2. La expresión de los genes *TP53, SERPINE1* e *IGFBP3* asociados a la senescencia en pacientes con VIH está disminuida en comparación con la de individuos sanos de control emparejados. Además, parece que los pacientes que están bajo terapias que contienen inhibidores de la transcriptasa inversa no nucleósidos muestran niveles más bajos de expresión de estos genes en comparación con aquellos que reciben otras clases de fármacos antirretrovirales.

3. En comparación con los controles sanos, las células mononucleares de sangre periférica obtenidas de pacientes con VIH tienen una expresión más baja de SIRT1, una proteína asociada a la longevidad, un efecto

intrínsecamente relacionado con la presencia de la infección por VIH en estos individuos, ya que no se encontró correlación con las características/variables de los pacientes y diferentes regímenes antirretrovirales.

4. La rilpivirina tiene un efecto antiinflamatorio en las células mononucleares de sangre periférica aisladas de pacientes con enfermedad hepática crónica tratadas *ex vivo*, al aumentar la expresión de STAT3 y sus citocinas activadoras y al regular a la baja STAT1 y sus genes diana de una manera dependiente de la concentración.

5. Las concentraciones clínicamente relevantes de rilpivirina disminuyen la expresión de CXCL10, un efecto observado tanto *in vivo* como *in vitro*. Los niveles reducidos de esta quimiocina podrían estar conectados con la señalización de STAT1 y NF-κB como se sugiere en las células Hep3B.

6. La rilpivirina disminuye la activación de p38 y JNK, efecto observado tanto en modelos de ratón como *in vitro*. Estos hallazgos pueden estar relacionados con una función antiapoptótica en los hepatocitos y un efecto antifibrogénico en las células estrelladas hepáticas.

ABSTRACT

The infection with the human immunodeficiency virus (HIV) remains a very important public health problem. Despite the fact that combined antiretroviral therapy (cART) has largely improved patients' quality of life, according to many epidemiological studies, these patients are aging earlier and diseases related with age such as cancer, osteoporosis and metabolic diseases are also developed earlier. The responsible mechanisms for these phenomena are still not known, although many studies have suggested a process of premature aging in these patients related with persistent inflammatory state and senescence.

Senescence is an irreversible cell cycle arrest driven by various stimuli (telomere shortening, genotoxic stress, oxidative stress, mitogen stimuli, and inflammatory cytokines), and senescent cells, whose proportion increases with age, secrete numerous factors, including inflammatory cytokines, chemokines and their regulators, thus generating low-grade inflammation, considered to be at the core of aging and age-related diseases. In particular, chronic liver disease (CLD) is becoming increasingly prominent in HIV patients. Our recent research has revealed a hepatoprotective effect of the antiretroviral rilpivirine (RPV) in various mouse models of chronic liver injury. However, the mechanisms responsible for these effects are still not fully elucidated.

The aims of this doctoral thesis were to investigate the interconnections among inflammation, chronic liver disease and HIV, as well as to explore the molecular mechanisms responsible for the hepatoprotective effects of RPV.

First, compared to uninfected matched controls, peripheral blood mononuclear cells (PBMCs) of HIV patients display increased expression of general inflammatory genes, some of which are related to certain demographical, biochemical and immunological parameters and the current cART regimen; while the expression of senescence-associated genes *TP53*, *SERPINE1* and *IGFBP3* in HIV patients is diminished, especially in those with non-nucleoside reverse transcriptase inhibitors (NNRTIs)-containing therapies. Second, PBMCs obtained from HIV patients have lower expression of SIRT1, an effect intrinsically linked to the presence of the HIV

infection in these individuals as no correlation was found with the patients' characteristics/variables and cART regimens. Finally, several anti-inflammatory effects that may be relevant for the hepatoprotective function of RPV were described. The expression of CXCL10, a potent pro-inflammatory chemokine, is diminished by RPV. Although the exact molecular mechanisms involved still remain unclear, CXCL10 down-regulation seems to be a result of RPV's effect on signal transducer and activator of transcription 1 and nuclear factor-kappa B transcription factors. Furthermore, RPV affects the regulation of mitogen activated protein kinase cascade, another potential contributor of the anti-inflammatory function of this antiretroviral drug in the context of CLD.

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Chapter I

INTRODUCTION

I.1. HIV AND ANTIRETROVIRAL THERAPY

I.1.1. HIV origin, life cycle and infection

Clinicians from Los Angeles and the Centre for Disease Control (CDC) in the United States of America (USA) reported in 1981, the first cases of *Pneumocystis jiroveci* pneumonia and candidiasis among previously healthy young men (1,2). The patients presented a depletion of $CD4^+$ T cells, which compromised their immune system, and unusual infections or/and cancers, such as Kaposi's sarcoma. Later, this clinical symptomatology became known as the Acquired Immunodeficiency Syndrome (AIDS), and in 1983, the pathological agent known as lymphoadenopathy-associated virus (LAV) was isolated from a lymph node biopsy of an AIDS patient and identified as the etiological agent causing this disease (3). In 1984, two research groups confirmed this novel retrovirus as the causative agent of AIDS (4,5) and two years later it was denominated as Human Immunodeficiency Virus (HIV) by the International Committee on the Taxonomy of Viruses (6).

It is believed that HIV first infected humans in the 1920s in Kinshasa (7–9) through multiple zoonotic infections, or cross-species transmissions, with simian immunodeficiency virus (SIV) which infects non-human primates (10). Moreover, other studies revealed two distinct lentiviruses with different SIV ancestors as the cause of AIDS in humans: HIV-1 and HIV-2 (11), which were described as members of reverse transcribing viruses from the *Retroviridae* family, *Orthoretroviridae* subfamily, and *Lentivirus* genus (10). The HIV species are differentiated by their replicative and pathogenic capacity, virus evolution and target of infection. HIV-1, most prevalent type of HIV, is spread all over the world, whereas HIV-2 is mainly restricted to West Africa. HIV-2 is less pathogenic compared to HIV-1 due to their differences in virulence, lower plasma viral load (VL) or better immune control of HIV-2 replication, which could also explain its more restrained distribution (11).

Recent medical and sociological advances have dramatically improved the natural course of the infection worldwide. Nevertheless, current statistics of the global HIV/AIDS burden reflect what continues to be an important pandemic, with 37.7 million infected, of whom 27.5 million are on treatment (12). In 2020, approximately 1.5 million people were infected with HIV and 680 000 lives were lost due to AIDS-related diseases (12). On a more positive note, there is a trend towards fewer new HIV infections, which have diminished by 31% since 2010 (12).

The most frequent route of HIV transmission globally is sexual transmission following exposure to cell-free or cell-associated infectious virus in semen or mucosal surfaces, while less common routes include transmission via injection drug use, exposure of blood and blood products via transfusions, and exposure of the foetus or infant to HIV from an infected mother (13).

On a molecular level, the infection with HIV is possible due to interaction of the envelope glycoproteins present on the surface of the virus (i.e. gp120 and gp41) with the CD4 receptor and the chemokine co-receptors (CXCR4 and CCR5) located on the primary target cells (activated $CD4^+$ T cells). The virus can also infect other cell types, such as resting $CD4^+$ T cells, macrophages, monocytes and dendritic cells (DCs) (14,15). Additionally, infection can occur independently of the CD4 receptor, as reported for astrocytes (16) and renal tubular epithelial cells (17). Inside the $CD4^+$ T cell, the virus releases RNA and enzymes, and uses reverse transcriptase (RT) to convert its genetic material - HIV RNA - into DNA, which enters the nucleus and combines with the host cell's DNA through the activity of viral integrase (Figure I.1.).

The natural course of HIV-1 infection – in the absence of antiretroviral therapy (ART) – consists of three different stages: acute infection, chronic phase and AIDS. Overall, HIV-1 causes a gradual decrease of the $CD4^+$ T cells, a chronic immune activation state and a subsequent exhaustion of the immune system, which leads to the manifestation of AIDS and in most cases, to the death of the patient (18).

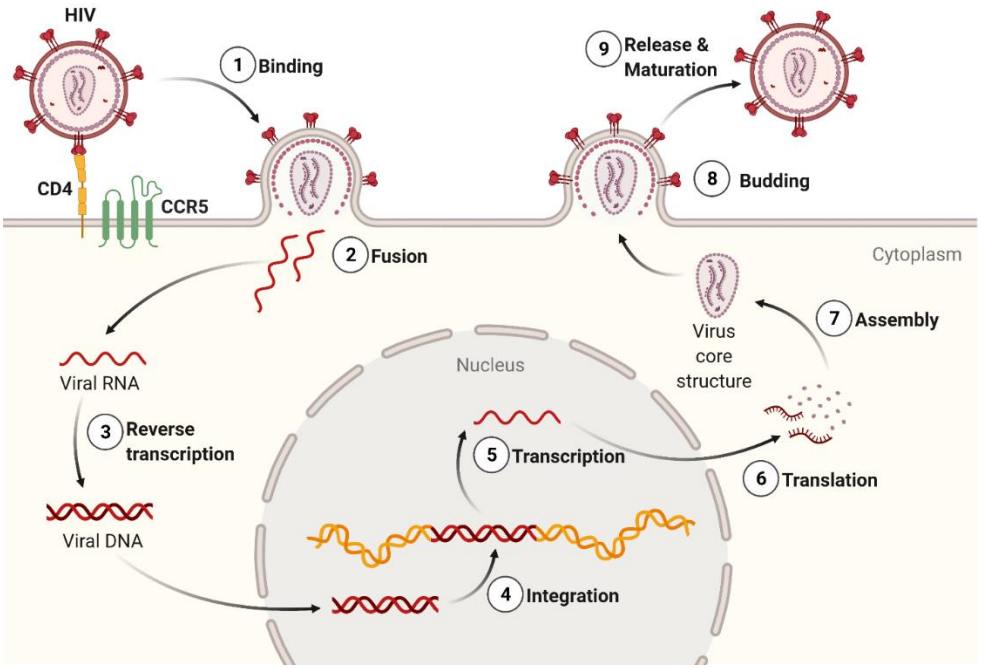


Figure I.1. Schematic representation of the HIV life cycle. *The stages in the process of viral infection are indicated with 1-7. Upon entering the cell, the viral core is released and disassembled in the cytoplasm, and its RNA is converted into DNA by the viral reverse transcriptase. The viral DNA is then transported to the cell nucleus, where it is integrated into the host genome by the viral integrase. The new viral RNA and HIV proteins move to the surface of the cell, where a new, immature HIV forms. Finally, the virus is released from the cell, and the HIV protease cleaves newly synthesized polyproteins to create a mature infectious virus. Created with BioRender.com.*

The acute HIV syndrome is characterized by flu-like symptoms associated with high VL as a result of high levels of HIV-1 replication (10^5-10^7 HIV-1 RNA copies/mL plasma), fever, lymphadenopathy, and rapid loss of $CD4^+$ T cells (18). This phase lasts from 1 to 6 weeks after infection, until anti-HIV-1 antibodies are detectable. Once the immune system develops a HIV-specific response, the viremia declines markedly for several months reaching the viral set point and most patients exhibit a

period of clinical latency that can last for 5-10 years. During this period, patients suffer a progressive depletion of the $CD4^+$ T cells with a deterioration of the immune system, due to the persistent exposure to HIV-1 antigens and the altered cytokine environment. Finally, this leads to the development of an immunodeficiency status, or AIDS, where the individuals are more susceptible to opportunistic infections (13,19). This stage is characterized by $CD4^+$ T-cell counts below 200 cells/μL, a dramatic increase of VL, and a complete failure of the immune system. The lowest $CD4^+$ T-cell count is known as $CD4^+$ nadir and in some patients decreases until 0 cells/μL (18).

I.1.2. Combined antiretroviral therapy

The first advance in anti-HIV therapy was made in 1987, when the US Food and Drug Administration (FDA) approved zidovudine, also known as azidothymidine (AZT) (20). AZT was originally developed as an anti-neoplastic, but it was observed that it was effective against HIV *in vitro* (21) and its use diminished opportunistic infections and mortality in patients with AIDS (22). However, HIV quickly developed viral resistance towards this monotherapy, thus becoming ineffective, which forced the development of newer anti-HIV compounds. The modern era of ART began in 1996 with the introduction of the combined ART (cART), or highly active ARV therapy (HAART), consisting in the combination of three different antivirals. cART effectively suppressed the HIV replication cycle, reduced HIV VL and increased circulating levels of $CD4^+$ T lymphocytes, resulting in a restoration of immune system (14).

The determination of the main steps in HIV life cycle helped to identify the potential biological targets for cART. So far, more than 30 ARV drugs and a monoclonal antibody (ibalizumab) have been approved for clinical use, and they are categorized in the following drug families: HIV fusion inhibitors, HIV entry inhibitors (CCR5 antagonists), attachment and post-attachment inhibitors, nucleoside and nucleotide

reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs) and protease inhibitors (PIs), as shown in Table I.1.

Table I.1. Pharmacological groups, their mechanism of action and the names of the FDA-approved antiretroviral drugs. *Drugs no longer available or recommended for use are not included (23). FDC, fixed-dose combination; gp, glycoprotein.*

DRUG CLASS	MECHANISM OF ACTION	DRUG GENERIC NAME AND ACRONYM	BRAND NAME
Fusion Inhibitors	Interfere with the entry of HIV into cells by inhibiting fusion of viral and cellular membranes	Enfuvirtide (T-20)	Fuzeon
Entry Inhibitors	CCR5 antagonist, prevents the interaction with HIV gp120 and prevents the virus from entering the cell	Maraviroc (MVC)	Selzentry
Attachment Inhibitors	Bind to the gp120 protein on the outer surface of HIV, preventing HIV from entering $CD4^+$ cells	Fostemsavir (FTR)	Rukobia
Post-Attachment Inhibitors	Inhibit the conformational changes in the CD4/gp120 complex that allow binding to CXCR4, thus inhibiting HIV fusion and entry	Ibalizumab-uiyk	Trogarzo

Reverse Transcriptase Inhibitors - Nucleoside Reverse Transcriptase Inhibitors (NRTIs) - Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Inhibit the HIV replication by binding directly to the reverse transcriptase enzyme	Zidovudine (AZT) Lamivudine (3TC) Emtricitabine (FTC) Abacavir (ABC) Tenofovir disoproxil fumarate (TDF) Tenofovir alafenamide (TAF) Nevirapine (NVP) Efavirenz (EFV) Doravirine (DOR) Etravirine (ETR) Rilpivirine (RPV)	Retrovir Epivir Emtriva Ziagen Viread *only FDC Viramune Sustiva Pifeltro Intelence Edurant
Integrase strand transfer inhibitors (INSTIs)	Block the strand transfer step of viral DNA integration into the host genome	Raltegravir (RAL) Dolutegravir (DTG) Elvitegravir (EVG) Cabotegravir (CAB) Bictegravir (BIC)	Isentress Tivicay *only FDC Vocabria *only FDC
Protease Inhibitors (PIs)	Inhibit HIV protease, thereby preventing cleavage of viral proteins and the subsequent generation of individual viral proteins	Ritonavir (RTV) Saquinavir (SQV) Atazanavir (ATV) Fosamprenavir (FPV) Tipranavir (TPV) Darunavir (DRV) Lopinavir (LPV)	Norvir Invirase Reyataz Lexiva Aptivus Prezista *only FDC

cART generally consists of a combination of drugs of the different families (in some cases together with a pharmacokinetic enhancer or booster, of which cobicistat and ritonavir are the most frequently chosen options) (24). The initial ARV regimen for a treatment-naïve patient generally consists of two NRTIs, usually abacavir/lamivudine (ABC/3TC) or either tenofovir alafenamide/emtricitabine (TAF/FTC) or tenofovir disoproxil fumarate/emtricitabine (TDF/FTC), plus a drug from one of three drug classes: an INSTI, an NNRTI, or a boosted PI. As shown in clinical trials and by retrospective evaluation of cohorts of patients in clinical care, this strategy for initial treatment has resulted in suppression of HIV replication and $CD4^+$ count increases in most people living with HIV (PLWH) (25). Additional data

now support the use of the two-drug regimen dolutegravir/lamivudine (DTG/3TC) for initial treatment of some PLWH (26).

cART was designed to limit the probability of drug resistance, to reduce specific drug side effects by diminishing the individual dosage of each compound, and to obtain a synergic effect between drugs that act on different molecular targets (27). Lack of drug adherence is one of the main problems of anti-HIV treatments and an important strategy to improve it is to use fixed-dose combinations (FDCs) which include ARV drugs of multiple classes in one single tablet taken once daily, known as a single tablet regimen (STR). FDCs can be co-administered with another ARV agent to create a multiple-tablet regimen that can be taken once or more times per day, depending on its components. Currently, there are 23 FDCs approved by FDA, of which 19 are STRs (Table I.2.).

I.1.2.1. Side effects of antiretroviral therapy

Toxicity and adverse effects have been described with all ARV drugs, are often enhanced due to drug-drug interactions, and are the most common reason for discontinuing therapy. Overall, gastrointestinal disturbances, hypersensitivity and skin reactions, neuropsychiatric disorders and liver toxicity tend to appear early after initiation of treatment, while other side effects, such as lipid and glucose metabolism abnormalities, kidney alterations, bone metabolic disorders and mitochondrial toxicity, generally develop after chronic exposure (28).

Due to the fact that cART achieves extended virologic suppression of HIV replication but does not eradicate it, patients are exposed to ARV compounds for decades, which can result in accumulated toxicity, one of the three factors that can contribute to a greater risk of chronic diseases in these patients.

Table I.2. FDA-approved combinations of anti-HIV medicines as fixed-dose combinations and their brand names *(29).* *FDC, fixed-dose combination;*

COMBINATION HIV MEDICINES AND FDCs	BRAND NAME
ABC / 3TC	Epzicom
ABC / DTG / 3TC	Triumeq
ABC / 3TC / AZT	Trizivir
ATV / COBI	Evotaz
BIC / FTC / TAF	Biktarvy
CAB / RPV, *extended-release injectable suspension	Cabenuva
DRV / COBI	Prezcobix
DRV / COBI / FTC / TAF	Symtuza
DTG / 3TC	Dovato
DTG / RPV	Juluca
DOR / 3TC / TDF	Delstrigo
EFV / FTC / TDF	Atripla
EFV / 3TC / TDF	Symfi and Symfi Lo
EVG / COBI / FTC / TAF	Genvoya
EVG / COBI / FTC / TDF	Stribild
FTC / RPV / TAF	Odefsey
FTC / RPV / TDF	Complera
FTC / TAF	Descovy
FTC / TDF	Truvada
3TC / TDF	Cimduo
3TC / AZT	Combivir
LPV / RTV	Kaletra

The other two include HIV itself and the effects of chronic viral infection and associated chronic, persistent inflammation; and the host, most notably the population and psychosocial context in which the HIV disease occurs.

For example, rates of hepatitis virus B and C infection, obesity, and tobacco use, all of which raise a patient's risk of suffering a variety of chronic illnesses, are high among HIV-infected patients, often substantially exceeding those in the general

population (30). Hence, treatment guidelines recommend regimens based not only on the anti-viral potency of the drugs, but also on their chronic toxicity.

There is reported evidence that the process of ageing and/or the development of illnesses that are typically associated with advanced age are affected by the virus itself and/or its treatment. On average, a 20-year old initiating cART may have already lost one-third of the expected remaining years of life compared with demographically similar HIV-uninfected persons (31). HIV-infected patients experience higher rates of non-AIDS malignancies, liver pathologies, renal disorders, cardiovascular diseases (CVD), bone disorders and dementia. Factors that drive ongoing morbidity include a combination of immune activation, ageing of the immune system and cART toxicity (32,33). These observations have led to growing concern that HIV-infected persons suffer from accelerated or premature "ageing" (34), a phenomenon that will be discussed in more detail further on.

I.1.2.2. Hepatotoxicity and liver disease in HIV patients on cART

Liver-related mortality ranks as second cause of AIDS-independent death in HIV-infected patients (35). HIV infection is thought to contribute to hepatic injury – a hypothesis supported by many clinical studies – and there is a special emphasis on the association between HIV RNA content and liver fibrosis (LF) development (36,37). Severe liver toxicity can be defined as the presence of an increase in plasma aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) levels over five-fold the normal upper limit (38). Following this criterion, many studies have shown that increased liver transaminase levels occur in 2-18% of patients after several months on cART (39), while a rate of up to 30% has been reported in other clinical trials/works (40,41). HIV infection itself elevates liver transaminase levels, and in some cases liver injury may be exacerbated when HIV patients undergo treatment (42,43). However, it is difficult to ascertain liver toxicity associated with each ARV drug, because they are administered in combination;

moreover, patients are also exposed to other liver toxins and/or often have pre-existing hepatic conditions. Of note, newer ARV drugs are safer for the liver (44,45) but due to the ageing of HIV-infected patients and their longer life expectancy, cART-associated liver toxicity will probably continue to be a major challenge in the treatment of HIV. Besides this, other risk factors have been associated with liver injury in HIV-infected patients.

Chronic hepatitis produced either by hepatitis C (HCV) or hepatitis B (HBV) virus promotes liver damage in HIV patients, as co-infected individuals display greater increases in plasma levels of liver enzymes compared to HIV mono-infected subjects. Other factors, like severe alcohol consumption, drug abuse, drug interactions, age, gender or ethnicity, also contribute to liver injury (46). The mechanisms by which liver injury occurs in HIV patients are numerous, but many converge in a common cellular outcome; i.e., the apoptosis of hepatocytes. Besides the direct effects exerted by the virus itself or HIV-encoded proteins, apoptosis of hepatocytes is clearly aggravated in these patients by the hepatotoxic insults mentioned above and by the cART itself (23).

The severity of hepatic injury in these patients ranges from transient rises in liver enzyme levels to more severe clinical events, such as hepatitis, non-alcoholic fatty liver disease (NAFLD) or steatohepatitis (NASH), non-cirrhotic portal hypertension or even acute liver failure, which can lead to death (47). These clinical events related to liver injury will be described in depth in the section on liver pathophysiology.

I.1.2.3. Rilpivirine

RPV is a second-generation NNRTI, approved by FDA in 2011. The recommended daily dose of RPV is 25 mg and it is primarily metabolized by CYP3A; for this reason, drugs that interact with these enzymes may modify the clearance of RPV (48). RPV is currently indicated by the FDA as part of "alternative initial treatment" or "recommended initial regimens in certain clinical situations" in cART-naïve

patients with HIV-RNA of <100,000 copies/mL and a CD4 count >200 cells/mm^3, as well as in treatment-experienced patients with virological suppression and without NNRTI-resistance mutations (49). In January 2021, FDA approved the first extended release injectable ARV regimen consisting of cabotegravir (CAB) and RPV, as an option to replace the current ARV regimen in adults with HIV (49).

Regarding RPV pharmacokinetics, after oral administration the maximum plasma concentration is generally achieved within 4-5 h, the absolute bioavailability is still unknown and approximately 99.7% of RPV *in vitro* is bound to plasma proteins. Moreover, RPV is primarily metabolised and eliminated by the liver and its plasmatic half-life is approximately 50 h (48). Data from animal models indicate that RPV distributes over the whole body, but higher concentrations are observed in liver, adrenal gland, brown fat and kidney (50).

The safety profile of RPV includes many frequently reported adverse reactions like headache, insomnia, rash and abdominal pain. However, RPV is considered safer than first-generation NNRTIs; for e.g., in the ECHO and THRIVE studies comparing RPV with EFV, the former was associated with a significantly lower incidence of grade 2–4 ALT and AST plasma elevations, with no serious treatment-related hepatic adverse events (51). Multiple studies have shown that RPV is safer than EFV regarding lipid abnormalities, and/or that it improves the lipid status (blood total cholesterol and triglycerides levels) of patients who have switched from EFV to RPV-containing therapies (52–54). A more recent lipidomic study confirmed these findings and identified different alterations in the plasma lipidome of HIV patients on stable cART who switched from EFV to RPV from those who continued on EFV in the cART regimen co-formulated with TDF/FTC. The distinct lipid classes significantly altered in patients who switched to RPV suggest that the use of this drug in the cART regimen could have a beneficial effect on the lipid profile compared to the effects of the EFV regimen. In particular, RPV may prevent excessive lipid droplets accumulation in multiple tissues, which represents a major

risk for CVD and NAFLD-associated atherosclerosis in these patients. In fact, biochemical parameters corroborated with these lipidomic results, suggesting an improved global lipid profile in patients taking RPV (55).

Mechanistic studies of hepatic cell injury demonstrated that exposure of cultured hepatocytes to clinically relevant plasma concentrations of RPV does not affect mitochondrial function or undermine cell viability (44). A recent study by our group suggested that RPV is hepatoprotective in conditions that are unrelated to HIV infection, as it exerts a protective role as an anti-inflammatory, anti-steatotic and anti-fibrotic agent in different mouse models of chronic liver injury (56). Specifically, these data demonstrated that RPV ameliorates LF through selective signal transducer and activator of transcription 1 (STAT1)-dependent induction of apoptosis in hepatic stellate cells (HSCs), while promoting liver regeneration. Furthermore, the results of the retrospective analysis of the Multicenter AIDS Cohort Study (MACS) public data set carried out within the same study were in line with its preclinical results and affirmed that RPV-treated patients with HIV display better liver function than HIV patients treated with RPV-free ARV regimens (56).

I.2. SENESCENCE AND INFLAMMATION IN HIV INFECTION

I.2.1 Ageing and senescence: general aspects

Ageing is a very complex and continuous process that affects almost all organ systems, causing qualitative and quantitative molecular and physiological alterations that lead to increased risk of morbidity and mortality (57). These mechanisms include genetic instability, telomere shortening, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication which includes abnormal endocrine and neuroendocrine signalling, as well as immune dysregulation (57). With age, the immune system undergoes a gradual process of

remodelling dictated by intrinsic events such as thymic involution as well as the response to environmental factors which synergize with age resulting in major alterations in immune function on cellular and systemic level (58).

Cellular senescence is a process that determines cell fate and can be considered a hallmark of ageing (57,59). Normal cells cannot proliferate indefinitely and after multiple rounds of cell division, an irreversible state of replicative senescence occurs known as the "Hayflick limit" (59). Senescence is triggered by developmental signals or different kinds of stress and intrinsic and extrinsic stimuli and, depending on the cell type, intensity and nature of the stress, cells respond by inducing repair, cell death or senescence. Triggers of senescence include progressive telomere shortening, changes in telomeric structure, mitogenic signals, oncogenic activation, radiation, oxidative and genotoxic stress, epigenetic changes, chromatin disorganization, perturbed proteostasis, mitochondrial dysfunction, inflammation, and/or tissue damage signals, irradiation, chemotherapeutic agents and nutrient deprivation (60).

Senescence is now considered to be a highly dynamic, multi-step process, during which the properties of senescent cells continuously evolve and diversify in a context-dependent manner (61). It is associated with multiple molecular changes and distinct phenotypic alterations including a stable and generally irreversible proliferation arrest, during which cells are viable but unresponsive to mitogenic stimuli and are usually resistant to apoptosis (60). Cells also undergo dramatic gene expression changes along with chromatin remodelling and engagement of a persistent DNA damage response (62). Characteristic features of senescent cells include increased lysosomal activity, macromolecular damage and a temporal cascade in the development of the complex senescence-associated secretory phenotype (SASP). Morphological and structural alterations include an enlarged, flattened, multinucleated morphology with enlarged vacuoles, altered composition of the plasma membrane and a remarkable nuclear enlargement (60) (Figure I.2.).

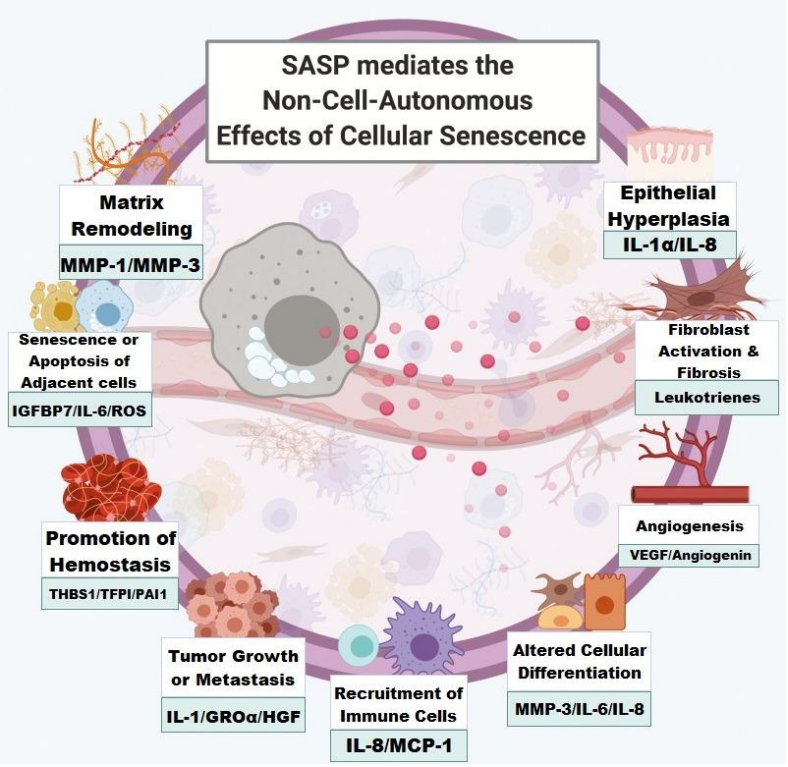


Figure I.2. Senescence-associated secretory phenotype mediates the non-cell-autonomous effects of cellular senescence *(63). The factors secreted as part of SASP mediate a variety of effects including epithelial hyperplasia, tumour growth and extracellular matrix remodelling. These factors also serve to reinforce cellular senescence by inducing senescence programming in adjacent cells. GROα, growth-regulated oncogene-alpha; HGF, hepatocyte growth factor; IGFBP7, insulin-like growth factor-binding protein 7; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; PAI1, plasminogen activator inhibitor 1; ROS, reactive oxygen species; TFPI, tissue factor pathway inhibitor; THBS1, thrombospondin-1; VEGF, vascular endothelial growth factor.*

SASP secretome is comprised of numerous factors, including inflammatory cytokines, chemokines and their regulators (interleukin (IL) 6 and 8; chemokine interferon-γ inducible protein 10 (CXCL10); insulin-like growth factor binding protein 3 (IGFBP3); tumour necrosis factor alpha (TNFα); plasminogen activator

inhibitor 1 (PAI1); transforming growth factor beta (TGFβ)), thus generating low-grade inflammation, considered to be at the core of ageing and age-related diseases (inflammageing) (64). A vast body of evidence has shown the association of these molecules with numerous human disorders including infectious, chronic inflammatory and autoimmune diseases, as well as tumour formation. Moreover, these mediators are closely inter-related. Increased expression of both IL-6 and the chemoattractant CXCL10 could be mediated by IL-18 (65), while TNFα and IL-6 are major contributors to the increases of the serine-protease PAI1 (66,67).

PAI1, which is a transcriptional target of p53, is employed as marker of hypercoagulability, a hallmark of advanced age and part of the well-established link between inflammation and fibrinolysis (68). Another molecule crucial in this regulatory triangle is IGFBP3, a downstream effector of PAI1-mediated senescence (69).

I.2.2. The effect of HIV on senescence and inflammation

Chronic viral infection and hyper-antigenemia, even at low to undetectable levels, cause permanent stress on the immune system (70). Regarding HIV, the persistent immune activation present in the infection leads to a subsequent exhaustion of T cells. Thus, several abnormalities are similar to what happens during ageing (71), including the shrinkage of the T cell repertoire, the accumulation of oligoclonal expansions (many T cells express an identical T-cell receptor) of memory/effector cells directed toward infectious agents, the involution of the thymus and the exhaustion of naïve T cells (72). Accelerated immune ageing in HIV patients is certainly multifactorial. Treated HIV patients need to rebuild their T cell repertoire at an age when thymic function has been already compromised by the thymic involution and has been impaired by the HIV infection itself. T cell diversity and subset distribution may be compromised even if normal $CD4^+$ T lymphocyte numbers are being reached after cART treatment (73). Homeostatic proliferation to

rebuild the repertoire will not only increase replicative stress to the system, but will also be associated with proliferation-induced differentiation even in the absence of antigen recognition (73). The innate immune system in younger HIV-infected patients shows similar changes as in healthy (HIV-uninfected) elderly individuals which include a higher baseline activation of DCs and monocytes/macrophages, modified toll like receptor (TLR) functions, and decreased effector functions such as phagocytosis and cytotoxicity. Moreover, DCs and monocytes/macrophages (mainly monocytes) may serve as a peripheral reservoir for the virus and convey their dissemination to various organs such as the brain (74,75). Taken together, these changes in the immune system due to a chronic HIV infection are linked to premature ageing of the immune response and to the appearance of chronic inflammatory diseases, such as CVD seen normally in elderly individuals. It has been hypothesized that non-immune organs sustain collateral damage due to the systemic inflammatory state either via circulating cytokines or from more direct insults when activation and senescent T cells are recruited to these organs at the site of infection or other injuries (73).

Additional problem that affects the progression of HIV infection is the rapid depletion of gut resident memory $CD4^+$ T cells, followed by the deterioration of the intestinal epithelium and an increased translocation of microbial products (76). The release in the circulation of bacterial products provokes a persistent, systemic activation of the innate immune system, that triggers and maintains inflammation (77).

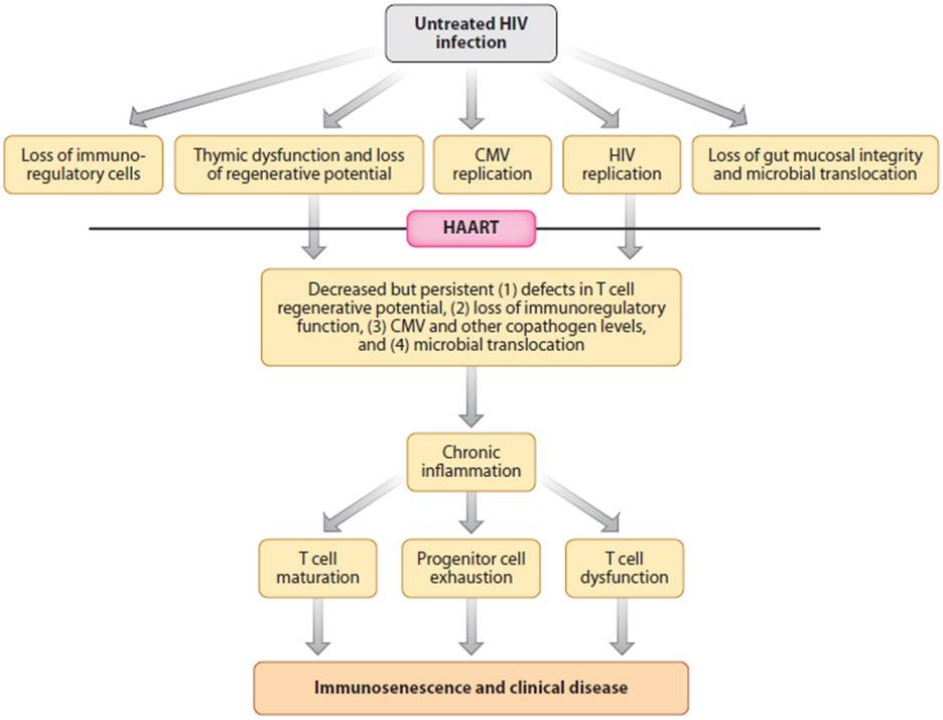


Figure I.3. The effect of HIV infection and its treatment on the development of chronic inflammation and immunosenescence *(34). CMV, cytomegalovirus; HAART, highly active antiretroviral therapy.*

The increasing amount of data supporting this concept has profoundly changed the view of HIV infection, which is also considered a disease with a major inflammatory component. As a consequence, successfully treated patients can experience several non-AIDS related complications that can be considered a direct or indirect consequence of chronic inflammation (78). Untreated HIV infected patients usually exhibit high plasma levels of inflammation molecules (IL-1β, IL-6, TNFα, C-reactive protein (CRP)), reduced vaccine efficacy and expansion of senescent T cells (79). With cART, the general inflammatory status is reduced, but the level of inflammation - as defined by IL-6, CRP, D-dimer (a fibrin degradation product that reflects activation of the coagulation cascade) remains elevated (Figure I.3.).

Co-pathogens are more common in PLWH, and they appear to have a harmful immunologic and clinical impact in HIV disease and may contribute to the "accelerated ageing" syndrome (34).

I.2.3. p53

p53 is a well-known tumour suppressor protein, which is activated by a plethora of stress signals. When activated, p53 regulates various cellular pathways that determine the cell fate, such as cell cycle arrest, differentiation, senescence and apoptosis (80). It is regarded as one of the most powerful tumour suppressor genes, due to its ability to halt cell proliferation and induce apoptosis, and its activity is pivotal to successful traditional chemotherapy, as many DNA-damage-inducing drugs target tumours via p53-mediated apoptosis (81). p53 is clearly involved in cancer, but the existence of p53 in short-living organisms that do not develop cancers, such as flies and worms, suggests that tumour suppression is not its only and, probably, original function. Indeed, recent studies have shown that p53 influences development, reproduction, metabolism and longevity (81). Evidence indicates that p53 has the ability to modulate the production and secretion of SASP by the senescent cells (82). SASP composition might be dependent on various factors, including tissue, cell type, and time point. Moreover, various signalling pathways, such as NF-κB, mTOR and p38 may promote the transcription of SASP factors (83). In contrast, p53 was shown to interfere with the induction of SASP, in particular *via* inhibition of NF-κB signalling (84). Recently a SASP transcriptional program that is specifically promoted by p53 activity was identified. This senescence phenotype was induced by cyclin-dependent kinases (CDK) 4/6 inhibitors, that are relevant as anti-cancer drugs. Namely, CDK4/6i-induced senescent cells do not acquire pro-tumorigenic and detrimental properties, but retain the ability to promote paracrine senescence and undergo clearance (85). Furthermore, p53 can have a role in restraining cell migration and angiogenesis, regulating immune responses and

mediating non-cell-autonomous interactions between stressed cells and surrounding tissue (86–89). Although each of these activities would limit tumour development, they are likely to have additional roles in health and disease (90).

Viral infection evokes cellular stress and infected cells harbour stabilized and activated p53. Consequently, to gain successful replication and spreading, viruses use different strategies to handle their host cells and manipulate p53's guardian role (80). Different viral families have evolved protein binding motifs and other mechanisms to either hijack or interfere with p53 functions. For instance, in order to spread, some viruses cause p53-mediated cell death of the host cell by different mechanisms, such as cell lysis and various types of programmed cell death (e.g., apoptosis), while other viruses stimulate host cell proliferation by attenuating p53 function, thus promoting the development of cancer (80).

Various HIV-1 proteins have been shown to interact with the host's p53 during the process of infection, to either attenuate or activate it, depending on the infection stage. In early phase, HIV-1 proteins such as Nef and HIV-1 long terminal repeat were suggested to inactivate p53. Nef interacts directly with p53, destabilizes it, and diminishes its transcriptional activity that results in apoptosis (91). In later stages, other HIV-1 proteins as Vif were suggested to induce p53 activity, by affecting p53 stability and blocking the mouse double minute 2 - p53 interactions thus mediating a G2 arrest, which positively supports HIV-1 replication (92).

I.2.4. SIRT1

Sirtuins belong to the III class of deacetylase enzymes and the mammalian sirtuin (SIRT) family is comprised of seven proteins (SIRT1-7). They share a nicotinamide adenine dinucleotide (NAD^+)-binding catalytic domain and may act specifically on different substrates depending on the biological processes in which they are involved (93,94).

Table I.3. Localization, substrates, functions and enzyme activities of different sirtuins. *Modified from Villalba and Alcaín, 2012 (94). FOXO, forkhead box O transcription factor; MnSOD, manganese superoxide dismutase; NAD, nicotinamide adenine dinucleotide; NF-κB, nuclear factor-kappa B; PARP1, poly (adenosine diphosphate-ribose) polymerase 1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1 alpha; PPAR-γ, peroxisome proliferator-activated receptor-gamma; rRNA, ribosomal RNA; TNFα, tumour necrosis factor alpha; UCP2, uncoupling protein 2.*

Sirtuin	Subcellular localization	Tissue expression	Main substrates	Functions	Enzyme activity
SIRT1	Nuclear and cytosolic	Brain, skeletal muscle, heart, kidney and uterus	Histones H1, H3 and H4 Transcription factors p53, FOXO family, Ku70, p300, NF-κB, PGC1α, PPARγ, UCP2 Acetyl-CoA synthetase 1	Cell survival, lifespan regulation, metabolism regulation, inflammation, oxidative stress response	NAD^+-dependent deacetylase
SIRT2	Cytosolic and nuclear	Brain	α-tubulin	Cell cycle regulation, nervous system development	NAD^+-dependent deacetylase
SIRT3	Mitochondrial, nuclear and cytosolic	Brain, heart, liver, kidney and brown adipose tissue	Acetyl-CoA synthetase 2 Isocitrate dehydrogenase 2 Ku70, FOXO 3a, MnSOD, Mitochondrial ribosomal protein L10 Long-chain acyl-CoA dehydrogenase 3-Hydroxy-3-Methylglutaryl CoA synthase 2 Succinate dehydrogenase NADH: quinone oxidoreductase	Regulation of mitochondrial metabolism	NAD^+-dependent deacetylase

SIRT4	Mitochondrial	Pancreatic β-cells, brain, liver, kidney and heart	Glutamate dehydrogenase	Regulation of mitochondrial metabolism,	ADP-ribosyl transferase
SIRT5	Mitochondrial	Brain, testis, heart muscle and lymphoblast	Cytochrome *c* Carbamoylphosphate synthetase 1	Apoptosis	NAD^+-dependent deacetylase Desuccinylase Demalonylase
SIRT6	Nuclear, associated to heterochromatin	Muscle, brain, heart, ovary and bone cells (absent in bone marrow)	Histone H3 TNFα PARP1	Genome stability, DNA repair	NAD^+-dependent deacetylase ADP-ribosyl transferase
SIRT7	Nucleolar	Peripheral blood cells, $CD33^+$ myeloid bone marrow precursor cells	RNA polymerase I p53	Regulation of rRNA transcription and cell cycle	NAD^+-dependent deacetylase

Acetylation and deacetylation are important mechanisms to regulate post-translationally the activity of proteins and SIRTs have great significance in the transduction pathways originating from energy sensing. Their ability to regulate systems that control the redox environment has the potential to help counteract oxidative damage that is associated with common diseases of ageing and that contributes to ageing itself (94,95).

SIRT1-7 appear to be important in suppression of such common ageing-related diseases as CVD, type 2 diabetes (DM) and dementia (95). Mammalian SIRT proteins have been found in a variety of subcellular locations, however several of them have been shown to shuttle between different cellular compartments (93–95) (Table I.3.).

SIRT1 was the first SIRT family member to be discovered and is still the most studied. Studies suggest that SIRT1 could regulate ageing mainly via the

deacetylation of target proteins, such as p53 (96), forkhead box O transcription factors (FOXOs) (97), nuclear factor-kappa B (NF-κB) (98), and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (99), which play key roles in oxidative stress and inflammation. SIRT1 also acts in oxidative stress resistance, autophagy, inflammation, metabolism and apoptosis due to its ubiquitous distribution and its role in mitochondrial function (100). With ageing, the expression of SIRT1 in animal tissues decreases, which dysregulates both the oxidative metabolism and the anti-oxidant defence system leading to oxidative stress (101).

SIRTs can potentially affect viral factors and influence the course of HIV infection (102). An example of this type of interaction is the deacetylation of the Tat HIV viral protein by SIRT1, which affects the efficiency of transcription of the integrated viral genetic material (102). Viral Tat protein influences chromatin modifying factors in a manner that is favourable to viral replication, including: recruiting histone acetyltransferase 1, increasing the level of Tat acetylation and the efficiency of the RNA transcription process, contributing to an increment in the VL (103). Tat also directly affects SIRT1, binding to its catalytic domain and thereby blocking its deacetylase activity relative to NF-κB, p53, p21 or BCL2-associated X protein (Bax), promoting the presence of HIV-specific status of chronic immune activation (102,104). By inducing a state of chronic immune activation due to HIV infection, the production of transcription factors and pro-inflammatory interleukins is intensified, which allows the integration of the newly formed viral DNA into the host genome (103).

I.3. LIVER PATHOPHYSIOLOGY

I.3.1. Liver physiology and functional structure

The liver is a central organ involved in a wide range of essential functions of the organism, such as metabolism, glycogen storage, drug detoxification, production of

serum proteins and bile secretion (105,106). Given that it is the largest and most toxin-exposed organ in the body, it is not surprising that liver pathological conditions, including LF, cirrhosis, hepatitis and hepatocellular carcinoma (HCC), are major contributors to morbidity and mortality (107).

There are two major liver cell populations: parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells, including liver sinusoidal endothelial cells (LSECs), HSCs and Kupffer cells (KCs), the liver's resident form of macrophages (105). HSCs are located in the space of Disse between the hepatocyte cords and sinusoids, and KCs are also found in sinusoids. Hepatocytes secrete bile salts into the bile canaliculi that lead to the bile ducts lined by the cholangiocytes (Figure I.4.) (107). Besides KCs, the liver contains many immune cells such as hepatic DCs, monocyte-derived macrophages (MDMs) and neutrophils. Additionally, innate lymphoid cells, including natural killer (NK) cells, natural killer T (NKT) cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells, as well as other T cells and B cells are also part of the liver immune cell population (108).

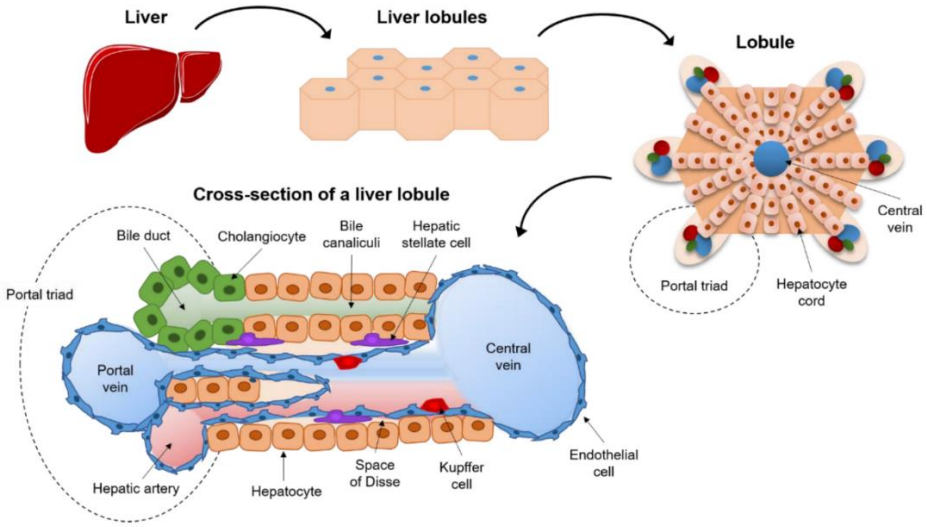


Figure I.4. Functional structure of the liver and cell types *(109). The liver is composed of many lobules. Each lobule, the functional unit of the liver, is composed of a central vein, from which hepatocyte cords, single-cell sheets of hepatocytes, radiate towards portal triads*

consisting of a portal vein, hepatic artery and biliary duct. Hepatocytes cords are separated by sinusoids that carry blood from the portal triads to the central vein. Within each lobule are a number of sinusoids, which are discontinuous vessels built of specialized fenestrated endothelial cells of the liver.

I.3.2. Main hepatic cells involved in liver health and injury

I.3.2.1. Hepatocytes

Hepatocytes constitute approximately 60% of the liver by cell number and approximately 80% by mass. They are cellular factories, equipped with abundant mitochondria and endoplasmic reticulum (ER) that produce large quantities of albumin, clotting factors and other serum proteins. Among other metabolic functions, hepatocytes secrete bile acids, the amphipathic products of cholesterol metabolism, and perform glutamine synthesis, urea formation and gluconeogenesis (106). In addition to these synthetic activities, hepatocytes play a major role in detoxification due to their large number of detoxifying enzymes (collectively termed the P450 enzymes) that recognize and modify a wide variety of chemicals, allowing for their elimination in bile or urine.

Prolonged liver injury of different etiologies such as viral hepatitis, drug toxicity, alcohol abuse and metabolic or cholestatic diseases can result in hepatic inflammation and this determines the clinical outcome - that is, resolution or progression to chronic liver disease (CLD) and LF (110). In many cases, this injury occurs due to direct damage of hepatocytes, resulting in hepatocyte death, which is considered the main driver of liver inflammation and fibrogenic response. Hepatocyte death has been categorized as inflammatory (necrosis, necroptosis and pyroptosis) and non-inflammatory (apoptosis) depending on its capacity to induce disruption of cellular integrity with a subsequent release of the cellular contents to the extracellular space and promotion of inflammatory response (111). However, it

is now known that apoptosis is associated with disease development and often leads to cellular leakage and inflammation (112).

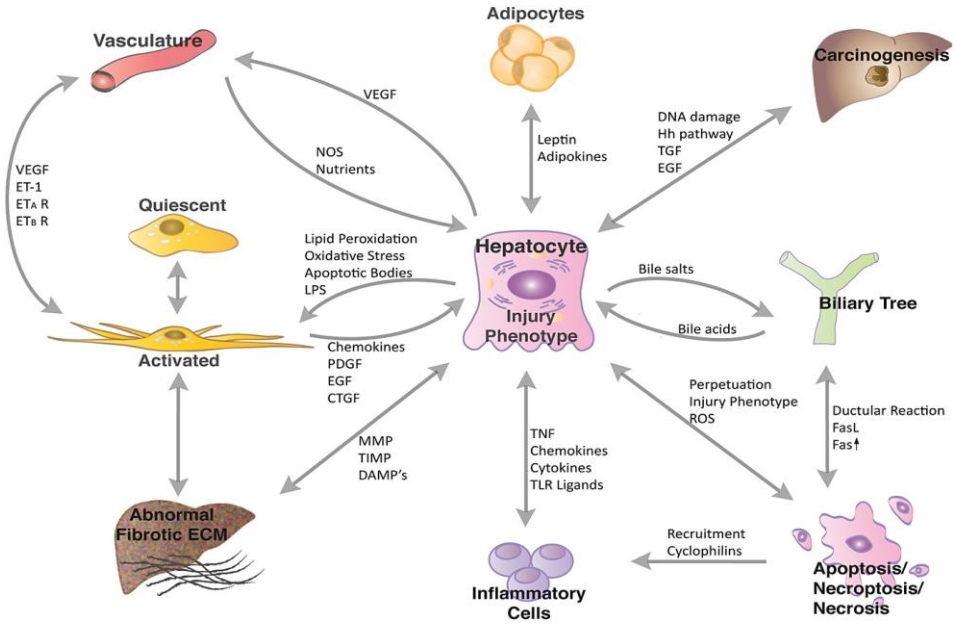


Figure I.5. Schematic overview of the interactions between hepatocytes and other cell types during liver injury *(113).* *CTGF, connective tissue growth factor; DAMP, damage-associated molecular pattern; ECM, extracellular matrix; EGF, epidermal growth factor; ET, endothelin; FasL, Fas ligand; Hh, hedgehog; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NOS, nitric oxide synthetase; ROS, reactive oxygen species; TIMP, tissue inhibitor of MMP; TNF, tumour necrosis factor; TLR, toll-like receptor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor.*

During injury, hepatocytes release damage-associated molecular pattern molecules (DAMPs) which act on multiple cellular subsets (including adipocytes, endothelial cells, HSCs, and infiltrating immune cells), resulting in progressive liver injury (110,113) (Figure I.5.).

DAMPs, a group of chemically heterogeneous molecules also known as alarmins due to their capability to alert the immune system, include, among other molecules,

reactive oxygen species (ROS), pro-inflammatory signals, proliferation-associated cytokines, and the activation of repair pathways (113). These danger signals are recognized by pattern recognition receptors (PRRs), that include TLRs and NOD-like receptors (NLRs), and non-PRRs, such as purinergic receptors, located on both non-immune and immune cells (114). ROS released by stressed and dying hepatocytes are major stimuli for ongoing injury via the activation of HSCs and immune cells. Furthermore, chronic ROS release leads to the development of LF and, if continued, eventually cirrhosis. ROS induce TGFβ and fibromodulin expression in hepatocytes, thereby promoting LF by inducing the migration and proliferation of HSCs (115,113).

Mounting evidence suggests that hepatocytes contribute more than just ROS and cell debris to activate pro-fibrotic responses from intrahepatic immune cells and HSCs. Studies focusing on chronic liver damage suggest that hepatocytes can directly alter extracellular matrix (ECM), as well as display an injury phenotype, which synergistically interacts with fibrogenesis mediators in surrounding cells, particularly HSCs (116). Moreover, hepatocytes can actively recruit inflammatory cells to the site of injury via the expression of chemokines such as CXCL10, CCL20, and CCL2 (117,118). The injury of hepatocytes and their complex interaction with surrounding cells suggests a pivotal role of the hepatocyte in the development of CLD.

I.3.2.2. Hepatic stellate cells

HSCs represent approximately 10% of all resident liver cells. Their location, together with their dendritic cytoplasmic processes, facilitates a direct contact between them, as well as with hepatocytes, endothelial cells, and KCs (119,120). The most characteristic hallmark of HSCs is the storage of retinyl esters in cytoplasmic lipid droplets and this property can be used for their easy isolation by density gradient centrifugation (119). In a healthy liver, HSCs have the following functions: retinoid

and drug metabolism and detoxification, balanced ECM production, preservation of hepatocyte mass, vasoregulation and normal hepatic development (120). During homeostasis, HSCs maintain a non-proliferative, quiescent phenotype which changes following liver injury or with their culture *in vitro*, thus HSCs become activated, transdifferentiating from vitamin A-storing cells to myofibroblasts, which are proliferative, contractile, inflammatory and chemotactic, and are characterized by enhanced ECM production (Figure I.6.) (121).

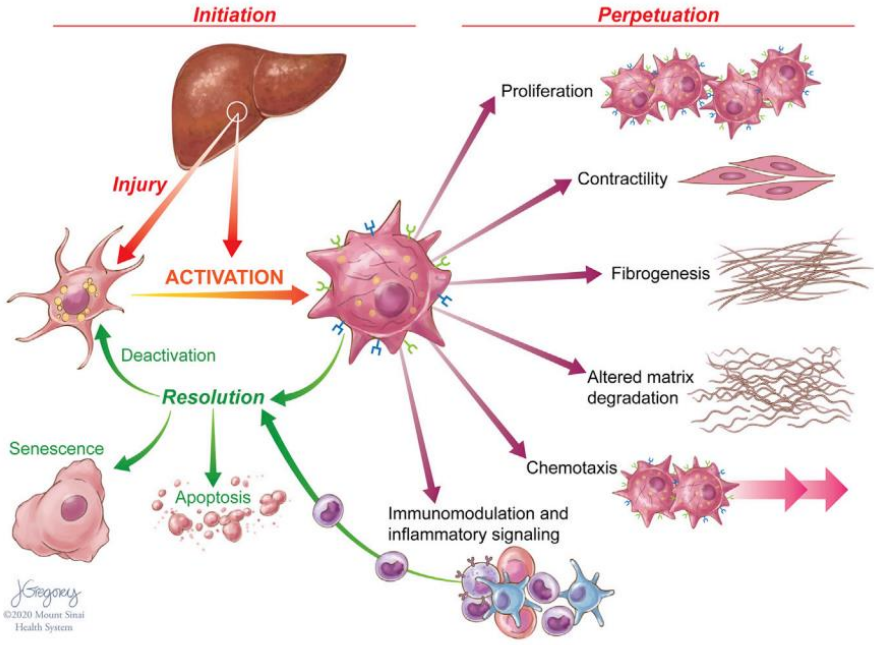


Figure I.6. Pathways of HSCs activation and fibrosis/liver injury resolution *(122). Following liver injury, hepatic stellate cells undergo activation including initiation and perpetuation phases. The major phenotypic changes after activation are shown. Once injury resolves, the fate of these cells is uncertain but may include apoptosis, senescence and/or deactivation to a quiescent cell phenotype.*

HSCs activation is triggered by numerous signals that serve as markers of cellular injury, including pro-inflammatory cytokines produced by infiltrating immune cells,

hepatocyte apoptotic bodies, endothelial cell-mediated growth factor activation, and increased ROS burden. HSCs respond to injury by potentiating the formation of paracrine and autocrine loops including fibrogenic signals such as TGFβ1 and connective tissue growth factor (CTGF). Upon activation, HSCs increase their sensitivity to platelet-derived growth factor (PDGF), due to the upregulated expression of the PDGF receptor, which results in enhanced proliferation and an increase of the total number of HSCs with a pro-fibrogenic phenotype (120). Another hallmark of activated HSCs is the elevated production of ECM, especially collagen I and III, alpha smooth muscle actin (αSMA), vimentin and desmin among others (123,124). Once injury resolves, HSCs undergo apoptosis, progressive senescence and/or deactivation to a quiescent, cell phenotype (123,122).

I.3.2.3. Macrophages

The liver represents 80% of all macrophages in the body, and they are divided in two main types depending on their origin: resident liver macrophages or KCs, which are positioned in liver sinusoids, and macrophages derived from infiltrating blood monocytes (110). During homeostasis, KCs express different scavenger receptors on their surface, molecules that allow the KCs to detect, bind and internalize pathogens and associated molecules (125). These receptors can activate KCs, which leads to production of cytokines and chemokines and allows them to function as an immune "guard", alerting other components of the immune system like lymphocytes and neutrophils to the presence of harmful pathogens (125). Apart from their role in hepatic homeostasis, liver macrophages also hold remarkable functional diversity, being implicated in progression and regression of acute and chronic inflammation and LF (110). Their pleiotropic actions and diverse roles in liver diseases are due to their high plasticity, as a response to environmental signals arising from parenchymal and immune cells (126).

During liver damage, hepatic macrophages expand dramatically and release additional inflammatory mediators enhancing the inflammatory response, which may increase hepatocyte cell death (127). Importantly, at the later stages of inflammation or once the liver insult has been eliminated, macrophages may undergo a functional switch towards pro-resolving or anti-inflammatory phenotype in order to repair damaged tissue (128,129).

I.3.3. Liver immunology

Liver homeostasis is regulated through an intense intercellular interplay between parenchymal and non-parenchymal cells. Hepatocytes and cholangiocytes, as well as LSECs, KCs and HSCs act as primary sensors for pathogens and pathogen-associated molecular patterns (PAMPs) from enterohepatic microcirculation. Moreover, LSECs and KCs are capable of antigen presentation, as well as of cytokine and chemokine production. Thus, it is easy to understand the importance of the liver to initiate and shape immune responses (105,110).

While the role of HSCs and macrophages in fibrogenesis is well described, the role of adaptive immune cells is less defined. Nevertheless, many *in vivo* studies underline the importance of lymphocytes in fibrogenesis showing that inhibition of lymphocyte recruitment in the liver induces a decrease in the fibrogenic responses (130). Lymphocytes are classically viewed as circulating immune cells that continuously traffic in blood, lymph nodes, and other secondary lymphoid tissues. Naïve T cells patrol the organs and are stimulated by antigen-presenting cells that migrate from the site of infection to lymph nodes in infectious conditions. Then, T cells undergo clonal expansion and differentiation into effector cells that can migrate to the sites of inflammation (131). Unlike other organs, inflammation in the liver is mainly sterile (non-infectious), excluding parasite-induced LF. Therefore, “innate-like” mechanisms (Th17, NK cells, NKT cells, MAIT cells, innate lymphoid cells) are major contributors to liver inflammation (132).

Chronic liver injury results in the production of pro-inflammatory mediators and the infiltration of leukocytes, including lymphocytes, in the sub-endothelial space. The recruitment of lymphocytes from the circulation is further triggered by interactions with endothelial cells, a process regulated by several chemokines. Importantly, lymphocytes can interact with ECM components and endothelial cells though cell surface integrins, which contribute to cell activation and differentiation as well as fibrogenic responses (133,134). After migration through the endothelium by a complex mechanism, lymphocytes are recruited at the injury site by chemoattractant molecules (135). It has been shown that CXCR3 activation by its ligands, including CXCL9, CXCL10, and CXCL11 produced by HSCs and endothelial cells, promotes lymphocytes trans-endothelial migration (136).

Apart from the recruiting lymphocytes, the liver contains multiple types of tissue-resident lymphocytes, including $CD8^{+}$TRM (tissue-resident memory) cells, NK cells, innate lymphoid cells (ILCs), γδT cells, and NKT cells. These liver-resident lymphocytes serve as the first line of defence in response to infection and non-infectious insults, and are important in immunosurveillance, immune regulation, and the maintenance of liver homeostasis (131).

I.3.3.1. Chemokines in liver disease

Chemokines are a family of low-molecular weight (7-13 kDa) heparin biding molecules which were originally recognized for structural similarities and for their main function as chemotactic cytokines. They have an important role in regulating and promoting the migration of the immune cells with relative specificity for leukocyte subpopulations (135,137). Overall, the chemokine family consists of at least 50 ligands and 19 receptors, which are expressed by multiple cells in both healthy and diseased liver (137). Chemokines segregate into four main families, which are defined by the number of amino acids (denoted as X) between cysteine residues at the N-terminus of the molecule. The largest groups of chemokines belong

to the CC-chemokine ligand (CCL) family and CXC-chemokine ligand (CXCL) family, while the remaining chemokines are part of either the C-chemokine ligand (CL) family or the CX3 C-chemokine ligand (CX3CL) family (135,138). The chemokine system has received considerable attention for its involvement in a number of biologic processes. Although members of this family have been initially recognized for their ability to recruit leukocytes to sites of injury, it has been shown that this system participates in the regulation of a wide number of conditions (139).

Chemokine receptors (seven transmembrane G-protein coupled receptors), are typically expressed in various leukocyte subsets and other immune cells. When chemokines bind to the corresponding receptors, the downstream intracellular cascades, such as phosphatidylinositol 3-kinase (PI3K), small Rho guanosine triphosphatase, and cellular calcium influx pathways, are activated, which increases the avidity of leukocyte integrins that promote leukocyte's interactions with adhesion molecules expressed on LSECs, thereby enabling leukocyte adhesion and subsequent extravasation (140). This interaction locally immobilizes and retains chemokines, creating a concentration gradient that allows a coordinated migration of leukocytes toward inflammatory sites (141).

In the context of liver diseases, leukocytes, hepatocytes, HSCs, endothelial cells, and vascular smooth muscle cells are capable of producing chemokines and their respective receptors are typically expressed in various leukocyte subsets (142). In addition, infiltrated leukocytes produce inflammatory cytokines that further stimulate hepatic immune cells including KCs and recruited circulating monocytes, HSCs, and hepatocytes, which enhances liver inflammation. Given that inflammation is a critical factor for the transition from simple steatosis to NASH and LF, the chemokine system play a prominent role in the pathogenesis of NAFLD (142); indeed, activation of the chemokine system has been reported in the presence of chronic inflammation and LF (139) (Figure I.7.).

Chemokines not only act as chemo-attractants, but also have potential to directly stimulate hepatocytes and HSCs to enhance their biological activities, such as lipid accumulation and collagen production, respectively (139).

A recent systematic review and network meta-analysis of some previous studies on the relationship between chemokines and NAFLD (143) revealed that there was a significant increase in the concentrations of CCL2 and CXCL8 in the NAFLD group when compared with the control group; and the concentrations of CCL3, CCL4, CCL20, CXCL8, and CXCL10 were increased in the NASH group when compared with the control group (143). This study pointed out the vital role of these chemokines in the development of the pathologies mentioned before and their utility as potential therapeutic targets for CLD (143).

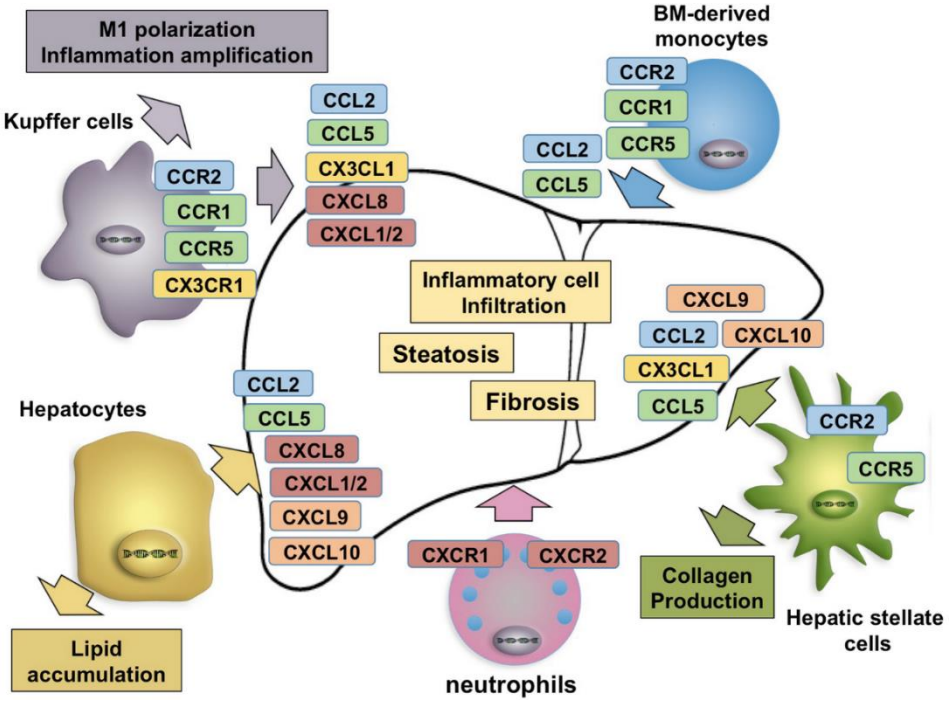


Figure I.7. Chemokines involved in the pathogenesis of non-alcoholic fatty liver disease (142). *BM, bone marrow.*

I.3.4. Chronic liver disease

I.3.4.1. General features: when liver injury becomes chronic

Upon acute liver injury, the immune system rapidly activates a controlled and self-limiting fibrotic and wound-healing response which functions as a repair mechanism. Through this response, ECM generated by active HSCs protect hepatocytes against several toxic stimuli and renders the liver more resistant to subsequent acute injuries. In this context, the liver can restore its complete mass and original architecture in a relatively short interval, even when a large fraction of the organ has been destroyed (144,145). However, the fibrotic response becomes pathological when dysregulated and excessive scarring occurs in response to persistent injury. Liver tissue undergoes an impaired regenerative response characterized by altered inflammatory infiltrate and chronic wound-healing response, including necrosis and/or apoptosis of parenchymal cells and their massive replacement by ECM, distortion of the liver vascular architecture and angiogenesis leading to organ dysfunction (144,145).

Once injury becomes chronic, perpetuation of wound-healing and inflammatory responses is mediated by several positive feedback loops that involve autocrine and paracrine effects of cytokines and growth factors, cell-cell and cell-matrix interactions. In this context, a wide range of pro-inflammatory and pro-fibrogenic mediators including IL-6, TNF, IL-1β, TGFβ, as well as ROS and other DAMPs, derived from recruited immune cells and damaged hepatocytes, rapidly activate HSCs and attract resident KCs. Both KCs and HSCs act as phagocytes of parenchymal-derived debris, and as enhancers of immune cells recruitment, thus contributing to pro-inflammatory and pro-fibrogenic paracrine signalling. At the same time, all these signals act in an autocrine manner and increase HSCs activation (mainly by TGFβ) and proliferation (PDGF) in a harmful loop (146). Additionally, the microenvironment in the damaged area leads to the inhibition of all the cellular mechanisms aimed to tissue repair. For instance, the macrophage capacity to degrade

newly synthetized scar matrix through the secretion of matrix metalloproteinase (MMP) is inhibited by the concurrent production of tissue inhibitor of MMP (TIMP) by active myofibroblasts and inflammatory-recruited macrophages, which results in increased ECM deposition and scar accumulation (145,147).

I.3.4.2. Non-alcoholic fatty liver disease

NAFLD is the most common cause of CLD in Western countries that is predicted to become also the most frequent indication for liver transplantation by 2030. It affects up to 25% of the entire adult population, and up to 70–90% of individuals with obesity or DM (148). According to the 2017 Global Burden of Disease Study, HCC and cirrhosis accounted for most of the liver-related deaths and, although chronic viral hepatitis remains the most common cause of these deaths worldwide, this study show that NAFLD is the most rapidly growing contributor to liver-related mortality and morbidity (149). Characterized by excessive fat accumulation without a definite liver damaging factor, NAFLD is an acquired metabolic stress liver injury closely related to obesity, insulin resistance, and genetic susceptibility (150).

The natural history of NAFLD mirrors the natural history of the metabolic syndrome and encompasses a histological spectrum ranging from simple steatosis (where an excess of fat accumulation can be observed in hepatocytes), with more benign course, to NASH, where besides fat accumulation, parenchymal inflammation and lipotoxicity drive to hepatocyte death. Depending on the duration and severity of this inflammation, NASH can undergo different degrees of LF, sometimes leading to clinically relevant LF, cirrhosis and eventually HCC (151) (Figure I.8.). It is estimated that about 44-64% of all patients with NAFLD develop NASH over 3-7 years and the risk of progression of NASH into cirrhosis is believed to be between 21% and 26% for a period of 8 years (152). The incidence of HCC has been increasing in parallel with the rise in NAFLD and its subsets and patients with NAFLD and cirrhosis have the highest risk to develop HCC (153).

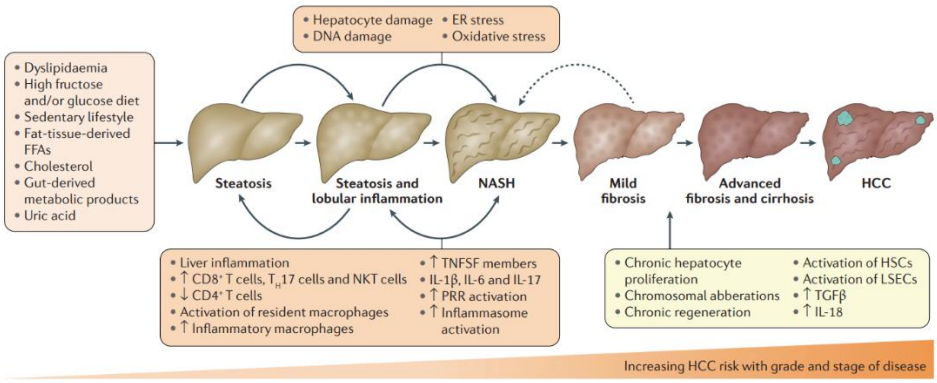


Figure I.8. Natural history of chronic liver disease *(154). Environmental and gut-derived factors in non-alcoholic steatohepatitis pathogenesis and the increased risk of liver tumorigenesis are represented. ER, endoplasmic reticulum; FFAs, free fatty acids; IL, interleukin; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; LSECs, liver sinusoidal endothelial cells; NKT, natural killer T; PRR, pattern recognition receptors; TGFβ, transforming growth factor beta; TNFSF, tumour necrosis factor super family.*

The underlying mechanisms for the development and progression of NAFLD are complex and multifactorial. According to the "multiple-hit hypothesis" (155), insulin resistance and gut microbiota are key factors that determine NAFLD progression. During insulin resistance, adipose tissue becomes dysfunctional and produces an altered pattern of adipokines and cytokines, which leads to inflammation and increased lipid accumulation in the liver through increased lipogenesis and impaired lipolysis. This altered interplay between adipose tissue and liver triggers lipotoxicity in hepatocytes and subsequent mitochondrial dysfunction and oxidative and ER stress (156).

The contribution of the gut-liver axis in this process is also very important, since altered gut microbiota leads to further production of fatty acids in the bowel, increased small bowel permeability and enhanced fatty acid and PAMPs absorption to the portal circulation, effects that, in summary, contribute to greater fat storage and inflammatory activation in the liver (150). Genetic predisposition and epigenetic

modifications have been also described as important features to determine the interindividual susceptibility to NAFLD development, affecting different parameters such as hepatocyte fat content, inflammatory microenvironment, HSCs activation and/or evolution to more severe phases of the disease (156).

I.3.4.3. Liver fibrosis and cirrhosis

Although fibrogenesis and LF may represent an attempt to limit the consequences of chronic liver injury within the so-called "chronic wound healing reaction", they represent key features of the progression of any form of CLD towards cirrhosis and hepatic failure. Moreover, liver fibrogenesis and CLD progression are linked to persisting pathological angiogenesis, with angiogenesis contributing to the expansion of tissue fibrosis (157). Most of the key features related to fibrogenesis progression have been already explained in the corresponding chapter describing HSCs (I.3.2.2.). On its hand, the angiogenic process leads to shunting of the portal and arterial blood supply directly into the hepatic outflow (central veins), compromising exchange between hepatic sinusoids and the adjacent liver parenchyma. In cirrhosis, the space of Disse is filled with scar tissue and LSECs lose their characteristic fenestrations and develop basal membrane in a process known as sinusoidal capillarization (158). Histologically, cirrhosis is characterised by vascularized fibrotic septa that link portal tracts with each other and with central veins, resulting in hepatocyte islands surrounded by fibrotic septa that are devoid of a central vein (159). The major clinical consequences of cirrhosis are liver dysfunction, portal hypertension, ascites and HCC development (160). Finally, it is important to note that a large body of clinical evidence, especially from patients effectively treated for chronic HBV or HCV infections, suggest that regression from LF occurs if the underlying liver injury is resolved or successfully treated (161). Even in the context of cirrhosis the natural history can be modified and patient outcome improved, as it has been demonstrated in patients with HCV-induced

cirrhosis who had been treated for the infection, thus presented a significant decrease in collagen content and cirrhosis regression, which led to the reduction of the clinical incidence of hard end-points like HCC and death (162).

I.4. SIGNALLING PATHWAYS INVOLVED IN HEPATIC INFLAMMATION AND FIBROSIS RELEVANT FOR THIS THESIS

I.4.1. JAK-STAT

The cytokine-activated Janus kinase (JAK)-STAT signalling pathway is one of the major pleiotropic cascades that transduce a multitude of signals for development and homeostasis. Its activation is involved in cell proliferation, differentiation, cell migration, apoptosis and many other cellular processes that regulate haematopoiesis, or adipogenesis (163). Furthermore, JAK-STAT signalling has a central role in the control of mammalian immune responses, thus its dysregulation is largely associated with various autoimmune disorders and pathologies where the functionality of the immune system is critical, like inflammatory and wound-healing disorders and cancer (164). Cytokines have essential roles in the control of immune responses and their biological functions mainly depend on their intrinsic capacity to activate or repress gene expression. JAK-STAT pathways have been described as common signalling pathways used by many cytokines and, given their intrinsic pleiotropic nature, the same JAK-STAT pathway can be activated in different cell types in response to the same or different cytokines triggering diverse cellular responses, often with opposite biological functions (165).

Intracellular activation of this pathway occurs when ligand binding induces the multimerization of receptor subunits: the binding of a cytokine to its cell-surface receptor results in receptor dimerization and the subsequent activation of JAK tyrosine kinases, which are constitutively associated with the receptor. Specific

tyrosine residues on the receptor are then phosphorylated by activated JAK and serve as docking sites for a family of latent STAT cytoplasmic transcription factors. STATs are phosphorylated by JAK, dimerize, subsequently leave the receptor and translocate to the nucleus, where they activate gene transcription. Therefore, the JAK-STAT cascade provides a fast and direct mechanism to translate an extracellular signal into a transcriptional response (164,165). The mammalian JAK family has four members: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) which selectively bind different receptor chains. In addition, there are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Different cytokines have the propensity to activate a particular STAT; however, unselective interaction between cytokines and any given STAT may occur (164).

In order to completely understand the function of JAK-STAT signalling in liver pathophysiology, it is essential to clarify which effect is induced by each STAT in each cell subtype. The biological effects induced by all the different STATs in hepatocytes and non-parenchymal cells in the liver are displayed in Table I.4. The most important STATs involved in CLD are STAT1 and STAT3, as they are able to induce different responses in liver parenchymal, non-parenchymal and immune cells.

I.4.1.1. Opposing roles of STAT1 and STAT3 in liver injury and repair

The activation of STAT1 and STAT3 in different liver cell types generally plays opposing roles in many aspects of liver pathophysiology (166–168). Interestingly, hepatic STAT1 and STAT3 not only functionally antagonize each other, but they also mutually inhibit each other's activation through the induction of suppressor of cytokine signalling (SOCS), SOCS1 and SOCS3, respectively, that inhibit both STAT1 and STAT3 activation (168,169). The involvement of STAT1 and STAT3 signalling pathways in induction of damage or in liver regeneration directly depends

on the cytokine microenvironment, which determines the type and the number of cells that overexpress STAT1 or STAT3 at a certain point of time (166).

Table I.4. Major activators and functions of STAT proteins in hepatocytes and non-parenchymal cells. *Modified from Gao et al., 2012 (166).*

STATs	Cell types	Major Activators	Major Functions
STAT1	Hepatocytes	IFN (α, β, γ, λ)	Promotes anti-viral response Promotes anti-tumour response Induces hepatocyte apoptosis Inhibits hepatocyte proliferation Promotes liver inflammation
	HSCs	IFNα, IFNβ, IFNγ	Inhibits fibrogenesis
	KCs	IFNγ	Promotes inflammatory response
	NK cells	IFNα, IFNβ, IFNγ	Promotes anti-viral, anti-tumour, and anti-fibrotic responses
STAT2	Hepatocytes	IFN (α, β, λ)	Promotes anti-viral response
	Non-parenchymal	Unknown	Unknown
STAT3	Hepatocytes	IL-6, IL-6 family cytokines, IL-22	Promotes hepatocyte survival and proliferation Ameliorates steatosis Induces expression of innate immunity proteins Promotes liver tumour cell survival and growth
	KCs	IL-10	Inhibits liver inflammation
	HSCs	Leptin, IL-6	Promotes fibrogenesis
	LSECs	Unknown	Inhibits liver inflammation
STAT4	Hepatocytes	Unknown	Unknown
	NK and NKT	IL-12, IFNα/β	Promotes liver inflammation

STAT5	Hepatocytes	Growth hormone	Upregulates metabolism enzymes, growth factors, etc. Promotes hepatocyte survival and proliferation Ameliorates steatosis
	HSCs	leptin	Promotes fibrogenesis
STAT6	Hepatocytes	IL-4, IL-13	Promotes liver injury and inflammation in T cell hepatitis Protects against ischemia/reperfusion and drug-induced liver injury
	HSCs	IL-4, IL-13	Promotes fibrogenesis

Although it is a simple and reduced explanation, to clearly understand this hepatic regulation it can be summarized that STAT3 activation in hepatic cells induces pro-survival and pro-proliferative pathways and increases the resistance of these cells to damage (167,170,171). Conversely, STAT1 activation triggers anti-proliferative pathways and induces cell cycle arrest, senescence and/or apoptosis (166,172). Consequently, STAT1 activation in hepatocytes is a pro-apoptotic signal that leads to increased liver damage, whereas STAT3 activation is a pro-survival and proliferative signal that protects against hepatocyte death (166). In contrast, STAT1 activation in HSCs inhibits their proliferation and induces cell cycle arrest and apoptosis, that limits the fibrogenic response (173) whilst, STAT3 activation in these same cells has a pro-fibrotic effect since it increases their activation and proliferation and enhances the ECM deposition in the scarring tissue (Figure I.9.) (171).

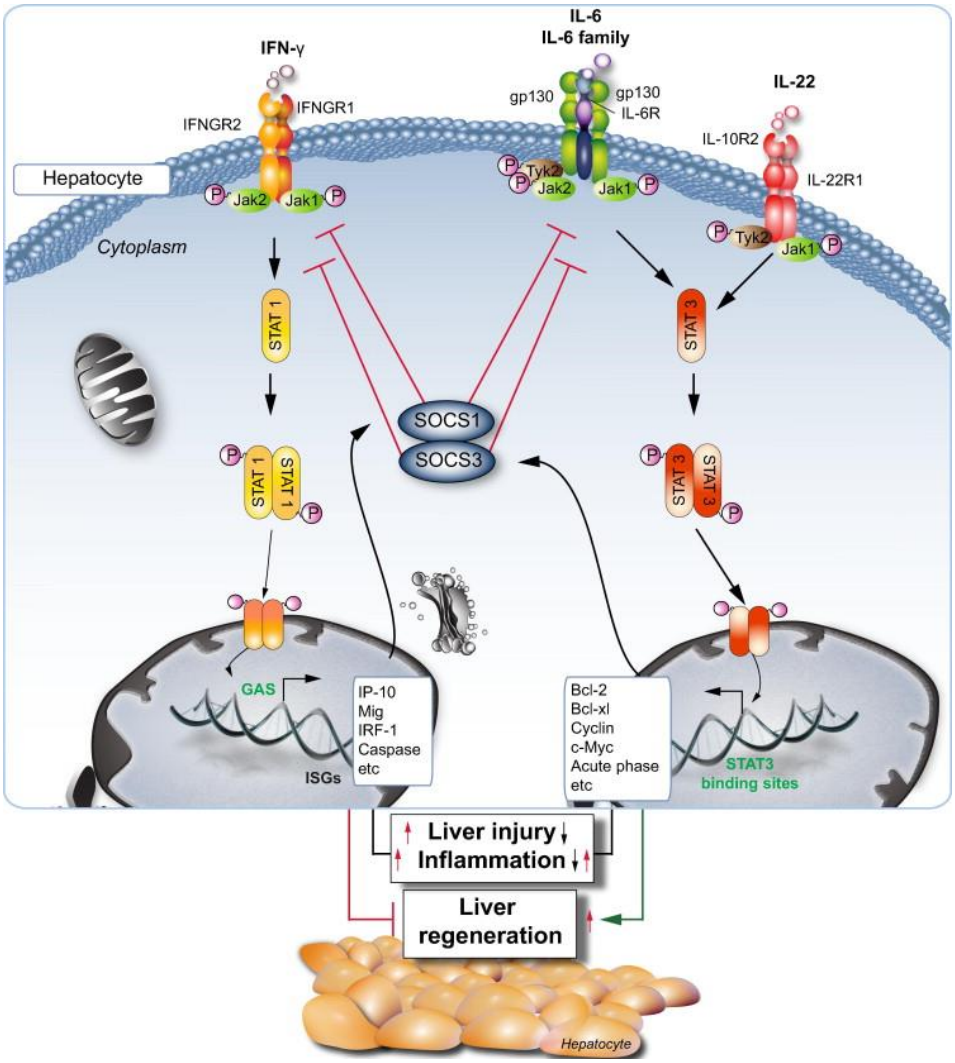


Figure I.9. Involvement of hepatocyte STAT1 and STAT3 balance in liver injury and regeneration *(166).* *Bcl, B-cell lymphoma; Bcl-xl, B-cell lymphoma-extra large; GAS, gamma interferon activation site; gp, glycoprotein; IFNγ, interferon gamma; IFNGR, interferon gamma receptor; IL, interleukin; IP-10, interferon gamma-induced protein 10; IRF-1, interferon regulatory factor 1; ISGs, interferon stimulated genes; Jak, Janus activated kinase; Mig, monokine induced by gamma; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; Tyk, tyrosine kinase.*

I.4.2. Nuclear factor-kappa B

NF-κB family of transcription factors are considered master regulators of pro-inflammatory signalling pathways in response to both infection and injury, as they induce an array of pro-inflammatory genes encoding for chemokines, cytokines, and adhesion molecules, together with the control of the expression of genes involved in cell cycle, cell growth, apoptosis, wound-healing, immune responses, development and acute phase responses (174). NF-κB protein family is comprised of the following members: RelA (p65), RelB, c-Rel, p50 (p105 precursor), p52 (p100 precursor), and Relish. They all share a highly conserved DNA-binding and dimerization domain termed Rel homology region (RHR) that enables them to homo- or heterodimerize (175).

As uncontrolled inflammation is deleterious, NF-κB activity is tightly regulated at several levels. In unstimulated cells, NF-κB is held in the cytoplasm in an inactive state, bound to an inhibitory protein of the IκB family (such as IκBα), which masks its nuclear localization site (176). NF-κB signalling is activated by a large number of extracellular stimuli, such as LPS, inflammatory cytokines (e.g. IL-1, TNFα), pathogens and stress conditions (e.g. oxidative stress, ER dysfunction), which are recognized by different receptors, including TLR, IL-1R and TNFR1 (176). The signalling cascades culminate in the phosphorylation of IκB by a complex consisting of two different catalytic IκB kinase (IKK) subunits and the protein NF-κB essential modulator. This phosphorylation marks IκB for subsequent degradation through the proteasome in ubiquitin-dependent pathway, and releases NF-κB from the inhibitory complex, allowing nuclear entry, where it binds to their target sequences and activates gene transcription (177).

The crucial role of NF-κB in the liver is underlined by the fact that genetic ablation of NF-κB regulators in mouse models leads to spontaneous liver injury, LF and HCC (178,179). NF-κB is a pivotal regulator involved in virtually every liver disease, including NAFLD, viral hepatitis and drug induced liver injury, due to its implication

in a wide range of functions in hepatocytes, KCs and HSCs (176). In response to damaging stimuli, NF-κB mediates both pro-inflammatory and anti-apoptotic activities in hepatocytes, in order to protect them from cell death while inflammatory responses are initiated. This dual role of NF-κB requires a delicate balance, since a dysregulated activation may lead to increased inflammation and subsequent enhanced liver injury and fibrogenesis (176). Inhibition of NF-κB in KCs resulted in reduced LF (180) and the activation of NF-κB in HSCs appears to promote LF via multiple mechanisms, including direct fibrogenic and anti-apoptotic effects, and the secretion of macrophage-recruiting chemokines (181–183).

I.4.3. Stress-activated protein kinases family

Mitogen-activated protein kinases (MAPK) transduce a variety of extracellular signals that are implicated in regulating cell proliferation, differentiation, apoptosis and metabolism (184,185). In mammals, three major groups of MAPK have been identified that are activated by a protein kinase cascade formed by a MKKK (MAPK kinase kinase), a MKK (MAPK kinase), and a terminal MAPK, ensuring signal amplification and fidelity (185). There are three major MAPK groups: extracellular signal-regulated kinases (ERK1/2), mainly activated by mitogens, and c-Jun N-terminal kinases (JNK) and p38 that are activated by stress and classified together as stress-activated protein kinases (SAPK) (184).

The stressors that activate SAPKs in humans and mice include ultraviolet radiation, genotoxic damage, hypoxia, oxidative stress, ER stress, long-chain saturated fatty acids, ceramides, inflammatory cytokines, hyperosmotic conditions, microbial bioproducts, hormones, cold and muscle contraction (186). In order to transduce the extracellular signals, each SAPK transforms specific stimuli into cellular responses via phosphorylation of several downstream substrates, which include transcription factors, cytoskeletal proteins, other kinases and proteins of the translational machinery (186).

The JNK kinase family includes three isoforms: JNK1, JNK2 and JNK3. While the JNK1 and JNK2 isoforms are commonly found in tissues and organs, JNK3 expression is restricted only to the brain, heart and testis (187). The p38 family has four isoforms: p38α, p38β, p38γ and p38δ (188). p38 family members are widely expressed, with p38α and p38β being expressed ubiquitously. Abundant expression of p38γ in skeletal muscle initially suggested that its expression was particular to this tissue, however, subsequent research revealed that it is expressed in humans and animals in almost all cells and tissues (including macrophages, liver, heart and adipose tissue). By contrast, p38δ is mainly found in secretory cells (such as the β-cells of pancreas), the small intestine and neutrophils (186).

In hepatocytes, JNK signalling is associated with cell death, survival, differentiation, proliferation and tumorigenesis while, in non-parenchymal cells including KCs, it is implicated in inflammation and polarization and, in HSCs, JNK is involved in inflammation and fibrogenesis (189). The JNK pathway also contributes to the pathogenesis of steatosis by inhibiting fatty acid oxidation via repression of PPARα (190). Both JNK1 and JNK2 are expressed in the liver and appear to contribute to experimental steatohepatitis, while more compelling evidence for JNK1 has been provided at a cellular level (191). JNK may be activated as part of the ER stress response or by oxidative stress, and results in upregulation of p53 upregulated modulator of apoptosis, which leads to cell death in association with Bim. Bax is another target downstream of JNK, and is also involved in the mitochondrial pathway to apoptosis (192). JNK activation also contributes to apoptosis induced by death receptors, via up-regulation of TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (193). Additionally, JNK interacts with mitochondrial Sab protein and promotes an intramitochondrial signalling pathway that impairs mitochondrial function and enhances ROS production, thus activating upstream kinases and sustain JNK activation (194) (Figure I.10.).

For the p38 family, cell-specific effects are unknown for many isoforms because, until recently, most research has focused on p38α, and little attention has been paid to the activation of the other family members. Thus, some described p38α functions might be due to the activation of other p38 kinases. Studies in mice both *in vitro* and *in vivo*, suggested that p38α stimulates hepatic gluconeogenesis (195,196). Inhibition of p38α with pharmacological inhibitors or small interference RNA reduced hepatic glucose production by blocking the expression of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and PGC1α (195). In addition, targeted deletion of p38α in hepatocytes reduced fasting glucose level and impaired gluconeogenesis by blocking 5' Adenosine monophosphate-activated protein kinase (AMPK) activation after fasting (196). p38α was activated in the livers of obese *db/db* mice (knockout for the leptin receptor), accompanied by AMPK inhibition and hyperglycaemia, and these changes were blocked by hepatic deletion of p38α in this mouse model (196). A recent study compared between hepatocyte-specific p38α knockout, macrophage-specific p38α knockout and wild-type mice fed with high fat diet (HFD), high fat/high cholesterol diet, or methionine and choline deficient diet (MCD) and suggested that hepatocyte p38α protects mice from the development of steatohepatitis (197). In particular, mice without p38α in hepatocytes demonstrated a decreased lipolysis and an induction of the hepatic ER stress signalling and pro-inflammatory cytokine production, whereas genetic deletion of p38α in macrophages led to ameliorated nutritional steatohepatitis through decreased pro-inflammatory cytokine secretion and increased M2 macrophage polarization (197) (Figure I.10.). The same study reported that p38α was significantly upregulated in the liver tissues of patients with NAFLD (197).

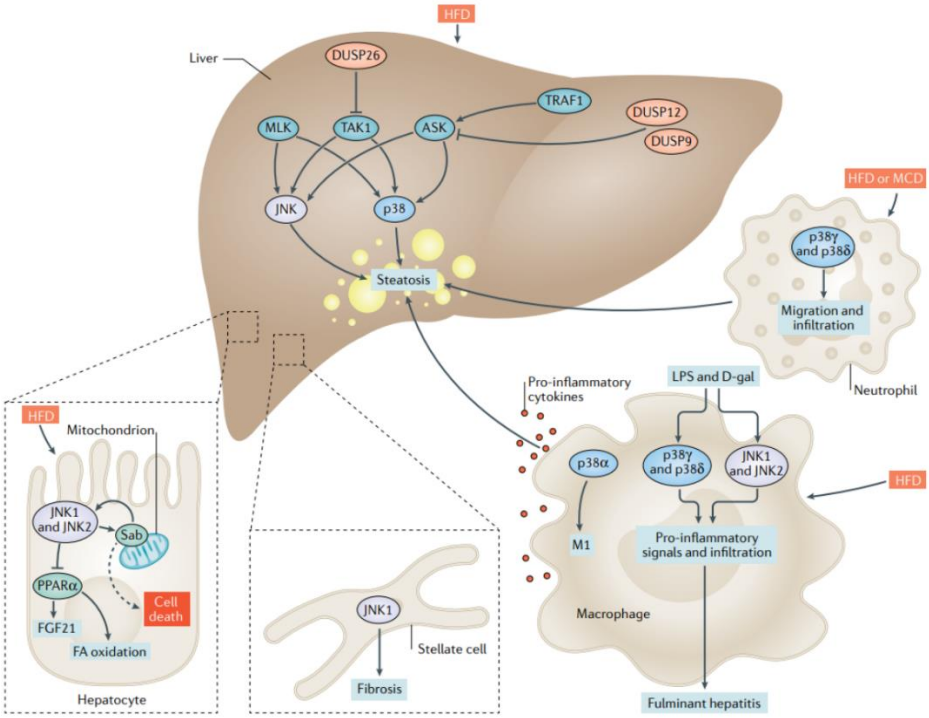


Figure I.10. Stress-activated protein kinases pathways in the liver *(186). Upstream kinases (MLK, TAK1 and ASK) regulate JNK and p38 activation in hepatocytes, whereas their inactivation is controlled by phosphatases of the DUSP family. Hepatic JNK1 and JNK2 inhibit PPARα activity and consequently repress FGF21 expression, leading to liver steatosis. In models of acute liver injury, JNK1 and JNK2 phosphorylate the mitochondrial protein Sab, triggering a feedback loop that ends in hepatocyte cell death. Neutrophil infiltration in the liver is mediated by p38γ and p38δ, thereby promoting liver steatosis. In macrophages, activation of JNK1 and JNK2 and of p38γ and p38δ induces pro-inflammatory signals that critically contribute to fulminant hepatitis. In activated hepatic stellate cells, JNK1 induces liver fibrosis. ASK, apoptosis signal-regulating kinase 1; DUSP, dual-specificity phosphatase; FA, fatty acid; FGF21, fibroblast growth factor 21; HFD, high-fat diet; LPS, lipopolysaccharide; MCD, methionine–choline-deficient diet; MLK, mixed lineage kinase; PPARα, peroxisome proliferator-activated receptor-α; TAK1, transforming growth factor beta-activated kinase*

Regarding the other p38 family members, p38γ/δ were found to be upregulated in liver biopsies from obese individuals with or without NAFLD or steatosis (198). In mice, both p38γ and p38δ are activated during liver steatosis and whole-body deletion of these kinases protects against diet-induced steatosis (198). Additionally, specific deletion of these kinases in myeloid cells reduces neutrophil recruitment to the liver, protecting animals against diet-induced steatosis and associated liver damage, an effect that is also partially mediated by the lack of p38δ in neutrophils (198).

Chapter II

AIMS

Chapter II AIMS

The general aims of this doctoral thesis were to investigate the interconnections among inflammation, chronic liver disease and HIV, as well as to explore the anti-inflammatory molecular mechanisms responsible for the hepatoprotective effects of the antiretroviral drug rilpivirine.

The specific aims were:

1. To compare the expression of inflammation and senescence markers in peripheral blood mononuclear cells isolated from HIV-infected patients under combined antiretroviral therapy and uninfected control population.

2. To study the correlation of these markers in the mentioned cohort of HIV-infected individuals with their clinical history, biochemical analysis and pharmacological treatment.

3. To analyse the inflammatory response in peripheral blood mononuclear cells isolated from patients with chronic liver disease and treated with rilpivirine *ex vivo*.

4. To study the effect of rilpivirine *in vivo*, focusing on pathways related to the inflammatory response.

5. To characterize the molecular mechanisms involved in the rilpivirine-induced anti-inflammatory effects observed *in vivo* using cultured hepatic cells.

Chapter III

MATERIALS AND METHODS

III.1. HUMAN SUBJECTS AND ETHICS STATEMENTS

In this thesis two clinical studies were performed. The first one included HIV-infected adults ≥18 years of age, men and women. All HIV subjects were virologically suppressed on cART (HIV RNA load, <40 copies/mL) for at least 6 months at the time of recruitment (naïve patients were excluded). The second study included patients with CLD from different etiology ≥18 years of age, men and women (patients with autoimmune conditions were excluded). Beside blood samples, general data about the patients were also provided that included date of birth, weight and height measurements. Alcohol, drug use, and cigarette smoking were self-reported. Clinical data for DM, co-infection with cytomegalovirus (CMV), HBV or HCV were also presented. Common blood test with analysed levels of glucose, lipids, cholesterol, transaminases etc., was performed. For every HIV-infected subject, the actual cART was listed, as well as $CD4^+$ and $CD8^+$ cell number count and progression to AIDS; Charlson comorbidity index (CCI) was calculated depending of the number and severity of the present diseases and pathologies.

Blood samples were also obtained from volunteers, men and women as control subjects for the HIV study. Age, sex, weight and height measurements, alcohol use and cigarette smoking data were obtained. The possibility of having some chronic pathology (e.g. DM, hypertension, dyslipidemia, CLD) that would interfere with the results was taken in consideration. Blood samples were collected at the Infectious diseases department and the Digestive medicine department for the first and the second study respectively, and for the control populations blood samples were collected in the transfusion department, all of them at *Hospital Clínico Universitario* of Valencia. The study's objectives and procedures were approved by the local investigational review board (*Comité Ético de Investigación Clínica del Hospital Clínico Universitario de Valencia*) (Annex 1, 2) and all participants provided informed consent (Annex 3-5).

III.2. DRUGS AND REAGENTS

III.2.1. General chemical reagents and drugs

All general chemical reagents were of analytical grade and were acquired from Sigma-Aldrich (Stenheim, Germany), Panreac Química S.L.U. (Barcelona, Spain), Merck Milipore (Darmstadt, Germany) and Roche Life Science (Penzberg, Germany). RPV used for the *in vitro* experiments was purchased from Biosynth Carbosynth (Compton, UK), and was dissolved in its corresponding vehicle dimethyl sulfoxide (DMSO). RPV used for the *in vivo* experiments was obtained from the Service of Pharmacy of the *Hospital Clínico Universitario de Valencia* (Spain). RPV pills were pulverized using a glass mortar, dissolved in DMSO and introduced in an ultrasound bath for 5 min until homogenous solutions were obtained, ready to be orally administered in animals.

III.2.2. Cell culture reagents

Media and supplements for cell culture (Table III.1.) were obtained from Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA), Sigma-Aldrich Chemicals and Lonza (Basel, Switzerland).

III.3. EXPERIMENTAL MODELS AND TECHNIQUES

III.3.1. *In vitro* approaches

III.3.1.1. Human cell lines

The human hepatoblastoma cell line Hep3B (86062703, European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK) was used as a human hepatocyte model (199,200). These cells were cultured in MEM supplemented with 10% heat-inactivated FBS (from now on abbreviated as FBS), 1x non-essential

amino acids solution (NEAA), 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U/mL penicillin/streptomycin.

Table III.1. Reagents used in cell culture and their supplier company.

REAGENT	COMPANY
Amphotericin B 250 µg/mL	Gibco
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dulbecco′s Modified Eagle′s Medium (DMEM) with high glucose concentration (4.5 g/L)	Gibco
Fetal bovine serum (FBS)	Sigma-Aldrich
Ficoll-Plaque™	Sigma-Aldrich
Hank's balanced salt solution (HBSS)	Sigma-Aldrich
L-glutamine 200 mM	Gibco
Minimum essential medium (MEM)	Gibco
Non-essential amino acids solution (NEAA) 100x	Gibco
Opti-MEM	Gibco
Penicillin/streptomycin 10000 U/mL	Gibco
Roswell Park Memorial Institute (RPMI) 1640	Sigma-Aldrich
Sodium pyruvate 100 mM	Gibco
Trypsin-Ethylenediaminetetraacetic acid (EDTA) 0.25%	Gibco

LX-2 cells, a human immortalized HSCs line, were purchased from Sigma-Aldrich and were routinely cultured in DMEM with high glucose concentration supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Cell cultures were maintained in a cell culture incubator (IGO 150, Jouan, Saint-Herblain Cedes, France) at 37 ºC, with a humidified atmosphere of 95% air and 5% CO_2 (AirLiquide

Medical, Valencia, Spain). The two lines were subcultured when they reached 90-95% confluence, using 0.25% Trypsin-EDTA. Sub-confluent cell cultures of passage number lower than 30 were used for all the experiments.

III.3.2. *Ex vivo* approaches

III.3.2.1. Isolation of PBMCs by density gradient centrifugation

Human PBMCs were isolated from peripheral venous whole blood samples obtained from healthy, uninfected donors and HIV-infected patients using density gradient centrifugation (201). Blood was collected in tubes containing sodium citrate as an anticoagulant and 25 mL were incubated for 45 min in 10 mL of 3% dextran (in physiological saline), thereby obtaining the pellet of most of the erythrocytes in the lower part of the falcon tube. The upper part was collected, slowly layered onto a Ficoll-Plaque™ density gradient medium and centrifuged (25 min, 500 g). Granulocytes and erythrocytes have a higher density than mononuclear cells and during centrifugation they sediment through the density medium layer. The less dense mononuclear cells remain at the plasma-density medium interface. PBMCs were obtained in the buffy coats (cells layer between plasma and Ficoll). This phase was then collected and transferred into a new tube; cells were washed with 1x phosphate buffered saline (PBS) buffer and centrifuged again (10 min, 400g). PBMCs were resuspended in 1 mL of physiological saline solution, centrifuged again and pellets were stored at -80 °C for further experiments.

III.3.2.2. PBMCs in culture

For establishing a cell culture for *ex vivo* experiments, PBMCs obtained from CLD patients were isolated as described before with some modifications. Briefly, the blood sample was diluted with the same amount of PBS and then, layered over Ficoll-Plaque™ medium and centrifuged (45 min, 400 g) thus obtaining PBMCs that

were then washed with PBS (10 min 400 g). Cell pellets were resuspended at $1x10^6$ cells/mL in RPMI 1640 medium with sodium bicarbonate and L-glutamine, supplemented with 100 U/mL penicillin/streptomycin, 250 µg/mL amphotericin B and 10% FBS (202).

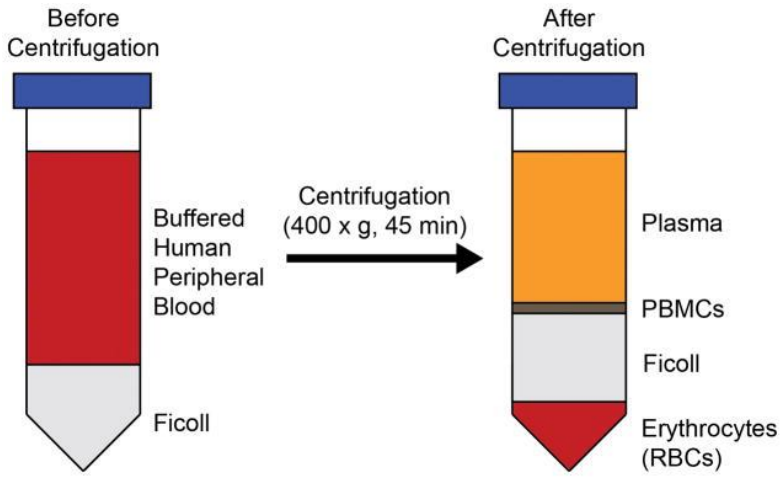


Figure III.1. Schematic depicting separation of human peripheral blood before and after centrifugation in Ficoll. *When subjected to a Ficoll gradient, whole blood separates into layers containing plasma, peripheral blood mononuclear cells (PBMCs), Ficoll, and red blood cells* (203).

III.3.3. Treatment

Cells were treated for 24 or 48 h with clinically relevant concentrations of RPV, chosen considering the interindividual pharmacokinetics variability reported in several clinical studies (48). Treatments were performed using sub-confluent cell cultures in medium supplemented with 10% FBS. Several concentrations of the drug were tested in order to analyse the concentration-dependence of the studied effect. Additionally, a negative control (untreated cells) and a vehicle control were used in every experiment. Also, several positive controls (see Table III.2.) were used depending on the biological process that was studied in each experiment.

Table III.2. List of pharmacological modulators used, including the specific concentration employed, description of their pharmacological actions and brand names. *E. coli, Escherichia coli; IFNγ, interferon gamma; LPS lipopolysaccharides; TGFβ, transforming growth factor beta; TNFα, tumour necrosis factor alpha.*

NAME	CONCENTRATION	ACTION	COMPANY
Human IFNγ	8-12.5 ng/mL	"LPS cocktail" (LPSc), a mixture composed of LPS, IFNγ and TNFα, was used as pro-inflammatory positive control.	Peprotech EC Ltd.
Human TNFα	12.5 ng/mL		Miltenyi Biotec
LPS from *E. coli* O26:B6 (LPS)	50 ng/mL		Sigma-Aldrich
Human TGFβ	2.5 ng/mL	Pro-fibrogenic activation	Miltenyi Biotec

III.3.4. *In vivo* approaches

III.3.4.1. Animal studies

Ten-week-old female C57BL/6J mice were supplied by Janvier Labs (Le Genest Saint Isle, France). Animals (20 ± 3 g of body weight) were given ad libitum access to water and chow diet (Envigo, Huntingdon, UK), and were kept at 21 ± 1 ºC under a standard light/dark regimen (12 h/12 h). Mice were always randomly divided into the different experimentation groups (ten mice per group). All animal procedures were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Valencia (Valencia, Spain), and were approved by the local ethics committee (authorization codes 2014/VSC/PEA/00188 and 2019/VSC/PEA/0112) (Annex 6).

III.3.4.2. RPV administration

Animal dosage was calculated using the normalized interspecies allometric scaling factor, based on interspecific body surface area calculations, stablished by FDA to

reach a dose for mice equivalent to the daily maximum therapeutic dose of RPV (25 mg) (204,205). Therefore, animals were orally treated (p.o.) on a daily basis with 0.1 mg of RPV dissolved in 10 µL of DMSO. All mice were sacrificed at the end of the protocol using isoflurane-inhaled anaesthesia. Blood samples were collected from the cave vein. Livers were weighed and fixed in 10% formalin for histological analysis or snap-frozen in liquid nitrogen and stored at -80 °C.

III.3.4.3. Mouse models of chronic liver disease

1. Nutritional model of non-alcoholic fatty liver disease

In order to study the implication of RPV in the development and progression of CLD (Figure III.2.), an overnutrition model of NAFLD was established in female C57BL/6J mice for 12 weeks. Aimed at recapitulating the pathogenesis of human NAFLD, obesogenic or the so-called HFDs are widely used to generate obesity and NAFLD in rodents (206). Specifically, we used a highly lipogenic and atherogenic diet ('high fat diet', HFD) specially prepared by Ssniff Spezialdiäten GmbH (Soest, Germany), composed of 59% of fat, 26% of carbohydrates, 15% of proteins and 2% of cholesterol (Ssniff® EFR/M D12330 mod.*/Surwit). In order to analyse the progression of NAFLD, and the effects of RPV regarding this process, animals were divided in different experimental groups:

- Normal diet (ND) control group: mice fed with ND.
- Vehicle+ND: mice fed with ND and daily administration of 10 µL DMSO p.o.
- RPV+ND: mice fed with ND and daily administration of 0.1 mg RPV in 10 µL DMSO p.o.
- HFD control group: mice fed with HFD.
- HFD+Vehicle: mice fed with HFD and daily administration of 10 µL DMSO p.o.

- HFD+RPV: mice fed with HFD and daily administration of 0.1 mg RPV in 10 µL DMSO p.o.

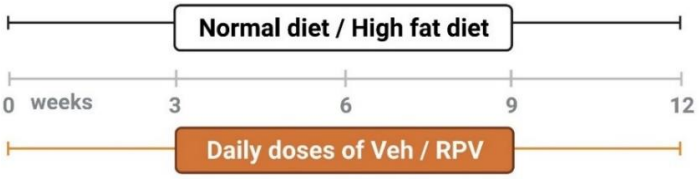


Figure III.2. Nutritional mouse model of non-alcoholic fatty liver disease. *Created with BioRender.com.*

2. Liver fibrosis models

We used CCl_4- and Bile duct ligation (BDL)-induced liver injury model in mice to study the capacity of the RPV to modulate the pathophysiological mechanisms involved in LF progression.

CCl_4 is metabolised in the liver by cytochrome P450 enzymes and converted to a highly reactive trichloromethyl (CCl3•) radical, ultimately leading to hepatotoxic damage, inflammation and LF (207). In this model, 0.5 mg/kg CCl_4 dissolved in 50 µL of corn oil were intraperitoneally injected (i.p.) in mice in alternate days for 4 weeks. RPV administration was performed exactly as in the nutritional model and RPV was administered (p.o.) on a daily basis during the whole duration of the experiment (see Figure III.3.). Three different experimental groups were defined for the study of each compound:

- Control group (Veh): daily administration of 10 µL DMSO p.o and corn oil injections in alternate days for 4 weeks.
- CCl_4+Veh: daily administration of 10 µL DMSO p.o and CCl_4 injections in alternate days for 4 weeks.
- CCl_4+RPV: daily administration of 0.1 mg RPV in 10 µL DMSO p.o. and CCl_4 injections in alternate days for 4 weeks.

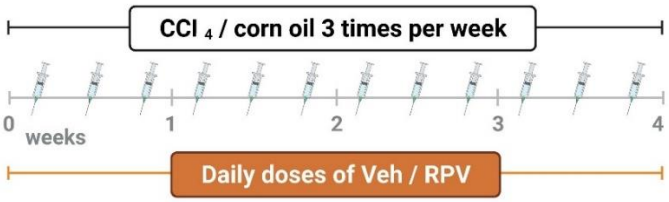


Figure III.3. CCl_4 mouse model to study the effect of RPV on the progression of liver fibrosis. *Created with BioRender.com.*

The surgical obstruction of the common bile duct causes bile to accumulate in the liver, leading to hepatic injury, inflammation and, ultimately, fibrosis and cirrhosis (206). BDL was performed by laparotomy through a midline incision following isoflurane anaesthesia. Once separated from the flanking portal vein and hepatic artery, two sutures were placed along the bile duct and secured, and it was then resected between them. The abdominal wall was closed after analgesic medication was administered. The sham operation was performed similarly without BDL. Animals were randomly divided in three groups, all of them with normal access to food and drink:

- Sham: daily manipulation without administration
- BDL+Vehicle: daily administration of 10 µL DMSO p.o.
- BDL+RPV: daily administration of 0.1 mg RPV in 10 µL DMSO p.o.

Treatments were administered for 15 days since the day of surgery (Figure III.4.).

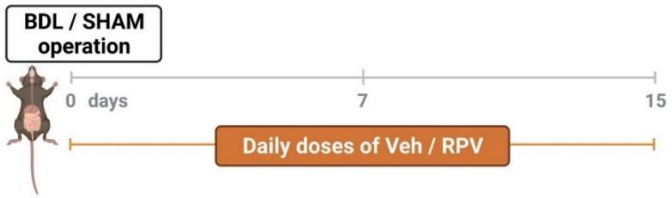


Figure III.4. Bile duct ligation mouse model to study the effect of RPV in the progression of liver fibrosis. *Created with BioRender.com.*

III.4. TRANSFECTION OF MAMMALIAN CELLS: TRANSIENT GENE SILENCING

Hep3B cells were transiently transfected using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Cells were seeded in 6-well plates and for the transfection, 100 pmol of STAT1 endo-ribonuclease prepared small interfering (si) RNA (esiRNA) (Sigma-Aldrich) and 5 μL Lipofectamine™ 2000 were diluted gently in 250 μL of serum-free Opti-MEM® (Invitrogen, Thermo Fisher Scientific). As a control, SignalSilence® control siRNA (Cell Signaling Technologies) was employed (siC). Diluted esiRNA/siC and Lipofectamine™ 2000 were mixed gently and incubated for 20 min at room temperature (RT). The transfection mixture (total volume 250 μL) was then added to Hep3B cells (70-80% confluence) in 6-well plates containing 450 μL of Opti-MEM® and mixed gently before incubation for 5 h at 37 ºC in a cell culture incubator. After 5 h, the medium was replaced by supplemented MEM. The following day, transfected cells were treated according to the different experimental conditions.

III.5. GENE EXPRESSION ANALYSIS

III.5.1. Quantitative RT-PCR

III.5.1.1. RNA extraction from cells

RNA isolation and purification from cells was performed using the illustra®RNAspin Mini RNA Isolation Kit (GE Healthcare), according to manufacturer's instructions. Briefly, cell pellets were resuspended in 350 μL lysis buffer and 3.5 μL β-mercaptoethanol (β-ME), and homogenized by passage through a 25 G needle. Next, 350 μL of 70% ethanol were added and the solution was transferred to a column which retains RNA. The following step included desalting

the silica membrane and digesting DNA. After washing three times, RNA was eluted in 30 µL RNase-free water, and its purity and concentration were determined spectrophotometrically, using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

III.5.1.2. cDNA synthesis

Complementary DNA (cDNA) was synthetized using 1 µg of total RNA using "PrimeScriptTM RT Reagent Kit" (Takara Bio, Kusatsu, Shiga, Japan). The reaction was performed as suggested in the manufacturer's protocol, in 20 µL final volume and the presence of 4 µL PrimeScript Buffer, 1 µL PrimeScript RT Enzyme Mix I, 1 µL Oligo dT Primer and 1 µL Random 6-mers. The reaction was carried out in SimpliAmp™ Thermal Cycler (Applied Biosystems™) under the following conditions: 37 °C for 15 min, 85 °C for 5 s, 4 °C ∞.

III.5.1.3. Quantitative RT-PCR

qRT-PCR was performed with SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio), containing TaKaRa Ex Taq HS, dNTP mixture, Mg^{2+}, Tli RNase H and SYBR Green I (DNA intercalator that is excited at 497 nm and emits fluorescence at 520 nm only when bound to dsDNA, and thus, detection of its fluorescent signal allows quantification of the amplification products). The reaction was performed by mixing 1 µL cDNA, 5 µL SYBR® Premix Ex Taq™, 0.2 µM primers (forward and reverse) and RNase-free water (10 µL final volume). qRT-PCR were carried out in a Lightcycler® 96 Real-Time PCR System (Roche Life Science) following this protocol: 95 ºC for 30 sec; 95 ºC for 5 sec, 60 ºC for 20 sec (40 cycles); 95 ºC for 1 sec; 65 ºC for 15 sec; 95 ºC for 1 sec and 40 ºC for 30 sec). All experiments were performed in duplicate, together with a negative control (RNase-free water instead of cDNA).

The primer pairs used (sequences shown in Table III.3.) were synthetized by Metabion (Planegg, Germany) or IDT® (Integrated DNA Technologies, Coralville, IA, USA). Before use, primers were tested by performing melting curve analysis and standard electrophoresis on 1.5% agarose gel. Agarose gels were prepared with TAE buffer (20 mM Tris pH 7.8, 0.5 mM EDTA and 10 mM sodium acetate), and GelRed™ Nucleic Acid Stain 10000X (Biotium Inc. Fremont, CA, USA), an ultra-sensitive, extremely stable and environmentally safe fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide for staining dsDNA, ssDNA or RNA in agarose gels or polyacrylamide gels. To determine the molecular weight of the DNA a mi-100 bp+DNA Marker Go (Takara Bio) was also loaded. To isolate DNA from the agarose gel in order to prepare a standard curve for quantification, an UltraClean® GelSpin® DNA Extraction Kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA) was used according to manufacturer's protocol. Briefly, the kit utilizes a silica-based spin filter membrane to isolate DNA from agarose gels. After electrophoresis, the desired DNA band is cut from an agarose gel and placed directly in the spin filter unit. The gel slice is melted irreversibly in a high molarity chaotropic salt. Then DNA is bound to the silica spin filter membrane, washed once to remove traces of agarose and salt and recovered from the spin filter membrane in elution buffer. DNA purity and concentration were determined spectrophotometrically, using a NanoDrop™ ND-1000 spectrophotometer.

Table III.3. Pairs of primers used for quantitative RT-PCR experiments. *Name of the gene, forward and reverse sequences and size of the amplified products (base pair) are presented.*

GENE	FORWARD PRIMER 5´ - 3´	REVERSE PRIMER 5´ - 3´	SIZE (bp)
ADIPOR1	TAATCGCCAACCCACCCAAA	TGAAGCAAGCCCGAAAGGAG	255
CCL2	AGCAGCAAGTGTCCCAAAGA	GGTTGTGGAGTGAGTGTTCAAG	152
CCL20	TTATTGTGGGCTTCACACGG	GGTTCTTTCTGTTCTTGGGCT	212
CD14	CCCGAGTCAACAGGGCATT	GATGTTTCAGGGAGGGGGAC	121

CD19	AGCGAATGACTGACCCCACC	AGCCCTCCCCTTCCTCTTCT	255
CD4	TTCCCAGAAGAAGAGCATACAA	TGGCAGTCAATCCGAACAC	254
CD8A	CCCTTTACTGCAACCACAGG	GGAAGGACTTGCTCCCTCAA	167
CDKN1A	TGTCTTGTACCCTTGTGCCT	GCGGATTAGGGCTTCCTCTT	157
CXCL10	GCTGCCTTATCTTTCTGACTCT	TTTTGCTCCCCTCTGGTTTT	290
CXCL9	TGTTCTTTTCCTCTTGGGCATC	TGGATAGTCCCTTGGTTGGT	111
CXCR3	TGCCTTTGTAGGGGTCAAGT	CTCACAAGCCCGAGTAGGAG	155
GAPDH	CTTCTTTTGCGTCGCCAGCC	TTCTCAGCCTTGACGGTGCC	232
IGFBP3	CCCTGCCGTAGAGAAATGGAAG	CTTGGTGGTGTAGCCTGGGA	201
IFNG	GGCTGAACTGTCGCCAGCAGC	GTTGGCTGCCTAGTTGGCCCC	356
IL18	GACCAAGGAAATCGGCCTCT	AATATGGTCCGGGGTGCATT	74
IL1B	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG	84
IL6	CACTGGTCTTTTGGAGTTTGAGG	ATTTGTGGTTGGGTCAGGGG	169
IL10	GTGATGCCCCAAGCTGAG	CACGGCCTTGCTCTTGTTTT	138
IL22	GCCCTATATCACCAACCGCA	GCGCTCACTCATACTGACTCC	118
RELA	ATCCCATCTTTGACAATCGTGC	CTGGTCCCGTGAAATACACCTC	153
SERPINE1	CGCTGTCAAGAAGACCCACA	ACCTGCTGAAACACCCTCAC	250
SIRT1	TGGGTACCGAGATAACCTTCT	TGTTCGAGGATCTGTGCCAA	181
SIRT2	CATCCCCGACTTTCGCTCTC	ATGGTTGGCTTGAACTGCCC	165
SIRT3	TCTGCCACCTGCACAGTCTGC	CAGCGGCTCCCCAAAGAACAC	138
STAT3	GGCGTCACTTTCACTTGGG	TCCGAATGCCTCCTCCTTG	201
TGFB1	CTTCAGCTCCACAAGAAGAACTG	CACGATCATGTTGGACAACTGCTC	297
TNFA	AGCCGAATCGCCGTCTCCTA	CAGCGCTGAGTCGGTCACCC	163
TLR4	CAACCTCCCCTTCTCAACCAA	AGATTTGTCTCCACAGCCACC	264
TP53	CCCATCCTCACCATCATCACA	AGTGCTCGCTTAGTGCTCC	176

For the HIV cohort study in order to quantify the result as number of amplified copies in each reaction, we generated standard curves by using serial dilutions of a

previously purified PCR product (DNA) for each primer pair. qRT-PCR data for the rest of the studies were analysed using the comparative CT method (208), obtaining the relative gene expression of the gene of interest. This method of presenting quantitative gene expression consists in this equation: Fold Change = $2^{-\Delta(\Delta CT)}$, where ΔCT = CT (target gene) - CT (housekeeping gene), and Δ(ΔCT) = ΔCT (treated) - ΔCT (control). *GAPDH* was used as a housekeeping gene (209) and results were normalized taking into consideration its expression.

III.5.2. Transcriptomic analysis

III.5.2.1. RNA extraction from liver tissue

RNA extraction from liver tissues was performed using TriPure Isolation Reagent (Roche Life Science). Liver samples (30-40 mg) were homogenized by MACS™ Dissociator (MACS Miltenyi Biotec) in 750 µL TriPure and samples were then centrifuged at 16000 g for 15 min at 4 ºC. Afterwards, 150 µL chloroform were added to the supernatant, in order to separate the different phases (aqueous, interphase and organic), and subsequently the samples were vigorously vortexed, incubated on ice for 15 min and centrifuged at 16000 g for 15 min at 4 ºC. The colourless aqueous upper phase, which contains RNA, was transferred to new tubes and RNA was precipitated by incubation with 500 µL isopropanol overnight at -20 ºC. Precipitated RNA was pelleted by centrifugation at 16000 g for 20 min at 4 ºC, washed with 1 mL 70% ethanol and pelleted again at 16000 g for 15 min at 4 ºC. Finally, RNA pellet was air-dried at RT and was resuspended in 50 µL RNase-free water. The purity and concentration of the RNA was determined using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific).

III.5.2.2. Affymetrix expression analysis

Total RNA from whole liver tissue was isolated as previously described (III.5.2.1). All the following steps for transcriptomic analysis were carried out by the Multigenic Analysis Unit at the Central Service for Experimental Research (Faculty of Medicine, University of Valencia). Genequant Pro Classic spectrophotometer (GE Healthcare) was used to determine the concentration and purity of RNA samples. The integrity analysis was performed using the RNA-6000 Nano Lab-on-a-Chip kit and the Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). The array used in this transcriptomic analysis was the GeneChip Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA). Before this, all samples were normalized to 300 ng of RNA and were hybridized by using the Hybridization Oven 645, also from Affymetrix, for 16 h at 45 °C. During this protocol, the Fluidics Station 450 (Affymetrix) was used in all required washing steps. Finally, all samples were scanned through the GeneChip Scanner 3000 7G (Affymetrix).

III.5.2.3. Data analysis

Bioinformatic analyses of the transcriptomic data were carried out by Dr. Francisco García García from the Biostatistics Unit at *Centro de Investigación Príncipe Felipe* (Valencia). Data were standardized using Robust Multi-array Average method (210) and quantile normalization. Differential gene expression was analysed using the limma (211) and maSigPro (212) packages from Bioconductor. Multiple testing adjustment of p-values was done according to Benjamini and Hochberg methodology (213). Gene set analysis was carried out using the Gene Ontology (GO) terms (214,215). This method detects significantly up- or down-regulated blocks of functionally related genes in lists of genes ordered by differential expression. Given that many functional terms are simultaneously tested, the results of the test are corrected for multiple testing to obtain an adjusted p-value. Gene set analysis returns adjusted p-values based on False Discovery Rate method (213,216). GO annotation

for the genes in the microarray were taken from Ensembl 78 release (http://www.ensembl.org).

III.6. PROTEIN EXPRESSION ANALYSIS

III.6.1. Protein extracts

III.6.1.1 Cell culture sample collection and preparation

After removing cell culture medium, cells were washed once with warm PBS and detached by adding trypsin-EDTA (at 37 ºC for 1 min). The resulting cellular suspension was centrifuged at 800 g for 3 min at RT, supernatant was then discarded and cell pellet was resuspended in 1 mL ice-cold PBS. This suspension was centrifuged again at 500 g for 5 min at 4 ºC. Finally, supernatants were discarded and cell pellets were obtained.

III.6.1.2. Whole-cell protein extraction with preserved phosphorylation

In order to preserve phosphorylated proteins, cell pellets were lysed in 40-70 µL of PhosphoSafe™Extraction Reagent (EMD Millipore Corp.), a lysis buffer that preserves the phosphorylation state of proteins, supplemented with 10x cOmplete Mini™ (Roche) protease inhibitor cocktail. The resulting lysates were vortexed for 15 sec, incubated for 5 min at RT and centrifuged at 4 °C, for 5 min, at 16000 g. Supernatants (whole-cell extracts) were collected and stored at -20 °C until future use.

III.6.1.3. Nuclear and cytosolic protein extraction

In order to obtain nuclear and cytosolic extracts, CHEMICON®'s Nuclear Extraction Kit (Merck Millipore) was used according to manufacturer's instructions. Cell pellets obtained from confluent t-25 flask cell cultures were resuspended in 70 µL ice cold 1x Cytoplasmic Lysis Buffer containing 0.5 mM DTT and 1/1000

dilution of Protease Inhibitor Cocktail with gentle pipetting avoiding foam production. Cell suspension was incubated on ice for 15 min and centrifuged at 200 g for 5 min at 4 ºC. Supernatants were then discarded and cell pellet were resuspended in 70 µL of ice cold 1x Cytoplasmic Lysis Buffer. This cell suspension was passed 4 times through a syringe with a small gauge needle (25 G), and the disrupted cell suspension was centrifuged at 8000 g for 10 min at 4 °C. The supernatant which contains the cytosolic portion of the cell lysate was transferred to a fresh tube and the remaining pellet (nuclear portion of the cell lysate), was resuspended in 70 µL ice cold Nuclear Extraction Buffer containing 0.5 mM DTT and 1/1000 Protease Inhibitor Cocktail. To disrupt the nuclei, cell suspension was gently passed 4 times through a syringe, with a 25 G needle. Next, the nuclear suspension was incubated on a rotator for 40 min at 4 ℃ and centrifuged at 16000 g for 5 min at 4 °C. Finally, the supernatant (nuclear fraction), was then transferred to a new tube and both, cytosolic and nuclear extracts, were stored at -80 °C for further use.

III.6.1.4. Total protein extracts from liver tissue

Mouse liver samples (20-35 mg) were homogenized in 900 µL extraction buffer (0.66 M Tris-HCl pH 7.5, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF and the protease inhibitor cOmplete Mini™) using a MACS™ Dissociator (MACS Miltenyi Biotec, Bergisch Gladbach, Germany). After adding 10 µL of 10% NP-40 SurfactAmps™, the homogenized samples were sonicated for 5 min at 15º C, and then centrifuged at 16000 g for 40 min at 4º C. The resulting supernatants (total protein extracts) were collected and stored at -80 ºC.

III.6.2. Protein quantification: bicinchoninic acid assay

The bicinchoninic acid (BCA) assay was used to quantify protein content in the extracts. This method combines the reduction of Cu^{2+} ions from Cu_2SO_4 to Cu^{+} by

proteins in an alkaline medium (the well-known Biuret reaction) with the highly sensitive colorimetric detection of Cu^{+}, based in the chelation of two molecules of BCA with one Cu^{+} ion. The purple-coloured reaction product of this assay exhibits a strong absorbance at 562 nm, which is approximately linear with increasing protein concentrations over a broad working range (0.02-2 mg/mL) (217). This assay was performed following the manufacturer's instructions (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). A standard protein curve was prepared by serial dilutions of BSA (0.03125-1 mg/mL) in H_2O. 20 µL of diluted samples (1:20 and 1:30 for *in vitro* and *in vivo* protein samples, respectively) or standard dilutions were used per well in a 96-well plate placed on ice, and immediately after 200 µL of working mixture reagent were added to each well. This working mixture was always prepared fresh before use, mixing 50 parts of Pierce™ BCA reagent A with 1 part of reagent B. Both the samples and standard curve dilutions were assayed in duplicate. Next, the plate was incubated (protected from light) at 37 ºC for 30 min with gentle rocking. Finally, absorbance was measured at 570 nm employing an Infinite® 200 PRO series spectrophotometer (TECAN Trading AG, Männendorf, Switzerland).

III.6.3. Western Blotting

III.6.3.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Samples were prepared by mixing extracts with equal total protein amounts (20-30 µg) with Laemmli sample buffer 6x (375 mM Tris-HCl, 9% sodium dodecyl sulphate (SDS), 50% glycerol, 9% β-ME and 0.03% bromophenol blue) and boiled at 98 ºC for 5 min in order to achieve protein denaturation. SDS- polyacrylamide gel electrophoresis (PAGE) was performed using the Mini-PROTEAN® Tetra Cell System (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels were made using a mixture of acrylamide/bis-acrylamide solution (PanReac AppliChem, Barcelona, Spain). Resolving gels were prepared with different percentage of polyacrylamide (8-15%) in 0.375 M Tris-HCl pH 8.8 and 0.1% SDS, whereas stacking gels were

always prepared with 3.75% polyacrylamide in 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. The polymerization reaction was catalysed by 0.1% ammonium persulfate (APS, Bio-Rad) and N,N,N′,N′-tetramethylethylenediamine (TEMED, Sigma-Aldrich). To determine the molecular weight of the polypeptides, a molecular weight marker (EZ-Run™ Pre-Stained Rec Protein Ladder, Fisher Bioreagents™, Thermo Fisher Scientific) was also loaded. Electrophoresis was performed with running buffer (25 mM Tris pH 8.3, 0.1% SDS and 192 mM glycine) at constant voltage of 90-140 V.

III.6.3.2. Protein transfer to nitrocellulose membrane

SDS-PAGE-resolved proteins were transferred from the resolving polyacrylamide gel to a 0.45 µm nitrocellulose blotting membrane (GE Healthcare Life Science) using a Mini Trans-Blot® Cell (Bio-Rad). The transfer was performed at 4 ºC for 1 h at a constant amperage of 0.4 A, in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol).

III.6.3.3 Ponceau and antibodies staining

In order to verify the transfer efficiency and quality, the nitrocellulose membrane was soaked in a 0.1% Ponceau/5% acetic acid solution (Sigma-Aldrich) for 1 min, which stains proteins. Ponceau staining was removed with Tris-buffered saline Tween (TBS-T, 20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1% Tween-20 v/v) before incubating the membrane in fresh blocking solution (5% fat-free milk powder or BSA, diluted in TBS-T) with continuous gentle shaking, for at least 1 h, at RT. Once the membrane was blocked, it was incubated with the primary antibody, prepared in blocking solution, overnight at 4 ºC with continuous gentle shaking. Subsequently, the membrane was washed three times in TBS-T for 10 min with vigorous shaking at RT and, incubated with a secondary antibody in fresh blocking

solution at RT for 1 h, and washed again. Primary and secondary antibodies used are listed in Table III.4.

III.6.3.4. Chemiluminescence detection

Immunolabeling was detected by enhanced chemiluminescence, employing Luminata™ Crescendo Western HRP substrate, Luminata™ Forte Western HRP substrate (Merck Millipore, Billerica, MA, USA), or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), following manufacturer's instructions. This detection method is based in an oxidation reaction of luminol catalysed by the enzyme horseradish peroxidase (HRP), conjugated to the secondary antibody, in the presence of hydrogen peroxide, and giving rise to 3-aminophthalate that emits light at 425 nm (218). Immunolabeling was visualized with a digital luminescent image analyser, Amersham ImageQuant 800 (GE Healthcare). Densitometric analyses were performed using ImageJ software (Fiji, Tokio, Japan). The protein expression was normalized versus that of GAPDH (employed as loading control).

Table III.4. List of primary and secondary antibodies employed in western blot assays.

PRIMARY ANTIBODIES				
PROTEIN	**SOURCE/TYPE**	**MW (kDa)**	**DILUTION**	**COMPANY (ref. number)**
GAPDH	rabbit/polyclonal	36	1:20000	Sigma-Aldrich (G9545)
SIRT1	rabbit/polyclonal	110	1:1000	Merck Millipore (07-131)
p53	rabbit/monoclonal	53	1:1000	Abcam (ab76242)
STAT3	rabbit/monoclonal	88	1:1000	Cell Signaling Technology (30835)
pSTAT3	rabbit/monoclonal	88	1:1000	Cell Signaling Technology (9145)
STAT1	rabbit/monoclonal	87	1:1000	Cell Signaling Technology (14994)

pSTAT1	mouse/monoclonal	87	1:1000	Abcam (ab29045)
acetyl Histone H3 (Ac-Lys9)	mouse/monoclonal	17	1:1000	Sigma-Aldrich (H0913)
NF-κB (p65)	rabbit/monoclonal	65	1:1000	Cell Signaling Technology (8242)
pNF-κB (pp65)	rabbit/monoclonal	65	1:1000	Cell Signaling Technology (3033)
Nucleolin	rabbit/polyclonal	105	1:2500	Sigma-Aldrich (N2662)
IκBα	rabbit/monoclonal	40	1:1000	Santa Cruz Biotechnology (sc-1643)
pIκBα	mouse/monoclonal	40	1:1000	Cell Signaling Technology (9246)
p38	rabbit/polyclonal	38	1:1000	Sigma-Aldrich (506123)
pp38	mouse/monoclonal	38	1:1000	Cell Signaling Technology (9216)
JNK 1/2	rabbit/polyclonal	46, 54	1:1000	Cell Signaling Technology (9252)
pJNK 1/2	rabbit/monoclonal	46, 54	1:1000	Thermo Fisher Scientific (700031)
pJNK 1/2	mouse/monoclonal	46, 54	1:1000	Cell Signaling Technology (9255)
PAI1	rabbit/polyclonal	45	1:1000	Abcam (ab66705)
CXCL10	mouse/monoclonal	10	1:1000	Abcam (ab8098)
αTubulin	mouse/monoclonal	50	1:1000	Sigma-Aldrich (T5168)
SECONDARY ANTIBODIES				
ANTIBODY		**LABELING**	**DILUTION**	**COMPANY (ref. number)**
Goat Anti-Mouse IgG Antibody		HRP	1:2000	Thermo Fisher (31430)
Goat Anti-Rabbit IgG Antibody		HRP	1:5000	Vector (PI-1000)

III.6.3.5. Stripping for reprobing

Stripping is a method that removes antibodies from a nitrocellulose membrane, enables reutilization of the membrane and its incubation with other antibodies. The stripping process was performed in two different ways depending on the experiment:

1. Incubation with a stripping solution (62.5 mM Tris-HCl pH 6.7, 100 mM β-ME and 2% SDS), at 56 ºC for 30 min with vigorous shaking. After that, the membrane was washed with TBS-T three times for 10 min, at RT.
2. Incubation with 0.5 M glycine pH 2.5 for 1 h at RT with vigorous shaking. Subsequently, the membrane was washed three times with TBS-T for 10 min, at RT.

Regardless of the protocol used, the membrane was blocked with the blocking solution again before incubating it with the antibodies, following the protocol described above (III.6.3.3).

III.6.4. Enzyme-linked immunosorbent assay

Hep3B cells were cultured and treated on 12-well cell culture plates. After treatment, cell culture media was recollected and IP-10 secretion was measured by enzyme-linked immunosorbent assay (ELISA), using a Human CXCL10/IP-10 Quantikine ELISA Kit (R&D Systems Europe, Abingdon, UK). Following the manufacturer's instructions, the standard (serial dilutions of a recombinant human IP-10) and (1:1.5) diluted cell supernatants were added in duplicate to 96-well polystyrene microplate, coated with a monoclonal antibody specific for human IP-10, and incubated for 2 h at RT. After washing three times with a washing solution, human IP-10 Conjugate-HRP was added and incubated for another 2 h at RT. Following a washing step to remove the excess HRP conjugate, ELISA was developed by the addition of the substrate solution containing stabilized H_2O_2 and 3,3',5,5'-tetramethylbenzidine solution (TMB, a chromogenic substrate) in each well, incubated for 30 min at RT protected from light. The reaction was stopped with 1 M H_2SO_4. Absorbance was measured at 450 nm within 30 min after adding the stop solution with an Infinite® 200 PRO series spectrophotometer (TECAN Trading AG). In order to obtain accurate values of IP-10, the presence of the coloured product was also detected at 620 nm and this background absorbance was subtracted from that obtained at 450 nm.

III.7. FLUORESCENT MICROSCOPY: IMMUNOCYTOCHEMISTRY

III.7.1. STAT1 expression

Hep3B cells were cultured on 12-well cell culture plates, treated and stained by means of immunocytochemistry whose specifications are detailed below. Cells were fixed with warm 4% paraformaldehyde (PFA) at RT for 10 min, and were then washed two times for 5 min with warm PBS. Cells were permeabilized with -20 ºC stored 95% EtOH and 5% Glacial acetic acid for 10 min and washed two times with PBS. To block non-specific binding sites, cells were incubated with blocking buffer (5% (w/v) BSA in PBS) at RT for 1 h, followed by an incubation overnight at 4 ºC with STAT1 rabbit monoclonal antibody (1:400, Cell Signaling Technologies) in antibody dilution buffer (5% (w/v) BSA in PBS). Afterwards, cells were washed three times with PBS and incubated with the secondary antibody Rabbit anti-goat IgG H&L Alexa Fluor® 488 (1:500, Abcam) at RT for 2 h in darkness, and washed again three times with PBS. Nuclei were stained by adding 1 µM Hoechst® 33342 (Sigma-Aldrich) in PBS in the last 30 min before taking the images.

LEICA DMI 3000-B (Leica Microsystems, Wetzlar, Germany) microscope was used for image recording. Images were recorded at 20x magnification and were analysed using Fiji by creating a macro plugin. Firstly, on the images that showed the Hoechst® 33342 dye, a mask was created for the nuclei and this mask was then subtracted from the mask that was created to mark the total fluorescence (cytoplasm and nuclei) of the protein in interest. These operations gave the integrated density (the sum of the values of the pixels in the image or selection, equivalent to the product of Area and Mean Gray Value) of both nuclei and cytoplasm which would define the expression of STAT1 protein in the two cellular compartments separately.

III.8. PRESENTATION OF DATA AND STATISTICAL ANALYSIS

Values are expressed as mean ± standard error of the mean (SEM) for parametric and median with interquartile range (IQR) for non-parametric data. The number of independent experiments (n) is indicated in the figure legends for each experiment. Data were analysed using GraphPad Prism® V6.01 (GraphPad Prism® Software Inc.). Normality was assessed by the D'Agostino and Pearson's omnibus normality test. Parametric and non-parametric variables were assessed by Student's unpaired *t*-test and Mann–Whitney U test, respectively. Significance was inferred from a p value <0.05 unless stated otherwise. Correlation coefficients were calculated to assess the strength and direction of the linear relationships between pairs of variables (Pearson's correlation coefficient was used when both variables were normally distributed, otherwise Spearman's correlation coefficient was employed). Only data with $r >0.4$ are considered co-related and are shown.

Chapter IV

RESULTS

SECTION I: ANALYSIS OF THE EXPRESSION OF INFLAMMATION AND SENESCENCE MARKERS IN PBMCs ISOLATED FROM HIV-INFECTED PATIENTS AND CONTROL POPULATION

IV.I.1. Anthropometric characteristics and general blood analysis of HIV patients and controls

The first cohort involved 70 HIV-infected men and women (≥18 years of age) and 43 uninfected age-, sex- and BMI-matched HIV-negative control subjects whose characteristics are represented in Table IV.1 and IV.2, respectively. Patients had median age of 52 years represented with the interquartile range (IQR). From the height and weight values obtained, body mass index (BMI) was calculated according the following formula: BMI= weight (kg)/height^2 (m^2). Median BMI value was 24.90 and, according to World Health Organization (WHO), this value is at the upper limit of the normal weight range (24.99). 35.72% of the HIV patients were smokers and only one of them declared that was consuming alcohol or drugs. 20 patients were HIV/HCV, and 2 were HIV/HBV co-infected.

Regarding the presence of metabolic comorbidities in the patients, 31.43% suffered dyslipidemia, 21.43% were diabetic, 28.57% and 22.86% had medium and high cardiovascular risk (CVR), respectively. For CVR, patients were stratified in those with high risk (patients with previous CVD, DM, more than 1 risk factor and Framingham calculated coronary risk for 10 years >20%), moderate risk (more than 1 risk factor and Framingham coronary risk for 10 years <20%) and low risk (1 risk factor). CVR factors involve hypertriglyceridemia, hypercholesterolemia, arterial hypertension, BMI over 25, familiar background for CVD and diagnosis for heart ischemia. The Framingham Risk Score is a gender-specific algorithm used to estimate the 10-year CVR of an individual. Beside sex, other variables used to

calculate Framingham risk include age, total and HDL cholesterol, systolic blood pressure and if the patient is receiving antihypertensive drugs, as well as if the patient is a smoker (219). CCI, which predicts 10-year survival, was calculated depending on the number and severity of the present pathologies—the higher the score the greater the comorbidity (220). Median CCI was 2, however ≥6 was registered in 30% of the patients.

HIV-related factors were also considered including mode of transmission, years since diagnosis, changes in the regimen of cART and previous development of AIDS-defined by internationally accepted criteria including the presence of an AIDS-defining condition or having a $CD4^+$ count lower than 200 cells/mm^3 regardless of the existence of an AIDS-defining condition.

As for their biochemical and haematological parameters, almost all median values were within the range considered normal, except glucose and LDL parameters, as well as the number of monocytes, whose median values were slightly above the upper limit of the normal range. The median CD4/CD8 ratio, also termed as the immune risk profile was 0.73 which is, as expected for HIV patients, lower than that considered normal for the general population (>1).

Table IV.1. Characteristics of HIV patients*. Information about the patients included general characteristics (age and BMI), clinical data for other diseases, and common biochemical and immunological blood tests, while alcohol consumption, drug use, and cigarette smoking were self-reported. Values are expressed as number of patients and percentage of total number of patients for non-numerical data, and median (1st and 3rd percentiles) for numerical data, which were all non-parametric. BMI, body mass index; HBV, Hepatitis virus B; HCV, Hepatitis virus C. HO/BI, homosexual/bisexual; HTSX, heterosexual; IDU, injection drug user; IIs, integrase inhibitors; IQR, interquartile range; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside and nucleotide reverse transcriptase inhibitors; PIs, protease inhibitors, TC, total cholesterol.*

GENERAL CHARACTERISTICS OF THE HIV PATIENTS					
GENDER	MALE 56 (80%)	FEMALE 14 (20%)			
AGE (years) median (IQR)	52.00 (45.75 – 56.00)				
BMI (kg/m^2) median (IQR)	24.90 (23.33 – 27.87)				
SMOKING	YES 25 (35.72%)	NO 33 (47.14%)	PREVIOUS SMOKER 12 (17.14%)		
ALCOHOL USE	YES 1 (1%)	NO 69 (99%)			
COMORBIDITIES-RELATED PARAMETERS					
HCV COINFECTION	YES 20 (28.57%)	NO 48 (68.57%)	NO DATA 2 (2.86%)		
HBV COINFECTION	YES 2 (2.86%)	NO 63 (90.00%)	NO DATA 5 (7.14%)		
DIABETES MELLITUS	YES 15 (21.43%)	NO 55 (78.57%)			
DYSLIPIDEMIA	YES 22 (31.43%)	NO 48 (68.57%)			
CARDIOVASCULAR RISC	NO 16 (22.86%)	LOW 16 (22.86%)	MEDIUM 20 (28.57%)	HIGH 16 (22.86%)	NO DATA 2 (2.86%)
CHARLSON INDEX median (IQR)	2.00 (0.00–6.00)				
HIV-RELATED PARAMETERS					
YEARS WITH HIV median (IQR)	19 (8.50-27.00)				
YEARS WITH THERAPY median (IQR)	15 (4.00–21.25)				
MODE OF TRANSMISSION	HO/BI 25 (35.71%)	HTSX 17 (24.29%)	IDU 21 (30.00%)	OTHER 7 (10.00%)	
AIDS	YES 18 (25.71%)	NO 52 (74.29%)			
THERAPY (in addition to NRTI back bone)	NNRTIs 11 (15.71%)	PIs 16 (22.86%)	IIs 28 (40%)	COMBINATION 15 (21.43%)	
THERAPY LINE median (IQR)	5 (2.00–8.25)				
MAX VIRAL LOAD (copies/mL) median (IQR)	79500 (5925–361000)				
CD4 NADIR COUNT (cell/mL) median (IQR)	207.5 (71.50–339.00)				
CD4/CD8 median (IQR)	0.730 (0.478–1.080)				

BIOCHEMICAL ANALYSIS		
PARAMETER	**MEDIAN (IQR)**	**REF.**
Glucose (mg/dL)	100.5 (91.75-112.3)	64-100
TC (mg/dL)	200 (174.3-228.3)	140-200

HDL cholesterol (mg/dL)	48 (40-54)	≥40
LDL calculated (mg/dL)	133.5 (110-152)	<130
TG (mg/dL)	139 (90-207.8)	40-150
ALT (U/L)	26 (19-34.25)	≤55
AST (U/L)	27.5 (22-32)	≤40
GGT (U/L)	31 (23-51.5)	≤48
Ferritin (ng/mL)	92 (57-148)	10-120
CRP (mg/L)	1.9 (0.9-4.2)	≤5
PARAMETER	**HEMATOLOGICAL ANALYSIS MEDIAN (IQR)**	**REF.**
Leucocytes *10^9/L	6.46 (5.29-7.74)	3.9-11
Neutrophils (%)	51.85 (46.73-58.40)	55-75
Lymphocytes (%)	35.20 (29.43-41.18)	17-45
Monocytes (%)	8.6 (7.30-9.93)	2-8
Eosinophils (%)	2.1 (1.4-3.8)	1-4
Basophils (%)	0.6 (0.4-0.8)	0.2-1.2
Platelets *10^9/L	198.5 (167.3-242.3)	140-400

Table IV.2. Characteristics of control subjects. *Information about the controls included general characteristics (gender, age and BMI), while alcohol consumption, and cigarette smoking were self-reported. Values are expressed as number of controls and percentage of total number of patients for non-numerical data, and median (1^{st} and 3^{rd} percentiles) for numerical data, which were all non-parametric. BMI, body mass index; IQR, interquartile range.*

CHARACTERISTICS OF THE UNIFECTED CONTROL POPULATION		
GENDER	MALE 33 (76.74%)	FEMALE 10 (23.26%)
AGE (years) median (IQR)	47 (42.00–54.25)	
BMI (kg/m^2) median (IQR)	26.10 (24.01–28.32)	
SMOKING	YES 10 (23.26%)	NO 33 (76.74%)
ALCOHOL CONSUMPTION	YES 33 (76.74%)	NO 10 (23.26%)

The characteristics of the healthy population are represented in Table IV.2. Controls had median age of 47 years and median BMI value of 26.1. According WHO, individuals with BMI values from 18.5 to 24.9 kg/m^2 are classified as normal weight. For BMI 25.0 to 29.9 (overweight) there is increased risk of comorbidities, and

moderate to severe risk of comorbidities exist for BMI greater than 30 (obesity). In addition, 10 of the controls were smokers and 76.74% declared that were consuming alcohol.

IV.I.1.1. Correlation between different parameters

We also explored the correlation between different parameters in our HIV cohort and those that displayed coefficient of correlation r>0.4 are shown in Table IV.3. As expected, age was positively correlated with the "years with HIV" parameter, and "glucose" was correlated with BMI. ALT showed a positive correlation with the others transaminases and CCI showed positive correlation with "therapy line", while negative correlation was detected between "CD4 nadir" and "max VL". Both the number of leucocytes and that of lymphocytes showed a negative correlation with monocytes and neutrophiles, respectively.

Table IV.3. Correlations between different parameters of the studied HIV population. *All HIV-related, biochemical and hematological parametres (shown in Table IV.1.) were assessed for the presence of correlations. Apart from the expected very high and high positive correlations in "Total Cholesterol/LDL" (r=0.920, not included in the table, an effect due to the fact that the value of serum LDL is not experimentally obtained - it is calculated with the total cholesterol value according to the formula: LDL=Total cholesterol – HDL cholesterol – TG/5) and "Years with HIV/Years with therapy (r=0.676) respectively, this table presents the pairs of parameters that displayed a correlation coefficient >0.4 (Pearson's correlation coefficient –r^p for parametric data and Spearman´s correlation coefficient -r^s, for non-parametric data). Negative correlation is shown in shaded boxes. Statistical significance is also shown, n=68-70. Bonferroni correction for multiple association was performed and p<0.002 was considered statisticaly significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; COL, cholesterol; GGT, gammaglutamyl transferase; HDL, high-density lipoprotein; TC, total cholesterol; VL, viral load.*

	Age	BMI	TC	ALT	Monocytes	Neutrophils	Therapy line	CD4 Nadir
Years with HIV	r^s=0.462 p<0.0001							
Glucose		r^s=0.403 p=0.001						
HDL			r^s=0.410 p<0.0001					
AST				r^s=0.735 p<0.0001				
GGT				r^s=0.434 p< 0.0001				
Leucocytes					r^p =-0.446 p< 0.0001			
Lymphocytes						r^p =-0.961 p< 0.0001		
Charlson Index							r^s=0.594 p<0.0001	
Max VL								r^s=-0.598 p< 0.0001

IV.I.2. Expression of inflammation and senescence markers in HIV patients and controls

We assessed the expression of a set of 17 genes related to inflammation and senescence in PBMCs from HIV patients and uninfected controls. In HIV patients, a statistically significant increase was detected for the inflammatory cytokines *IL6*, *IL18* and *CXCL10* (IP-10) while, interestingly, *SERPINE1*, *IGFBP3*, *TP53* and *SIRT1* were downregulated (Table IV.4). mRNA levels of *STAT3* were also significantly lower in HIV-infected individuals.

Table IV.4. Differences in the expression of selected genes in PBMCs between HIV patients and controls. *Name and function of the protein encoded by the gene are displayed. The detected statistically significant upregulation or downregulation of the mRNA levels in samples from HIV population vs controls are indicated with an arrow. Data are shown as mean ± SEM for parametric and median (IQR) for non-parametric data. The number of samples for each gene studied (for control and HIV patients) is displayed. Statistical analysis*

(SA): Student´s unpaired t-test as parametric and Mann-Whitney test (MW) as non-parametric test, p<0.05 was considered statistically significant.

GENE	FUNCTION	HIV *vs* C	HIV *vs* control	SA
IL6	Pro-inflammatory cytokine; maturation of B cells	↑	**0.4504 ± 0.01131 n=69** **0.4146 ± 0.009253 n=43**	**p=0.0278**
IL18	Pro-inflammatory cytokine	↑	**1.278 (1.233-1.313) n=69** **1.268 (1.211-1.289) n=42**	**p=0.0317 (MW)**
IL1B	Pro-inflammatory cytokine	-	1.118 ± 0.01412 n=69 1.160± 0.01825 n=43	p=0.0643
TNFA	Pro-inflammatory cytokine; cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation	-	0.7544 ± 0.01133 n=70 0.7771 ± 0.01413 n=43	p=0.2166
CCL2 (MCP1)	Chemotactic activity for monocytes and basophils	-	0.5385 (0.4807-0.6060) n=70 0.5394 (0.5031-0.5937) n=43	p=0.8069 (MW)
CCL20 (MIP3A)	Chemotactic activity for lymphocytes and macrophages	-	0.6270 ± 0.01553 n=70 0.6144 ± 0.01688 n=43	p=0.5989
CXCL10 (IP10)	Stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression	↑	**0.5593 ± 0.01472 n=65** **0.5099 ± 0.01504 n=36**	**p=0.0322**
ADIPOR1	Receptor for adiponectin, a hormone secreted by adipocytes which regulates fatty acid catabolism and glucose levels	-	0.7757 (0.7588-0.8034) n=70 0.7811 (0.7680-0.8008) n=43	p=0.4384 (MW)
RELA (p65)	Transcription factor; inflammatory signalling pathways in response to infection or injury	-	0.7144 (0.6947-0.7337) n=69 0.7050 (0.6981-0.7295) n=43	p=0.6209 (MW)
SERPINE1 (PAI1)	Inhibitor of fibrinolysis Involved in inflammation and senescence	↓	**0.3083 ± 0.01024 n=70** **0.3395 ± 0.01010 n=42**	**p=0.0448**
STAT3	Plays a key role in many cellular processes such as cell growth and apoptosis	↓	**0.7313 (0.7146-0.7400) n=70** **0.7397 (0.7203-0.7539) n=43**	**p=0.0192 (MW)**

IGFBP3	Hormonal regulation of cell growth, differentiation and metabolism	↓	**0.5788 (0.5439-0.6164) n=69** **0.5977 (0.5728-0.6503) n=43**	**p=0.0022 (MW)**
TP53 (p53)	Transcription factor; cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism	↓	**0.9450 ± 0.003198 n=70** **0.9643 ± 0.006061 n=41**	**p=0.0026**
CDKN1A (p21)	Cell cycle inhibitor-promotor of replicative senescence	-	0.6352 ± 0.009105 n=70 0.6477 ± 0.007662 n=43	p=0.3413
SIRT1	Protein deacetylase - cell survival, lifespan and metabolism regulation	↓	**0.7387 ± 0.003033 n=68** **0.7688 ± 0.005302 n=43**	**p<0.0001**
SIRT2	Protein deacetylase - cell cycle regulation, nervous system development	-	0.6578 ± 0.006038 n=65 0.6585 ± 0.01009 n=41	p=0.9528
SIRT3	Protein deacetylase - Regulation of mitochondrial metabolism	-	1.008 ± 0.005900 n=69 1.020 ± 0.01181 n=43	p=0.2851

Given that HIV patients have altered CD4/CD8 ratio, we wished to analyse whether the different cell type proportions within PBMCs accounted for the observed differences between the genes in Table IV.4. To this aim, firstly, we assessed the expression of *CD4* and *CD8A* (one of the two CD8 genes) levels and the expected result was obtained with HIV patients showing increased *CD8A* expression. The other major cell populations within PBMCs, i.e. monocytes and B lymphocytes using *CD14* and *CD19* as their markers respectively, were also compared and no significant differences were found between controls and HIV patients (Figure IV.1.).

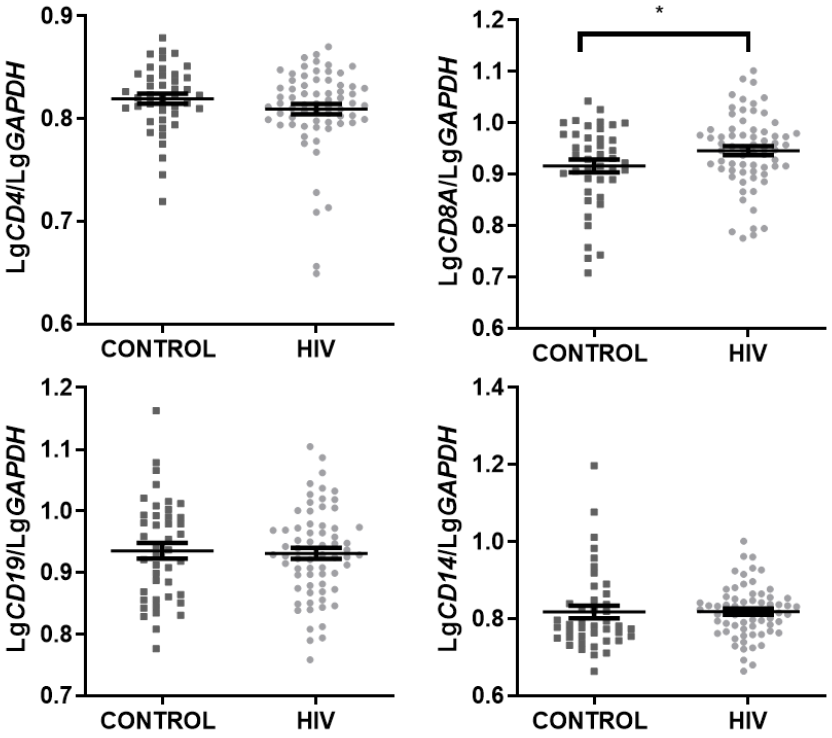


Figure IV.1. mRNA levels (*CD4, CD8A, CD14* and *CD19*) in PBMCs of uninfected controls and HIV patients. *Data (mean ± SEM), for control individuals n=42-43 and for HIV patients n=67-68. Gene expression was calculated as number of copies of the gene normalized with the number of copies of the housekeeping gene (GAPDH). Statistical analysis between groups: Student´s unpaired t-test as parametric and Mann-Whitney test as non-parametric test, $p<0.05$ was considered statistically significant.*

IV.I.3. Gene expression in different categories of the HIV-infected patients

For a more detailed analysis of the HIV patients and the possible changes in the specific gene expression in PBMCs, we assessed their expression in relation to a variety of parameters including anthropometric factors, comorbidities, HIV-

associated parameters and biochemical variables. The genes that presented statistically significant differences are displayed in Figure IV.2.

Regarding the inflammatory genes, women showed higher expression reaching statistical significance for *IL1B* and *TNFA*. Age and BMI were also associated factors and increased levels were observed with greater age or BMI in almost all genes, being the difference statistically significant for *IL1B*, *IL6* and *TNFA* (for age), and IL6 (for BMI). Presence of AIDS was associated with lower expression of *IL18*, but higher levels of *CCL2* and *CXCL10* mRNA. Interestingly, some inflammatory genes - *IL1B, IL6, TNFA* and *CCL2* displayed decreased expression in patients with dyslipidemia, and this effect may be due to the anti-inflammatory capacity of the lipid-lowering drugs that these patients are taking (statins). On the contrary, diabetic patients had higher mRNA levels of *CCL20* and *CXCL10* chemokines. In the categories where we divided the patients taking into consideration the level of transaminases as indicators for liver function, we detected a significant upregulation of *IL6, IL18, TNFA* and *CCL20* mRNA amounts in the subgroup where the transaminases were higher than the reference limit values (ALT > 55), pointing to functional alteration in the mentioned organ.

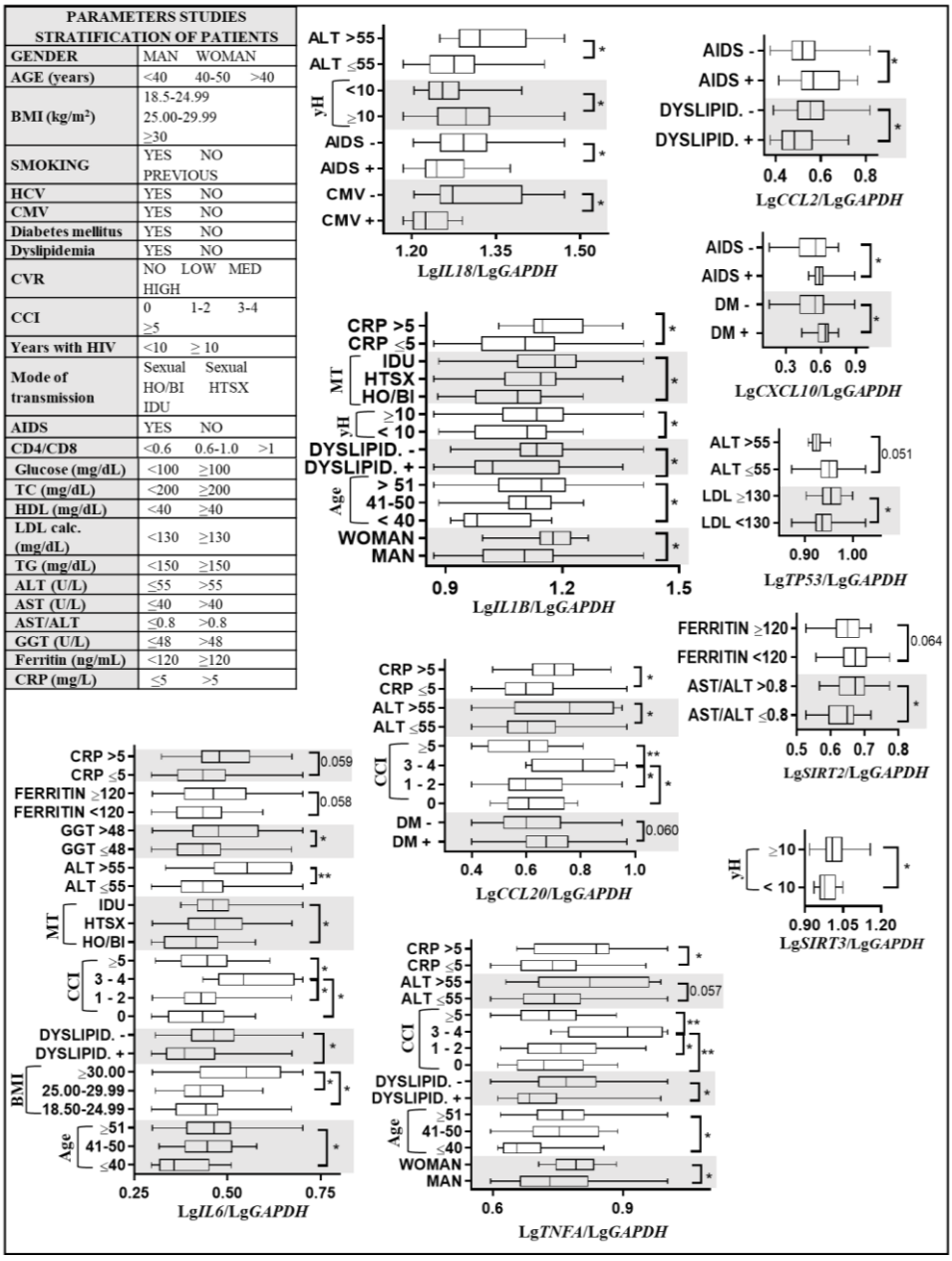


Figure IV.2. mRNA levels of the inflammatory and senescence markers in PBMCs of HIV patients that showed statistically significant differences in relation to different anthropometric, HIV-associated parameters, comorbidities indicators, and blood biochemical parameters. *The classification was performed according to parameters of*

*anthropometry (BMI, gender, age), cigarette smoking, presence of comorbidities, co-infection, development of AIDS, source of infection and duration of antiretroviral treatment. Data (mean ± SEM) of gene expression were calculated as number of copies of the gene of interest normalized with the number of copies of the housekeeping gene (GAPDH). Statistical analysis between groups: Student unpaired t-test as parametric and Mann Whitney test as a non-parametric test (*p<0.05, **p<0.01); for comparing three or more groups: one-way ANOVA multiple comparison test for parametric data followed by a multiple comparison test, Bonferroni post-test and Kruskal-Wallis's test followed by a multiple comparison Dunn's test for non-parametric data (*p<0.05). Data of the genes whose expression did not show statistically significant differences in any of the parameters are not displayed. BMI, body mass index; CCI, Charlson comorbidity index; CMV, cytomegalovirus; CRP, C-reactive protein; CVR, cardiovascular risk; DM, diabetes mellitus, HCV, hepatitis virus C; IDU, injection drug user; MT, mode of transmission refers to HO/BI, patients who got infected through homosexual/bisexual intercourse; HTSX, patients who got infected through heterosexual intercourse; TC, total cholesterol, yH, years with HIV.*

The mode of transmission was also relevant in this analysis, showing that injection drug users had increased *IL6* and *IL1B* gene expression, compared to the patients who got infected through homosexual/bisexual intercourse. Finally, patients that had CRP >5 also had higher expression of *IL6, IL1B, TNFA* and *CCL20*.

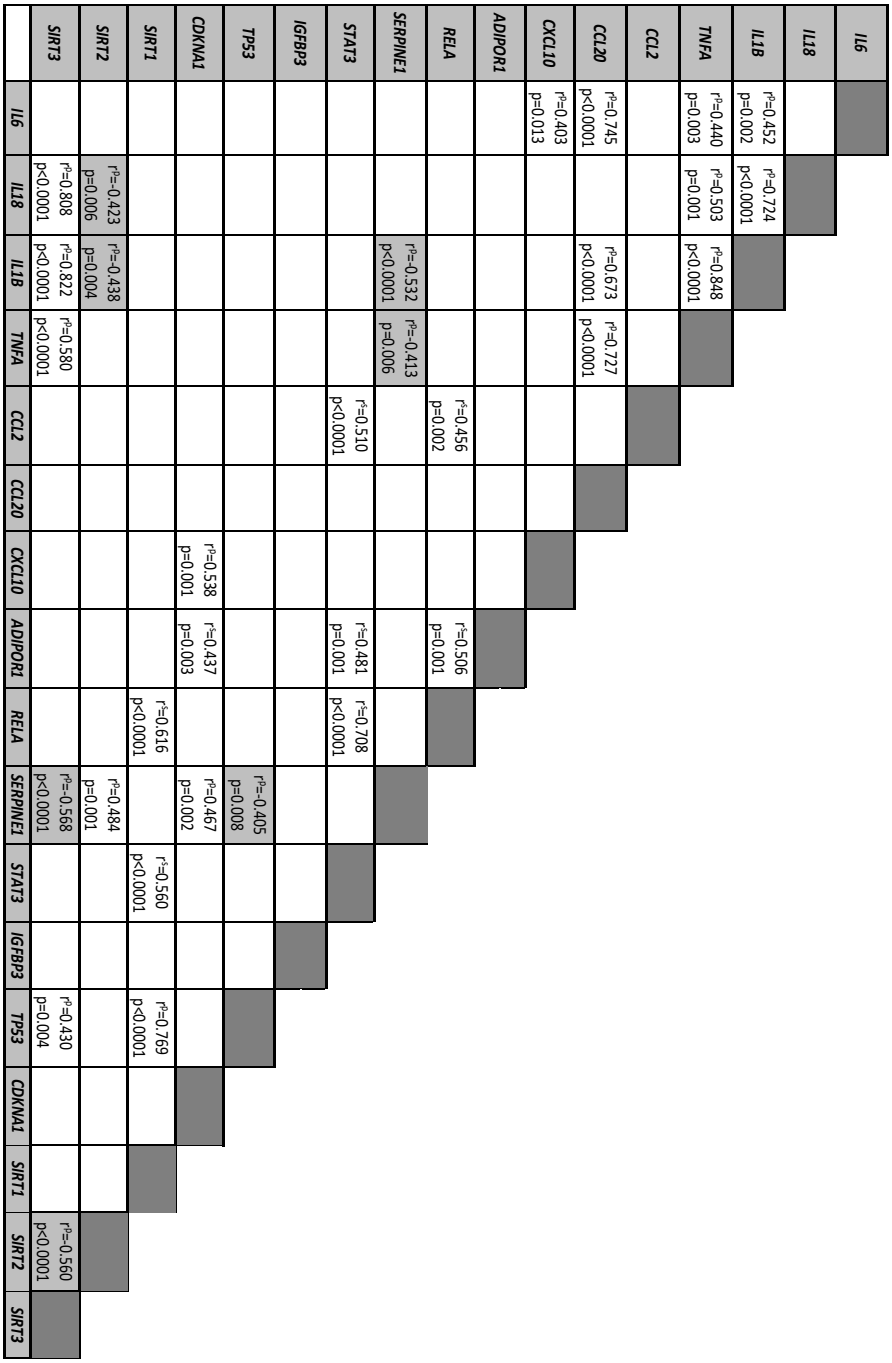
IV.I.4. Correlation between different genes´ expressions in HIV patients and controls

Further, we analysed the existence of correlation between the expressions of different genes in the control population (Figure IV.3.A.) and in HIV patients (Figure IV.3.B.). In the controls, a positive correlation with r>0.4 was found in 25 gene pairs while a negative one was detected in 7 pairs. Among the inflammatory genes, *TNFA* and *IL1B* correlated with each other and with other inflammatory genes including *IL6, IL18* and *CCL20*. Noteworthy, *SERPINE1* displayed correlation with 6 genes including a negative correlation with some inflammatory genes (*IL1B* and *TNFA*). It

also negatively correlated with *TP53* and *SIRT3*, while a positive correlation was detected with *SIRT2* and *CDKNA1*. *CDKNA1* displayed negative correlation with *TP53* and *TP53* was positively correlated with *SIRT1* and *SIRT3*. Regarding the SIRTs, apart from the correlations mentioned before, *SIRT1* was correlated with *RELA* and *STAT3*, *SIRT2* displayed a negative correlation with *IL1B* and *SIRT3*, and *SIRT3* had a positive correlation with various inflammatory markers (*IL18, IL1B, TNFA*).

When the correlations between the expressions of different genes were assessed in the HIV-infected patients (Figure IV.3.B.), a positive correlation with r>0.4 was found in 23 gene pairs and 1 gene pair had a negative one. Inflammatory genes showed highest correlation with *TNFA* and *IL1B*. Apart from being inter-related, they both positively correlated with *IL6* and *CCL20*. Senescence genes also showed high grade of correlation and in this case, 4 genes were very closely related: *SIRT1, TP53, IGFBP3* and *STAT3*. Several differences were detected when we compared the analysis between patients and controls. Of note, *SERPINE1* did not show the correlations it displayed in the control population, except for *CDKN1A*. *SERPINE1* and *TP53* seemed to be associated in a distinct manner. While in the control population *SERPINE1* negatively correlated with *TP53* (r=-0.405), in HIV-infected patients this correlation disappeared (r=0.061).

A


IL6
IL18
IL1B
TNFA
CCL2
CCL20
CXCL10
ADIPOR1
RELA
SERPINE1
STAT3
IGFBP3
TP53
CDKNA1
SIRT1
SIRT2
SIRT3
r²=0.452 p=0.002
r²=0.440 p=0.003
r²=0.745 p<0.0001
r²=0.403 p=0.013
r²=0.724 p<0.0001
r²=0.503 p=0.001
r²=0.848 p<0.0001
r²=0.673 p<0.0001
r²=0.727 p<0.0001
r²=0.456 p=0.002
r²=0.506 p=0.001
r²=0.708 p<0.0001
r²=0.481 p=0.001
r²=0.510 p<0.0001
r²=-0.532 p<0.0001
r²=-0.413 p=0.006
r²=0.538 p=0.001
r²=0.437 p=0.003
r²=0.616 p<0.0001
r²=-0.405 p=0.008
r²=0.467 p=0.002
r²=0.560 p<0.0001
r²=0.484 p=0.001
r²=-0.568 p<0.0001
r²=0.769 p<0.0001
r²=0.430 p=0.004
r²=-0.423 p=0.006
r²=-0.438 p=0.004
r²=0.808 p<0.0001
r²=0.822 p<0.0001
r²=0.580 p<0.0001
r²=-0.560 p<0.0001

B

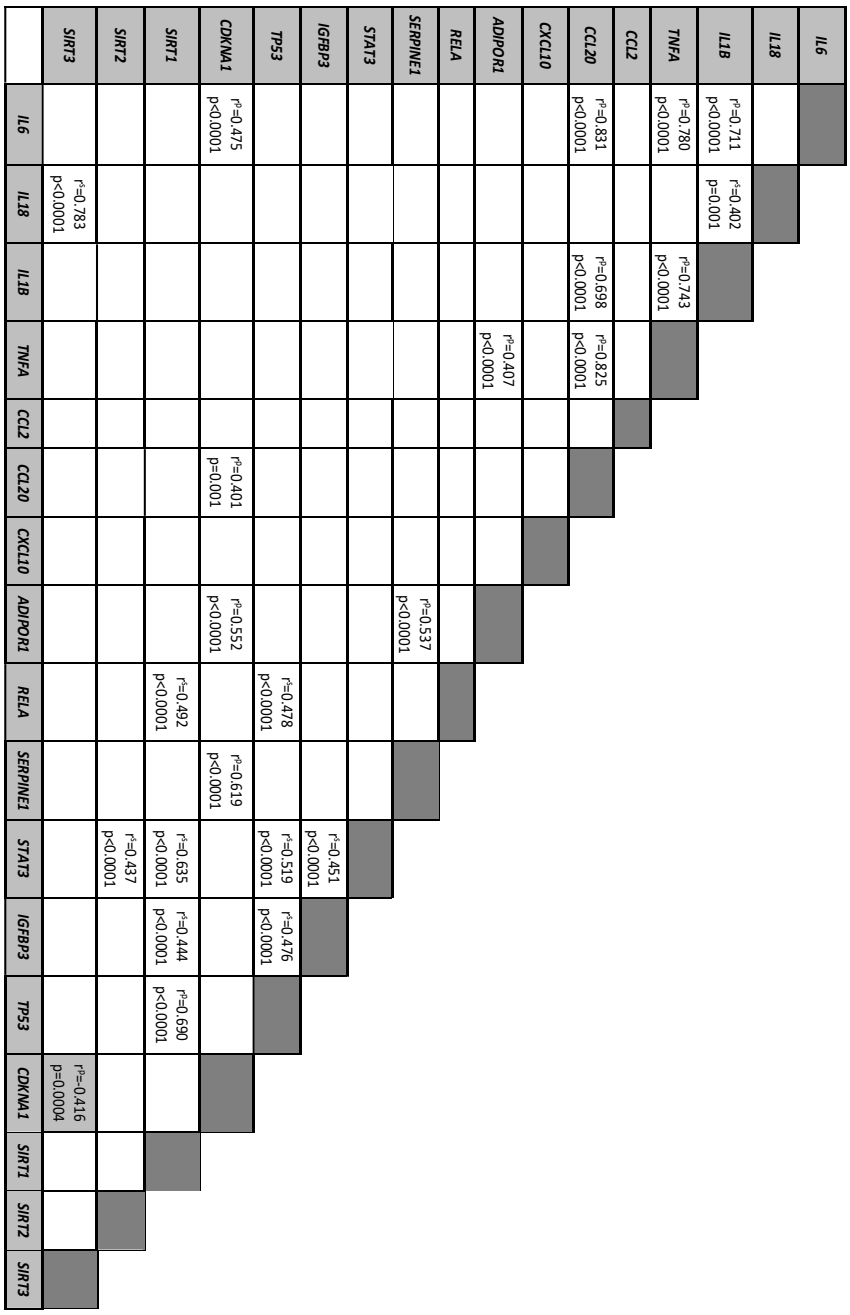


Figure IV.3. Correlations between the expression of various genes in PBMCs of controls (A) and HIV-infected patients (B). *The figure presents the pairs of genes that displayed a*

correlation coefficient >0.4 (Pearson's correlation coefficient (r^p) for parametric and Spearman's correlation coefficients (r^s) for non-parametric data). Negative correlations are shown in shaded boxes. Statistical significance is also shown. The highest correlation in HIV patients between genes was detected between IL6 and CCL20 (r^p=0.831, p<0.0001, n = 69). For controls, n=41-43 except for CXCL10 (n=36); for HIV patients n=66-70.

IGFPB3 did not show any correlations in the control population, but it correlated positively with *TP53*, *STAT3* and *SIRT1* in HIV patients. In summary, these results reveal a dysregulation in the p53-PAI-1 and p53-IGFBP-3 axes in HIV-infected individuals.

IV.I.5. Western blot analysis of several proteins in whole PBMCs extracts

We further analysed some of the markers at protein level by WB using whole-cell protein extracts. As mentioned before, STAT3 is a transcription factor and its activity is regulated post-translationally rather than on a transcriptional level. For that matter, we assessed the protein content of its active (phosphorylated) form and discovered that HIV patients did not display altered pSTAT3 (quantified as pSTAT3/STAT3 ratio, Figure IV.4.A.). Conversely, the decreased gene expression of *TP53* and *SERPINE1* in HIV patients was also corroborated at protein level (Figure IV.4.A.). Analysis of the correlation between the protein content of p53 and PAI1 revealed that while in uninfected controls, there was no correlation (r^s=-0.1608), p53 and PAI1 were positively correlated (r^s=0.4176) in HIV patients (Figure IV.4.B). This result again points to the presence of altered p53-PAI1 signalling in HIV individuals.

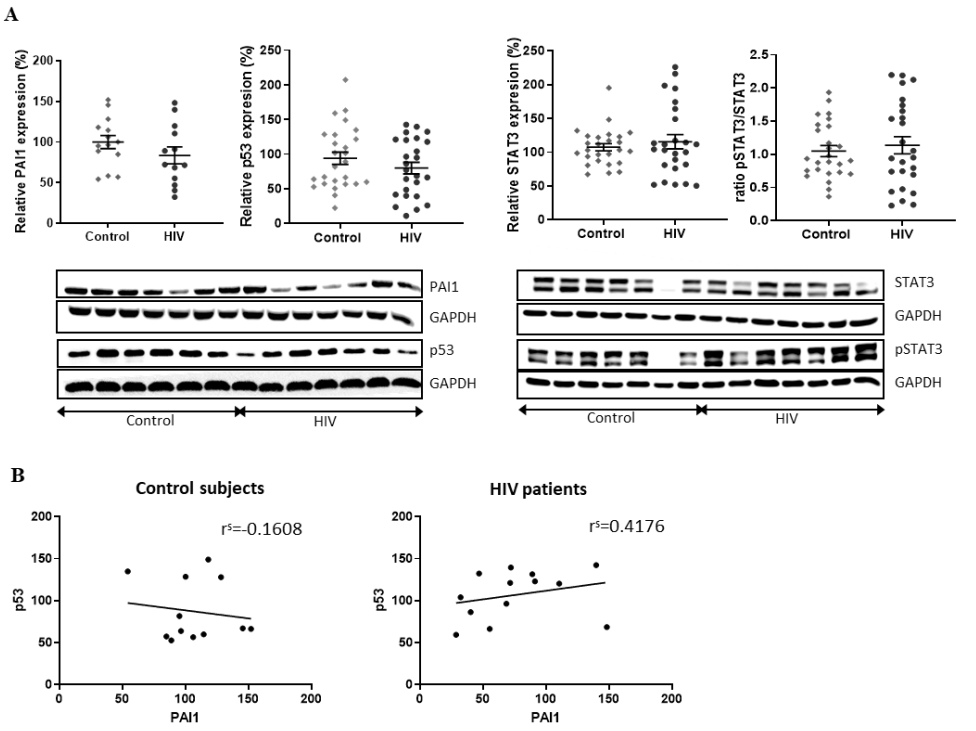


Figure IV.4. Western blot analysis of p53, PAI1, STAT3 and pSTAT3 protein expression in whole-cell extracts from PBMCs obtained from HIV patients and uninfected controls. ***A.*** *Graphical representations of the quantified data and representative images are shown. Data represent mean ± SEM (n=13 and n=14 for PAI1, n=26 and n=25 for p53, STAT3 and pSTAT3, in control and HIV patients respectively) and were calculated as % of control (mean value of the protein expression in control group was considered 100%). GAPDH was used as loading control. Statistical analysis between groups: Student´s unpaired t-test, p<0.05 was considered statistically significant.* ***B.*** *Graph representing correlation between the expression of PAI1 and p53 in control subjects and HIV patients.*

The lower expression of SIRT1 in HIV patients was also corroborated at the protein level (Figure IV.5.). Histones are among the main targets of the deacetylating activity of SIRT1. We found that HIV patients have higher levels of the acetylated form of

histone H3 (H3K9) (Figure IV.5.), which is in line with the finding of lower SIRT1 expression in these individuals.

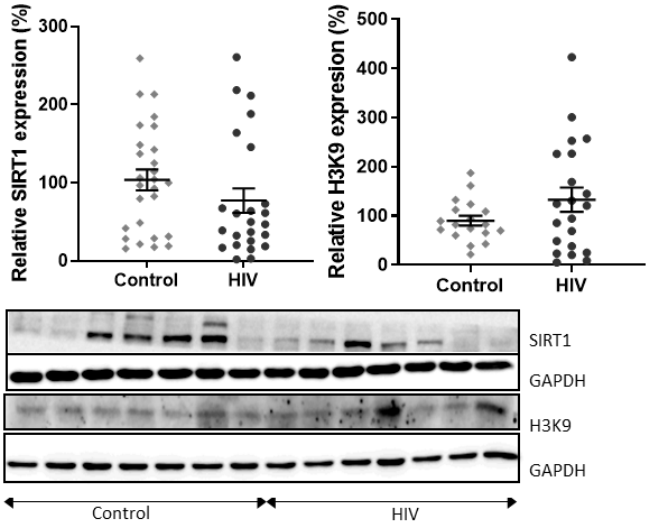
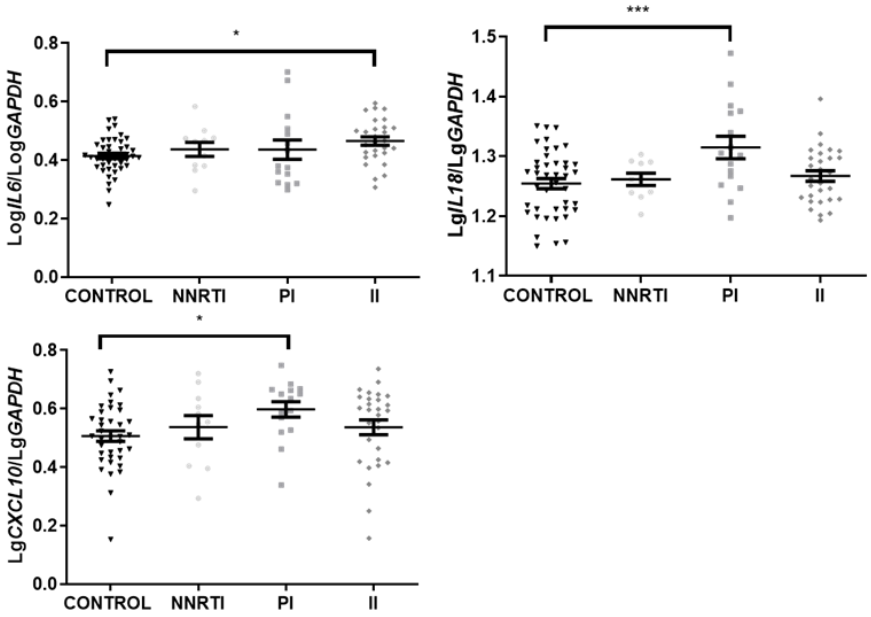


Figure IV.5. Western blot analysis using whole-cell protein extracts for SIRT1 and acetylated histone 3 (H3K9). *Graphical representations of the quantified data and representative images are shown. Data represent mean ± SEM (n=27 and n=24 for SIRT1; n=18 and n=21 for H3K9, in control and HIV patients respectively) and were calculated as % of control (mean value of the protein expression in control group was considered 100%). GAPDH was used as loading control. Statistical analysis between groups: Student´s unpaired t-test, $p<0.05$ was considered statistically significant.*

IV.I.6. Expression of inflammation and senescence markers in HIV patients in relation to cART

The chronic exposure to cART has also been attributed a role in the development of age-related pathologies. In light of this, we analysed the expression of the aforementioned panel of genes in relation to the current treatment (received for at least 6 months). While all patients had NRTI as backbone (emtricitabine/tenofovir, abacavir/lamivudine) with or without booster drug (cobicistat), they differed in the

additional drug in their cART and could thus be grouped in those who received a NNRTIs (efavirenz, rilpivirine or etravirine), PIs (ritonavir, atazanavir or darunavir) and IIs (raltegravir, dolutegravir or elvitegravir). Patients treated with more than one drug from different pharmacological groups were not analysed. Figure IV.6. displays the results that were statistically significant in the genes whose expression increases or decreases in HIV patients compared to uninfected controls. While we did not detect any particular pattern in the inflammatory genes (*IL6*, *IL18* and *CXCL10*), in the case of those related to senescence/aging (*TP53*, *SERPINE1*, *IGFBP3*), NNRTIs were the most affected drug class. Of note, *SIRT1* mRNA levels were lower in all HIV patients independently of their current cART.



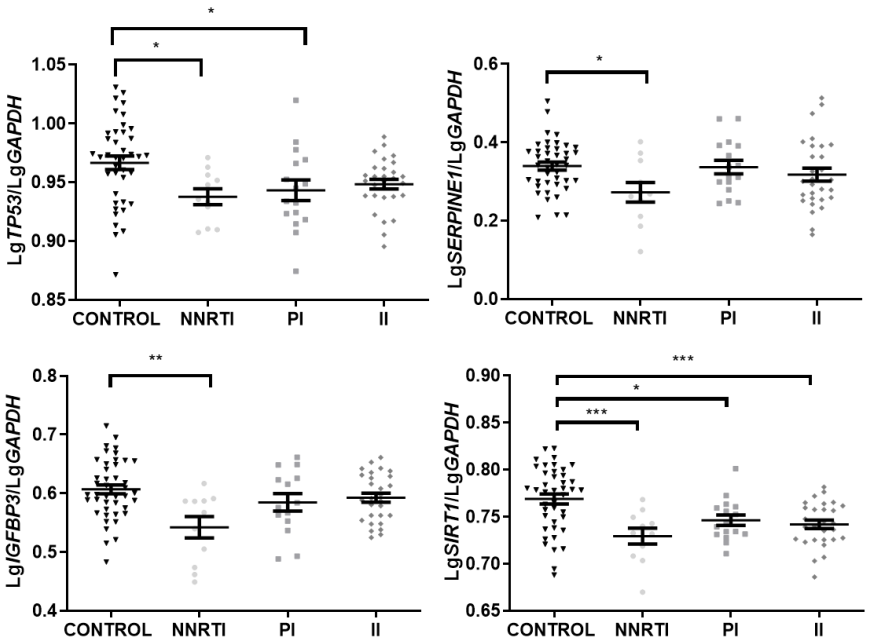


Figure IV.6. mRNA levels (IL6, IL18, CXCL10, SERPINE1, IGFBP3, TP53 and SIRT1) in PBMCs of uninfected controls and patients previously grouped considering their present cART. *Data were mean ± SEM, for Control n=42-43 except for n=38 (CXCL10), n=40 (TP53); for NNRTIs n=10-11; for PIs n=15-16 and for IIs n=28-29. Gene expression was calculated as number of copies of the gene normalized with the number of copies of the housekeeping gene (GAPDH). Statistical analysis between groups: one-way ANOVA multiple comparison test for parametric data followed by a multiple comparison test, Bonferroni post-test and Kruskal-Wallis test followed by a multiple comparison Dunn's test for non-parametric data (*p<0.05, **p=0.01, ***p<0.001 vs control).*

IV.I.7. The expression of inflammation and senescence genes in relation to the different cell type population within PBMCs

Next, we evaluated the correlation of inflammation and senescence gene expression with the mRNA levels of *CD4*, *CD8A*, *CD14* and *CD19* (Table IV.5.). We found that *IL18* correlated positively with all PBMCs subpopulation markers in the control

group, except for *CD4*, while in the HIV group, *IL18* maintained the correlations with *CD14* and *CD19*. The rest of the specific inflammatory markers showed correlations in the control population that were absent in the patients group. The levels of *SERPINE1* mRNA lacked correlation with either *CD4* or *CD8A* in both controls and patients. *IGFBP3* only showed correlation with *CD8A* and only in HIV patients (r^s=0.499, p<0.0001). Interestingly, no correlation was detected between *TP53* and *CD4* in neither controls nor patients, while a positive correlation was observed in both controls and patients between *TP53* and *CD8A* to a similar extent (r^p=0.458, p=0.002 and r^p=0.524, p<0.0001 respectively). These may be expected results given that CD8 lymphocytes undergo significant replicative senescence. Nevertheless, it does not explain/account for the finding of *TP53* and other senescence-related genes expression being diminished in PBMCs from HIV patients considering that HIV patients have more CD8 cells than uninfected individuals. In addition, *SIRT1/CD4* and *SIRT1/CD14* did not show correlation in neither HIV patients nor controls. Interestingly, uninfected individuals exhibited moderate positive *SIRT1*/*CD8A* and *SIRT1*/*CD19* correlations (r^p=0.634, p<0.0001 and r^p=0.599, p<0.0001, respectively), while these correlations had r^p<0.4 in HIV patients. *SIRT2* showed negative correlation with *CD14* in the control group, while this correlation was absent in HIV patients and a new correlation appeared with *CD4*. *SIRT3* maintained similar correlations with *CD14* and *CD19* in both control and HIV groups; however, there was positive correlation with *CD8A* in the patient group (r^p=0.502 p<0.0001) that was not present in the control group. Whether these correlations between sirtuins and specific leukocytes markers are causal needs to be further explored.

Table IV.5. Correlation between the expression of various genes and PBMCs subpopulation markers in controls (A) and HIV-infected patients (B). *Only the correlation coefficients>0.4 are shown - Pearson's correlation coefficient (r^p) for parametric and Spearman's correlation coefficient (r^s) for non-parametric data. Statistical significance is also shown (n=37-43 for the control group and n=65-70 for the HIV group).*

A

	CD4	*CD8A*	*CD14*	*CD19*
IL18	r^s=-0.432 p=0.004	r^p=0.436 p=0.003	r^s=0.503 p=0.001	r^p=0.692 p<0.0001
IL1B			r^s=0.609 p<0.001	r^p=0.532 p<0.0001
TNFA			r^s=0.434 p=0.004	
CXCL10			r^s=-0.481 p=0.003	
SERPINE1			r^s=-0.632 p<0.001	r^p=-0.532 p<0.0001
TP53		r^p=0.458 p=0.002		r^p=0.698 p<0.0001
SIRT1		r^p=0.634 p<0.0001		r^p=0.599 p<0.0001
SIRT2			r^s=-0.602 p<0.0001	
SIRT3			r^s=0.598 p<0.0001	r^p=0.804 p<0.0001

B

	CD4	*CD8A*	*CD14*	*CD19*
IL18			r^s=0.616 p<0.0001	r^s=0.554 p<0.0001
SERPINE1			r^p=-0.448 p=0.0001	
IGFBP3		r^s=0.499 p<0.0001		
TP53		r^p=0.524 p<0.0001		r^p=0.438 p<0.0001
SIRT2	r^s=0.556 p<0.0001			
SIRT3		r^p=0.502 p<0.0001	r^p=0.627 p<0.0001	r^p=0.655 p<0.0001

SECTION II. ANALYSIS OF THE MOLECULAR MECHANISMS INVOLVED IN THE HEPATOPROTECTIVE EFFECTS OF THE ANTIRETROVIRAL DRUG RILPIVIRINE

IV.II.1. Anthropometric characteristics and general blood analysis of chronic liver disease patients

The characteristics of the patients from the second cohort are represented in Table IV.6. Regarding the etiology of the CLD, the majority of patients had HCV infection (34.2%), followed by the patients that suffered from a hepatic disease as a result of persistent alcohol consumption (21.5%), and the same number of patients were into mixed etiology group, meaning that they were affected at the same time by at least two of the chronic liver conditions studied here. Seven patients were diagnosed with NASH, one was HBV-infected and no data were available for another patient.

As for the presence of metabolic comorbidities, 34.2% had arterial hypertension, 36.8% were diabetic and 14 of them suffered from dyslipidemia. The data about hepatic fibrosis detected by FibroScan® showed that 39.5% had the lowest grade, F0-F1, while 23.7% were F2 and 31.6% had the most severe, F3-F4 grade.

Table IV.6. Characteristics of the patients with chronic liver disease. *Variables include general characteristics (anthropometry, tobacco and alcohol use), disease etiology, comorbidities-related parameters and liver fibrosis-related parameters. Data from general biochemical and hematological analyses are also included. Values are expressed as number of patients and percentage of total number of patients for non-numerical data, and median (1^{st} and 3^{rd} percentiles) for numerical data. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; CRP, C-reactive protein; GGT, gammaglutamyl transferase; INR, international normalized*

ratio; Hg, hemoglobin; HBV, hepatitis virus B; HCV, hepatitis virus C; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TC, total cholesterol, TG, triglycerides.

GENERAL CHARACTERISTICS OF THE PATIENTS						
GENDER	MALE 28 (72.5%)	FEMALE 10 (27.5%)				
AGE (years) median (IQR)	58.5 (55.0 – 68.3)					
BMI (kg/m^2) median (IQR)	26.5 (24 – 31)					
SMOKING	YES 8 (21.1%)	NO 22 (57.9%)	PREVIOUS SMOKER 8 (21.1%)			
ALCOHOL USE	YES 9 (23.7%)	NO 23 (60.5%)	PREVIOUS 6 (15.8%)			
ETIOLOGY	NASH 7 (18.4%)	ALCOHOL 8 (21.5%)	HCV 13 (34.2%)	HBV 1 (2.6%)	MIXED 8 (21.5%)	OTHER 1 (2.6%)
COMORBIDITIES-RELATED PARAMETERS						
ARTERIAL HYPERTENSION	YES 13 (34.2%)	NO 25 (65.8%)				
DIABETES MELLITUS	YES 14 (36.8%)	NO 24 (63.2%)				
DYSLIPIDEMIA	YES 14 (36.8%)	NO 24 (63.2%)				
LIVER FIBROSIS-RELATED PARAMETERS						
FIBROSIS (grade)	F0-F1 15 (39.5%)	F2 9 (23.7%)	F3-F4 12 (31.6%)	NO DATA 2 (5.3%)		
ELASTICITY (kPa) median (IQR)	7.80 (5.45 – 11.35)					
CAP (dB/m) median (IQR)	278 (212.8 – 305)					
HEPATIC CIRRHOSIS	COMPENSATED 11 (28.9%)	DECOMPENSATED 6 (15.8%)	NO 21 (55.3%)			

BIOCHEMICAL ANALYSIS		
PARAMETER	**MEDIAN (IQR)**	**REF.**
Glucose (mg/dL)	104.5 (93-136.8)	64-106
Urea (mg/dL)	38.5 (31.3-47.5)	20-50
Creatinine (mg/dL)	0.8 (0.7-1)	0.51-0.95
Ferritin (ng/mL)	128 (65.3-226)	10-120
TC (mg/dL)	175.5 (155-204.3)	140-200
HDL cholesterol (mg/dL)	51 (46-63.5)	>35
LDL calculated (mg/dL)	109.5 (93.3-133.8)	<130
TG (mg/dL)	89 (75.3-131.8)	40-160
ALT (U/L)	31 (18.3-56.8)	1-31
AST (U/L)	32 (27-45.5)	1-31
GGT (U/L)	67 (31-101.8)	1-38
ALP (mU/mL)	85 (64.3-102.5)	30-120
Hg (g/dL)	14.4 (13.5-15.9)	11.2-15.5
Bilirubin (mg/dL)	0.6 (0.5-0.8)	0.1-1.0
Albumin (g/dL)	4.4 (4.2-4.6)	3.5-5.2
INR	1.04 (1-1.1)	0.85-1.35
CRP (mg/L)	2.1 (1.1-4)	0-5

PARAMETER	HEMATOLOGICAL ANALYSIS MEDIAN (IQR)	REF.
Leucocytes *10^9/L	5.46 (4.9-7.9)	3.9-11
Neutrophils *10^9/L	3.1 (2.2-4.5)	2.5-7.5
Lymphocytes *10^9/L	1.7 (1.3-2.4)	1.5-4.5
Monocytes *10^9/L	0.5 (0.4-0.7)	0.2-0.8
Eosinophils *10^9/L	0.1 (0.1-0.25)	0.05-0.5
Basophils *10^9/L	0.04 (0.02-0.06)	0.01-0.15
Platelets *10^9/L	176 (112.8-253.5)	140-400

Liver stiffness (LS) (elasticity) measurement is a widely used non-invasive tool for the diagnosis of LF with high accuracy. It is quantified by ultrasound based transient elastography using FibroScan® and is not only sensitive to fibrosis but is also affected by various factors such as congestion and inflammation (221). LS is normally between 2 and 6 kPa and in the present cohort of hepatic patients this value is above the normal range. Controlled attenuation parameter (CAP) quantifies liver steatosis; the parameter measured is ultrasound attenuation, expressed in dB/m. It describes the decay of the ultrasound signal according to depth. The higher the steatosis load in the liver, the greater this decay. Values range from 100 to 400 dB/m and in this cohort, the median value was 278 dB/m.

As mentioned before, cirrhosis is a late-stage CLD. There are 2 clinical stages of cirrhosis: compensated and decompensated. Patients with compensated cirrhosis are asymptomatic and overall have median survival times of more than 12 years. Patients with decompensated cirrhosis have at least one complication including ascites, jaundice, variceal hemorrhage or hepatic encephalopathy, and overall, they have a median survival time of 2 years. In this study, we had data about 17 patients with cirrhosis: 11 patients with compensated and 6 with decompensated cirrhosis. Regarding the biochemical and hematological parameters of all the patients, all median values were within the range considered normal, except for the ferritin and GGT levels, whose values were above the upper limit of the normal range.

IV.II.1.1. Correlation between different parameters

We also explored the correlation between different parameters in the second cohort and those patients' parameters that displayed correlation coefficient of r>0.4 are shown in Table IV.7.

***Table IV.7. Correlations between different parameters of the studied patients with chronic liver disease**. All liver fibrosis-related parameters, biochemical and hematological parameters (shown in Table IV.7.) were assessed for the presence of correlations and this table presents the pairs of parameters that displayed a correlation coefficient >0.4 (Pearson's correlation coefficien –r^p for parametric data and Spearman´s correlation coefficient -r^s, for non-parametric data). For reasons mentioned in section IV.I.1.1., "Total Cholesterol/LDL" (r=0.917), is not included in the table and since fibrosis grade is calculated from the elasticity parameter, "Elasticity/Fibrosis" (r=0.939) is also not included. Negative correlation is shown in shaded boxes. Statistical significance is also shown (n=34-38). Bonferroni correction for multiple association was performed and p<0.002 was considered statistically significant. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gammaglutamyl transferase; INR, international normalized ratio; Hg, hemoglobin; HDL, high density lipoproteins TC, total cholesterol.*

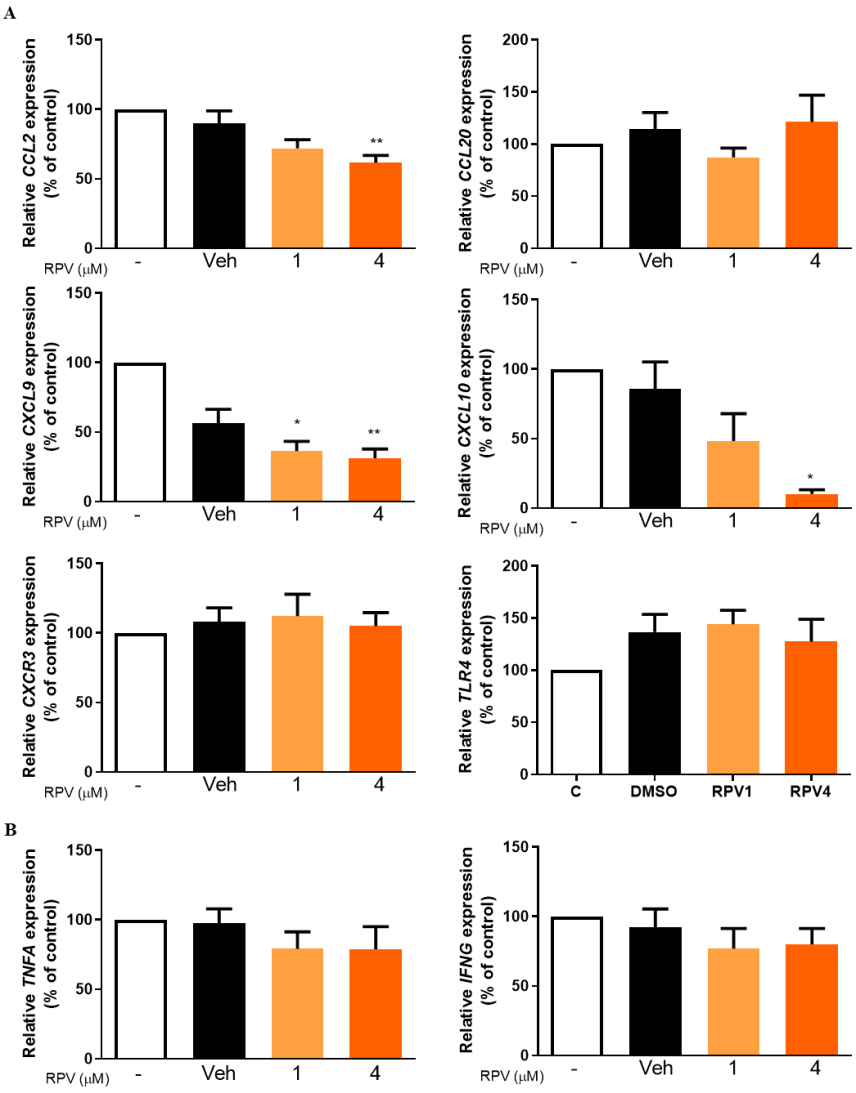
	Age	Elasticity	Fibrosis	Hg	TC	ALT	ALP	Bilirubin	Eosinophiles	Basophils	Platelets
Glucose	r^s=0.583 p<0.001										
HDL					r^p=0.543 p=0.001						
AST		r^s=0.583 p<0.0001				r^s=0.820 p<0.0001					
GGT		r^s=0.630 p<0.0001	r^s=0.603 p<0.0001								
ALP		r^s=0.523 p=0.001	r^s=0.525 p=0.001					r^s=0.512 p=0.001			
Albumin											r^s=0.652 p<0.0001
Lymphocytes											r^p=0.532 p=0.001
Monocytes				r^p=0.533 p=0.001							r^p=0.632 p<0.0001
Platelets		r^s=-0.531 p=0.001	r^s=-0.562 p=0.001						r^s=0.448 p<0.0001	r^s=0.636 p<0.0001	
INR							r^s=0.531 p=0.001	r^s=0.517 p=0.001	r^s=-0.734 p=0.001	r^s=-0.578 p<0.0001	r^s=-0.585 p<0.0001

As expected, liver transaminases and ALP showed positive correlation between them and were also correlated with elasticity and the grade of fibrosis. ALP also showed positive correlation with bilirubin (r^s=0.512, p=0.001) and international normalized ratio (INR) (r^s=0.531, p=0.001). The INR is an international standard for the prothrombin time test and measures the time needed for a clot to form in a blood sample. The number of platelets was correlated with another 8 parameters, apart from albumin, positive correlations were present with the hematological parameters (number of lymphocytes, monocytes, eosinophiles and basophils), while negative correlations were noted with INR, elasticity and LF.

IV.II.2. Treatment of PBMCs with RPV *ex vivo*

Nowadays, RPV is the most frequently used NNRTI and, considering the previous studies where anti-inflammatory and anti-fibrotic effects in mouse livers were demonstrated, we aimed to uncover the molecular mechanisms responsible for these hepatoprotective effects. For this, we chose to study the regulation of some of the inflammatory genes that showed different expression in controls and patients in the previous cohort in this thesis (section IV.I.2.), as well as other genes encoding for proteins closely related to STAT1 and STAT3 signalling. In order to do this, we isolated PBMCs from the CLD patients (described in section IV.II.1.) and treated them with clinically relevant concentrations of RPV (1 and 4 μM) *ex vivo* for 24 h (Figure IV.7.). We studied the gene expression of several chemokines including *CXCL10* and its receptors *CXCR3* and *TL4*, *CXCL9*, *CCL2* and *CCL20* (Figure IV.7.A). Concentration-dependent decrease in *CXCL10* and *CXCL9* mRNA levels was observed, with no change in their receptor *CXCR3*. Next, we explored gene expression of the pro-inflammatory cytokines TNFα and IFNγ and no effect on mRNA levels was observed after the *ex vivo* treatment, except for the pro-fibrotic marker *SERPINE1*, whose expression was downregulated with RPV (Figure IV.7.B). Finally, different anti-inflammatory mediators were analysed and a

concentration-dependent tendency for an increase in their expression was observed in *IL6* and *IL22*, being statistically significant for *IL10* (Figure IV.7.C).



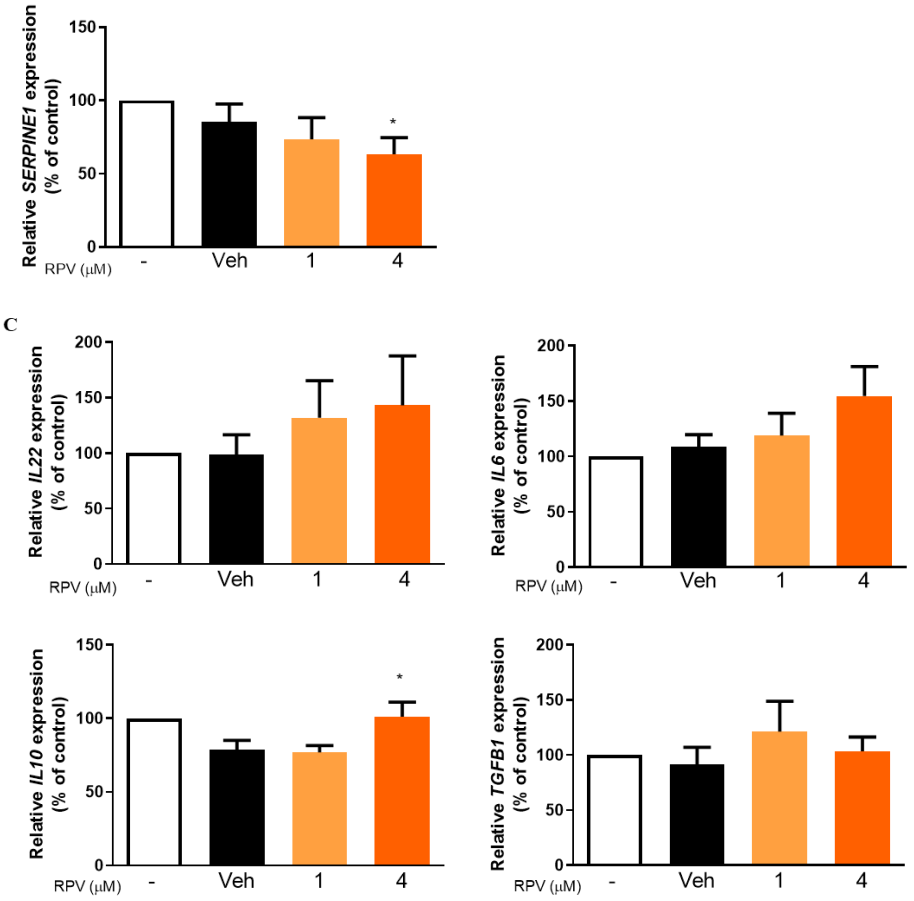


Figure IV.7. Expression of selected inflammation and immunoregulatory genes in PBMCs isolated from CLD patients after treatment with RPV ex vivo. A. *Chemokines and chemokine receptors.* ***B.*** *Cytokines and other pro-inflammatory mediators.* ***C.*** *Anti-inflammatory mediators. Cells were treated for 24h with RPV (1 and 4 μM), vehicle (DMSO) or left untreated (control). CXCL10, CCL20 n=7; TLR4, n=8; TGFB n=9; IFNG n=10; CXCL9, IL22, TNFA n=11; IL6, SERPINE1 n=12; IL10 n=14; CCL2 n=16; CXCR3 n=17. Relative mRNA expression was analysed by qRT-PCR. Data (mean ± SEM) were normalized versus the expression of the housekeeping gene GAPDH and expressed as percentage of control (gene expression in the untreated cells were considered 100%). Statistical analysis was performed by repeated measures (RM) one-way ANOVA with Bonferroni's multiple comparisons test (*p<0.05, **p<0.01, vs Veh).*

In addition, we studied the activation of STAT1 and STAT3 by WB, assessing the ratio between phosphorylated STAT1 (pSTAT1) and phosphorylated STAT3 (pSTAT3) and the total STAT1 or STAT3 protein, respectively (Figure IV.8). These experiments showed that RPV significantly diminished the activation of STAT1 protein and slightly upregulated the activation of STAT3 in these cells, which is in line with the results shown previously. Apart from STAT1 and STAT3 protein levels, we also studied one regulator of NF-κB transcription factor, IκBα, whose protein expression showed tendency to increase in the RPV-treated PBMCs, confirming again the anti-inflammatory effect of RPV in this model.

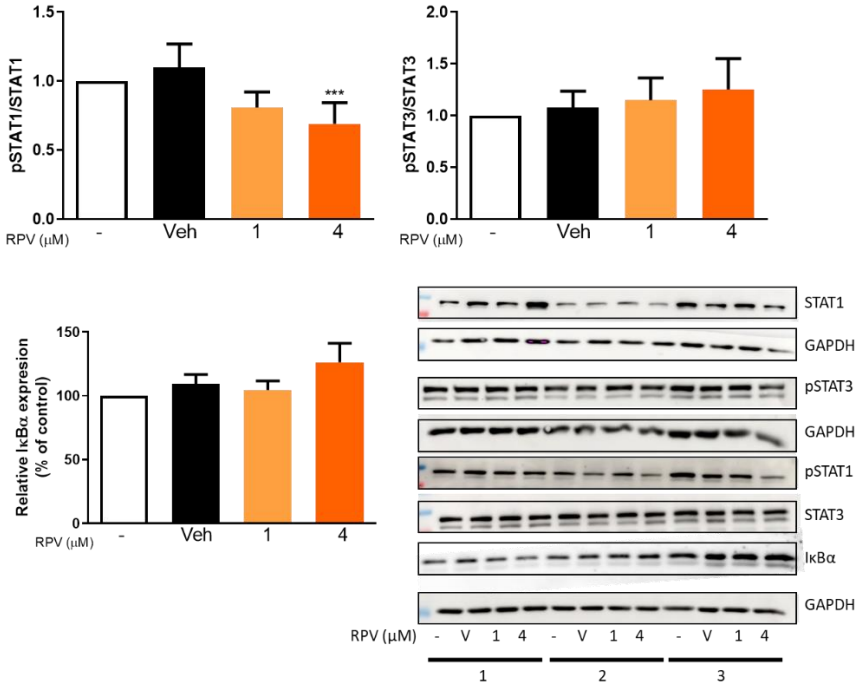


Figure IV.8. Western blot analysis of STAT1, pSTAT1, STAT3, pSTAT3 and IκBα in whole-cell protein extracts from PBMCs isolated from CLD patients after treatment with RPV ex vivo*. Cells were treated for 24 h with RPV (1 and 4 μM), vehicle (DMSO) or left untreated (control) (n=13-17). 1, 2, 3 are three different patients. Graphical representations of the quantified data and representative images are shown. Data (mean ± SEM) were*

*calculated as % of control (protein expression in the untreated cells was considered 100%) and normalized versus the levels of GAPDH which was used as loading control. Statistical analysis was performed by repeated measures (RM) one-way ANOVA with Bonferroni's multiple comparisons test (***p<0.001, vs Veh). V, vehicle.*

IV.II.3. Effects of RPV on the inflammatory responses in mouse models of CLD

In order to better understand the anti-inflammatory effect that RPV showed *in vivo* in previous studies by our group (56) and in the *ex vivo* treated PBMCs, a transcriptomic analysis was performed in liver samples obtained from HFD and HFD+RPV mice, in a NAFLD mouse model of chronic liver injury. During the bioinformatic analysis, a gene set enrichment analysis was performed in order to detect significantly up- or down-regulated blocks of functionally related genes, grouped as biological processes of functions. The comparison of the data between the two groups revealed 1536 significant (p<0.05) "GO biological process propagated". Figure IV.9. represents the top 30 statistically significant GO terms related to inflammation, leukocyte and lymphocyte activation, and cell migration, sorted by the negative log of the adjusted p value (the longer the bar the smaller the p value). The term size is also represented next to the corresponding graph bar. All these processes had negative log odds ratio (LOR), meaning down-regulation of the functional class in HFD+RPV group. The process with the smallest p value was "inflammatory response (GO:0006954)", that had a "term size" (number of genes grouped) of 452 genes, which is amongst the 5 GO terms with highest number of functionally related genes significant in this study. The first 3 are "response to cytokine (GO:0034097)" with 474 genes, followed by "lymphocyte activation (GO:0046649)" with 464 and "regulation of cell migration (GO:0030334)" with 459.

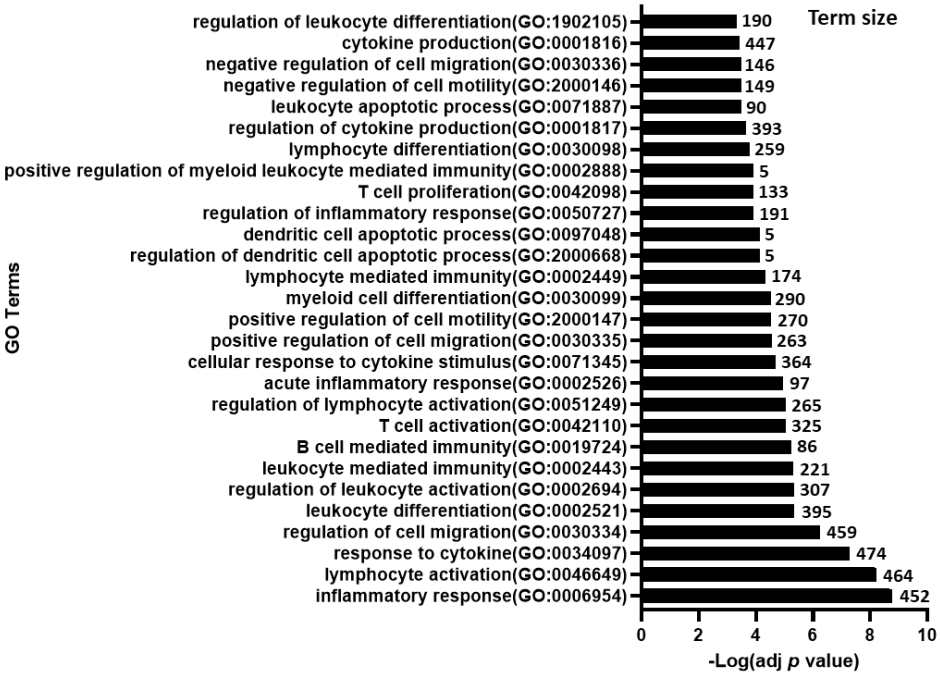


Figure IV.9. Transcriptomic analysis of whole liver samples from a NAFLD mouse model related to inflammation. *Data were obtained from bioinformatics gene set enrichment analysis performed after transcriptomic analysis of whole-liver tissue RNA from HFD and HFD+RPV mice groups (n=3).*

As the transcriptomic analysis revealed the presence of a number of down-regulated biological processes that were associated with lymphocyte activation and cell migration, we hypothesized that chemokines may have a potential role in the molecular mechanisms responsible for the hepatoprotective effects of RPV. Considering the results in Figure IV.7.A. and the importance of CXCL10 as a recruiter of immune cells to the liver parenchyma, thus promoting hepatic inflammation, we next analysed the CXCL10/IP-10 protein levels in the same mouse model of HFD-induced liver injury (Figure IV.10.A.).

It was observed that there was no significant alteration of CXCL10 expression in the livers of the animals fed with HFD compared to those with ND, however RPV significantly reduced CXCL10 protein expression in the HFD+RPV group. In addition, protein levels of this chemokine were analysed in other two mouse models of liver injury, CCl_4 and BDL. Hepatic expression of CXCL10 was elevated in Veh+CCl_4 and Veh+BDL group compared to their control groups respectively and RPV reduced hepatic CXCL10 protein expression in the two models employed (Figure IV.10.B and C).

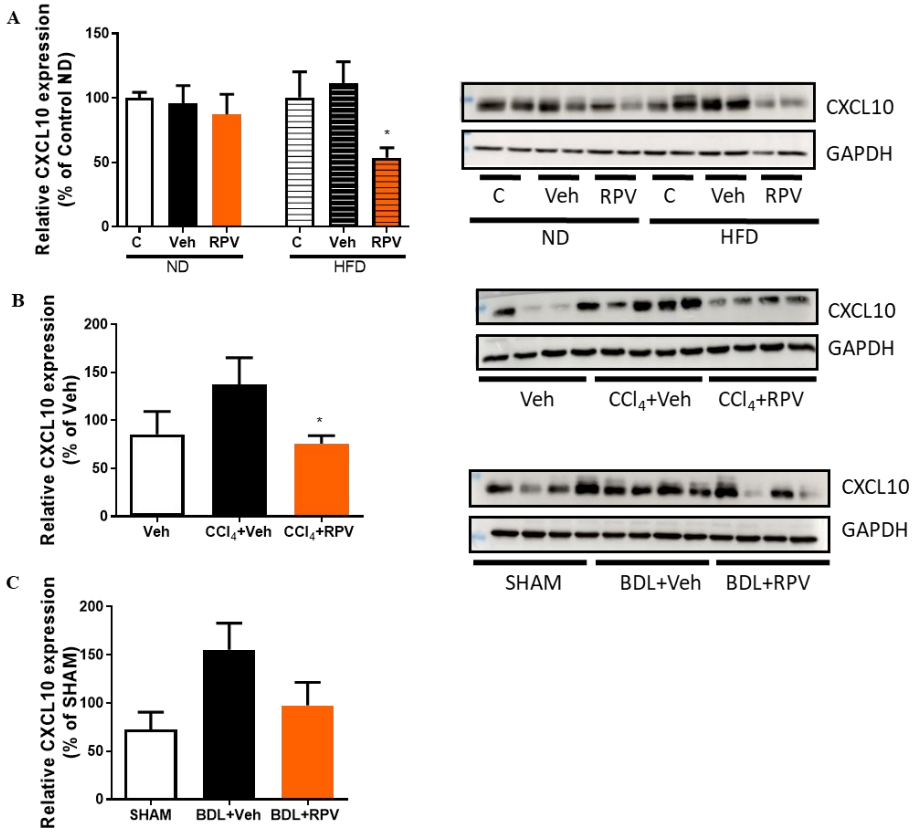


Figure IV.10. Western blot analysis of hepatic CXCL10 protein levels in mouse models of (A) HFD-, (B) CCl_4- and (C) BDL-induced liver injury. *Densitometry of the WB analysis (n=7-8) and representative images are shown. Data (mean ± SEM) were analysed by a*

*Student's t-test: *p<0.05 vs Veh-HFD (A) or vs CCl_4+Veh group (B). C, control; ND, normal diet; HFD, high fat diet.*

IV.II.4. Effect of RPV on CXCL10 and STAT1 expression in Hep3B cells

According to the existing literature about STAT1 regulation, which points out that one of its most prominent targets is CXCL10, and the previously obtained results by our group (56), we hypothesized that part of the hepatoprotective effects of RPV are related to the downregulation of STAT1 activation in hepatocytes, thus leading to decreased *CXCL10* expression. In order to prove this, an *in vitro* study was performed, where Hep3B were pre-treated for 24 h with a relatively low concentration of IFNγ (8 ng/mL), one of the main activators of STAT1, and 2 or 4 μM of RPV were added for another 24 h.

The activation of STAT1 was studied through its translocation to the nucleus and the expression and secretion of CXCL10. As it can be observed in Figure IV.11., RPV significantly reduced mRNA expression (Figure IV.11.A) and extracellular levels of this chemokine (Figure IV.11.B). In the presence of IFNγ, its expression was stimulated, while it was slightly reduced with RPV's treatment. These effects were paralleled with a modest decrease in the nuclear translocation of STAT1 (Figure IV.11.C).

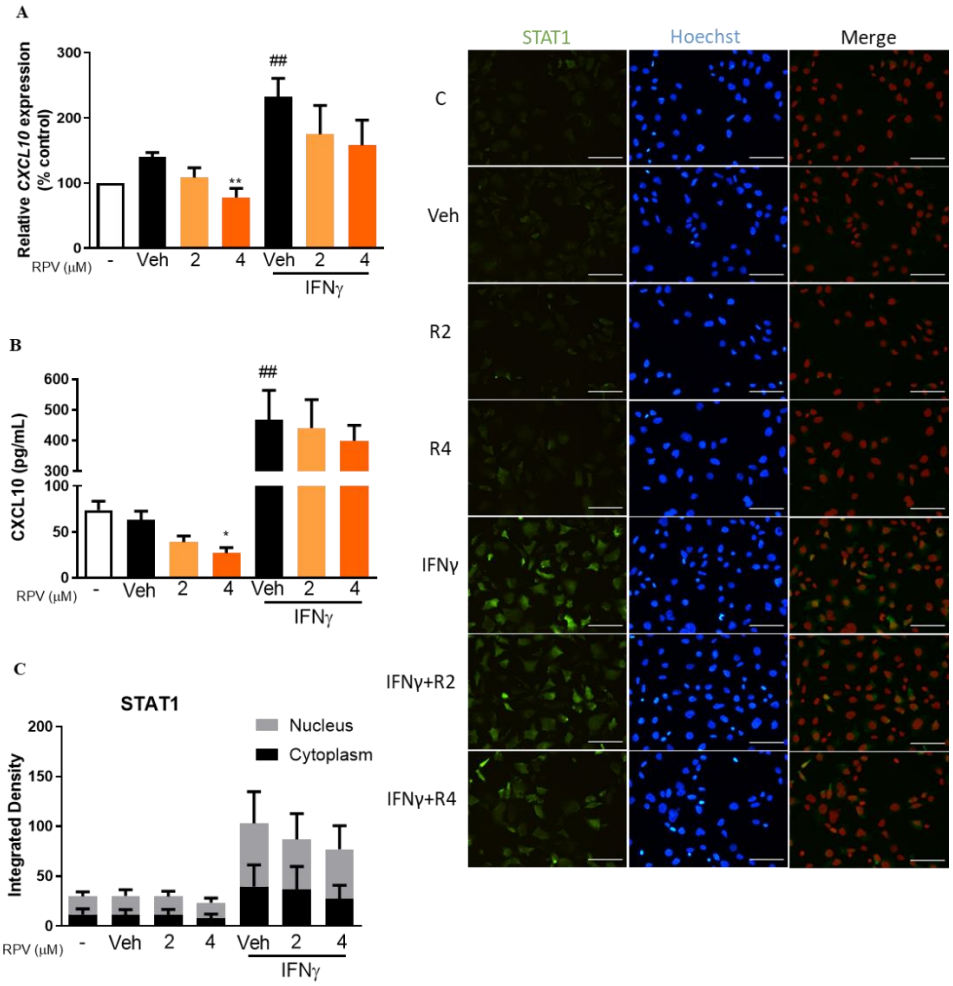


Figure IV.11. Effect of RPV on CXCL10 and STAT1 expression in Hep3B cells. *Cells were pre-treated with IFNγ for 24 h and RPV or Veh (DMSO) were added for additional 24 h.* ***A.*** *qRT-PCR analysis of CXCL10 mRNA levels (n=6-8).* ***B.*** *Levels of secreted CXCL10 measured by ELISA (n=2-4).* ***C.*** *Analysis of the subcellular localization of STAT1 by immunofluorescence. Graphical analysis of the integrated density from immunofluorescent staining of STAT1 (n=5) and representative images. Data (mean ± SEM) are expressed as percentage of untreated cell (control) which was considered 100% and were analysed by one-way ANOVA with Bonferroni post-test *p<0.05 **p<0.01 vs Veh, or Student´s t test ##p<0.01 Veh vs IFNγ+Veh. Scale bar 0.1 mm.*

IV.II.5. STAT1 silencing in Hep3B cells

In order to evaluate if reduced CXCL10 expression depends directly on STAT1 activation, we performed transient silencing of Hep3B cells with siRNA for STAT1 (Figure IV.12.).

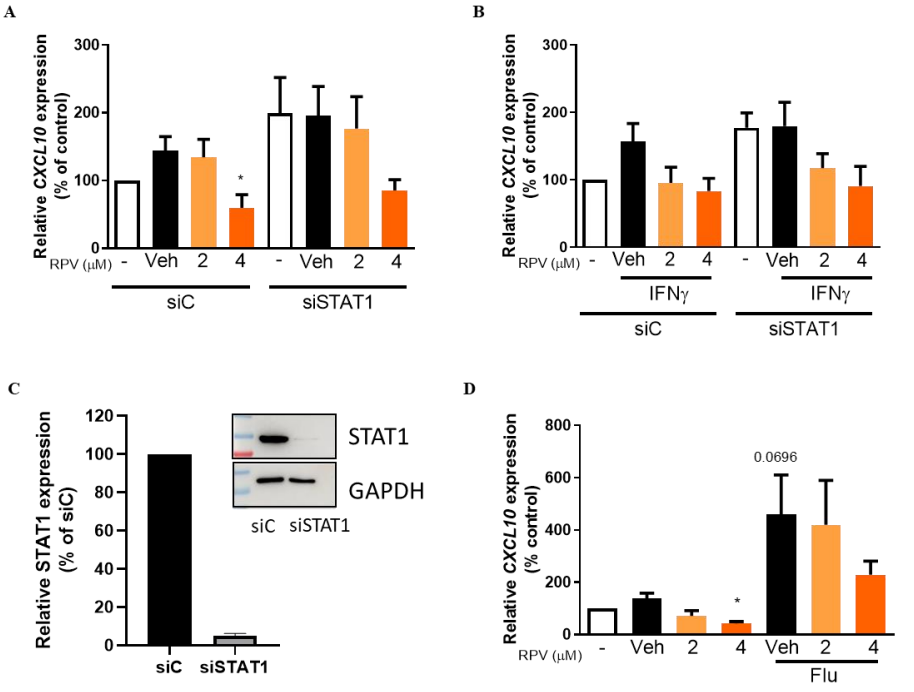


Figure IV.12. RPV´s effect on CXCL10 expression in Hep3B cells lacking active STAT1. *Cells were pre-treated with IFNγ or fludarabine (Flu) for 24 h and RPV or Veh were added for another 24 h. qRT-PCR analysis of CXCL10 (n=4) after transient transfection with siC or siSTAT1 of unstimulated cells* ***(A)*** *or cells previously stimulated with IFNγ* ***(B)*** *(n=3).* ***C.*** *Analysis of the protein levels of STAT1 by WB - densitometry and a representative image are shown (n=3). Data were normalised vs those of the housekeeping protein GAPDH.* ***D.*** *qRT-PCR analysis of CXCL10 (n=2-3) in Hep3B cells co-treated with Flu. Data (mean ± SEM) are expressed as percentage of untreated cells (control) which was considered 100% and were analysed by one-way ANOVA with Bonferroni post-test, *p<0.05 vs Veh.*

Interestingly, it was observed that *STAT1* silencing under basal conditions (without IFNγ stimulation) led to an increase in *CXCL10* expression in Hep3B, while RPV's effect on *CXCL10* expression was preserved (Figure IV.12.A). As expected, it was noted that IFNγ treatment had not increased *CXCL10* expression in Hep3b cells silenced for *STAT1*, however RPV´s effect on *CXCL10* expression was preserved again (Figure IV.12.B). Additionally, Hep3B cells were treated with the chemical inhibitor of STAT1, Fludarabine and similar results were obtained. Fludarabine's treatment enhanced *CXCL10* gene expression, while once again RPV-induced down-regulation was preserved (Figure IV.12.D.). These results suggest that there may be other transcription factors involved in *CXCL10* expression, which are probably closely related to STAT1 activity.

IV.II.6. The effect of RPV on NF-κB signalling assessed *in vivo* and *in vitro*

Another transcription factor that could be implicated in the regulation of CXCL10 expression is NF-κB (222). Previous studies revealed that activation of NF-κB complex protein induced by HFD, CCl_4 and BDL was decreased after RPV's treatment *in vivo* (56), thus reducing hepatic inflammation. These findings were also present in the transcriptomic analysis of whole liver samples from our NAFLD mouse model. In Figure IV.13.A. the top 10 statistically significant GO terms related to NF-κB signalling are represented, with their term size, sorted by the negative log of the adjusted *p* value (the longer the bar the smaller the *p* value). All these processes had negative LOR, meaning downregulation of the functional class in HFD+RPV group.

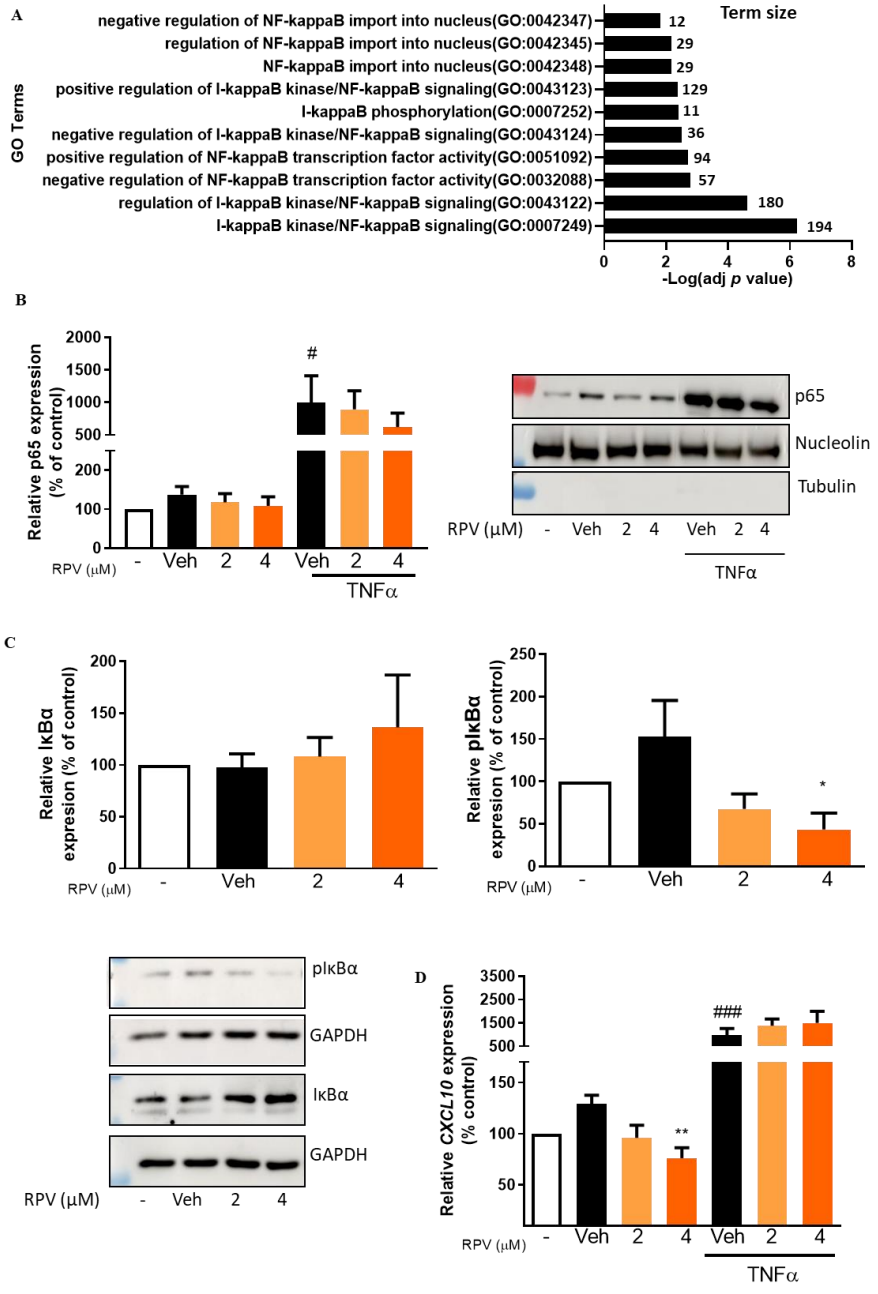


Figure IV.13. The effect of RPV on NF-κB signalling assessed in vivo and in vitro. A. *Transcriptomic analysis of whole liver samples from NAFLD mouse model related to NF-κB*

signalling pathway (n=3). ***B.*** *WB analysis of nuclear p65 expression in Hep3B cells. Cells were pre-treated with TNFα for 24 h and RPV or Veh (DMSO) were added for another 24 h. Data were normalised vs the housekeeping protein Nucleolin (n=4-8) and representative images of WB are shown.* ***C.*** *WB analysis of NF-κB regulator IκBα (n=4) - densitometry and representative images. Cells were treated with RPV or Veh for 48 h.* ***D.*** *qRT-PCR analysis of CXCL10 (n=6-8). Treatment conditions were the same as in panel B. Data (mean ± SEM) are expressed as percentage of untreated cell (control) which was considered 100% and were analysed by one-way ANOVA with Bonferroni post-test *p<0.05, **p<0.01 vs Veh or Student's t-test, #p<0.05, ###p<0.001 Veh vs TNFα+Veh.*

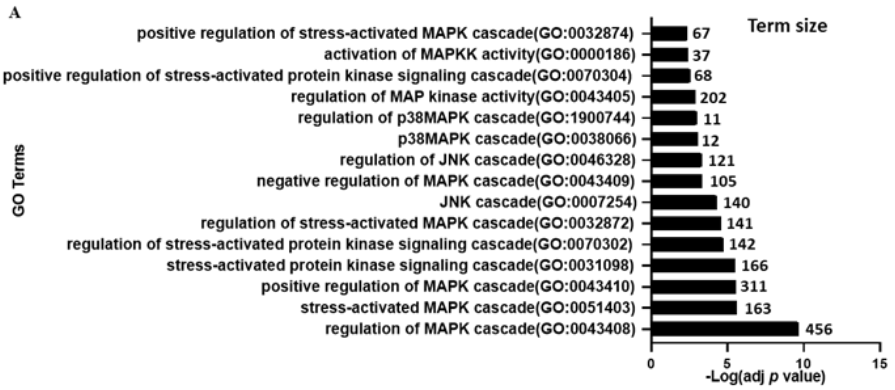
In order to uncover the molecular mechanism behind these observations, RPV´s effect on nuclear p65 expression was analysed *in vitro.* It was demonstrated that there was a decreasing tendency in its expression under basal conditions. Nevertheless, when Hep3B cells were stimulated with TNFα (12.5 ng/mL), a classic NF-κB inducer, there was a modest reduction in p65 nuclear expression with RPV's treatment (Figure IV.13.B). In addition, RPV's effects on NF-κB complex were also observed in its regulator IκBα, that changed its expression under treatment. Data revealed that treatment with RPV increased IκBα protein levels while, under the same conditions, its phosphorylated form was significantly reduced (Figure IV.13.C), meaning that IκBα is less degraded and its able to inhibit NF-κB transcriptional activity. This is in line with previous findings pointing out the anti-inflammatory effect of RPV (56). We observed that treatment with TNFα significantly increased *CXL10* expression, but no down-regulation was seen in cells treated with RPV (Figure IV.13.D.).

IV.II.7. The effect of RPV on p38 and JNK signalling assessed *in vivo*

Transcriptomic analysis of the NAFLD mouse model revealed differences between HFD+Veh and HFD+RPV groups regarding MAPK/SAPK signalling, being this another pathway that may have relevance for CLD and RPV-induced anti-

inflammatory effects demonstrated *in vivo*. Figure IV.14.A. represents the top 15 statistically significant GO terms related to this signalling pathways, sorted by the negative log of the adjusted *p* value. Similarly to the previous results above, all of these processes had negative LOR, meaning down-regulation of the functional class in HFD+RPV group. The top term with the smallest *p* value, "regulation of MAPK cascade (GO:0043408)" had 456 functionally grouped genes being the top 4 term by "term size" in the whole transcriptomic analysis.

In order to confirm these results, WB analysis of total and phosphorylated (active) form of p38 and JNK expression was performed. These experiments showed that the levels of the phosphorylated forms of these kinases were enhanced in the HFD groups while RPV's treatment significantly diminished them (Figure IV.14.B. and C), which is in line with the results observed in the transcriptomic analysis. In addition, we also explored protein expression of these protein kinases in the rest of the mouse model employed in this thesis.



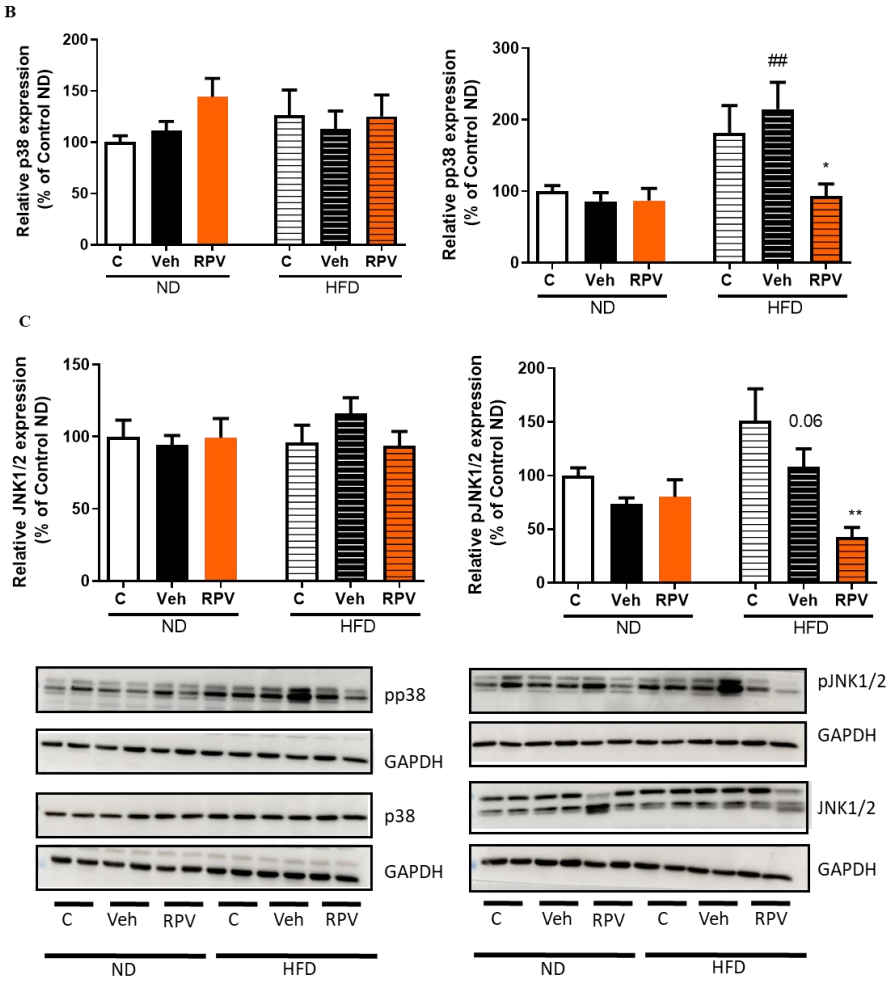


Figure IV.14. The effect of RPV on p38 and JNK signalling assessed in vivo. A. *Transcriptomic analysis of whole liver samples from NAFLD mouse model related to MAPK cascade (n=3). WB analysis of p38 (**B**) and JNK (**C**), (n=7-8) – densitometry and representative images are shown. Data were normalised vs those of the housekeeping protein GAPDH. Data (mean ± SEM) were analysed by a Student's t-test: *p<0.05, **p<0.01 vs HFD+Veh; ##p<0.01 vs ND+Veh. ND, normal diet; HFD, high fat diet.*

Similar results were obtained in the CCl_4 model of toxic injury. Namely, pp38 and pJNK1/2 were increased in the CCl_4+Veh group and showed a tendency to decrease in the CCl_4+RPV group (Figure IV.15.).

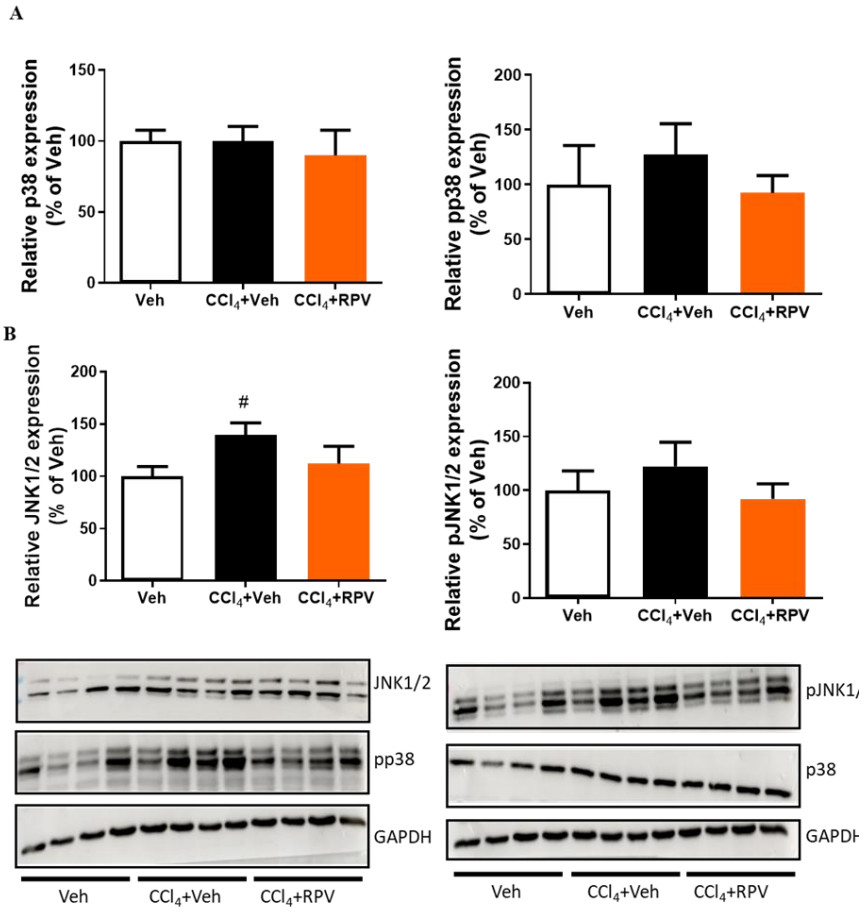


Figure IV.15. Western blot analysis of hepatic p38, pp38 JNK and pJNK protein expression in mouse model of CCl_4-induced liver injury. *WB analysis (n=7-8) of p38* ***(A)*** *and JNK* ***(B)*** *levels - densitometry and representative images are shown. Data were normalised vs the levels of the housekeeping protein GAPDH. Data (mean ± SEM) were analysed by a Student's t-test. #p<0.01 vs Veh.*

Regarding the BDL model, total protein amount of p38 and JNK1/2 were increased in the BDL+Veh group, and treatment with RPV significantly reduced hepatic pp38 protein expression while enhancing pJNK protein levels (Figure IV.16.).

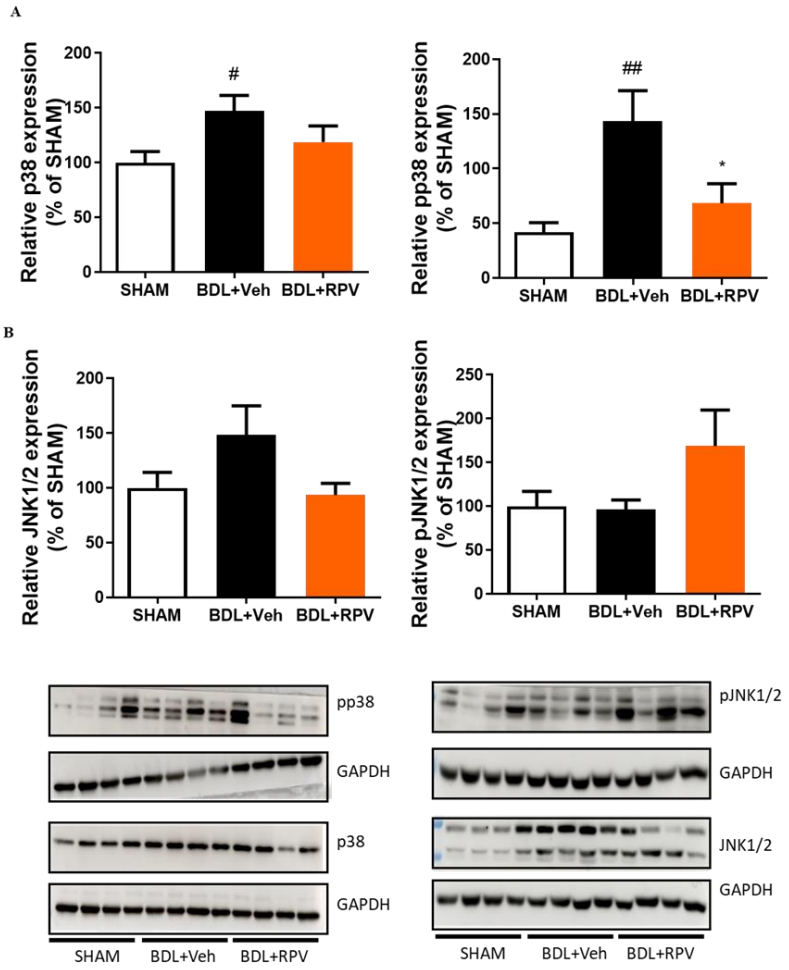
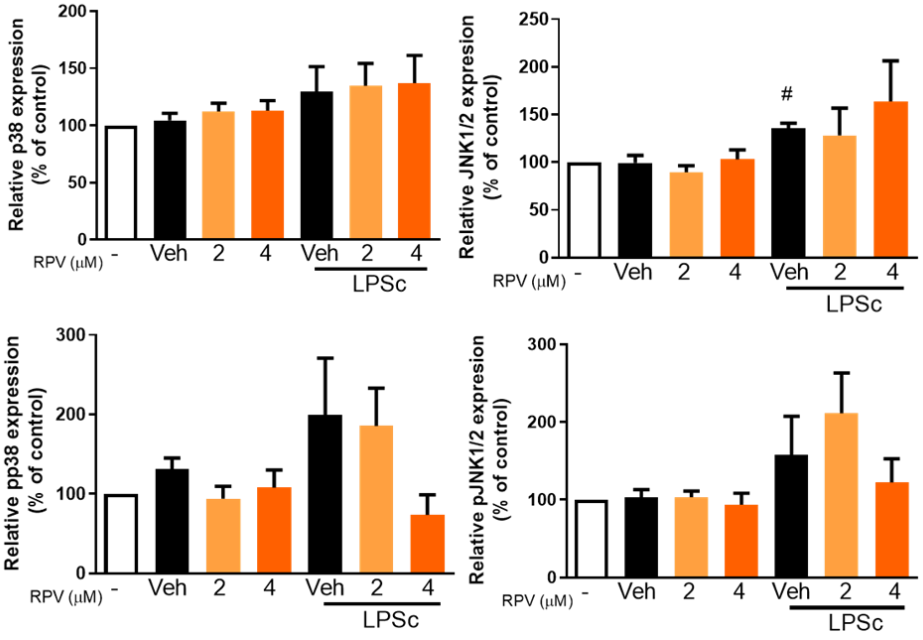


Figure IV.16. Western blot analysis of hepatic p38, pp38, JNK and pJNK protein expression in a mouse model of BDL-induced liver injury. *WB analysis (n=7-8) of p38* ***(A)*** *and JNK* ***(B)*** *levels - densitometry and representative images are shown. Data were normalised vs the expression levels of the housekeeping protein GAPDH. Data (mean ± SEM) were analysed by a Student's t-test. *p<0.05, **p<0.01 vs BDL+Veh, #p<0.05, ##p<0.01 vs SHAM.*

IV.II.8. The effect of RPV on p38 and JNK signalling assessed *in vitro*

To better understand these *in vivo* effects, *in vitro* studies in both Hep3B and LX-2 cells were performed (Figure IV.17 and IV.18). Hep3B cells were pre-treated for 24 h with LPSc, a pro-inflammatory stimulus that contains LPS, IFNγ and TNFα, and RPV was added for another 24 h.

Under basal conditions (without LPSc treatment) there were not changes in the p38 and JNK1/2 protein levels, however, the pro-inflammatory stimulus increased pp38 and total JNK1/2 expression. RPV reduced this increment in a concentration-dependent mode in the case of pp38, and it induced a modest decrease in pJNK1/2 protein expression only at the highest concentration employed (4 µM).



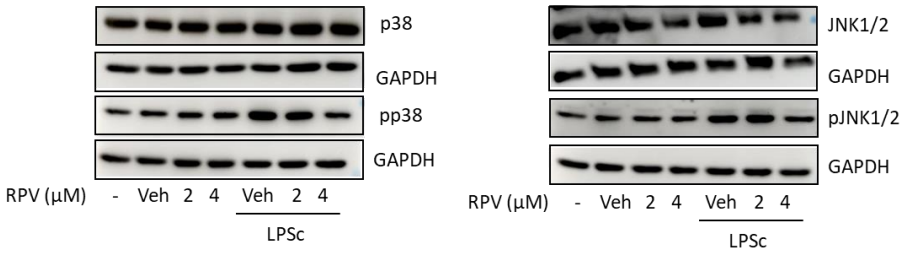
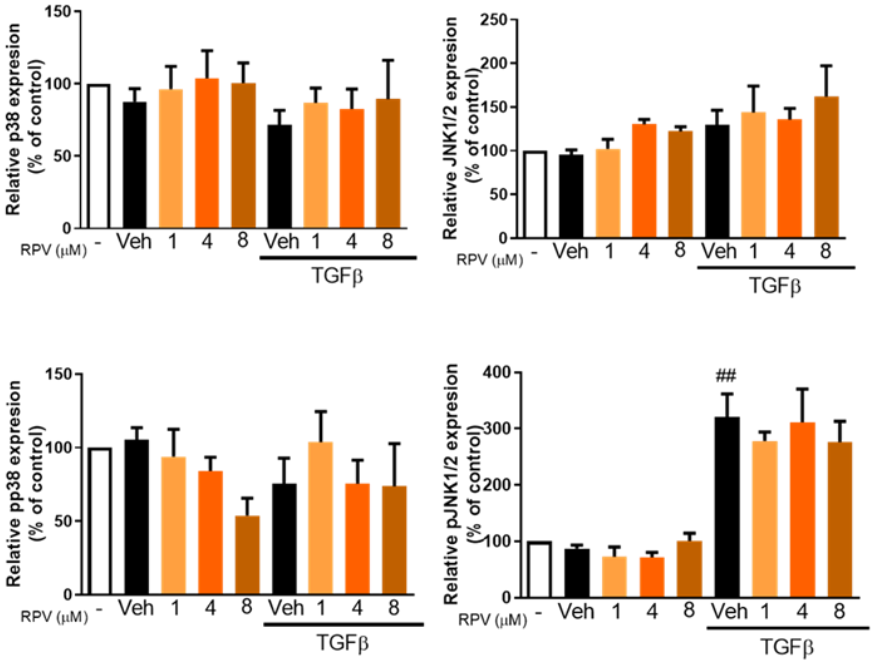


Figure IV.17. Western blot analysis of p38, pp38, JNK and pJNK protein expression in Hep3B cells stimulated with LPSc. *Cells were pretreated with LPSc for 24 h, and RPV or Veh were added for another 24 h. WB analysis (n=7-8) and representative images are shown. Data were normalised vs those of the housekeeping protein GAPDH (n=4-9). Data (mean ± SEM) are expressed as percentage of untreated cells (control) which was considered 100% and were analysed by one-way ANOVA with Bonferroni post-test or with Student's t-test: #p<0.05 vs Veh. LPSc, lipopolysaccharides cocktail.*



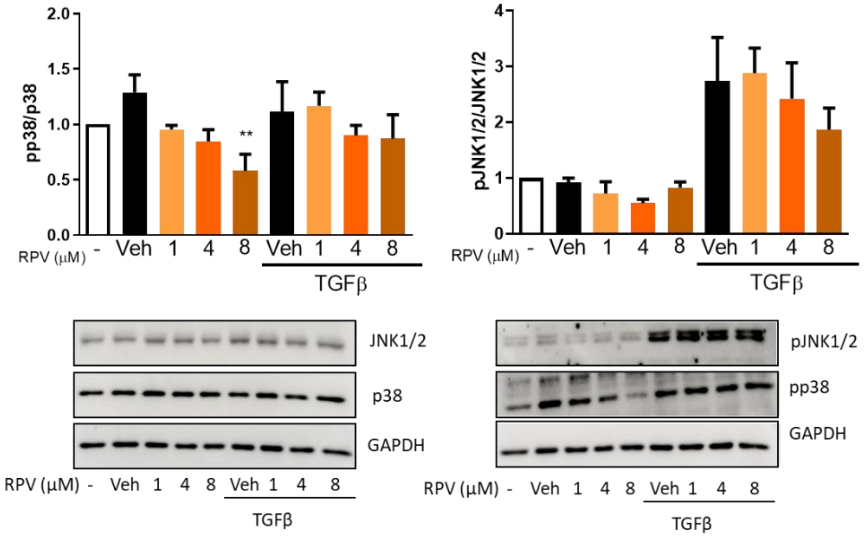


Figure IV.18. Western blot analysis of p38, pp38, JNK and pJNK protein expression in TGFβ-stimulated LX-2 cells. *Cells were co-treated with TGFβ and RPV or Veh for 48 h. WB analysis (n=3-4) densitometry and representative images are shown. Data were normalised vs those of the housekeeping protein GAPDH. Data (mean ± SEM) are expressed as percentage of untreated cells (control) which was considered 100% and were analysed by one-way ANOVA with Bonferroni post-test **p<0.01 vs Veh or Student's t-test: ##p<0.01 Veh vs Veh+TGFβ. TGFβ, transforming growth factor beta.*

Regarding LX-2 cells, TGFβ (2.5 ng/mL) was used as a positive, pro-fibrogenic challenge that was added as a co-treatment with RPV. As expected, TGFβ significantly enhanced phosphorylated JNK protein level and RPV induced a concentration-dependent decrease in its expression. Phosphorylated p38 expression was not increased with TGFβ stimulation; however, RPV significantly decreased its activation under basal conditions.

Chapter V

DISCUSSION

The current pharmacological approach for the treatment of HIV infection, cART, has significantly prolonged the survival of HIV-infected people in developed countries, transforming HIV infection into a chronic, rather than a life-threatening disease. Compared to previous combined regimens, current options are associated with increased viral suppression and lower rates of treatment discontinuation due to their improved convenience, lower rate of resistance and increased tolerability. However, the agents used in this treatment are also responsible for the development of different side effects. This, together with the fact that the treatment once started must be taken throughout life, has generated growing interest in the long-term adverse effects and the mechanisms responsible for them. Additionally, many studies have reported that, despite taking suppressive cART, HIV-infected patients still show abnormally high levels of plasma biomarkers related to immune activation, inflammation and coagulation that can predict increased morbidity and mortality (223).

Historically, inflammation was regarded as a passive pathological consequence of injury and infection. More recently, it has also been considered a mechanism of immune defence and repair. Immune activation usually encompasses changes in the levels of cellular markers and soluble factors associated with inflammation, such as TNFα, IL-6, and other cytokines and chemokines (224). The remodelling of the immune system occurs as part of the "natural" ageing process and in the elderly people, it results in functional impairment of the immune function and a reduced ability for adaptation to metabolic stress. Moreover, HIV is considered a relevant contributor to the increased age-related risk of cardiovascular, bone, and neurocognitive comorbidities as a major source of persistent low-level inflammation and immune activation, both of which are linked to 'inflammageing', a concept that attributes a pro-inflammatory milieu to the ageing process (224,225). In addition to the comorbidities mentioned before, HIV patients have high prevalence of metabolic

alterations that result in liver damage, consequently leading to NAFLD and its more severe form NASH, making CLD a major cause of death in these patients (226).

In the clinical studies performed in this thesis, PBMCs were isolated from: i) HIV-infected patients and their sex-, age- and BMI-matched uninfected controls, and ii) patients with CLD, with the aim to analyse gene and protein expression of different inflammatory markers. PBMCs are circulating immune cells including lymphocytes T, B and NK cells in the range of 70–90%, monocytes from 10 to 20% and DCs accounting for only 1-2% (227). They are often used in gene expression studies because they can be easily collected and their expression profiles have been shown to be remarkably similar with those of other cell types, including those of brain, colon, heart, kidney and liver, as PBMCs express approximately 80% of the genes encoded by the human genome (228,229). These circulating blood cells meet every cell in the human body and provide an active defence against insult and injury and any macro- and micro-environment changes affect gene expression in blood cells. Therefore, circulating blood cells may provide information as to the health or disease of any particular tissue by the change of expression pattern of their transcriptome (229). PBMCs have been used in biological studies and biomarker exploration studies (230–233), and may undergo immune reactions similar to *in vivo* situations, even under *in vitro* culture conditions (234–236).

In the first study we: i) compared the expression of a panel of 17 genes related to inflammation and senescence in PBMCs of HIV patients *vs* matched uninfected controls, ii) analysed the expression of these genes in HIV patients in association with several demographic, biochemical and immunological parameters and iii) compared the expression in relation to the current cART they received. The HIV cohort in this thesis comprises a very heterogeneous population, representative of the majority of successfully treated HIV patients in Spain. However, we cannot

exclude the fact that patients were only recruited from one hospital that may pose certain bias to the patients' population and treatment. Furthermore, this work is subject to a number of limitations common to cross-sectional studies, such as difficulty to separate cause and effect, the measurements are from a unique time point and changes over time cannot be examined.

The median values of the blood parameters in HIV-infected individuals were within the normal range, except for the median number of monocytes, glucose, LDL levels - minimally above the upper limits - and the CD4/CD8 ratio. This ratio is considered a reliable marker of the overall immune function, disease progression, response to treatment, morbidity and mortality, as well as age-related diseases such as kidney disease, arterial stiffness and carotid atherosclerosis in virally suppressed HIV patients (237). In the general population, inverted CD4/CD8 ratio (<1) is a surrogate marker of immunosenescence and independently predicts all-cause mortality. 70% of the patients in our cohort presented inverted CD4/CD8 ratio, which is very similar to previous studies who investigated aging and co-morbidities in Spanish HIV patients (237).

We also provide evidence of increased expression of several pro-inflammatory marker genes in HIV patients compared to healthy controls including *IL6*, *IL18* and *CXCL10,* which is in line with the findings of other groups who have also studied transcript levels in PBMCs of cART-treated HIV patients (238). In particular, IL-6, whose concentration in plasma was enhanced in HIV patients treated for 1 year, has been shown to correlate with non–AIDS-related morbidity and mortality (239) and is viewed as a universal marker of inflammation-mediated pathologies. As previously mentioned, CXCL10 exerts a strong chemotactic and activating effect for T and B cells through its specific receptor CXCR3, and it plays an important role in the recruitment of lymphocytes during inflammation (240). This heightened inflammation can drive further viral replication, establishing a vicious circle of

further immune stimulation that results in premature immune aging (241). Furthermore, our data show diminished expression of p53 and p53-dependent mediators PAI1 and IGFBP3 in HIV patients, particularly in those treated with NNRTI-containing regimens.

Within PBMCs populations, p53 is predominantly expressed in monocytes and lymphocytes (242). Under physiological conditions, cells have low levels of p53 that increase in response to cellular stress (DNA damage, activation of oncogenes or viral infection, hypoxia, or depleted ribonucleoside triphosphate pools). However, p53 is a master regulator not only of cell cycle, senescence and programmed cell death as suggested initially, but also of a much broader biological portfolio of processes including autophagy, cell differentiation, free radical production and both carbohydrate and lipid metabolism, as shown in the last decade (243). This second life-rather than death-promoting function of p53 may be related to its anti-inflammatory capacity, which probably occurs via opposing the NF-κB signalling (244) and may play a role in the survival of cells exposed to modest stress (245), which is of relevance in HIV patients who suffer permanent (and increasing with age) low-grade inflammation. It has been reported that p53 inhibits STAT1 and with that the expression of IFN-inducible genes and pro-inflammatory cytokines (246). p53 can directly repress the activation of the IL-6 promoter and NF-κB-dependent promoters, and inhibit the transcription of a set of TNF-inducible genes (246). p53 deficiency in macrophages enhances the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF, that are targets of NF-κB. Among the genes which are induced by p53 are *IGFBP3* or *SERPINE1*. In our cohort, both *IGFBP3* and *SERPINE1* are downregulated in HIV patients compared to matched controls which is in line with the decreased expression of p53 we observed in these patients.

PAI1 is a serine protease inhibitor that is synthesized by a variety of cells including vascular endothelial cells, adipocytes, macrophages, cardiomyocytes and fibroblasts (247). Several studies have shown that HIV replication alters the endothelial function and some of the excess CVR associated with HIV may be explained by immune activation and inflammation (248). Consequently, HIV-infected individuals may also be in a hypercoagulable state due to the presence of antiphospholipid-anticardiolipin antibodies, decreased activities of natural anticoagulants (especially protein S), and increased platelet activation, as well as the use of PIs (249). In one study, HIV/HCV-coinfected patients had higher PAI1 values, as a coagulopathy marker, than healthy controls and HIV-monoinfected patients, associating coagulopathy to an increased risk of progression and death in HIV-infected people (223). In contrast to this, HIV patients employed in our study had lower *SERPINE1* mRNA levels than the healthy controls and we suggest that this effect may be p53-dependent. Besides, PAI1 is commonly regarded as a marker of senescence and aging, and has been suggested to have a double-sided role for longevity, negative as risk factor for CVD and cancer, and positive as inhibitor of the PI3K-AKT pathway (250). This pathway is also part of the insulin signalling axis whose downregulation has beneficial effects on longevity as demonstrated in flies, worms and mice (251). IGFBP3 plays a major role in hormonal regulation of growth, differentiation and metabolism in mammals (252). p53 is able to inhibit insulin-like growth factor network at various stages including the stimulation of the transcription of IGFBP3, which inhibits ligand-induced activation of its receptor (253). The liver is the main source of circulating IGFBP3 and its receptor, and according to previous studies in HCV-viremic/HIV patients, low levels of IGFBP3 are related to progressive liver disease (254,255). Moreover, low concentrations of this hormonally modulated protein in HIV-infected individuals are linked to functional impairment associated with low bone mineral density and muscle mass (256). Additionally, a recent *in vitro* study using IMR-90 Human Caucasian fetal lung fibroblast, referred to IGFBP3 as

"ageing marker" as a result of the relation between senescent cells and the decrease of IGFBP3 expression through p53 signalling pathway (252).
In addition, abundant but somewhat conflictive evidence links HIV with p53. HIV downregulates p53 as HIV protein Tat inhibits p53 transcription (257), Nef protects against p53-mediated apoptosis (91) while Vif stabilizes and activates p53 to induce G2 arrest in infected cells which positively supports HIV-1 replication (92). p53 has been suggested as an important host restriction factor against HIV-1 replication (258).

Moreover, in our cohort, presence of positive correlation between the expression of *SIRT1* and *TP53* was observed within the two populations employed. As previously mentioned, SIRT1 is a protein deacetylase, one of the key epigenetic regulators and tumor suppressors, which regulates transcriptions of multiple target substrates including p53, and plays an important role in many cellular events, especially longevity, cancer and cellular reprogramming. Depending on the DNA damage, cellular fates to either undergo repair or senescence/apoptosis are governed through acetylated p53 (the activated form of p53). Inhibition of SIRT1 results in increased active form of p53 that is associated with senescence-like phenotype (259). Nevertheless, our HIV patients had downregulation of SIRT1, both at gene and protein level, but not an increase in p53. It has been reported that only virus-expressing cells have upregulated p53 and the DNA damage induced by HIV-1 integration is required for p53 accumulation (260), suggesting that maybe p53 is downregulated in the HIV patients of the present study as a result of the cART efficacy and viral suppression. In this case, it would not be related with SIRT1, since this deacetylase showed lower expression under all three classes of antiretroviral drugs (NNRTIs, PIs and IIs), suggesting that this effect is related with the underlaying (inflammatory) condition of otherwise well-controlled HIV patients. The diminished presence in HIV patients is specific for SIRT1 as we detected no

alterations in the expression of two other sirtuins, SIRT2 and SIRT3. Our results support previously published data of diminished *SIRT1* mRNA in $CD4^+$ cells from HIV patients (261), however, in this article no information about their comorbidities, HIV-related parameters or the cART they received was described. In another study, $CD4^+$ cells from HIV patients under cART displayed significantly higher expression of SIRT1 in comparison with patients that did not receive treatment (262), and this may be the result of lower presence of Tat due to the effective therapy. The decrease in SIRT1 levels in HIV patients may be of clinical relevance for the pathogenesis of various comorbidities. A study in HIV transgenic rats and human astrocytes, described that miR-34a and -138 inhibit SIRT1 expression and subsequently activate astrocytes, that affects aging and HIV-associated neurocognitive disorder (263). Another study in HIV transgenic mice reported that the expression of HIV genes at a low level in kidney cells accelerates diabetic kidney disease through enhanced cellular senescence and inflammation, in part as a consequence of reduced SIRT1 expression (264). Furthermore, clinical studies have suggested that downregulation of SIRT1 gene and protein expression in circulating PBMCs has been associated with the development of metabolic syndrome and insulin resistance pointing to its importance as a life-span determinant gene (265).

Accumulating evidence points to a fundamental role of SIRT1 as a regulator of both the innate and the adaptive immune responses. For instance, it plays a crucial role in human $CD8^+$ T cell differentiation and function, and its expression is markedly down-regulated in terminally differentiated $CD8^+CD28^-$ memory T cells, a population that accumulates during ageing (266). Loss of SIRT1 and enhanced proteasomal degradation of the downstream transcription factor FOXO1 enhance glycolytic capacity and granzyme B secretion under resting conditions, pointing to the SIRT1–FOXO1 axis as an important mechanism for preserving resting memory T cell metabolism and function (266). We observed a moderate but significant

SIRT1/*CD8A* correlation in uninfected controls while it was lower in HIV patients and, given that $CD8^+$ T cells are fundamental in HIV-infection, this finding may be highly relevant for HIV-infected individuals. A similar result was obtained with *SIRT1*/*CD19* (marker of B lymphocytes). SIRT1 is important for B cells and in the context of HIV, B cell hyperactivity and dysfunction has been reported in some organs in HIV-infected individuals (i.e., intestine), with a possible role for the miR-34a–SIRT1–p65 pathway (267). Also, activated B cells express low SIRT1 protein levels along with high levels of pro-inflammatory cytokines in patients with multiple sclerosis (268) and SIRT1 seems to increase B cell survival, as reported in mouse B lymphocytes (269).

In summary, we provide evidence that compared to uninfected matched controls, PBMCs of HIV patients display increased expression of general inflammatory genes, some of which are related to certain demographical, biochemical and immunological parameters and the current cART regimen, while the expression of senescence-associated genes *TP53*, *SERPINE1* and *IGFBP3* in HIV patients is diminished. This is a very interesting observation, more so as it seems that NNRTIs-containing therapies show lower levels in comparison with other antiretroviral drug classes. PBMCs obtained from HIV patients have lower expression of SIRT1, an effect intrinsically linked to the presence of HIV infection in these individuals as no correlation was found with the patients' characteristics/variables or cART regimens. Also, HIV patients exhibit altered relation of SIRT1 and certain lymphocyte subpopulations, namely $CD8^+$ T and B cells (Figure V.1.). Of note, these findings may help to better understand the development of ageing-associated comorbidities and decreased longevity in HIV patients. This study is a clear base for further investigation in order to explain the molecular mechanisms that happen in PLWH after successful implementation of cART and how the therapy affects the development of HIV-independent comorbidities.

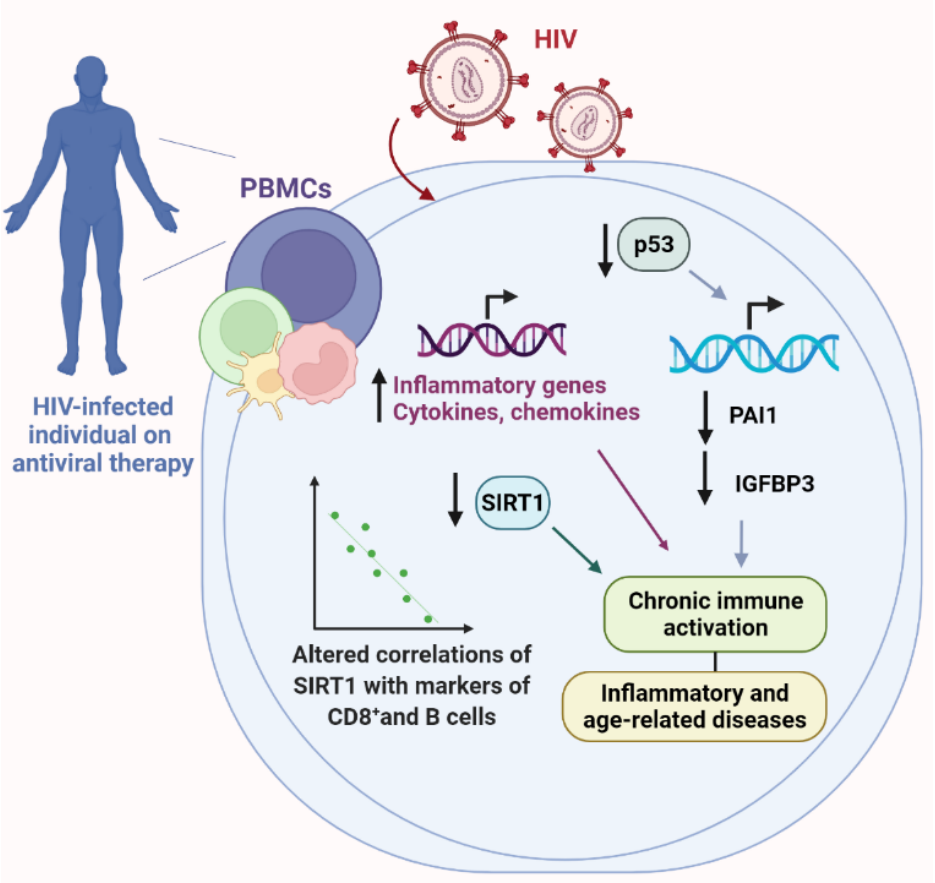


Figure V.1. Overview of the findings from the study of inflammatory and senescence markers in HIV patients. *Compared to controls, HIV-infected patients displayed increased expression of inflammatory genes, had lower SIRT1 gene and protein expression, and those on NNRTIs-containing therapies displayed decreased expression of p53 and its downstream mediators PAI1 and IGFBP3. These findings may help to better understand the development of aging-associated comorbidities and decreased longevity in HIV patients. Created with BioRender.com.*

The processes of immune activation are poorly understood, and the impact of ageing in treated long-life HIV is not yet clear. Thus, in order to reduce morbidity and mortality, there is an urgent need to clearly understand the causes of such

inflammation and to develop interventions for attenuating the effects of chronic inflammation and immune activation in PLWH.

As mentioned before, aging-related comorbidities, including CLD, represent the main drivers of morbidity and mortality in PLWH (226,270), representing 14-18% of all deaths in this population (271). HIV patients with controlled disease (suppressed HIV RNA and restored CD4 counts) are prone to developing liver diseases from simple and common causes such as alcohol use, viral hepatitis, and ageing, in addition to more HIV-specific processes including cART-related toxicity and direct injury to the liver by the HIV virus itself. (270–272). Studies suggest that PLWH are at higher risk for both NASH and NASH-related LF compared to uninfected subjects (272). Typical mechanisms of liver disease in these patients include oxidative stress, mitochondrial injury, lipotoxicity, immune-mediated injury, cytotoxicity, toxic metabolite accumulation, gut microbial translocation, systemic inflammation, senescence and nodular regenerative hyperplasia (270). During the evaluation of several antiretrovirals on the development of CLD, it was found that the second-generation NNRTI RPV, displays substantial and to that moment unforeseen anti-inflammatory and anti-fibrotic actions in different mouse models of CLD, pointing to a direct protective mechanism in the injured liver, regardless of the disease's ethiology (56). Data from clinical studies have suggested that RPV is associated with a good safety profile, especially regarding lipid metabolism (273–275). Given the evidences of the hepatoprotective action of RPV, the second part of this thesis was focused on elucidating the molecular mechanisms involved in these phenomena, particularly those related to the inflammatory response. Importantly, it was already demonstrated by our group that RPV reduced liver inflammation and LF progression when administered in combination with the toxic insult, and also when administered after consolidation of LF, thus highlighting the potential of this compound to reverse fibrosis. The subsequent mechanistic study revealed an

interesting and selective pro-apoptotic effect of RPV in activated HSCs through interference with STAT1 signalling, which was not observed in hepatocytes. Furthermore, an interesting interplay between HSCs and hepatocytes via JAK-STAT signalling was described, which may lead to liver regeneration: RPV's treatment restored the number of proliferative hepatocytes through STAT3 activation, thereby re-establishing parenchymal homeostasis. Nevertheless, this activation was secondary to and dependent on the RPV-mediated apoptosis of HSCs (56).

One of the aims of this doctoral thesis was to explore the molecular mechanisms responsible for the hepatoprotective effects of RPV seen *in vivo* and investigate the interrelations between the expression of specific, inflammatory markers in CLD patients. For this reason, we recruited 38 patients with CLD of different origin, from which PBMCs were isolated and treated with RPV *ex vivo*. We found elevated levels of GGT and ferritin in these patients, while the rest of the biochemical and hematological analysis were within normal ranges. Common causes of elevated GGT activity in plasma include liver disease, obesity, excessive alcohol consumption, the use of certain medications, congestive cardiac failure and smoking (276). GGT is widely used as a marker of excessive alcohol intake in patients with alcoholic liver disease and serum GGT levels are often increased in patients with NAFLD (277). A study that used GGT as a marker of NAFLD in patients with metabolic syndrome reported a GGT value of 57.31 ± 5.00 U/L (mean ± SE) in the patients with most severe form of the disease (278). In our population, GGT levels were even greater, with a median of 67 U/L and a third quartile of 101.8 U/L. This may be due to the fact that in our cohort not only patients with NAFLD, but patients with different etiology of CLD (HCV, NASH, etc.) were considered and every analysis was done for all of them together. Ferritin is an acute-phase protein that is commonly increased in patients with NAFLD and metabolic syndrome and previous studies have shown an association between hyperferritinaemia and advanced LF in patients with NAFLD

(279). For the 38 CLD patients of the present cohort, ferritin median value was slightly above the upper limit, however the median of the third quartile was almost a double of the normal value. CAP is a simple, non-invasive technique that allows diagnosing and quantifying liver fat content simultaneously to liver stiffness measurement using FibroScan® (280). A meta-analysis study confirmed that CAP is a reliable method to diagnose significant steatosis (281). Liver stiffness measurement is higher in patients with higher CAP, and, in turn, patients with liver stiffness measurement >10 kPa show higher CAP values even in the absence of severe steatosis, suggesting a complex interaction between the two (281,282). In line with the last two works cited, in the patients of the present study, these parameters were closely correlated. The cut-off for the detection of liver steatosis reported in the literature ranges from 222 dB/m in a cohort of HCV-patients, to 294 dB/m in a meta-analysis of NAFLD patients (283). The patients in the present study had a median CAP value of 278 dB/m, meaning that in half of them some level of steatosis was detected. Further studies with a higher number of patients that will allow to group them for severity and etiology are necessary in order to better analyse the present population of patients with CLD.

Recent evidence indicates that immunologic imbalance in the liver supports the maintenance and progression of inflammation in NAFLD. In general, NASH is characterized by a robust recruitment of immune cells into the liver where they become activated and release molecules that cause inflammation (284). During the pathogenesis of NAFLD, multiple T cell subsets are involved in exerting differential effects on hepatic inflammation, injury and fibrosis. Depending on the cytokine environment, T helper cells can assume a pro-inflammatory Th1 phenotype, characterized by the release of IFNγ and TGFβ, or an anti-inflammatory Th2 phenotype, characterized by the release of IL-4, IL-5, and IL-10 (285). Th17 cells, another subtype of T helper cells, facilitate leukocyte recruitment through the

secretion of various cytokines including IL-17, IL-21 and TNFα and its hepatic cell infiltration is found in NASH (286). The marked increase in Th1 and Th17 responses in NASH is complemented by a loss in Treg in the adipose tissues, peripheral blood, or livers of NASH patients (287,288). A vast body of evidence supports the use of PBMCs in studies of liver and metabolic diseases due to the fact that PBMCs have the ability to migrate across the endothelium and establish a dialogue between cells in solid organs. This explains why the transcriptome changes observed in PBMCs often parallel the changes observed in solid organs, such as the liver (289,290). Therefore, transcriptomic analysis of human PBMC constitutes a promising tool to study lipid metabolism as well as the pathogenesis of obesity without the need of performing invasive biopsies of liver or adipose tissue (289).

After PBMCs isolation from CLD patients and their subsequent treatment with RPV *ex vivo*, we first studied the gene expression of the main activator cytokines of STAT1- and STAT3-mediated pathways, namely, IFNγ, IL6, IL22 and IL10. In addition, the gene expression of some of the STAT1 target genes were also analysed, as *CXCL10*, *CXCL9*, their receptor *CXCR3*, other chemokines and pro-inflammatory cytokines. PBMCs of CLD patients showed enhanced gene expression of the main STAT3-activating cytokines (*IL6*, *IL22* and *IL10*) and downregulated those in STAT1-mediated pathways (*CXCL10* and *CXCL9*). We also demonstrated that pSTAT3 protein expression was increased, whereas that of pSTAT1 was significantly decreased in PBMCs treated with RPV *ex vivo*. Furthermore, in the present study was demonstrated that RPV downregulated *CCL2*, a major inflammatory chemokine that plays pivotal role in monocyte recruitment (291), and *SERPINE1*, whose functions have already been discussed. The pro-inflammatory or anti-inflammatory effect, in some cases even both, that a cytokine may produce depends on distinct factors, including which cell type has produced it and which cell type will respond to it, as well as the current pathological condition. However, some

cytokines can have opposing effects as well. For instance, IL-6 or IL-22, which prevent fibrogenesis also promote HCC in mouse models of CLD (292). It was reported that IL-6 *via* STAT3 activation can be anti-apoptotic and tumorigenic in certain conditions, due to its role in the progression of several cancers; but IL-6 also has the potential to promote liver tissue regeneration, enhancing hepatocyte growth and preserving T cell function during chronic antigen exposure (293). Another contrasting attribute of this pleiotropic cytokine is that it not only acts as a classical inflammatory cytokine, but can also function as a potent inducer of anti-inflammatory pathways (293). Several studies have demonstrated that IL-22, mainly by activating STAT3, induces the expression of anti-apoptotic and anti-oxidant genes in hepatocytes (294). Furthermore, this activation protects against different types of liver damage such as T cell-mediated liver injury, chemical/drug-induced liver injury, and alcoholic liver disease (295). Similar to IL-6, IL-22 is also a promoter of liver regeneration, an inducer of antimicrobial genes and a suppressor of lipogenic genes (294). However, IL-22 may have a pathogenic role in context-dependent manner, since it has been reported to be elevated in CLD patients with viral hepatitis and advanced LF (296,297). IL-10 is the prototypic anti-inflammatory interleukin that controls neutrophil infiltration and possesses tissue-protective functions during CLD and fibrogenesis (292). Regarding STAT1, JAK1-STAT1 pathway is responsible for the pro-inflammatory phenotype markers on KCs isolated from rat liver (298). Furthermore, STAT1 signalling is increased in the livers of obese patients with NAFLD (299) and STAT1 signalling in hepatocytes promoted T cell accumulation, inflammation, NASH, and LF in HFD-fed mice (299). Another work suggested that STAT1 plays a harmful role in a model of T cell–mediated hepatitis by activation of $CD4^+$ and NKT cells and directly inducing hepatocyte death (168). All this being said, we suggest that RPV exerts anti-inflammatory effects on PBMCs isolated from CLD patients treated *ex vivo*, showed by STAT1 lower

expression and its target genes, as well as by the enhanced STAT3 expression and its activating cytokines.

An important chemokine responsible for the recruitment and localization of inflammatory cells to sites of tissue damage or infection is CXCL10 (140). It binds CXCR3, a receptor shared by CXCL9 and CXCL11, which is expressed on CD4 and CD8 T cells, NK cells, B cells and DCs (300). It is suggested that CXCL10 plays a role in the development of hepatic inflammation and fibrosis, and its expression correlates with liver inflammation and histological severity in chronic HCV infection (118,301). In the liver, hepatocytes are a major source of CXCL10 (222). We demonstrate that RPV-treated Hep3B cells show diminished expression of CXCL10 and reduced STAT1 translocation in the nucleus. However, after transient silencing of *STAT1* we discovered a much more complex scenario. Namely, cells silenced for *STAT1* had greater *CXCL10* mRNA levels and RPV's effect was preserved in both experimental designs, with or without IFNγ stimulation. *STAT1* silencing only halted IFNγ-stimulated expression of *CXCL10*. These findings suggest that other transcription factors may be involved in *CXCL10* regulation.

A vast body of evidence supports the crucial role that NF-κB plays in CXCL10 transcription induced by cytokines (302–305). Previous to this thesis, it was already demonstrated that p65 protein expression was downregulated under RPV's treatment in our *in vivo* models (56), however, an *in vitro* analysis in hepatocytes was not performed. For this reason, we analysed nuclear p65 and the NF-κB regulator IκBα protein expression in Hep3B treated with RPV. Under basal conditions, the degradation of IκBα was reduced and nuclear p65 behaved similarly as STAT1, a minimal tendency to decrease was shown. When Hep3B cells had previously been stimulated with TNFα, RPV reduced nuclear p65 protein expression. Nonetheless, this reduction was not enough to diminish TNFα-induced CXCL10 expression.

These findings suggest that RPV reduces CXCL10 levels under basal and IFNγ-stimulated conditions, but not under the more potent TNFα stimulation. Although the current study focused on p65 and STAT1, involvement of other transcription factors (activator protein 1, CCAAT/enhancer-binding protein beta, interferon regulatory factor) in the induction of CXCL10 cannot be ruled out.

A better understanding of the underlying mechanisms of CLD is mandatory for the design of new therapeutic approaches. Animal models represent a fundamental preclinical tool to further elucidate the pathophysiology of CLD and to define effective treatments and potential biomarkers. Over the years different experimental approaches have been developed in rodents as models of different liver diseases, from NAFLD and NASH to LF, cirrhosis and HCC. However, no model to date can completely recapitulate the "corresponding" human disorder. Limiting factors include: i) the time frame required in humans to establish a certain liver disease, ii) the fact that rodents possess a distinct immune system compared with humans, iii) different metabolic rates affecting liver homeostasis (306). In the present study three animal models were employed: HFD-, CCl_4- and BDL-induced liver injury. The severity of HFD-induced NAFLD may depend on the: species (rats are more sensitive then mice and need shorter time for severe histological NAFLD manifestation (307)), gender (male mice are more susceptible than females, as oestrogen is protective against NAFLD (308)) and strain of the animals (309). Our NAFLD model was obtained after 12 weeks of HFD intake, and hepatic steatosis, inflammation and even an incipient fibrotic response with evident collagen deposition were observed (56). Regarding CCl_4 and BDL models, LF was induced after 4 and 2 weeks respectively, avoiding more severe stages like cirrhosis that would require longer experimental procedures (56).

In line with our *in vivo* data and regarding the relevance of CXCL10, it was reported that neutralization of CXCL10 directly induced hepatocyte proliferation, provided sinusoidal scaffolds, and protected mice from massive hepatocellular loss in acute liver injury induced by CCl_4 (310). These findings indicate that inducible CXCL10 is a critical factor that inhibits remodelling of hepatic tissue structure during injury and provides a novel therapeutic approach which stimulates liver regeneration (310). Another study suggested that CXCL10 was responsible for autophagic impairment during the development of steatohepatitis. Upregulated CXCL10 in both *in vitro* and *in vivo* models of steatohepatitis inhibited the fusion of autophagosomes and lysosomes, thereby attenuating the autophagic flux. This attenuation led to the inhibition of autophagic protein degradation and the prominent accumulation of undegraded autophagy substrates, such as p62/SQSTM1 aggregates and ubiquitinated proteins, ultimately resulting in steatohepatitis development (311). Moreover, administration of the anti-CXCL10 monoclonal antibody could prevent MCD-induced steatohepatitis both *in vitro* and *in vivo* as indicated by reduced lipid accumulation and inflammatory cell infiltration in MCD-fed mice administrated with anti-CXCL10 monoclonal antibody (312). Here we show that RPV diminished CXCL10 protein expression in all three mouse models of liver injury employed, and this action may be of relevance for the molecular mechanisms of hepatoprotection exerted by RPV.

Another signalling cascade implicated in CXCL10 regulation and CLD-related inflammation is MAPK signalling pathway. It was demonstrated that under lipotoxic stress CXCL10 expression in hepatocytes is regulated by mixed lineage kinase (MLK) 3 (313). The molecular mechanism described was activation of MLK3 that activates phosphorylation of MKK3/6, p38 resulting in STAT1 phosphorylation at Ser727 and its nuclear localisation following binding and activation of the CXCL10 gene promoter (313). In addition, these hepatocytes release chemotactic extracellular

vesicles enriched with CXCL10 in an MLK3-dependent fashion that involves CXCL10 induction through a STAT1 and CXCL10 trafficking into EVs through a JNK-dependent mechanism (314). Moreover, JNK activation is crucial in the development of hepatocyte apoptosis accompanying NASH (192,315). In the present study we show that clinically relevant concentrations of the antiretroviral drug RPV diminish p38 and JNK activation, an effect observed both *in vivo* and *in vitro*. Based on previous studies that reported the association of p38 and JNK with apoptosis of hepatocytes (316–318), we hypothesize that RPV exerts an anti-apoptotic, thus anti-inflammatory effect through the downregulation of p38 and JNK in whole liver tissue (mostly constituted of hepatocytes) and in Hep3B cells. RPV's anti-apoptotic effect in parenchyma was already described in our *in vivo* models (56), which is in line with the new findings observed.

In regard to LX-2 cells, JNK importance for the activation of HSCs and its role in fibrogenesis has been well documented (319,320). Here we demonstrate the ability of RPV to diminish the phosphorylation of JNK in TGFβ-activated LX-2 cells. In addition, p38 has been found to be associated with enhanced collagen expression and increased stability of α1(I) collagen mRNA in activated HSCs (321), confirming the anti-fibrotic effect RPV has on activated LX-2 cells.

However, several limitations in our *ex vivo*, *in vivo* and *in vitro* studies should be discussed and considered. Firstly, CLD patients' number was relatively low and any grouping of the patients (i.e. by severity or etiology) was limited. We analysed all patients together, independently of the etiology, but we are well aware that etiology and type of hepatic injury are very important and context-dependent. Secondly, although animal models are still the most adequate to comprehensively study the pathophysiology of liver diseases and to develop new drugs, sometimes translating the findings from animals to humans can be challenging. Thus, complementary advanced 3D human cellular culture systems such as spheroids, organoids, liver-on-

a-chip, (*in vitro*) and precision-cut liver slices and decellularized liver scaffold (*ex vivo*) have been developed to enable researchers to study specific cellular functions and regulation, as well as drug efficacy and toxicity, in a liver-centered environment. Further studies on identification of therapeutic targets of RPV should be performed using this advanced *in vitro* and *ex vivo* approaches.

There are still many questions that need to be answered if we wish to describe the whole sequence of events leading to the hepatoprotective effects of RPV. For instance, functional assays such as migration assay both for PBMCs and HSCs should be performed to evaluate CXCL10's chemotactic activity under treatment. CXCL10 receptors (CXCR3 and TLR4) and their relevance in our models need to be studied in the future. Given the importance of the MAPK cascade, up-stream kinases of p38 and JNK including MLK3 and TAK1 need to be analysed in our *in vivo* and *in vitro* models as possible therapeutic targets of RPV.

In conclusion, this thesis describes several pathways involved in the anti-inflammatory response that may be relevant for the hepatoprotective function of RPV (Figure V.2.). Although the exact molecular mechanisms involved still remain unclear, RPV's effect on STAT1 and NF-κB transcription factors seems to have an important role for the diminished expression of CXCL10. Furthermore, RPV affects the regulation of MAPK cascade, another potential contributor of the anti-inflammatory function of this antiretroviral drug in the context of CLD.

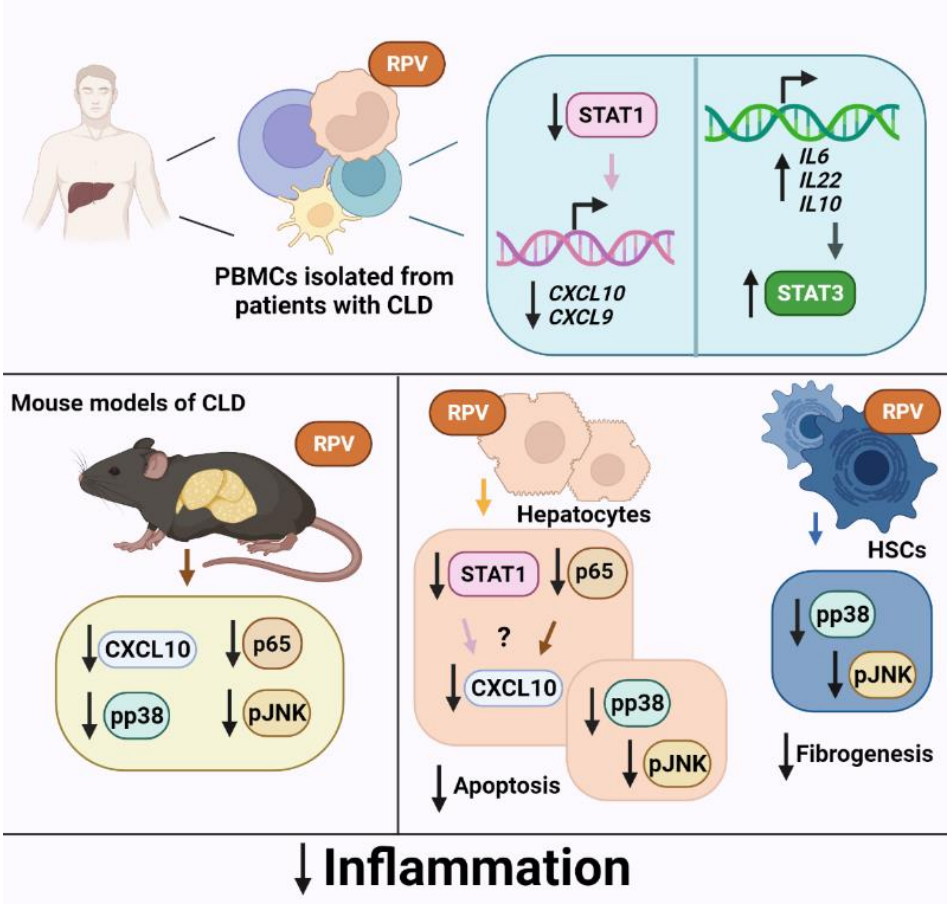


Figure V.2. Overview of the anti-inflammatory and hepatoprotective effects exerted by RPV. *RPV exerts anti-inflammatory effects on PBMCs isolated from CLD patients treated ex vivo, showed by STAT1 lower expression and its target genes, as well as by the enhanced STAT3 expression and its activating cytokines. The expression of CXCL10, a potent pro-inflammatory chemokine, is diminished by RPV. Although the exact molecular mechanisms involved still remain unclear, the implication of STAT1 and NF-κB transcription factors seems to have an important role in CXCL10's down-regulation. Furthermore, RPV affects the regulation of MAPK cascade, another potential contributor of the anti-inflammatory function of this antiretroviral drug in the context of CLD. Created with BioRender.com.*

We believe that the findings of this doctoral thesis are clinically relevant both for the management of HIV patients and for the improvement of the pharmacological therapy for hepatic diseases. Moreover, the obtained results may serve as the basis for discovering novel targets for clinical intervention, as a new therapeutic strategy itself, and improving our current understanding of the molecular mechanisms involved in CLD.

Chapter VI

CONCLUSIONS

1. Compared to uninfected matched controls, peripheral blood mononuclear cells of HIV patients display increased expression of inflammatory genes *IL6*, *IL18* and *CXCL10*, some of which are related to certain demographical, biochemical and immunological parameters and the current combined antiretroviral treatment.

2. The expression of senescence-associated genes *TP53*, *SERPINE1* and *IGFBP3* in HIV patients is diminished compared to healthy matched control individuals. Furthermore, it seems that patients that are under therapies that contain non-nucleoside reverse transcriptase inhibitors show lowest levels of these genes in comparison with those that receive other antiretroviral drug classes.

3. Compared to healthy controls, peripheral blood mononuclear cells obtained from HIV patients have lower expression of SIRT1, a longevity associated protein, an effect intrinsically linked to the presence of the HIV-infection in these individuals, as no correlation was found with the patients´ characteristics/variables and different antiretroviral regimens.

4. Rilpivirine has an anti-inflammatory effect in peripheral blood mononuclear cells treated *ex vivo*, isolated from patients with chronic liver disease; this occurs by enhancing the expression of STAT3 and its activating cytokines and downregulating STAT1 and its target genes in a concentration-dependent manner.

5. Clinically relevant concentrations of rilpivirine downregulate CXCL10 expression, an effect observed both *in vivo* and *in vitro*. The reduced levels

of this chemokine could be connected to STAT1 and NF-κB signalling as suggested in Hep3B cells.

6. Rilpivirine diminishes p38 and JNK activation, an effect observed both in mouse models and *in vitro*. These findings may be related with an anti-apoptotic function in hepatocytes and an anti-fibrogenic effect in hepatic stellate cells.

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ANNEXES

Annex 1. Ethics committee approval certificate of the first clinical study

GENERALITAT VALENCIANA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

INFORME DEL COMITE ETICO DE INVESTIGACION CLINICA DEL HOSPITAL CLINIC UNIVERSITARI DE VALENCIA

D. Antonio Peláez Hernández, Presidente del Comité Ético de Investigación Clínica del Hospital Clínic Universitari de Valencia

CERTIFICA

Que en este Comité, en su reunión de fecha 24 de noviembre de 2016, y según consta en el acta de la misma, se han analizado los aspectos éticos y científicos relacionados al proyecto de investigación que lleva por título:

ANÁLISIS DE BIOMARCADORES DE COMORBILIDADES VIH-INDEPENDIENTES EN LOS PACIENTES CON VIH CON ESPECIAL INTERÉS EN ENFERMEDADES HEPÁTICAS Y METABOLICAS.

Mismo que será llevado a cabo en el Servicio de Medicina Interna y cuyo investigador principal es la Dra. Maria José Galindo en coaboración con la Dra. Nadezda Apostolova del departamento de Farmacología de la Universitat de Valencia, acordando que reúne las características adecuadas referentes a información a los pacientes y cumplimiento de los criterios éticos para la investigación médica y biomédica establecidos en la ***Declaración de Helsinki*** (Junio 1964, Helsinki, Finlandia) de la Asamblea Médica Mundial, y sus revisiones (Octubre 1975, Tokio, Japón), (Octubre 1983, Ve necia, Italia), (Septiembre 1989, Hong Kong), (Octubre 1996, Somerset West, Sudáfrica), (Octubre 2000, Edimburgo), (Octubre 2008 Seúl, Corea) y (Octubre 2013 Fortaleza, Brasil) y en la ***Declaración Universal sobre el Genoma Humano y los Derechos del Hombre de la UNESCO*** y los acuerdos del ***Protocolo Adicional del Consejo de Europa para la protección de los Derechos del Hombre y de la dignidad del ser humano frente a la aplicaciones de la biología y de la medicina*** (Paris 12-1-1998, ratificado el 23-7-1999).

Lo que certifico a efectos oportunos.

Valencia, 24 de noviembre de 2016.

Fdo. : D. Antonio Peláez Hernández
Presidente del Comité Ético de Investigación Clínica

Annex 2. Ethics committee approval certificate of the second clinical study

GENERALITAT VALENCIANA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

INFORME DEL COMITE ETICO DE INVESTIGACION CLINICA DEL HOSPITAL CLINIC UNIVERSITARI DE VALENCIA

Doña Cristina Gomis Gozalbo, Vicepresidenta del Comité Ético de Investigación del Hospital Clínic Universitari de Valencia

CERTIFICA

Que en este Comité, en su reunión de fecha 20 de diciembre de 2018, y según consta en el acta de la misma, se han analizado los aspectos éticos (Protocolo versión 1 de 21 de noviembre de 2018; HIP/CI Paciente Cirugía Pancreática o Biliar versión 2 de 21 de diciembre de 2018; HIP/CI Paciente con Enfermedad Hepática versión 2 de 21 de diciembre de 2018) y científicos relacionados al proyecto de investigación que lleva por título:

Nuevas dianas farmacológicas para el tratamiento de la enfermedad hepática crónica: rutas de señalización JAK-STAT1 y JAK-STAT3 como punto de partida. (2018/272)

Mismo que será llevado a cabo en el Servicio de Gastroenterología y en la Universitat de València y cuyos investigadores principales son el Dr. Joan Tosca Cuquerella, la Dra. Nadezda Apostolova y la Dra. Ana García Blas, acordando que reúne las características adecuadas referentes a información a los pacientes y cumplimiento de los criterios éticos para la investigación médica y biomédica establecidos en la ***Declaración de Helsinki*** (Junio 1964, Helsinki, Finlandia) de la Asamblea Médica Mundial, y sus revisiones (Octubre 1975, Tokio, Japón), (Octubre 1983, Venecia, Italia), (Septiembre 1989, Hong Kong), (Octubre 1996, Somerset West, Sudáfrica), (Octubre 2000, Edimburgo), (Octubre 2008 Seúl, Corea) y (Octubre 2013 Fortaleza, Brasil) y en la ***Declaración Universal sobre el Genoma Humano y los Derechos del Hombre de la UNESCO*** y los acuerdos del ***Protocolo Adicional del Consejo de Europa para la protección de los Derechos del Hombre y de la dignidad del ser humano frente a la aplicaciones de la biología y de la medicina*** (París 12-1-1998, ratificado el 23-7-1999).

Lo que certifico a efectos oportunos.

Valencia, 20 de diciembre de 2018.

Fdo. : Doña Cristina Gomis Gozalbo
Vicepresidenta del Comité Ético de Investigación Clínica

F-CE-GEva-15 v1.1 (23-Julio-2015) Dictamen proyecto Página 1 | 1

Annex 3. Information sheet for the HIV-infected patient and patients' informed consent

HOJA DE INFORMACIÓN AL PACIENTE

Se le ofrece la posibilidad de participar en el proyecto de investigación titulado "**ANALISIS DE BIOMARCADORES DE COMORBILIDADES VIH-INDEPENDIENTES EN LOS PACIENTES DE VIH CON ESPECIAL INTERES EN ENFERMEDADES HEPATICAS Y METABOLICAS**" que está siendo realizado por la **Dra Nadezda Apostolova** del Departamento de Farmacología de la Facultad de Medicina y Odontología de la Universidad de Valencia en colaboración con la **Dra Mª José Galindo** del Hospital Clínico Universitario de Valencia y que ha sido ya evaluado y aprobado por el Comité Ético de Investigación Clínica del Hospital Clínico Universitario de Valencia.

Antecedentes

La infección por el VIH afecta a más de 35 millones de personas en el mundo, habiendo casi 2 millones de nuevos infectados cada año. Aunque en la actualidad disponemos de una terapia muy eficaz que ha conseguido disminuir significativamente la mortalidad asociada a esta enfermedad, existen evidencias que afirman que los pacientes infectados con el HIV envejecen más rápidamente que los no infectados y desarrollan las enfermedades relacionadas con la edad en mayor número y antes que la población control, incluidas las enfermedades hepáticas y metabólicas. Los factores que contribuyen a dicho fenómeno son múltiples siendo los fármacos antirretrovirales uno de ellos.

¿Cuál es el objetivo de este estudio?

El objetivo de este estudio es buscar marcadores tempranos y fiables, y estudiar los mecanismos involucrados en el desarrollo de enfermedades no transmisibles, VIH-independientes (las denominadas enfermedades de la edad) en los pacientes infectados que se encuentran bajo tratamiento antirretroviral.

¿Por qué se le ha pedido que participe?

Se le pide su participación en este estudio ya que es un paciente infectado con el virus de la inmunodeficiencia humana (VIH) que actualmente está recibiendo tratamiento antirretroviral.

¿En qué consiste su participación? ¿Qué tipo de pruebas o procedimientos se le realizarán?

Se le solicita permiso para utilizar con fines científicos una muestra de sangre. La participación en el presente proyecto no modificará bajo NINGÚN concepto el criterio médico en relación a tratamiento y seguimiento. En todo momento usted va a recibir exactamente el mismo tratamiento decida o no participar en el estudio.

¿Cuáles son los riesgos generales de participar en este estudio?

No se prevé ningún riesgo adicional para usted, ya que utilizaremos una muestra de sangre obtenida dentro del programa habitual de seguimiento hospitalario. El riesgo previsible de la obtención de una muestra de sangre será mínimo e incluye molestias, dolor, enrojecimiento e hinchazón y/o pequeños hematomas en el lugar del brazo donde se ha producido la extracción.

¿Cuáles son los beneficios de la participación en este estudio?

Es muy posible que los resultados obtenidos en esta investigación tengan poco valor diagnóstico o predictivo para usted, pero podrá ayudar a conocer mejor su enfermedad y mejorar el pronóstico y el tratamiento de futuros pacientes. Su participación en este estudio nos ayudará a conocer en desarrollo de distintas enfermedades (diabetes, enfermedad hepática, envejecimiento prematuro) que aparecen en la población de pacientes infectados con el VIH-1.

¿Qué pasará si decido no participar en este estudio?

Su participación en este estudio es totalmente voluntaria. En caso de que decida no participar en el estudio, esto no modificará el trato y seguimiento que de su enfermedad realicen ni su médico ni el resto del personal sanitario que se ocupa de su enfermedad. Así mismo, podrá retirarse del estudio en cualquier momento, sin tener que dar explicaciones.

¿A quién puedo preguntar en caso de duda?

Es importante que comente con cualquiera de los investigadores de este proyecto los pormenores o dudas que surjan antes de firmar el consentimiento para su participación. Así mismo, podrá solicitar cualquier explicación que desee sobre cualquier aspecto del estudio y sus implicaciones a lo largo del mismo contactando con los responsables del estudio, La Dra

María José Galindo del Hospital Clínico (tel: 961973500) y la Dra Nadezda Apostolova de la Facultad de Medicina (tel: 963983767/963544147).

Confidencialidad:

Todos sus datos, así como toda la información médica relacionada con su enfermedad será tratada con absoluta confidencialidad por parte del personal encargado de la investigación. Así mismo, si los resultados del estudio fueran susceptibles de publicación en revistas científicas, en ningún momento se proporcionaran datos personales de los pacientes que han colaborado en esta investigación.

Tal y como contempla la Ley Orgánica 15/1999 de Protección de Datos de carácter personal, podrá ejercer su derecho a acceder, rectificar o cancelar sus datos contactando con el investigador principal de este estudio.

¿Qué pasará con las muestras biológicas obtenidas durante la investigación?

Durante su participación en este estudio, se le extraerá una muestra de sangre. Estas muestras serán siempre utilizadas con fines científicos, pudiéndose utilizar si usted así lo autoriza en el marco de otros proyectos de investigación que previamente hayan sido evaluados y aprobados por el Comité Ético de Investigación Clínica del Hospital. Dicha muestra será conservada en el Departamento de Farmacología hasta que sea procesada (no más de un mes), tras lo cual se destruirá.

Además, este material no será bajo ningún concepto ni en ningún momento motivo de lucro, bien sea por la venta del material o de los derechos para realizar estudios sobre los mismos.

CONSENTIMIENTO INFORMADO

Título del Proyecto titulado: "**ANALISIS DE BIOMARCADORES DE COMORBILIDADES VIH-INDEPENDIENTES EN LOS PACIENTES DE VIH CON ESPECIAL INTERES EN ENFERMEDADES HEPATICAS Y METABOLICAS**"

Investigador principal: **Nadezda Apostolova**, Universitat de Valencia

Investigador clínico: **Mª José Galindo**, Servicio: Medicina interna, Hospital Clínico de Valencia

Yo, _____ he sido informado por la **Dra Mª José Galindo**, colaborador del proyecto de investigación arriba mencionado, y declaro que:

- He leído la Hoja de Información que se me ha entregado
- He podido hacer preguntas sobre el estudio
- He recibido respuestas satisfactorias a mis preguntas
- He recibido suficiente información sobre el estudio

Comprendo que mi participación es voluntaria

Comprendo que todos mis datos serán tratados confidencialmente

Comprendo que puedo retirarme del estudio:

- Cuando quiera
- Sin tener que dar explicaciones
- Sin que esto repercuta en mis cuidados médicos

SE RECUERDA QUE EL APARTADO SIGUIENTE ÚNICAMENTE SE DEBE MANTENER CUANDO PROCEDA.

Autorizo a que las muestras obtenidas durante el proyecto de investigación sean utilizadas con fines científicos en otros proyectos de investigación que tengan por objeto el estudio de mi enfermedad y que hayan sido aprobados por el Comité de Ética de Investigación Clínica del Hospital Clínico Universitario de Valencia

-----Sí ----- No

Quiero que se me pida autorización previa para utilizar mis muestras biológicas para futuros proyectos de investigación

-----Sí ----- No

Con esto doy mi conformidad para participar en este estudio,

Firma del paciente:	Firma del Investigador:
Fecha:	Fecha

Annex 4. Controls informed consent

CONSENTIMIENTO INFORMADO

Título del estudio: "**ANALISIS DE BIOMARCADORES DE COMORBILIDADES VIH-INDEPENDIENTES EN LOS PACIENTES DE VIH CON ESPECIAL INTERES EN ENFERMEDADES HEPATICAS Y METABOLICAS**"

Investigador principal: Dra. Nadezda Apostolova

Centro: Departamento de Farmacología, Facultad de Medicina y Odontología, Universidad de Valencia

Teléfono de contacto: 963 983 767

Yo

,…….

he sido informado debidamente de los objetivos del estudio que se está realizando y de la confidencialidad de mis datos por lo que **Autorizo** que se me tome una muestra sanguínea para que sea utilizada con fines científicos.

Firma del donante:

Fecha:

Por favor proporcione la siguiente información.

Edad: Talla:

Sexo: Peso:

Fumador: Si No

Ingesta de alcohol: Nunca Esporádicamente Habitualmente

Patología crónica que padece:

- Hipercolesterolemia
- Hipertrigliceridemia
- Hipotiroidismo
- Hipertiroidismo
- Hipertensión
- Artritis
- Diabetes
- Enfermedad hepática
- Otra. Indique cual. ……

Annex 5. Informed consent for the chronic liver disease patients

HOJA DE INFORMACIÓN AL PACIENTE CON ENFERMEDAD HEPÁTICA

TÍTULO DEL ESTUDIO: Nuevas dianas farmacológicas para el tratamiento de la enfermedad hepática crónica: rutas de señalización JAK-STAT1 y JAK-STAT3 como punto de partida	
PROMOTOR	
INVESTIGADOR PRINCIPAL	**Joan Tosca Cuquerella**
SERVICIO	**Gastroenterología**
CENTRO	**Hospital Clínico Universitario**

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por el Comité de Ética de la Investigación de su centro, de acuerdo a la legislación vigente, Ley 14/2007, de 3 de julio, de Investigación biomédica. Nuestra intención es que usted reciba la información correcta y suficiente para que pueda decidir si aceptao no participar en este estudio. Lea esta hoja de información con atención y nosotros le aclararemoslas dudas que le puedan surgir. Además, puede consultar con las personas que considere oportuno.Así mismo, podrá solicitar cualquier explicación que desee sobre cualquier aspecto del estudio y sus implicaciones a lo largo del mismo contactando con el investigador principal del proyecto, el Dr.**Joan Tosca Cuquerella** en el teléfono **961 97 35 00 Ext: 436 448**.

1. Participación voluntaria

Le invitamos a participar en el estudio porque ha sido diagnosticado de **enfermedad hepática crónica**.

Debe saber que su participación en este estudio es totalmente voluntaria y que puede decidir NO participar. Si decide participar, puede cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en suatención sanitaria. Así mismo, podrá retirarse del estudio en cualquier momento, sin tener que darexplicaciones.

2. Justificación y Objetivo del estudio

La enfermedad hepática crónica y, particularmente, la enfermedad de hígado graso no alcohólico (EHGNA) representa actualmente un problema creciente en nuestra sociedad, especialmentedebido al importante aumento en la incidencia de diabetes mellitus y obesidad. De hecho, se ha convertido en la causa más frecuente de disfunción hepática en los países desarrollados - se estimaque en España una de cada cuatro personas padece esta enfermedad. Aunque en algunos pacientesse manifiesta solo como acumulación de lípidos en el hígado, en muchos otros progresa a estadios

más graves como es la esteatohepatitis, la fibrosis, la cirrosis y el cáncer de hígado. Desgraciadamente, no existen tratamientos específicos ni efectivos para su diagnóstico y pronóstico. Por todo ello, existe un gran interés en la identificación de posibles moléculas útiles para el tratamiento y el diagnóstico de la enfermedad. El objetivo de este estudio es buscar estas dianas moleculares con potencial diagnóstico, pronóstico o terapéutico para la enfermedad hepática crónica.

3. Descripción del estudio

El estudio involucrará pacientes con enfermedad hepática crónica de distintos orígenes y en distintos estadios. Se reclutarán adultos, sin límite superior de edad y de ambos sexos. Puesto quese trata de una población bastante amplia y heterogénea, el número total de sujetos involucrados en el estudio no debería ser menor de 100. El estudio consiste en la utilización de muestras humanas (sangre y tejido hepático) con el fin de estudiar los mecanismos celulares de la enfermedad. Además, de dichas muestras se aislarán células que se tratarán con distintos fármacospara evaluar su posible capacidad terapéutica.

4. Actividades del estudio

Se prevé obtener el número total de muestras a lo largo de 1 año. A partir de la última muestra recogida se añadirán 6 meses para el análisis y publicación de los datos. En un principio, no se prevéque su participación en el estudio conlleve la realización de más visitas y más pruebas de las que serealizarían si no participara. Tampoco se prevé la realización de exploraciones y actividades complementarias o extraordinarias por su participación en el estudio. En el caso de que sean necesarias por el desarrollo del proyecto, Ud. será informado debidamente.

5. Riesgos y molestias derivados de su participación en el estudio

En el caso de la extracción de sangre: el riesgo previsible de su participación únicamente será el mínimo riesgo que conlleva la extracción de una muestra de sangre. Para la mayoría de las personas, las punciones con agujas para la extracción de sangre no suponen ningún problema. Sin embargo, en ocasiones, pueden provocar hemorragias, hematomas, enrojecimiento, hinchazón, infecciones y/o dolor en el punto de extracción de sangre. También puede sentirse mareado. De todos modos, no se trata de ningún riesgo adicional para usted, ya que no va a realizar ningún procedimiento fuera de la práctica clínica habitual.

En el caso de la donación de una muestra de tejido hepático: el riesgo previsible es la presencia dehemorragia en la zona de extracción del tejido hepático, pero cabe destacar que se trata de una reacción cuya frecuencia es muy baja. De todos modos, no se trata de ningún riesgo adicional parausted, ya que dicha extracción formará parte del procedimiento habitual y no supondrá realizar ningún procedimiento fuera de la práctica clínica habitual.

6. Posibles beneficios

Es muy posible que usted no obtenga ningún beneficio para su salud por participar en

este estudio, pero podrá ayudar a conocer mejor su enfermedad y mejorar el pronóstico y el tratamiento de futuros pacientes.

7. Protección de datos personales

El investigador/promotor y el centro son responsables respectivamente del tratamiento de sus datos y se comprometen a cumplir con la normativa de protección de datos en vigor, la Ley Orgánica3/2018, de 5 de diciembre, de Protección de Datos Personales y Garantía de los Derechos Digitales,el Real Decreto que la desarrolla (RD 1720/2007) y el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD).

Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no incluya información que pueda identificarle, y solo su médico del estudio/colaboradores podrá relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a persona alguna salvo excepciones en caso de urgencia médica o requerimiento legal.

El acceso a su información personal identificada quedará restringido al médico del estudio/colaboradores, autoridades competentes, al Comité de Ética de la Investigación y personal autorizado por el promotor (monitores del estudio, auditores), cuando lo precisen para comprobarlos datos y procedimientos del estudio, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

De acuerdo a lo que establece la legislación de protección de datos, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual deberá dirigirse a su médico del estudio. Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos, pero sí se utilizarán los que ya se hayan recogido.

Además, puede limitar el tratamiento de datos que sean incorrectos, solicitar una copia o que se trasladen a un tercero (portabilidad) los datos que usted ha facilitado para el estudio. Para ejercitar sus derechos, diríjase al investigador principal del estudio o al Delegado/a de Protección de Datos del centro/institución en dpd@gva.es. Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

Los datos codificados pueden ser transmitidos a terceros y a otros países, pero en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, iniciales,dirección, nº de la seguridad social, etc. En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito o para su uso en publicaciones científicas, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

8. INFORMACION RELATIVA A MUESTRAS BIOLÓGICAS

Su participación en este estudio conlleva la obtención y utilización de muestras biológicas con finesde investigación, para lo que se observará la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011 de Biobancos, normativas que garantizan el respeto a los derechos que le asisten.
Al firmar este documento, revisado y evaluado favorablemente por el Comité de Ética de Investigación de su centro, usted acepta que se utilicen sus muestras para las finalidades del presente estudio.

8.1 Procedimientos de obtención de muestras, molestias y posibles riesgos

Las muestras serán obtenidas durante el seguimiento habitual de su enfermedad y formarán partede la práctica clínica habitual y el proceso asistencial. No se prevé ningún riesgo adicional para usted, ya que no va a realizar ningún procedimiento fuera de la práctica clínica habitual. Los riesgos que conllevan los procedimientos en cuestión son los mismos que tendrá si decide no participar enel estudio.
Se le solicita permiso para utilizar con fines científicos **una muestra de su sangre** y/o, en el caso deque usted sea intervenido quirúrgicamente a nivel hepático, **una muestra de su hígado**.

- Muestras de sangre: se obtendrá una muestra de sangre entera de 25 ml de sangre. El riesgo previsible de su participación únicamente será el mínimo riesgo que conlleva la extracción de una muestra de sangre.
- Muestras de hígado: se le pedirá que done una parte de la muestra de una biopsia o de una intervención quirúrgica que se le haya realizado. El riesgo previsible es de hemorragia en la zona deextracción.

Las muestras estarán asociadas a un código que solo podrá ser relacionado con su identidad por personal autorizado, de la misma manera que se ha explicado previamente con los datos obtenidosdurante el estudio.

Los datos que se deriven de la utilización de estas muestras se tratarán del mismo modo que el resto de datos que se obtengan durante este estudio en cuanto a la protección de datos.
Las muestras y los datos asociados se mantendrán bajo las condiciones de seguridad adecuadas y se garantiza que los sujetos no podrán ser identificados a través de medios considerados razonablespor personas distintas a las autorizadas.
Es posible que sea necesario algún dato o muestras adicionales. En ese caso, su médico se pondrá en contacto con usted para solicitarle de nuevo su colaboración. Se le informará de los motivos y se le solicitará de nuevo su consentimiento.

8.2 Beneficios esperados

No se espera un beneficio directo por su participación en el estudio. No obstante, los conocimientos obtenidos gracias a los estudios llevados a cabo a partir de sus muestras y de muchas otras puedenayudar al avance científico.
No percibirá ningún beneficio económico por la donación de las muestras y la cesión de los datos proporcionados, ni tendrá derechos sobre posibles beneficios

comerciales de los descubrimientos que puedan conseguirse como resultado de la investigación efectuada.

8.3. Lugar de análisis y almacenamiento de muestras

Durante el desarrollo del estudio sus muestras pueden ser analizadas en la Facultad de Medicina de la Universidad de Valencia. Durante este proceso el responsable de las muestras será elpromotor/investigador del estudio.

8.4. Implicaciones de la información obtenida al analizar las muestras

En el caso de que usted lo solicite, se le podrá facilitar información acerca de los estudios generalesdel presente estudio.

En el caso de que en este estudio se obtengan datos que pudieran ser clínica o genéticamente relevantes para usted, e interesar a su salud o a la de su familia, podrá solicitar que le sean comunicados por su médico del estudio.

No obstante, si usted manifiesta su negativa a ser informado, pero según criterio del médico responsable, la información obtenida sea necesaria para evitar un grave perjuicio para su salud o lade sus familiares biológicos, se informará a un familiar próximo o a un representante, previa consulta al Comité de Ética Asistencial del centro. La comunicación de esta información se llevará acabo por profesionales que le podrán explicar adecuadamente su relevancia y las opciones que se pudieran plantear. En caso de información genética clínicamente relevante podrá recibir el preceptivo consejo genético.

8.5. Uso futuro de las muestras

Las muestras serán conservadas en el Departamento de Farmacología de la Facultad de Medicina de la Universidad de Valencia hasta que sean procesadas (no más de un mes), tras lo cual se destruirán.
Una vez finalizado el estudio, las muestras sobrantes serán destruidas. Además, este material no será bajo ningún concepto ni en ningún momento motivo de lucro, bien sea por la venta del materialo de los derechos para realizar estudios sobre los mismos.

CONSENTIMIENTO INFORMADO

TÍTULO DEL ESTUDIO: Nuevas dianas farmacológicas para el tratamiento de la enfermedad hepática crónica: rutas de señalización JAK-STAT1 y JAK-STAT3 como punto de partida	
PROMOTOR	
INVESTIGADOR PRINCIPAL	**Joan Tosca Cuquerella**
SERVICIO	**Gastroenterología**
CENTRO	**Hospital Clínico Universitario**

Yo,_____<<nombre y apellidos del participante>>(Nombre de puño y letra por el paciente)

He leído la hoja de información que se me ha entregado sobre el estudio. He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con_____<<nombre del investigador>>(Nombre de puño y letra por el paciente)

Comprendo que mi participación es voluntaria.

Comprendo que puedo retirarme del estudio:

- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

Consiento al uso y tratamiento de mis datos personales para esta investigación en las condicionesexplicadas en esta hoja de información.

<u>Uso de Muestras</u>

Consiento al almacenamiento y uso de las muestras y de los datos asociados para esta investigaciónen las condiciones explicadas en esta hoja de información.

☐ SI ☐ NO

Deseo que el médico del estudio me comunique la información derivada de la investigación (genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable parami salud o la de mis familiares:

☐ SI ☐ NO Teléfono o e-mail de contacto

_____

Consiento a ser contactado en el caso de necesitar más información o muestras biológicasadicionales.

ˠ SI NO Teléfono o e-mail de contacto

_____

Recibiré una copia firmada y fechada de este documento de consentimiento informado

Firma del participante
Fecha:_____/_____/
_____

Firma del investigador
Fecha:_____/_____/
_____

(Firma y fecha de puño y letra por el paciente)

CONSENTIMIENTO INFORMADO

REPRESENTANTE LEGAL

TÍTULO DEL ESTUDIO: Nuevas dianas farmacológicas para el tratamiento de la enfermedad hepática crónica: rutas de señalización JAK-STAT1 y JAK-STAT3 como punto de partida	
PROMOTOR	
INVESTIGADOR PRINCIPAL	**Joan Tosca Cuquerella**
SERVICIO	**Gastroenterología**
CENTRO	**Hospital Clínico Universitario**

Yo,_____<<nombre y apellidos del representante>>, en calidad de _____

(Nombre de puño y letra por el representante)

_____<<indicar parentesco>>, de_<<nombre y apellidos del participante>>(Nombre de puño y letra por el representante)

He leído la hoja de información que se me ha entregado sobre el estudio. He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.
He hablado con_____

_____<< nombre del investigador>> (Nombre de puño y letra por el representante)

Comprendo que su participación es voluntaria.
Comprendo que puede retirarse del estudio:

- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en sus cuidados médicos.

Presto libremente mi conformidad para su participación en el estudio
Consiento al uso y tratamiento de sus datos personales para esta investigación en las condicionesexplicadas en esta hoja de información.

Uso de Muestras
Consiento al almacenamiento y uso de las muestras y de los datos asociados para esta investigaciónen las condiciones explicadas en esta hoja de información.

Ɣ SI □ NO

Deseo que el médico del estudio me comunique la información derivada de la investigación(genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable para mi salud o la de mis familiares:

 SI NO Teléfono o e-mail de contacto:

Consiento a ser contactado en el caso de necesitar más información o muestras biológicasadicionales.

 SI NO Teléfono o e-mail de contacto

Recibirá una copia firmada y fechada de este documento de consentimiento informado

Firma del representante legal, familiar o persona vinculada de hecho

Fecha:_____/____/_____

(Firma y fecha de puño y letra por el representante)

Firma del investigador

Fecha:_____/____/_____

CONSENTIMIENTO INFORMADO ORAL ANTE TESTIGOS

TÍTULO DEL ESTUDIO: Nuevas dianas farmacológicas para el tratamiento de la enfermedad hepática crónica: rutas de señalización JAK-STAT1 y JAK-STAT3 como punto de partida	
PROMOTOR	
INVESTIGADOR PRINCIPAL	**Joan Tosca Cuquerella**
SERVICIO	**Gastroenterología**
CENTRO	**Hospital Clínico Universitario**

Yo,_____<<nombre y apellidos del testigo>>,(Nombre de puño y letra por el testigo)

como testigo, afirmo que en mi presencia se ha informado a D/Dª _____<<nombre y apellidos del participante>>(Nombre de puño y letra por el testigo)

y se ha leído la hoja de información que se le ha entregado sobre el estudio, de modo que:

Ha podido hacer preguntas sobre el estudio.
Ha recibido suficiente información sobre el estudio.
Ha hablado con_____

_____<<nombre del investigador>>(Nombre de puño y letra por el testigo)

Comprende que su participación es voluntaria.
Comprende que puede retirarse del estudio:
- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en sus cuidados médicos.

Presta libremente su conformidad para su participación en el estudio.
Consiente al uso y tratamiento de sus datos personales para esta investigación en las condicionesexplicadas en esta hoja de información.

Uso de Muestras

Consiente al almacenamiento y uso de las muestras y de los datos asociados para esta investigaciónen las condiciones explicadas en esta hoja de información.

☐ SI ☐ NO

Desea que el médico del estudio me comunique la información derivada de la investigación (genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable parami salud o la de mis familiares:

☐ SI ☐ NO Teléfono o e-mail de contacto ___ Consiente a ser contactado en el caso de necesitar más información o muestras biológicasadicionales.

☐ SI ☐ NO Teléfono o e-mail de contacto ___ Recibirá una copia firmada y fechada de este documento de consentimiento informado

Firma del testigo
Fecha:_____/_____/_____
(Firma y fecha de puño y letra por el testigo)

Firma del investigador
Fecha:_____/_____/_____

Annex 6. Ethics committee approval certificates for the mouse models

30 anys D'IDENTITAT
GENERALITAT VALENCIANA
CONSELLERIA DE PRESIDENCIA Y AGRICULTURA, PESCA, ALIMENTACION Y AGUA

Unión Europea

AUTORIZACION PROCEDIMIENTO 2014/VSC/PEA/00188

Vista la solicitud realizada en fecha **31/10/14** con nº reg. entrada **20463** por D/Dª. **Pilar Campins Falcó**, Vicerrectora Investigación y Política Científica, centro usuario **ES462500001003**, para realizar el procedimiento:

"Estudio del efecto de la terapia antirretroviral sobre un modelo vivo de esteatohepatitis inducida con dieta rica en grasa "

Teniendo en cuenta la documentación aportada, según se indica en el artículo 33, punto 5 y 6, y puesto que dicho procedimiento se halla sujeto a autorización en virtud de lo dispuesto en el artículo 31 del Real Decreto 53/2013, de 1 de febrero,

Vista la propuesta del jefe del servicio de Sanidad y Bienestar Animal.

AUTORIZO:

la realización de dicho procedimiento al que se le asigna el código: **2014/VSC/PEA/00188** tipo **2,** de acuerdo con las características descritas en la propia documentación para el número de animales, especie y periodo de tiempo solicitado. Todo ello sin menoscabo de las autorizaciones pertinentes, por otras Administraciones y entidades, y llevándose a cabo en las siguientes condiciones:

Usuario: **Universitat de València**

Responsable del proyecto: **Ana Blas García**

Establecimiento: **Animalario Unidad Central de Investigación**

Necesidad de evaluación restrospectiva:

Condiciones específicas:

Observaciones:

Valencia a, 26 de noviembre de 2014

El director general de Producción Agraria y Ganadería

José Miguel Ferrer Arranz

GENERALITAT VALENCIANA
Conselleria d'Agricultura, Medi Ambient, Canvi Climàtic i Desenvolupament Rural

Dirección General de Agricultura, Ganadería y Pesca
Ciutat Administrativa 9 d'Octubre
Calle de La Democracia, 77 · Edif. B3 P2
46018 València

AUTORIZACION PROCEDIMIENTO 2019/VSC/PEA/0112

Vista la solicitud realizada en fecha **16/04/19** con nº reg. entrada **263068** por D/Dª. **Carlos Hermenegildo Caudevilla**, **Vicerrector de Investigación y Política Científica**, centro usuario **ES462500001003**, para realizar el procedimiento:

"Estudio del efecto de la terapia antirretroviral sobre la progresión del daño hepático crónico de tipo colestásico inducido por ligadura de conducto biliar."

Teniendo en cuenta la documentación aportada, según se indica en el artículo 33, punto 5 y 6, y puesto que dicho procedimiento se halla sujeto a autorización en virtud de lo dispuesto en el artículo 31 del Real Decreto 53/2013, de 1 de febrero,

Vista la propuesta del jefe del servicio de Producción y Sanidad Animal.

AUTORIZO:

la realización de dicho procedimiento al que se le asigna el código: **2019/VSC/PEA/0112** tipo **2,** de acuerdo con las características descritas en la propia documentación para el número de animales, especie y periodo de tiempo solicitado. Todo ello sin menoscabo de las autorizaciones pertinentes, por otras Administraciones y entidades, y llevándose a cabo en las siguientes condiciones:

Usuario: **Universidad de Valencia-Estudio General**

Responsable del proyecto: **Ana Blas García**

Establecimiento: **Animalario Unidad Central de Investigación (Fac. Medicina y Odontología) Valencia**

Necesidad de evaluación restrospectiva:

Condiciones específicas:

Observaciones:

Valencia a, fecha de la firma electrónica
El director general de Agricultura, Ganadería y Pesca

Firmat per Rogelio Llanes Ribas el 25/04/2019 14:19:13
Càrrec: Dirección General

Annex 7. Bibliographic production of the PhD candidate

1. **Gruevska A**, Moragrega AB, Galindo MJ, Esplugues JV, Blas-Garcia A, Apostolova N. "Down-regulation of the longevity-associated protein SIRT1 in peripheral blood mononuclear cells of treated HIV patients". *Cells.* 2022;11(3):348. https://doi.org/10.3390/cells11030348.
2. Lucantoni F, Benedicto A.M, **Gruevska A,** Moragrega AB, Fuster-Martínez I, Esplugues JV, Blas-Garcia A, Apostolova N. "Implication of autophagy in the antifibrogenic effect of Rilpivirine: when more is less". *Cell Death & Disease*. 2022;13(4):385. doi: 10.1038/s41419-022-04789-7.
3. Lucantoni F, Martínez-Cerezuela A, **Gruevska A,** Moragrega AB, Victor VM, Esplugues JV, Blas-Garcia A, Apostolova N. "Understanding the implication of autophagy in the activation of hepatic stellate cells in liver fibrosis: are we there yet?". *The Journal of Pathology*. 2021;254(3):216-228. doi: 10.1002/path.5678. (Editor's Choice Review, Front page).
4. **Gruevska A**, Moragrega AB, Cossarizza A, Esplugues JV, Blas-Garcia A, Apostolova N. "Apoptosis of Hepatocytes: Relevance for HIV-Infected Patients under Treatment". *Cells*. 2021;10(2),410. https://doi.org/10.3390/cells10020410.
5. **Gruevska A,** Moragrega AB, Galindo MJ, Esplugues JV, Blas-Garcia A, Apostolova N. "p53 and p53-related mediators PAI-1 and IGFBP-3 are downregulated in peripheral blood mononuclear cells of HIV patients exposed to non-nucleoside reverse transcriptase inhibitors". *Antiviral Research* 2020; 178:104784. doi:10.1016/j.antiviral.2020.104784.
6. Apostolova N, Iannantuoni F, **Gruevska A**, Muntane J, Rocha M, Victor VM. "Mechanisms of action of metformin in type 2 diabetes: Effects on mitochondria and leukocyte-endothelium interactions". *Redox Biology.* 2020;34:101517. doi:10.1016/j.redox.2020.101517
7. Martinez-Arroyo O, **Gruevska A**, Victor VM, González-Polo RA, Yakhine-Diop SMS., Fuentes JM., Esplugues JV., Blas-Garcia A., Apostolova N.

“Mitophagy in human astrocytes treated with the antiretroviral drug Efavirenz: Lack of evidence or evidence of the lack”. *Antiviral Research*. 2019; 168:36-50. doi: 10.1016/j.antiviral.2019.04.015.