UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural

Mecanismos moleculares implicados en los efectos antiinflamatorios y hepatoprotectores del fármaco antirretroviral Rilpivirina

Trabajo Fin de Grado

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UNIVERSITAT POLITÈCNICA DE VALÈNCIA

MOLECULAR MECHANISMS INVOLVED IN THE ANTI-INFLAMMATORY AND HEPATOPROTECTIVE EFFECTS OF THE ANTIRETROVIRAL DRUG RILPIVIRINE

Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural

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Abstract

Drug repurposing is a common therapeutical approach widely used in the pharma industry that consists of the discovery of additional indications for already-approved drugs. Previous studies performed in animal models and in cultured cells revealed that Rilpivirine (RPV), an antiretroviral drug widely used in the treatment of the Human Immunodeficiency Virus (HIV) infection, exerts anti-inflammatory and anti-fibrotic effects in the liver. This action can be exploited in the search of novel drug targets for the treatment of chronic hepatic diseases such as non-alcoholic fatty liver disease (NAFLD), a very common condition that can give rise to non-alcoholic steatohepatitis (NASH) and liver fibrosis, and can ultimately lead to end-stage liver disease such as cirrhosis. In this Final Degree Project, the anti-inflammatory function of RPV has been evaluated in peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals, and some of the molecular mechanisms that might be implicated in its hepatoprotective, anti-inflammatory and anti-fibrotic roles at hepatic level that act on the mitogen-activated protein kinases (MAPK) signalling have been described using primary human hepatic stellate cells (HSCs). The results obtained showed that in PBMCs, RPV enhanced the expression of anti-inflammatory mediators while reducing that of chemokines and pro-inflammatory molecules. Moreover, RPV inhibited HSCs proliferation and migration under PDGF-ββ stimulation while reducing MAPKs activation.

Resumen

El reposicionamiento farmacológico es una estrategia terapéutica ampliamente usada en la industria farmacéutica que consiste en el descubrimiento de indicaciones alternativas en fármacos ya aprobados. Estudios previos realizados en modelos animales y cultivos celulares revelan que la Rilpivirina (RPV) , un fármaco antirretroviral ampliamente utilizado en el tratamiento de la infección por el virus de la inmunodeficiencia humana (VIH), ejerce efectos antinflamatorios y antifibróticos en el hígado. Esta acción puede ser aprovechada para la búsqueda de nuevas dianas terapéuticas para el tratamiento de enfermedades hepáticas crónicas como la enfermedad de hígado graso no alcohólica (EHGNA), una condición muy común que pueden derivar en esteatohepatitis no alcohólica (EHNA) y fibrosis hepática, y en última instancia producir una enfermedad hepática terminal, como la cirrosis. A lo largo de este Trabajo de Fin de Grado se ha evaluado el efecto antiinflamatorio de la RPV en células mononucleares de sangre periférica (PBMCs) aisladas de controles, y se han descrito algunos de los mecanismos moleculares que podrían estar implicados en sus efectos hepatoprotectores, antiinflamatorios y antifibróticos a nivel hepático, utilizando células estrelladas hepáticas (HSCs) y centrándonos en la ruta de señalización de las kinasas activadas por mitógenos (MAPK). Los resultados obtenidos mostraron que en PBMCs, la RPV estimuló la expresión de mediadores antiinflamatorios a la vez que redujo la de quimiocinas y moléculas proinflamatorias. Además, RPV inhibió la proliferación y migración de HSCs estimuladas con PDGF-ββ al mismo tiempo que disminuyó la activación de MAPK kinasas.

Keywords: Inflammation / Chronic Liver Disease / Fibrosis / Rilpivirine / Antiretroviral therapy

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ABBREVIATIONS & ACRONYMS

AIDS	Acquired immunodeficiency syndrome
APS	Ammonium persulfate
ARV	Antiretroviral
cART	Combined antiretroviral therapy
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
βME	Beta mercaptoethanol
CD4	Cluster of differentiation 4
COVID-19	Coronavirus disease 2019
cDNA	Complementary DNA
CCL2	CC motif chemokine ligand 2
CXCL9	C-X-C motif chemokine ligand 9
CXCL10	C-X-C motif chemokine ligand 10
CXCR4	C-X-C chemokine receptor type 4
CCR5	C-C motif chemokine receptor 5
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FDA	Food and drug administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hanks′ balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus

HRP	Horseradish peroxidase
HSC	Hepatic stellate cell
IL1-β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
LGBT	Lesbian gay bisexual transgender
MF	Myofibroblasts
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium
PAI-I	Plasminogen activator inhibitor 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKB	Protein kinase B
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPV	Rilpivirine
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
ssRNA	Single-stranded ribonucleic acid
SDS	Sodium dodecyl sulphate
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
TEMED	N,N,N′,N′-tetramethylethylenediamine
TSB-T	Tris-buffered saline-Tween
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
Veh	Vehicle
WHO	World health organization

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1. INTRODUCTION

1.1. HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION AND ANTIRETROVIRAL THERAPY

1.1.1. Historical context and phases in the progression of the disease

The AIDS covers a range of conditions caused by the infection of human immunodeficiency virus (HIV). The chronic infection of this retrovirus provokes a reduction in the T-lymphocytes population, resulting in a severe reduction in the patient immune system. The first cases were reported in 1981 in the USA when LGBT community members presented pneumonia-like symptoms and developed rare skin cancer after a short period of time after infection (de Cock *et al*., 2021). So far, HIV infection has affected more than 70 million people worldwide and has been the cause of death of approximately 39 million people according to the Joint United Nations Programme on HIV/AIDS (UNAIDS). Viral particles are transmitted via sexual intercourse, vertical transmission, and persistent contact with body fluids and tissues. While HIV-1 was firstly described for infection processes in the USA and Europe and has a higher prevalence, HIV-2, which is less aggressive, was attributed to infections in Africa (Clavel *et al*., 1986).

The overall process distinguishes three clear phases until the development of the syndrome: an acute infection process, a latency period, and finally the development of AIDS. The acute infection (also called primary HIV) is characterised by the development of influenza- or mononucleosis-like symptoms 2-4 weeks after the exposure to HIV and lasts for about 1-2 weeks. The duration of the latency period (chronic HIV) varies greatly, from 3 to 20 years, and this is influenced by the HIV subtype and the patient´s age. Near the end of the latency phase patients experience weight loss, muscular pain and, in most cases, persistent generalized lymphadenopathy, characterised by the enlargement of lymph nodes. The AIDS stage is characterised by a CD4+ T-lymphocytes count below 200 cells per uL and the appearance of HIV-associated diseases like pneumocystis pneumonia, cachexia or esophageal candidiasis (Vergis and Mellors, 2000).

1.1.2. HIV life cycle

HIV belongs to the group VII of viruses according to the Baltimore classification, which contains single-stranded +RNA viruses that replicate through a DNA intermediate. More specifically, HIV is a *Lentivirus* inside the *Retroviridae* family. Its main target are T-lymphocytes, but HIV can also infect other cell types such as tissue macrophages or dendritic cells.

HIV infection starts by the fusion of the viral envelope with the T-lymphocyte membrane through the interaction between the viral envelope trimeric complex formed by glycoproteins gp120 and gp41 with the host membrane receptor CD4 and the chemokine coreceptors CXCR4 and CCR5 (Melikyan, 2014). After membrane fusion, the viral nucleocapsid escapes from the endocytosis process. Once free, both copies of viral ssRNA are retrotranscribed into dsDNA thanks to the enzyme reverse transcriptase in a process known as retrotranscription. The generated dsDNA is integrated in the host genome with the help of the viral integrase. After a latency period, viral dsDNA is transcribed and viral proteins are generated. Transcription results in early instance in the expression of Tat and Rev through short transcripts. Tat facilitates the transcription of bigger transcripts by interaction with the Tat Response Element (TAR) site, and Rev enhances viral maturation. Long messenger RNAs (mRNAs) are translated in the cytoplasm and generate the rest of the viral proteins coded by genes *Gag*, *Pol* and *Env*. *Env* codes for gp160, cleaved by the protease to generate gp120 and gp41. Finally, two viral RNA+ strands are assembled inside the immature nucleocapsid and, by exocytosis, the immature virion is released. Maturation takes place thanks to a viral protease, generating the entire set of viral particles.

1.1.3. Classification of antiretroviral drugs

HIV infection is treated with antiretroviral (ARV) drugs which are not usually administered individually but in a combination called combined antiretroviral therapy (cART), the main responsible for a dramatic reduction in AIDS-derived deaths during the last years. More recently, due to the appearance of several resistances and adverse events, the need of new ARV drugs has increased.

Notwithstanding, HIV is not thought to be eradicated by the use of drugs due to its incredible mutagenic capacity, as reverse transcriptase does not possess a proofreading subunit. Consequently, the heterogenous viral population inside the same individual marks the degree of response of each patient to the therapy, and ARV drugs are mostly used to reduce the associated morbidity and mortality. In addition, as people need to take ARVs for longer periods of time, the observed toxicity has increased over the years.

The different types of ARV drugs are designed to target each of the different steps involved in the HIV life cycle: binding and fusion, reverse transcription, integration in the host genome and virion maturation (Benedicto *et al*., 2021) (Figure 1).

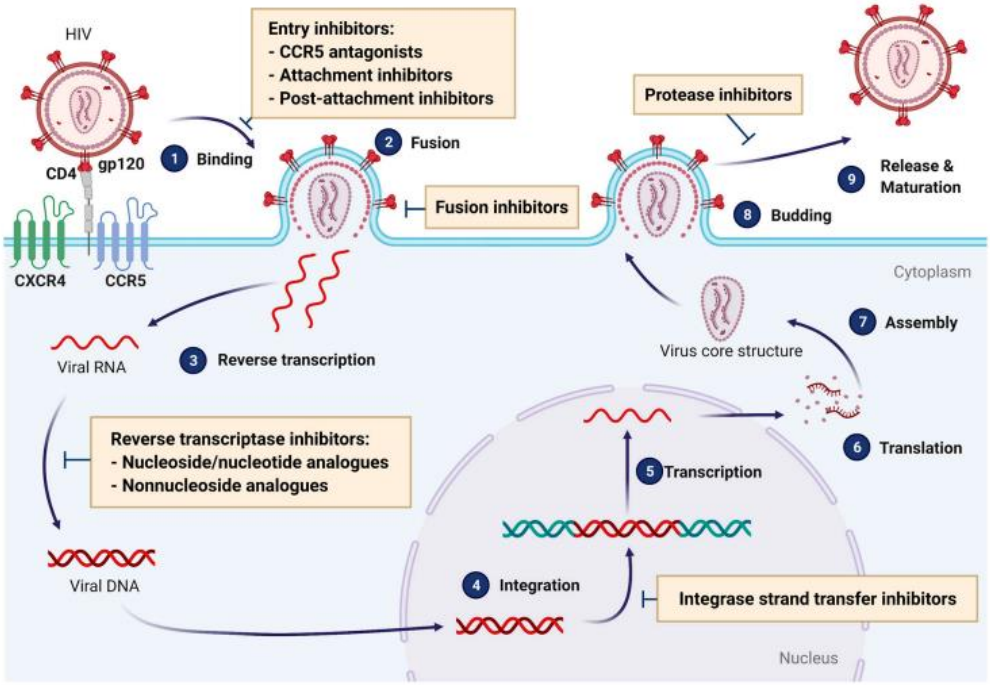


Figure 1. Available anti-HIV drug families and their mechanisms of action depending on the viral cycle step (Benedicto *et al*., 2021).

Binding/Fusion inhibitors

Blocking the CD4 receptor is not a feasible approach due to the great importance of this receptor in the immune system. However, by blocking the coreceptors with CXCR4 and CCR5 antagonists, the virus is unable to bind and fuse with the membrane. Bicyclam derivatives such as AMD3100 have proved to block X4 and dual-tropic (R5/X4) variants (De Clercq and Schols, 2001), and Maraviroc is a CCR5 antagonist (Fadel and Temesgen, 2007). Besides, the fusion step per se can also be targeted by the use of synthetic peptides such as Enfuvirtide, which, thanks to its affinity towards the gp41, is able to block the fusion of viral and cell membranes (Dando and Perry, 2003).

Reverse transcriptase inhibitors

These molecules bind the reverse transcriptase, inhibiting the reverse transcription and thus, HIV replication. This family of compounds comprises two groups: nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI).

NRTI are pro-drugs as, in order to exert its function, they need to be phosphorylated by cellular kinases. Thanks to its structural homology with normal nucleosides, NRTIs avoid the synthesis of the cDNA. The prototype from which the rest are derived is Zidovudine (Sperling, 1998), the first approved anti-HIV drug. There are 7 commercially available NRTIs and their biggest drawback is the derived mitochondrial toxicity and hepatic steatosis (Margolis *et al*., 2014).

NNRTIs inhibit the retrotranscription process in a non-competitive way. They bind closely to the active site, in a hydrophobic pocket, provoking a conformational change that disrupts the ability of the enzyme to add nucleosides effectively to the generating DNA strand. RPV, the drug of study, belongs to this group (Margolis *et al*., 2014).

Integrase inhibitors (IIs)

These drugs impede insertion of integrase in the synthesized DNA duplex, unabling the endonucleolytic processing of dsDNA ends, and thus, the generation of the sticky ends needed for the integration process in the CD4+ lymphocyte genome (Bhatti *et al*., 2016).

They are all based on the presence of a chelating motif which, depending on the generation of the II, is located somewhere in the conformational structure. In the case of last generation IIs, this motif is located inside on a tricyclic scaffold and recent advances have focused on reducing the drug-derived resistance thanks to a chemical linker (Wang *et al*., 2021).

Protease inhibitors (PIs)

These drugs prevent HIV replication by inhibiting the action of the viral protease. They bind the active site of this enzyme and avoid virion maturation, provoking, among other actions, that gp160 does not generate gp120 and gp41 and thus, HIV is unable to enter the CD4+ cell. PIs are generally well-tolerated by the organism and its major toxicity has been reported after a long-term exposure (Bhatti *et al*., 2016).

Currently used drugs of each family of ARV drugs are shown in Table 1.

Table 1. Currently used antiretroviral drugs depending on their family (WHO, 2021)**.**

ARV drug family	Currently used drugs
Binding/Fusion inhibitors	Enfuvirtide
Reverse transcription inhibitors	
Nucleoside reverse transcriptase inhibitors	Abacavir, Emtricitabine, Didanosine, Lamivudine, Stavudine,Tenofovir alafenamide, Tenofovir disoproxil fumarate, Zidovudine.
Non-nucleoside reverse transcriptase inhibitors	Efavirenz, Etravirine, Neviparine, Rilpivirine.
Integrase inhibitors	Cabotegravir, Dolutegravir, Elvitegravir, Raltegavir.
Protease inhibitors	Atazanavir, Lopinavir/Ritonavir, Saquinavir, Fosamprenavir, Darunavir, Indinavir.

1.1.4. cART-related side effects and pharmacological guidance

Because of the combinatorial use of ARV drugs, it is difficult to identify the drug which causes the adverse effects. However, second-generation ARVs show better results than first-generation ones. In patients exposed to cART, lower high-density lipoprotein and higher low-density lipoprotein and triglyceride levels have been reported (Kovari and Weber, 2011). This obviously depends on the type of inhibitor that is administered to the patient (Benedicto *et al*., 2021). For example, PIs are thought to be the main responsible for dyslipidemia.

The liver has been shown to be affected after continuous exposure to ARV drugs, generating cholestatic, hepatocellular and mixed damage (Neff *et al*., 2006). In HIV-infected patients, the major hepatopathy is consequence of HCV and HBV chronic infection. However, AIDS-related opportunistic infections together with alcohol intake or gender have shown to be another major cause of liver disease in these patients (Kovari and Weber, 2011).

WHO 2021 guidelines recommend the use of two NRTI and one II for HIV treatment, being Tenofovir disoproxil fumarate combined with Lamivudine (or Emtricitabine) and Dolutegravir the preferred first-line regimen in adults and adolescents. This combination can be changed under special circumstances. The combination of Emtricitabine/Tenofovir disoproxil fumarate has previously showed to reduce in a 92% the acquisition of HIV by HIV-negative individuals as pre-exposure prophylaxis (Kojima and Klauser, 2016). ARV drugs are metabolised in the hepatic cytochrome P450. However, common adverse effects have also been described (Table 2).

Table 2. Side effects associated with different families of ARV drugs (Adapted from OARAC, 2021).

ARV drug type	Side effects
Binding/Fusion inhibitors	Headache, fatigue, nausea, nasopharyngitis,
Reverse transcription inhibitors	
Nucleoside reverse transcriptase inhibitors	Nephrotoxicity, hypersensitivity reactions, lactic acidosis, lipoatrophy, loss of bone mineral density, bone marrow suppression, macrocytic anemia, Fanconi syndrome, myopathy, increased risk of cardiovascular events, diarrhea, pancreatitis, neuropathy, non-cirrhotic portal hypertension, hepatic steatosis, hyperpigmentation,
Non-nucleoside reverse transcriptase inhibitors	Drug interactions, rash, neuropsychiatric side effects, hepatic effects, dyslipemia, Stevens-Johnson syndrome, teratogenicity, cardiovascular diseases,
Integrase inhibitors	Fatigue, diarrhea, insomnia, nasopharyngitis, upper respiratory infections
Protease inhibitors (PI)	Gastrointestinal problems (diarrhea, nausea, vomiting), hypertriglyceridemia, insulin resistance, hyperbilirrubinemia,

1.1.5. Rilvipirine

RPV (Edurant ®) was approved by the FDA in 2011. It is a second generation NNRTI administered as 25 mg tablets taken once a day and is metabolised by the cytochrome P450 3A4 (CYP3A4), with a half-life of approximately 50 h (Jansen, 2021). It is currently indicated for cART-treatment naïve patients "12 years of age and older and weighing at least 35 kg with HIV-1 RNA less than or equal to 100,000 copies/mL" (Jansen, 2021).

RPV´s plasma concentration ranged from 12 to 255 ng/ml (Aouri *et al*., 2017), being the maximum of this concentration achieved within 4-5 h after oral administration. It is distributed throughout the whole body, but higher concentrations are present in liver, adrenal gland, brown fat and kidney (EMA, 2021).

Regarding its safety profile, some commonly reported side effects of RPV are abdominal pain, headache and insomnia, which usually appear during the first months of treatment. Despite not being one of the most potent NNRTI, it is one of the most used due to its incredible safety profile, in comparison with other NNRTI like Efavirenz, regarding lipid abnormalities and blood total cholesterol and triglycerides levels (Viciana, 2017). It has been seen that RPV also prevents excessive lipid droplet accumulation in preclinical models of chronic liver disease (Martí-Rodrigo *et al*., 2020), the main threat for cardiovascular disease and NAFLD-associated atherosclerosis in cART-patients. Besides, some cases of improved lipid profiles after patient's treatment with RPV have been reported (Curran *et al*., 2020).

For these reasons, RPV is considered key for long-term HIV treatments (Viciana, 2017), when the patient is continuously exposed to ARV drug concentrations.

Regarding hepatic toxicity, studies with cultured hepatocytes exposed to RPV at relevant plasma concentrations have revealed that the drug does not alter neither mitochondrial functions nor cell viability (Blas-Garcia *et al*., 2014). Furthermore, RPV has shown a protective role as an anti-inflammatory, anti-steatotic and anti-fibrotic agent in different mouse models of chronic liver injury (Martí-Rodrigo *et al*., 2020). This study demonstrated that fibrosis was reduced by the action of RPV through selective STAT1-dependent apoptosis in HSCs, which activates STAT3 in hepatocytes, increasing their proliferation and promoting liver regeneration.

1.2 DRUG REPURPOSING

The average global drug development cost has been set in around 2-3 billion $ (Pushpakom *et al*., 2018) and the number of new drugs approved per billion US dollars spent on R&D has halved roughly every 9 years since 1950. However, less than a 12% of drugs making it to Clinical stage 1 get final approval (Pushpakom *et al*., 2018).

Drug repurposing is a common commercial approach used in the pharma industry that consists in the discovery of alternative indications for already-approved drugs. The advantage of this approach is an earlier development and commercial launch, since data of drug's security and efficacy are available from previous clinical trials, thus overcoming the two main drawbacks of drug development: price and failure (Pushpakom *et al*., 2018). It is considered that around 75% of approved drugs can be repositioned (Singh *et al*., 2020). Clear examples of drug repurposing appeared along COVID-19 pandemic. Some drugs such as remdesivir, ribavirin, hydroxychloroquine, tocilizumab and interferons, among others, have shown inhibitory effects against SARS-CoV2 infections (Singh *et al*., 2020).

There are three different approaches for drug repurposing: computational, biological (experimental) and mixed approaches. Serendipity, the main biological experimental approach, is the most common way for drug repurposing and it is based in retrospective clinical observations. Free drug samples given by pharma companies (off-label drug use) and used under clinician's approach to be given to a patient with

a rare disease is a common practise as well. Mixed approaches comprise the purposeful screening of known compounds of the so-called compound libraries of different pharma companies, for which the access is clearly limited. Finally, computational methods involve gene expression, chemical structure, patient´s genotype and proteomic data analysis, and are covered in drug development through rational drug design (Pushpakom *et al*., 2018).

1.3. LIVER PATHOPHYSIOLOGY

1.3.1. Liver: structure and physiology

The liver is a key metabolism coordinator as it performs essential functions for the organism such as detoxification, storage of reserve proteins like glycogen, metabolism and vitamin production, among others. This organ is highly involved in growth and digestion by the synthesis of the necessary proteins and biochemicals. Besides, it is an important element of the endocrine system thanks to the production of hormones. Due to its great importance, diseases that affect this organ are usually associated with high morbidity and mortality.

Anatomically, the liver is formed by two lobes divided by the falciform ligament and it is located in the right upper quadrant of the abdominal cavity, protected by the rib cage. Microscopically, each lobe is formed by several hepatic lobules formed by plates of hepatocytes, the main hepatic cell population, and sinusoids that emerge from a central vein towards the portal triads, located in the corners. Each triad is formed by a branch of the hepatic artery, a branch of the portal vein, and a common bile duct.

Two different groups of cells can be found in the liver: parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells. The latter group is formed by hepatic stellate cells (HSCs), Kupffer cells (KCs), which are the hepatic resident macrophages, and liver sinusoidal endothelial cells (LSECs). HSCs locate between hepatocytes and sinusoids (space of Disse), where KCs are also found, while cholangiocytes are present in the bile ducts (Figure 2).

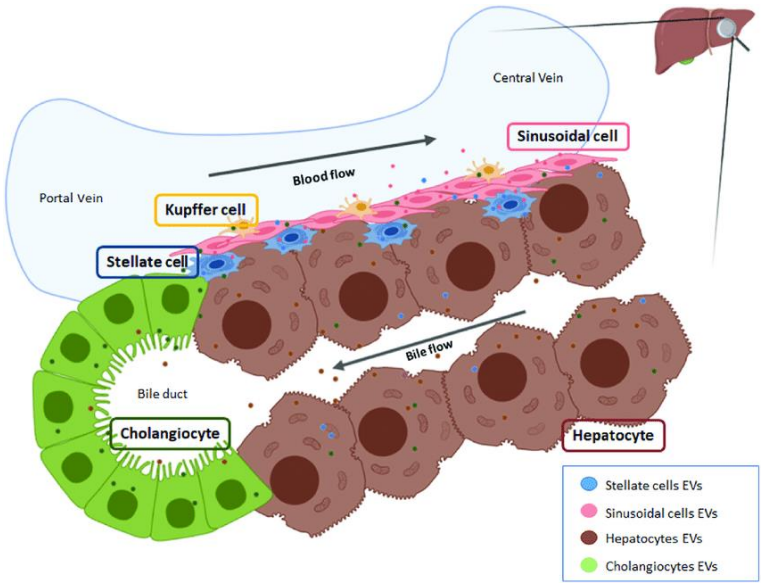


Figure 2. Cell types and functions and distribution among the liver (Azparren-Angulo *et al*., 2020).

Hepatocytes are the main effector cells in the liver: they represent around the 80% of the total cell population and actively participate in carbohydrate, protein and lipid metabolism, protein production, bile secretion, hormone production and detoxification processes. LSECs mainly participate in the clearance of blood borne waste while KCs remove foreign debris and particles that reach the liver from the gastrointestinal tract via hepatic portal system, by phagocytosis or pinocytosis depending on the size of the particle to be digested. HSCs are in charge of the regulation of sinusoid contractibility, vitamin storage and, when activated, they differentiate into MF-like HSCs, cells that cause serious hepatic damage by the excessive deposition of collagen, leading to several diseases. Lastly, cholangiocytes contribute to bile secretion via release of bicarbonate and water.

Under hepatic damage, an immune response that promotes fibrosis and enhances tissue regeneration is triggered: active HSCs generate ECM that isolates hepatocytes and protects them from acute damage. This is why the liver is able to regenerate in a short period of time even when a big portion of it has been damaged, through the activation of cyclin-dependent kinases (Michalopoulos and Bhushan, 2020). The problem resides when this regulatory mechanism becomes dysregulated under the presence of a continuous damage and becomes chronic, generating a condition called chronic liver disease (CLD). The excessive amount of ECM leads to a distortion in liver sinusoidal structure and, finally, hepatic dysfunction and cell death. This chronic condition is maintained by positive loops of cytokines and growth factors as well as cell-cell and cell-matrix interactions. Pro-inflammatory and pro-fibrogenic molecules like IL-6, TNFα, IL-1β, TGFβ, together with ROS, activate HSCs and induce HSCs and KCs-derived phagocytic activity. Other mediators like TGFβ and PDGF autocrinally increase HSCs activation and proliferation, respectively. In conclusion, there is a sustained activation of a pro-inflammatory response as well as persistent liver fibrogenesis and wound healing response (Parola and Pinzani, 2018).

1.3.2. Non-alcoholic fatty liver disease (NAFLD)

NAFLD is the main cause of chronic liver disease worldwide, with an estimated prevalence of the 20-30% in the general population, being the number in men higher than that in women (Byrne and Targher, 2015). This number is likely to continue increasing in the near future mainly due to the evolution to a sedentary lifestyle and to a fattier diet. Over last years, it has gained importance the evidence of NAFLD being a multisystem disease, since extra-hepatic tissues such as kidneys or heart are usually affected. Importantly, 70% of people suffering from type 2 diabetes mellitus (T2DM) also present NAFLD and most of these patients are also obese (75%) (Byrne and Targher, 2015).

NAFLD is usually diagnosed by radiological imaging techniques through the observation of more than a 5% of hepatic fat accumulation in the absence of other causes for this accumulation like alcohol, viral infection, drugs or autoimmunity. NAFLD is not just a particular image of the hepatic tissue, but the starting point of a progression through different conditions that lead to serious damage in the liver. It begins with lipid accumulation, leading to a high percentage of fat accumulation (hepatic steatosis). Fat presence is likely to trigger an associated inflammatory response (non-alcoholic steatohepatitis, NASH) accompanied by liver fibrosis (LF). LF develops in 40-50% of the patients with NASH, being NASH reached in the 30-40% of NAFLD cases (Byrne and Targher, 2015). Advanced disease stages can develop, being the final stage hepatocellular carcinoma (HCC) (Figure 3).

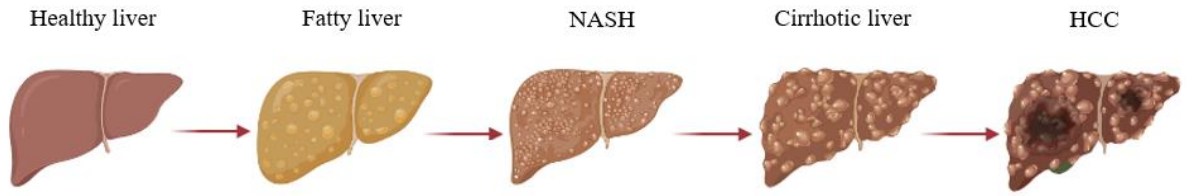


Figure 3. Progression of non-alcoholic fatty liver disease.

Recent studies also describe the importance of the gut-liver axis and epigenetics in the development of NAFLD. On the one hand, microbiome dysregulations have shown to increase fatty acids production in the bowel and, on the other hand, the epigenome is likely to alter the interindividual susceptibility to NAFLD development. This dysregulation in the microbiome leads to an increase in the intestinal permeability, which facilitates the transport of microbial metabolites into the liver, increasing local levels of inflammation (Song and Zhang, 2022).

Recently, the concept of NAFLD has been proposed to be changed to the term "metabolic dysfunction-associated fatty liver disease" (MAFLD) since this last term is likely to better reflect the pathogenesis and public understanding of the disease (Eslam *et al*., 2020).

1.3.3. Signalling pathways involved in hepatic inflammation and fibrosis relevant for this final degree project.

1.3.3.2. Stress-activated protein kinases

Mitogen-activated protein kinases (MAPK) are a big family of enzymes that act as transductors in signalling pathways which regulate cell proliferation, differentiation, apoptosis and overall metabolism (Sun *et al*., 2015). Three major groups of MAPKs have been identified in eukaryotic cells and named according to the order in which they act: the first in the cascade is the MKKK (MAPK kinase kinase), followed by the MKK (MAPK kinase), and a terminal MAPK. This system of three different enzymes ensures the correct signal amplification and the efficiency of the transduction throughout the cell.

The generic MAPK structure is shared by three different groups of kinases: extracellular signal-related kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and p-38 MAPK. While the first group is mainly activated by mitogens, JNK and p-38 MAPK are activated by stress signals and are named as stress-activated protein kinases (SAPK) (Fang and Richardson, 2005).When activated, these kinases catalyse the phosphorylation of downstream mediators, which include other enzymes like kinases or transcription regulators, cytoskeletal proteins and transcription factors (Fang and Richardson, 2005).

SAPKs activation has shown to be associated with metabolic alterations like insulin resistance and obesity, where these enzymes enhance tissue inflammation. Moreover, chronic activation of these kinases has been described in NASH and liver cancer (Fang and Richardson, 2005). Therefore, the targeting of the MAPK pathways is a crucial and needed potential therapeutic approach for the prevention and treatment of not only metabolic syndrome, but a wide range of diseases in the liver.

In the liver, JNK1/2 signalling has shown to regulate cell death, differentiation and proliferation of hepatocytes, while in non-parenchymal cells like HSCs, its activation is mainly associated with inflammation and fibrogenesis. p38 family functions are still unknown but some members like p38α are thought to be involved in gluconeogenesis (Cuadrado and Nebreda, 2010).

PDGF is one of the most potent mitogens: by binding to its receptor (PDGFr), this molecule promotes cell proliferation, survival and migration, primarily of cells of mesenchymal origin. However, the

dysfunction in the PDGF/PDGFr pathway has been associated with a wide range of conditions like cancer or fibrosis and it has been proposed as an alternative drug target for the treatment of these diseases (Papadopoulus and Lennartsson, 2017).

In HSCs, PDGF-derived growth has been previously described (Pinzani, 2002). More concretely, the isophorm formed by two B-chains (PDGF-ββ) is the most potent in terms of HSC growth and intracellular signalling, thanks to the higher expression of PDGF-receptor β (or type B) subunits compared to PDGF-receptor α (or type A) subunits in activated HSC (Pinzani *et al*., 1995), and this molecule has shown to be pro-fibrogenic in some conditions like experimental cholestatic liver injury (Pinzani, 2002). PDGF signalling starts with the phosphorylation of its receptor and the creation of high-affinity sites withing the receptor is the starting point for the sequential activation of downstream elements. The subsequent activation of phosphatidylinositol 3-kinase (PI3K), Ras or protein kinase C (PKC) triggers the cell towards cell growth, chemotaxis and survival signal production (Figure 4).

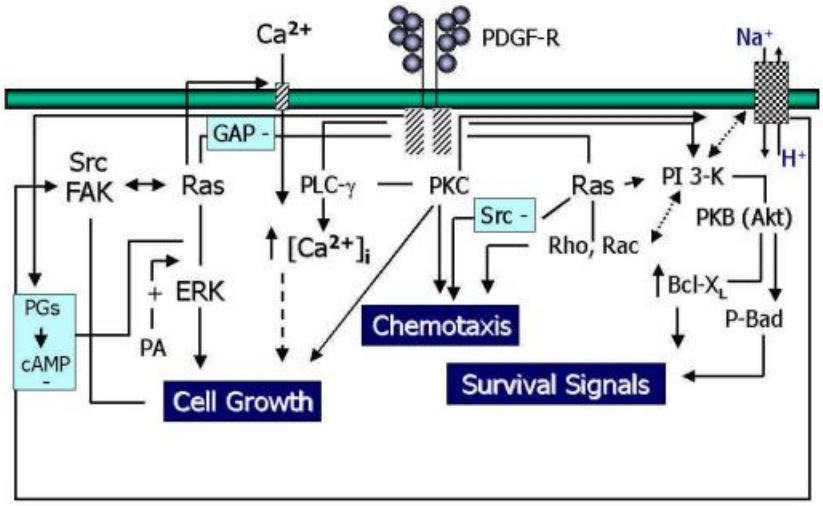


Figure 4. PDGF signalling. PDGF binds to PDGFr, provoking the phosphorylation of the receptor at its cytosolic tails, generating SH2 or PTB domains to which downstream elements can bind. PDGF signalling is not just one straight route, but a group of three different signalling pathways that lead to an increase in cell growth, chemotaxis and the release of survival signals (Pinzani, 2002).

Despite the fact that overexpression of PKD is not associated with LF and NAFLD progression (Pinzani, 2002), the activation of both the MAPK/ERK and protein kinase B (AKT) pathway is linked with hepatic damage and collagen deposition by HSCs. Because of this, it would be crucial to therapeutically reduce the population of the active form of these kinases.

2. OBJECTIVES

The general aim of this final research project was to evaluate the mechanisms that underline the hepatoprotective effect of the antiretroviral drug Rilpivirine regarding its anti-inflammatory and antifibrogenic action.

The specific aims were:

- To analyse the expression of inflammation markers in peripheral blood mononuclear cells isolated from control individuals treated with Rilpivirine *ex vivo.*

- To study the effect of Rilpivirine on the proliferation of primary hepatic stellate cells *in vitro*.

- To characterise the anti-inflammatory effect of Rilpivirine on mitogen-activated protein kinase signalling cascade *in vitro*.

- To study the effect of Rilpivirine on primary hepatic stellate cells chemotactic migration *in vitro*.

3. MATERIAL AND METHODS

3.1 GENERAL EQUIPMENT, DRUGS AND REAGENTS

All the experimental procedures in which sterility was required were performed inside a Telstar Bio-II-A Laminar flow cabinet. Medium and cells were pipetted using Pipetum PROFILLERTm 446 pipettor (Socorex, Ecublens, Switzerland). Centrifugation was performed in a Universal 320/320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), whereas that involving smaller volumes and higher speed was done in an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany).

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 was purchased from Biosynth Carbosynth (Compton, UK), and was dissolved in its corresponding vehicle dimethyl sulfoxide (DMSO).

Cell cultures including HSC were performed using 25, 75 and 150 cm^2 flasks (SPL life sciences, Pochon, South Korea) using *Dulbecco's Modified Eagle's Medium* (DMEM) medium (Gibco BRL, Grand Island, NY, USA) supplemented in most cases with FBS (Gibco BRL, Grand Island, NY, USA), antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin) and amphotericin. Cultures were maintained in a humid atmosphere at 37ºC 5% CO2 (AirLiquide, Medical, Valencia, Spain) in a cell culture incubator IGO 150 (Jouan, Saint-Herblain Cedes, France).

3.2. HUMAN SUBJECTS AND ETHICS STATEMENTS

To evaluate the overall inflammation status, peripheral blood mononuclear cell (PBMC) were used as proxy. Blood samples were obtained from volunteers, men and women, at the transfusion department of the *Hospital Clínico Universitario* of Valencia. Besides sex, age, weight and height measurements, alcohol use and cigarette smoking data were also collected. The presence of chronic conditions such as hypertension, which could interfere with the results obtained, was also considered.

For the development of this Final Degree Project, HSCs were isolated from resected liver tissue obtained from patients that had undergone pancreatic or biliary surgery. The study's objectives and procedures were approved by the local investigational review committee *(Comité Ético de Investigación Clínica del Hospital Clínico Universitario de Valencia*) (Annex 1) and all participants provided informed consent (Annex 2-3).

3.3. PERIPHERAL BLOOD MONONUCLEAR CELL ($PBMC_S$) ISOLATION, CULTURE AND TREATMENT

3.3.1. PBMC isolation by density gradient centrifugation and culture

Human PBMCs were isolated using density gradient centrifugation. This technique is based on the fact that, during the centrifugation of a particular mix of macromolecules and cells (e.g. blood) in a density gradient medium, each particle will move through the established density gradient until they find a density equal to that of the particle. As a result, particles are separated based on their corresponding densities, creating a solution layered by densities from least to most looking from top to bottom (Harwood, 1974). Blood was collected in tubes containing sodium citrate as an anticoagulant. The total

volume of citrated blood was collected in a single tube and the same volume of sterile PBS was added. The diluted blood was next slowly deposited into 15 mL of Ficoll-Plaque™ density gradient medium (Figure 5) and centrifuged during 40 min at 400 g. After carefully isolating PBMCs from the buffy coat, cells were washed with PBS and centrifuged (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.

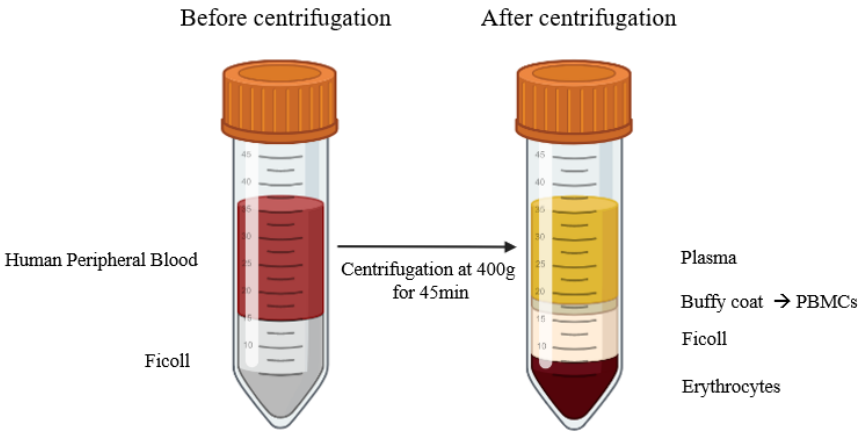


Figure 5. Schematic representation of the process of separation of human peripheral blood before (left) and after (right) centrifugation in Ficoll.

3.3.2. PBMC treatment

The following day after isolation, PBMCs were treated for 24 h with RPV at clinically relevant concentrations: 1 and 4 µM. These concentrations were chosen based on the interindividual pharmacokinetics variability observed in treated patients (Jansen, 2021). Cells were treated in RPMI medium supplemented with 10% FBS. A negative control (untreated cells) and a vehicle control (DMSO) were used in every experiment together with two different RPV concentrations.

3.4. HUMAN HEPATIC STELLATE CELLS (HSCs) ISOLATION, CULTURE AND TREATMENT

3.4.1. HSCs isolation

The isolation protocol was previously described by Casini *et al.*,1993. After washings with HBSS at 37 °C, liver samples were carefully minced with the use of a sterile razor blade inside a sterile flask containing 0.5% pronase (Sigma-Aldrich), 0.05% type IV collagenase (Sigma-Aldrich), 5 µg/mL of deoxyribonuclease I (Dnase) (Sigma-Aldrich) in 100 mL of HBSS without the cations Ca^{2+}/Mg^{2+}, and shaken at 37 ºC for 30 min. The obtained cell suspension was filtered with the help of two metal strainers that were placed one above the other. This filtration process is repeated through a Falcon® 100 µm cell strainer. The undigested material was digested using 0.05% pronase, filtered again, and pooled with the remainder of cell suspension, as the objective is to isolate the maximum number of cells. All the digest was washed several times and centrifuged for 5 min at 500 g in 5 µg/mL Dnase in HBSS solution. The obtained cell pellet was resuspended in 25 mL of the same solution.

To separate HSCs from the rest, a three-step Percoll (GE Healthcare Life Sciences, Chicago, IL, USA) gradient centrifugation (100%, 50% and 35% (v/v)) was performed at 274 g for 30 min at 4 °C. HSCs were located in the interphase between the medium and the 35% gradient.

To initially culture HSCs, they were plated onto cell culture flasks in DMEM medium with high glucose concentration, 20% FBS, 100 U/mL penicillin and streptomycin and 0.25 µg/mL amphotericin B at 37 °C in 5% CO_2. Medium was changed 24 h after initial plating and 72 h from thereafter. If the confluence reached maximum levels, cells were subcultured by trypsinisation.

2.4.2. HSCs treatment

When cells were confluent, they were trypsinized, counted and the corresponding volume was plated into plastic cell flasks or cell plates. In all cases, the experimental procedure lasted 5 days. The second day, 20% FBS DMEM medium was removed and serum-free DMEM medium was added. The following day, cells were treated for 48 h with RPV at clinically relevant concentrations (1, 2, 4 and 8 µM) in DMEM medium. These concentrations were chosen based on the interindividual pharmacokinetics variability (Jansen, 2021). On the fifth day, the correspondent experiment took place.

3.5. PROTEIN EXPRESSION ANALYSIS

3.5.1. Sample collection from cell cultures

Cell culture medium was removed and cells were washed with warm PBS and trypsinized with trypsin-EDTA during 1 min at 37 ºC. The employed volume of trypsin-EDTA depends on the volume of the cell culture: for t-25, t-75 and t-150 flasks, 0.5, 1.5 and 3 mL were added respectively. Medium containing FBS was added to neutralise the catalytic activity of the previous chemical. Cells were then collected, centrifuged at 1200 rpm for 3 min at RT and supernatant was discarded with the help of a void pump. The pellet was resuspended in 1 mL ice-cold PBS and centrifuged again at 500 g for 5 min at 4 ºC. Finally, PBS was discarded again and pellets were ready to be submitted to protein extraction.

3.5.2. Total protein extraction with preserved phosphorylation modifications.

To ensure the maximum purity, pellets were centrifuged at 500 g for 1.5 min at 4 ºC. Later, 50 µL of the lysis buffer PhosphoSafe™Extraction Reagent (EMD Millipore Corp.) were added together with 10x cOmplete Mini™ (Roche) protease inhibitor cocktail (10:1 solution). This solution preserves phosphorylation modifications in the proteins and avoids protein digestion. Cell lysates were vortexed for 30 sec and later incubated at RT during 5 min. Next, they were vortexed again for 20 sec and centrifuged at 4 °C, for 5 min, at 16000 g. Supernatants were collected and stored at -20 ºC until their quantification.

3.5.3. Protein quantification

Proteins were quantified by the bicinchoninic acid assay. In an alkaline medium, cooper ions are reduced from Cu^{+2} to Cu^{+} (biuret reaction), producing a strong purple colour in reaction with the BCA, which forms a tetrameric complex with Cu. This method can be done in one single step while other quantification methods like the Lowry method require two steps. Concentration of the measured Cu depends on protein concentration and incubation time and protein concentration of unknown samples

can be determined by comparison with known protein standards (standard curve), both measured at an absorbance of 562 nm.

To perform this assay, a standard protein curve was prepared by serial dilutions of BSA (2 mg/L) from 0.015 to 1 mg/L. 20 µL of each dilution were placed in a 96-well plate with 20 µL of the diluted samples (1:30 dilution). To measure each well intensity, 200 µL of the mix prepared by adding 50 parts of Pierce TM reagent A and 1 part of reagent B were introduced inside each well. The plate was incubated under dark conditions for 30 min at 37 ºC. After the incubation period, absorbance was measured at 570 nm in an Infinite® 200 PRO series spectrophotometer (TECAN Trading AG, Männendorf, Switzerland).

3.5.4. Western Blotting (WB)

a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Mini-PROTEAN® Tetra Cell System. To prepare the gels, a mixture 37.5:1 of acrylamide/bis-acrylamide solution (Ultrapure ProtoGel® supplied by National Diagnostics) was employed. The percentage ranged from 10 to 15% and depended on the molecular weight of the proteins to be analysed. 0.375 M Tris-HCl pH 8.8 and 0.1% SDS were used for the resolving gel preparation. In order to prepare the stacking gel once the resolving has solidified, the corresponding volume of 3.75% polyacrylamide, 0.125 M Tris-HCl pH 6.8 and 0.1% SDS was added. The polymerisation was enabled by adding 0.1% APS (SERVA) and TEMED (Sigma-Aldrich).

To prepare the samples, a minimum of 6 µg of protein with the optimum being between 20-30 µg was mixed with the corresponding volume of 1X and 6X Laemmli sample buffer (375 mM Tris-HCl, 9% SDS, 50% glycerol, 9% β-ME and 0.03% bromophenol blue) and samples were boiled at 98 ºC for 5 min in order to achieve protein denaturation. After boiling, samples were pipetted (volume being dependent on the comb size) and the molecular weight marker was added on the first race preferably. The employed molecular weight marker is the EZ-RunTM Pre-Stained Rec Protein Ladder, from Thermo Fisher Scientific. Electrophoresis was executed in a buffer tank containing running buffer (25 mM Tris pH 8.3, 0.1% SDS and 192 mM glycine) at a constant voltage of 90-120 V.

b) Protein transfer to nitrocellulose membrane

Once proteins were separated in the resolving gel, they were transferred to a 0.45 µm nitrocellulose blotting membrane (GE Healthcare Life Science) using a Mini Trans-Blot® Cell (Bio-Rad). Transfer proceed for one hour at 4 ºC at a constant amperage of 0.4 A, being this period 10 min longer in case of transferring proteins with a molecular weight higher than 100 kDa (e.g. Collagen). The process takes place in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol).

c) Ponceau staining, antibodies incubation periods and washings

To ensure sample integrity, correct loading and transfer effectiveness, the nitrocellulose membrane is submerged in a 0.1% Ponceau/5% acetic acid solution (Sigma-Aldrich) for 30 sec and then cleared with TBS-T (20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1%Tween-20 v/v) until bands can be clearly visualized. After Ponceau staining, membranes were blocked with 5% fat-free milk powder or BSA, diluted in TBS-T (depending on the corresponding antibody solution) with continuous gentle shaking, for 1 h, at RT. Membranes were then incubated overnight with the primary antibody prepared in blocking solution supplemented with 0.02% NaN_3 (Sigma-Aldrich) at 4 ºC with continuous gentle shaking. On the following day, membranes were washed three times with TBS-T for 10 min at RT under vigorous shaking conditions and then incubated with the secondary antibody at RT for 1 h with continuous gentle shaking. Finally, washings were repeated as described.

d) Detection by chemiluminescence

Detection was achieved using the substrates 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 and using the digital luminescent image analyser Amersham ImageQuant 800 (GE Healthcare). Detection in all cases is based in luminol's oxidation catalysed by the enzyme HRP, which is conjugated to the secondary antibody. HRP in presence of hydrogen peroxide, gives rise to the product 3-aminophthalate that emits light at 425 nm. Densitometric analyses were performed using Multi Gauge V3.0 software (Fujifilm, Tokio, Japan). The protein expression was normalized versus GAPDH (employed as loading control).

e) Stripping for reprobing

Stripping allows us to visualize more than one band on the same membrane by eliminating the signal corresponding to the previously analysed protein. The stripping process was performed by incubating the membrane with 0.5 M glycine pH 2.5 for 30 min at 37 ºC with robust shaking and then washing it three times with TBS-T for 10 min, at 37 ºC. After washings with TBS-T, the membrane was pre-blocked again and incubated with the antibodies following the protocol described above.

The primary and secondary antibodies used for the development of all the experiments are described in Table 3.

Table 3. List of primary and secondary antibodies employed in WB experiments. *HRP, horseradish peroxidase.*

PRIMARY ANTIBODIES				
PROTEIN	SOURCE/TYPE	MW (kDa)	DILUTION	COMPANY (ref.number)
GAPDH	rabbit/polyclonal	36	(1:20000)	Sigma-Aldrich (G9545)
pJNK 1/2	rabbit/monoclonal	46, 54	(1:1000)	Thermo Fisher Scientific (700031)
pERK 1/2	rabbit/polyclonal	44,42	(1:1000)	Cell signaling (ref.: 9101)
pAkt	rabbit/polyclonal	65	(1:1000)	Cell signaling (ref.: 9271)
Col1a1	rabbit polyclonal	130	(1:1000)	Cell signaling (ref.: 84336)
p-p38 SAPK	mouse/monoclonal	38	(1:1000)	Cell signaling (ref.: 9216)
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)

3.6. GENE EXPRESSION ANALYSIS

3.6.1. RNA isolation from cultured cells

RNA isolation from cells and its purification was performed using the illustra®RNAspin Mini RNA Isolation Kit (GE Healthcare). Following manufacturer's instructions, cell pellets were resuspended in 350 µL of lysis buffer and 3.5 µL of β-ME, and homogenized by passage through a 25 G needle. Next, 350 µL of 70% ethanol were added and the solution was introduced through a column that retains RNA. Membrane was desalted, DNA was digested enzymatically and the membrane was washed three times. Finally, RNA was eluted using 30 µL Rnase-free water, and its purity and concentration were determined spectrophotometrically, using a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.6.2. RNA retrotranscription

Synthesis of cDNA was performed using the "PrimeScriptTM RT Reagent Kit" (Takara Bio, Kusatsu, Shiga, Japan), using 1µg of total RNA and following manufacturer's instructions. 4 µL PrimeScript Buffer, 1 µL PrimeScript RT Enzyme Mix I, 1 µL Oligo dT Primer, 1 µL Random 6-mers in a final volume of 20 µL Rnase free water were mixed. Retrotranscription reaction was carried out in a SimpliAmp™ Thermal Cycler (Applied Biosystems™) under the following conditions: 37 °C for 15 min, 85 °C for 5 s and 4 °C for undefined time.

3.6.3. Quantitative RT-PCR (qRT-PCR)

The technique was performed using the kit 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. SYBR Green I is a DNA intercalator molecule that is excited at 497 nm and emits fluorescence at 520 nm only when bound to dsDNA, therefore the fluorescent signal detected will be proportional to the amount of synthesized dsDNA after each amplification round. The reaction was performed by adding 1 µL of sample's cDNA, 5 µL SYBR® Premix Ex Taq™, 0.2 µL primers (forward and reverse) and Rnase-free water, in a final volume of 10 µL, and using the 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).

Primer pairs used (Table 4) were synthetized by Metabion (Planegg, Germany) or IDT® (Integrated DNA Technologies, Coralville, IA, USA), and were tested by assessing the amplified fragment using 1.5% agarose gel electrophoresis.

Table 4. List of forward and reverse primers employed in qRT-PCR.

GENE	FORWARD PRIMER (5´ - 3´)	REVERSE PRIMER (5´ - 3´)
CCL2	AGCAGCAAGTGTCCCAAAGA	GGTTGTGGAGTGAGTGTTCAAG
GAPDH	CTTCTTTTGCGTCGCCAGCC	TTCTCAGCCTTGACGGTGCC
IL6	CACTGGTCTTTTGGAGTTTGAGG	ATTTGTGGTTGGGTCAGGGG
IL10	GTGATGCCCCAAGCTGAG	CACGGCCTTGCTCTTGTTTT
TGFB1	CTTCAGCTCCACAAGAAGAACTG	CACGATCATGTTGGACAACTGCTC
HGF	GCAGAGGGACAAAGGAAAAGAAG	TGCTATTGAAGGGGAACCAGAG
CXCL9	AGTGGTGTTCTTTTCCTCTTGG	TGGATAGTCCCTTGGTTGGTG
CXCL10	TTGCTGCCTTATCTTTCTGACT	GACCTTTCCTTGCTAACTGCT
TNFA	AGCCGCATCGCCGTCTCCTA	CAGCGCTGAGTCGGTCACCC
SERPINE1	CGCTGTCAAGAAGACCCACA	ACCTGCTGAAACACCCTCAC

To quantify the amount of dsDNA, the CT comparative method was used. CT analysis relies on the difference in the amplification cycle where the gene of study was detected in comparison with the cycle of detection of the housekeeping gene (*GAPDH*). The equation in which this method is based is the following:

Fold Change = 2-Δ(ΔCT), where ΔCT = CT (target gene) – CT (housekeeping gene), and Δ(ΔCT) = ΔCT (treated) – ΔCT (control)

3.7. MICROSCOPIC DETERMINATIONS: MIGRATION ASSAY

Primary HSCs were plated in t-25 flasks at a concentration of 0.06 million cells/mL in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin/streptomycin and amphotericin B (250 µg/mL). When confluent, HSCs were serum-starved for 24 h and after exposed to different RPV concentrations for 48 h. After the incubation period, cells were washed, trypsinized, resuspended in serum-free medium (5 mL) and centrifuged and resuspended in 1 mL.

Cells were counted and 30.000 cells were resuspended in 200 µL of final volume. Chemotaxis was assessed in modified Boyden chambers (Neuro Probe, Inc. Gaithersburg, MD, USA) with 8 µm pore filters (Cytiva, Marlborough, MA USA), previously coated with rat tail collagen (20 µg/ml) (Sigma-Aldrich) and incubated at 37 ºC for 30 min. Cell suspension (200 µL) was placed in the upper compartment, while PDGF-ββ (10 ng/mL) was placed in the lower compartment (200 µL) in serum-free medium. After incubation (6 h at 37 °C in 5% CO_2), the cell suspension was discarded, and the upper part of the filter was cleaned with Q-tips. Cells that migrated to the underside of the filters were fixed (15 min in 100% methanol), stained (15 min in haematoxylin 100%), mounted and images were acquired at 10X magnification (Figure 6). Data are the average of haematoxylin area obtained in ten randomly chosen high-power fields. LEICA DMI 3000-B (Leica Microsystems, Wetzlar, Germany) microscope was used for imaging.

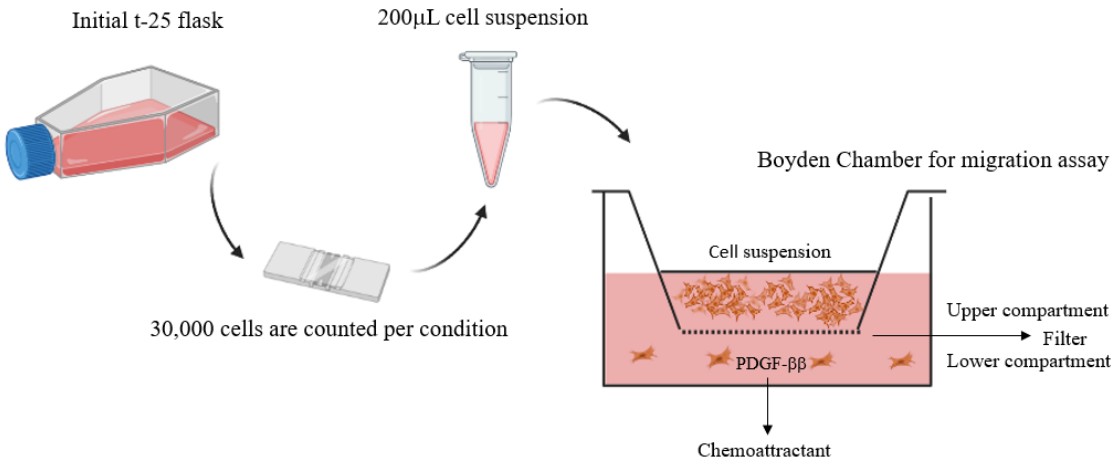


Figure 6. Experimental procedure of the hepatic stellate cell migration assay.

3.8. DETERMINATION OF CELL NUMBER AND VIABILITY

After 48 h of treatment in a 12-well plate, cells were washed with PBS, trypsinized with 100 µl trypsin-EDTA during one minute at 37 ºC and resuspended in 1 mL of DMEM 10% FBS cell culture medium. Cells were subsequently transferred to their corresponding tubes and centrifuged at 500 g for 5 min at RT in order not to produce cell lysis. The supernatant was discarded and cells were resuspended in 200 µL of the same culture medium. Immediately after, cells were counted in a 1:1 solution with Trypan Blue (Gibco BRL, Grand Island, NY, USA) in a total volume of 20 µL employing the Corning Cell Counter (CytoSmartTM, Eindhoven, Germany) and images were acquired using its corresponding software. To count, 10 µL of the total cellular suspension mixed with Trypan Blue were pipetted on each side of the counting chamber provided by the equipment. Four images were taken per side of the

counting chamber and data of total cell number, live cell number, death cell number, viability and cell sizes in both death and live cells were obtained.

Trypan Blue viability assay is based on the fact that only dead cells will stain due to damage present at the plasma membrane, while live cells will not present the bluish colour.

3.9. DATA PRESENTATION AND STATISTICAL ANALYSIS

Values are represented as mean ± standard error of the mean (SEM). The number of independent experiments (n) is indicated in each figure legend. Data is represented as percentage of control (considered 100%), number of cells, viability or area. Data were analysed using the software GraphPad Prims ® V8.0.2 (GraphPad Prim ® Software Inc).

Regarding statistical tests, in cases when comparing two different groups a Student *t*-test versus the vehicle or control treatment was performed ($P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***)). When the effects of the different drug concentrations were compared, a one-way ANOVA with Bonferroni's multiple comparisons analysis between the vehicle + inducer treatment and the different RPV concentrations was performed ($P<0.05$ (*), $P<0.01$ (**)).

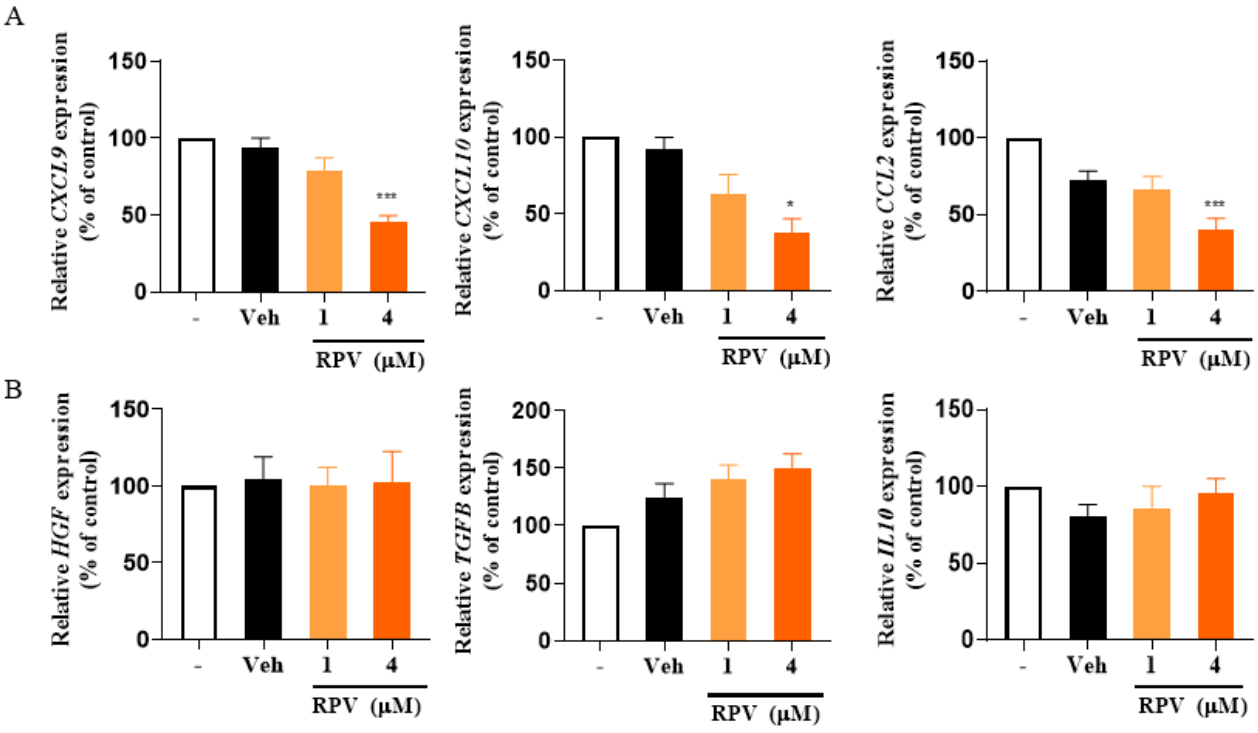
4. RESULTS

SECTION I: ANALYSIS OF THE ANTI-INFLAMMATORY EFFECTS OF THE ANTIRETROVIRAL DRUG RILPIVIRINE IN PBMCS ISOLATED FROM CONTROLS

4.1. Treatment of PBMCs with RPV ex vivo

Considering previous studies where RPV performed anti-inflammatory and anti-fibrotic actions in several mouse models of hepatic injury (Marti-Rodrigo *et al*., 2020), we aimed to explore the effect of the drug on the expression of several general inflammatory markers using PBMCs isolated from healthy subjects as a model. The justification for the use of PBMCs is their ability to act as a proxy system of the overall immune system and their easy isolation from blood samples.

To perform the experiment, PBMCs were isolated as described in the "Material and Methods" section and treated with relevant concentrations of RPV (1 and 4 µM) ex vivo for 24 h. Next, expression of chemokines *CXCL9*, *CXCL10* and *CCL2* was evaluated via qRT-PCR, observing a concentration-dependent decrease in all their corresponding mRNA levels (Figure 7.A). This decrease was statistically significant in all cases for the treatment with RPV 4 µM. Anti-inflammatory mediators *HGF*, *TGFB* and *IL10* were also analysed: *HGF* mRNA levels showed no change under the different concentrations of the RPV. However, regarding *TFGB* and *IL10* expression levels, a concentration-dependent increase from the vehicle treatment was observed (Figure 7.B). It is important to note that, despite its pro-fibrotic activity in hepatic tissue, due to its pleiotropy, TFG-β has been reported to perform anti-inflammatory functions such as the immune suppression (Sanjabi *et al*., 2009). Finally, the pro-inflammatory genes *IL6* and *TNFA* together with the pro-inflammatory marker *SERPINE1* showed a concentration-dependent down-regulation in the presence of RPV, being this decrease statistically significant in the case of *SERPINE1* (Figure 7.C).



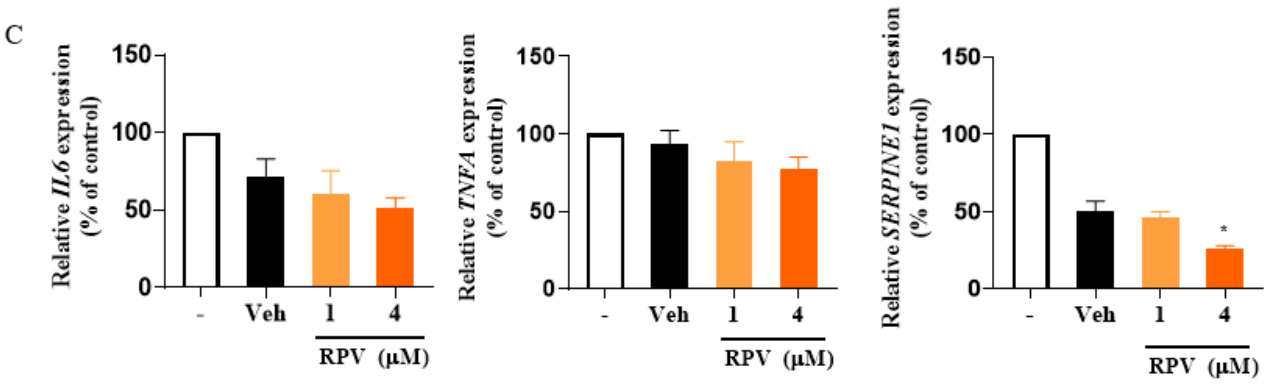


Figure 7. Expression of inflammation and immunoregulatory genes in PBMCs isolated from controls after 24h RPV ex vivo treatment. A. Chemokines. B Anti-inflammatory mediators. C. Pro-inflammatory mediators. Cells were isolated and treated with RPV (concentrations 1 and 4 μM), vehicle (DMSO) or left untreated (control) for 24h. Relative mRNA expression was analysed by qRT-PCR. *CXCL10*, *IL6*, n=7; *IL10*, n=8; *CCL2*, *SERPINE-1* (PAI-1), *TNFA* n=9; *TGFB*, *HGF*, *CXCL9* n=10. Data (plotted as mean ± SEM) were normalized considering the expression of *GAPDH* (housekeeping gene) and expressed as percentage of control (untreated) which was considered 100%. For statistical analysis, repeated measures (RM) one-way ANOVA using with Bonferroni's multiple comparisons test (*p< 0.05, ***p< 0.001, vs Veh) was performed.

SECTION 2: MOLECULAR MECHANISMS OF THE ANTI-FIBROTIC AMD HEPATOPROTECTIVE EFFECTS OF THE ANTIRETROVIRAL DRUG RILPIVIRINE IN HSC$_S$ ISOLATED FROM CLD-PATIENTS

In order to better understand the anti-fibrotic properties of RPV, the following experiments were focused on the population of HSCs. Cells were obtained by hepatic resections provided in all cases by patients submitted to pancreatic or biliary surgery who authorised their use (see Annex1). More specifically, the experiments focused on the effect of RPV over the physiological effects of the PDGF-ββ signalling cascade and its roles in the activation and transdifferentiation to MF-like HSCs.

4.2. The effect of RPV on PDGF-ββ–stimulated proliferation in human HSCs *in vitro*

Previous studies revealed that RPV diminished the profibrotic effect of TGF-β in HSCs (Martí-Rodrigo *et al*., 2020), since the number of activated HSCs showed to be indirectly proportional to RPV concentration in HSCs stimulated with TGF-β. Besides, RPV reduced the viability of HSCs under TGF-β stimulation *in vitro* (Lucantoni *et al*., 2022). As previously mentioned, PDGF-ββ has been postulated as the most potent stimulus for HSC growth and also described as pro-fibrogenic (Pinzani, 2002). Our initial hypothesis was that TGF-β and PDGF-ββ may affect the population of HSCs and their activation cascade in different ways. Therefore, the possible effects that RPV may exert on PDGF-ββ-mediated HSC activation signalling pathway is a key piece of study.

The results obtained showed that treatment with PDGF-ββ compared to Veh significantly stimulated cell growth, as expected, and that co-treatment with increasing concentrations of RPV (1-8 μM) was able to reduce cell growth in a concentration-dependent way (Figure 8.A). Simultaneously, cell viability could be monitored in this same assay using Trypan blue staining. The results showed that, despite the

decrease in the cell number, cell viability was maintained with the different RPV concentrations employed (Figure 8.B).

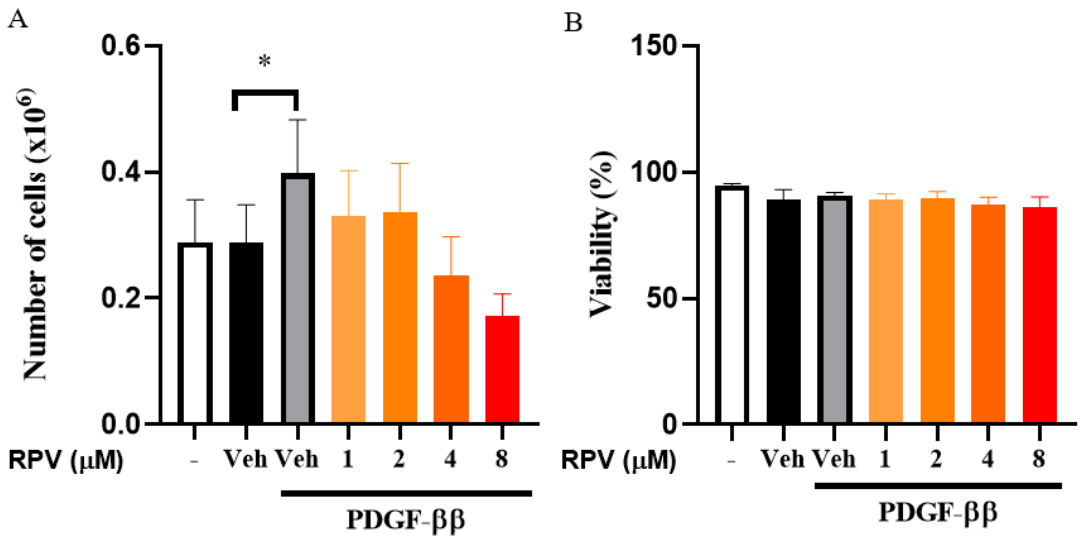


Figure 8. Effect of Rilpivirine on platelet derived factor beta (PDGF-ββ)-stimulated proliferation in hepatic stellate cells. A. Cell number data. B. Cell viability. Cells were isolated and treated with RPV (concentrations 1 to 8 μM) with 0,1% PDGF-ββ, vehicle (DMSO) with or without 0.1% PDGF-ββ or left untreated (control) for 48h. Number of cells (cell count) and viability data (n = 5) were plotted as mean ± SEM. In the case of cell viability, data were referred to those of the control which were considered 100%. For statistical analysis, repeated measures (RM) one-way ANOVA with Bonferroni's multiple comparisons test vs Veh and a Student's t-test: *p<0.05 vs Veh + PDGF-ββ was performed.

4.3. The effect of RPV on PDGF-ββ-induced intracellular signalling in human HSCs *in vitro*

As RPV was able to reduce HSC proliferation, being PDGF-ββ the most potent mitogen for HSC (Foglia *et al*., 2019), we sought to study several signal transducers of the MAPK signalling routes, one of the main HSCs activation pathways (Fang and Richardson, 2005).

As the pathway is formed by many different protein kinases, we assessed the effect of RPV on their phosphorylated (active) forms. These kinases when activated, participate in the subsequent phosphorylation steps resulting with modulation of the gene expression and increased proliferation. They are part of the mammalian MAPK cascades pERK1/2, pJNK1/2, pAkt and pp38. Besides, the levels of collagen α-1, an ECM protein accumulated in fibrotic tissue, was also analyzed to confirm the anti-fibrotic role of RPV in PDGF-ββ-stimulated HSCs.

Western blotting experiments revealed that collagen α-1 expression was not increased with the treatment with PDGF-ββ however RPV diminished it in a concentration-dependent way. pAkt was induced by PDGF-ββ and increases in RPV concentration provoked the decrease of this enzymatic form in a concentration-dependent manner. The phosphorylated form of the protein kinase JNK was initially increased with the treatment with the growth factor and, despite the slight increases in the 1μM and 4μM RPV treatments, it could be affirmed that a decrease between the lowest (1μM) and the highest (8μM) RPV concentration was observed. In the case of the protein pp38, PDGF-ββ did not produce an increase

in the pp38$_{00}$ enzyme levels and RPV treatments seemed to increment them in a concentration-dependent manner. However, a decrease with the highest RPV concentration (8μM) is observed. Finally, the initial exposure with PDGF-ββ increased pERK1/2 levels and subsequent RPV treatments did not seem to affect the levels of the phosphorylated form.

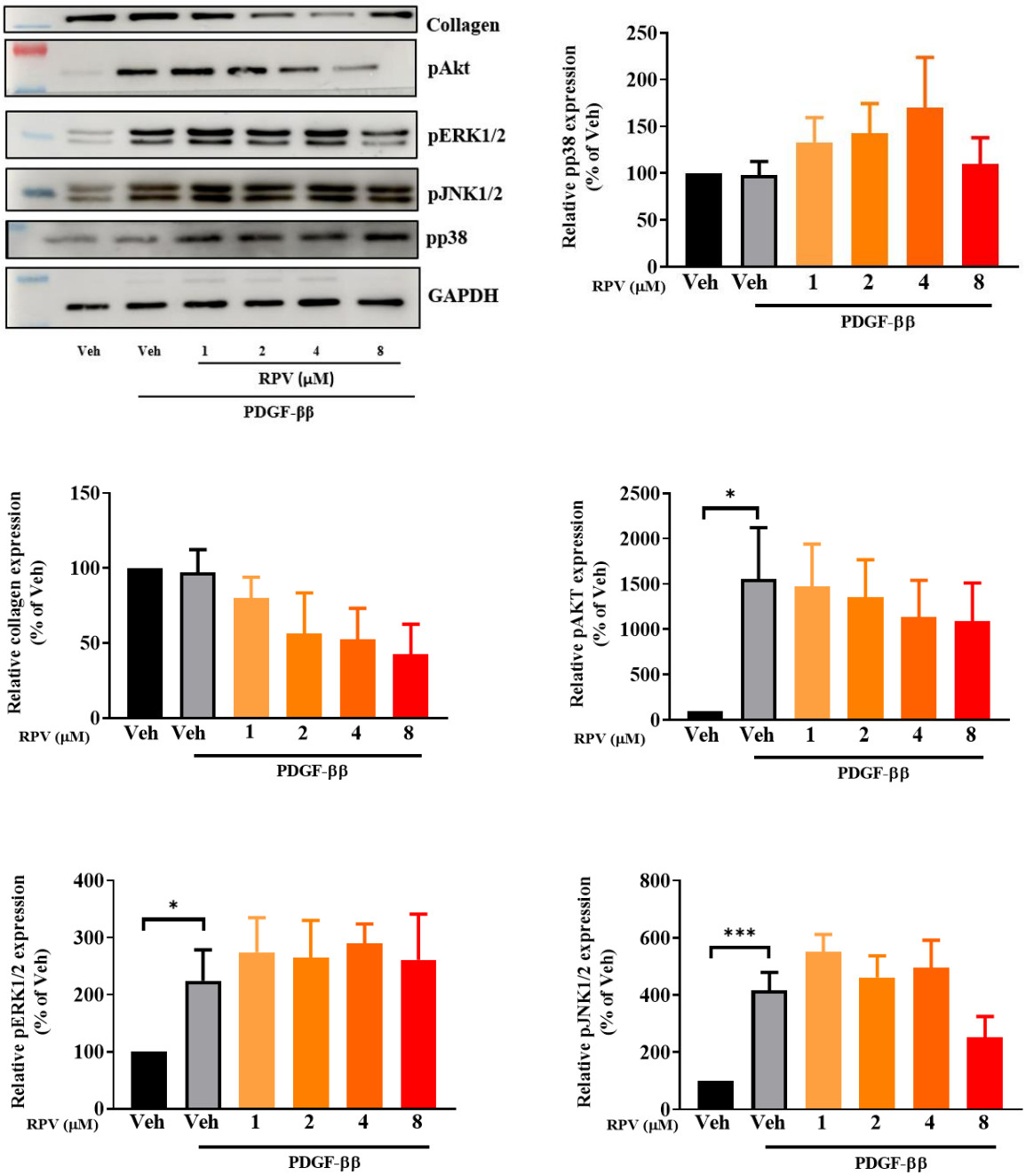


Figure 9. Western blot analysis of pAkt (PKB), pp38, pJNK1/2, pERK1/2 and collagenα1 protein expression in HSCs cells stimulated with PDGF-ββ. Cells were isolated and treated with RPV (concentrations 1 to 8 μM),

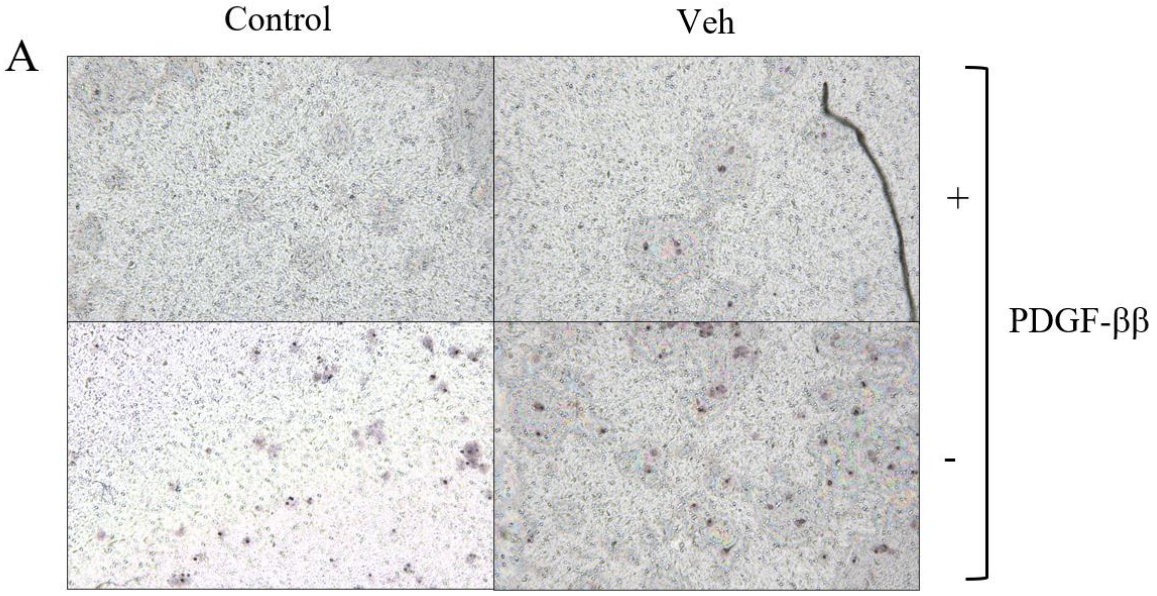
vehicle (DMSO) for 48 h and were finally exposed to 0.1% PDGF-ββ during the last 10 min. WB analysis (n=4-7) and representative images are shown. Data were normalised against the obtained values of the housekeeping protein GAPDH (n=7). Data (mean ± SEM) are expressed as the percentage of untreated cells (Veh), considered 100%. For statistical analysis, repeated measures (RM) one-way ANOVA using with Bonferroni's multiple comparisons test (*p< 0.05, **p< 0.01, vs Veh) and Student's t-test: *p<0.05, **p<0.01, **p<0.001 vs Veh + PDGF-ββ was performed.

4.4. The effect of RPV on PDGF-ββ-induced chemotaxis in human HSCs *in vitro*

HSCs migration is an important process in CLD progression and maintenance, since it has been described that migration of these cells towards affected areas help maintain the positive pro-inflammatory loops in fibrotic septa (Foglia *et al.*, 2019). PDGF-ββ has been described as the most potent chemoattractant signalling molecule in HSCs and MAPK signalling route has also been reported to influence HSC chemotaxis (Marra *et al.*, 1999; Ye *et al.*, 2021).

To further understand RPV's effect on MAPK signalling cascade, we analyzed the effect of RPV on PDGF-ββ-induced chemotaxis using a transwell chemoattraction assay in Boyden chamber, as described in the "Material and Methods" section.

Migration experiments revealed PDGF-ββ stimulated HSC migration after 48h of exposure, as expected. Besides, a clear decrease between both vehicle (with or without the inducer) and RPV (2 μM RPV 4 μM) treatment was observed (Figure 10).



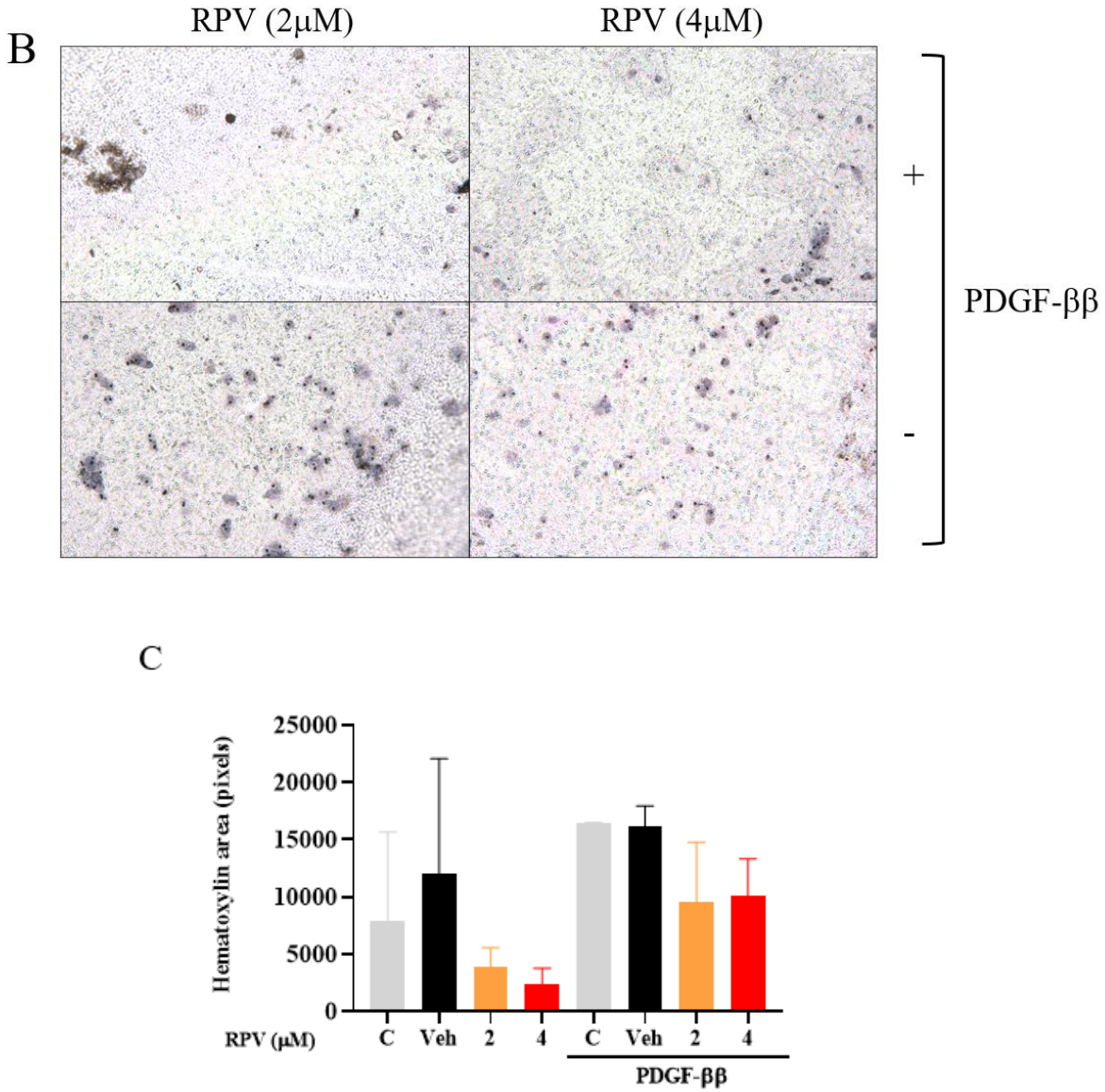


Figure 10. Effect of RPV on PDGF-ββ-induced chemotaxis in human HSCs. A. Microscopic visualization of the membranes in which cells were not exposed to RPV. B. Microscopic visualization of the membranes in which cells were exposed to RPV. C. Representation of hematoxylin area in the different treatments. Cells were isolated and treated with RPV (concentrations 2 and 4 μM), vehicle (DMSO) or left untreated (control) for 48h. 30.000 cells of each treatment were exposed for 6h to 0.1% PDGF-ββ, whereas the other 30.000 were not exposed to the inducer. Data (mean ± SEM) are expressed as the calculated hematoxylin area (n=2). For statistical analysis, repeated measures (RM) one-way ANOVA using with Bonferroni's multiple comparisons test vs Veh and a Student's t-test vs Veh + PDGF-ββ was performed.

5. DISCUSSION

Since the development of cART, survival of patients suffering from HIV infection in developed countries has significantly increased, thus transforming this disease into chronic rather than a high-mortality disease. The wide range of drugs that are available for therapy has made possible the design of many different combinations which can adapt better to different patients. However, cART-related side effects continue being, together with drug resistance, the main drawbacks of this therapy. The fact that the average life of HIV patients has increased during last years and that these drugs must be taken throughout the entire life once the treatment begins has generated a growing interest regarding the possible long-term effects of cART.

Without any doubt, the liver is the major organ affected by the continued use of drugs. Studies describing the molecular effects of anti-HIV drugs such as Efavirenz have confirmed that its continuous use causes a persistent drug-derived liver damage (Benedicto *et al*., 2021). HIV-infected patients are at greater risk of developing CLD in comparison with the uninfected population not only due to the continuous exposure to both HIV and ARV drugs, but also because of AIDS-associated viral infections. Because of the increasing prevalence of CLD along last years, it is essential not only to describe the molecular mechanisms that underline the progression of this disease, but also to explore therapeutical targets able to palliate this incurable condition.

Knowing the drug's security and efficacy data obtained from its clinical trials, the strategy of drug repurposing facilitates the description of alternative indications for already-approved drugs. Following this line, RPV, a second generation NNRTI, has previously showed anti-inflammatory, anti-steatotic and hepatoprotective effects (Martí-Rodrigo, 2018). However, the molecular mechanism of this drug that is responsible for those actions is still unknown. It has been seen that RPV clearly affects the relationship between hepatocytes and HSCs, and that it selectively activates STAT1, leading to activated HSC apoptosis thereby attenuating liver fibrosis (Martí-Rodrigo *et al*., 2020).

In the presented final degree project, RPV was used to treat PBMCs isolated from control individuals and HSCs isolated from patients undergoing pancreatic or biliary surgery, with the aim to study the anti-inflammatory and hepatoprotective roles of this ARV drug.

PBMCs include T and B lymphocytes together with Natural Killer cells (70-90%), monocytes (10-20%) and dendritic cells (1-2%) (Kleiveland, 2015). Their easy collection and the similarities in the gene expression profiles compared to other cell types make their use as proxy of the overall *in vivo* immune state of the organism (Reimann *et al*., 2000), thus constituting a good option for immunological studies. In the former model, a total of 9 genes related to inflammation were explored which act as pro-inflammatory or anti-inflammatory molecules. On the one hand, we provided evidence of statistically significant decreased expression of chemokines *CXCL9, CXCL1*0 and *CCL2*, naturally described as molecules able to increase tissue inflammation by stimulating cellular chemotaxis and inflammation (Li *et al*., 2017; Tokunaga *et al*., 2018). Also, the pro-inflammatory genes *IL6* and *TNFA* were reduced by RPV administration. On the other hand, expression of genes coding for the anti-inflammatory molecules such as *TGFB* and *IL10* were increased with RPV treatments in a concentration-dependent manner. The pleotropic nature of TGF-β should be considered when analysing this result since, while in hepatic tissue TGF-β is clearly a pro-fibrotic molecule, outside hepatic tissue, the same molecule act in the regulation of immunosuppression and G1 phase arrest, being both clearly anti-inflammatory processes (Tzavlaki and Moustakas, 2020). Contrasting our initial expectations, *HGF* did not change among treatments. However, this might be due to the experimental limitation of the use of PBMCs for the analysis of this gene because, since obtained cells reflect the overall immune state of the whole organism, the tissue-specific function of this factor could be better analysed using tissue resident immune cells. Another option could be that, as healthy individuals do not present liver damage and the subsequent need for tissue regeneration (mainly by hepatocyte proliferation), the levels of this mediator are low and fail to be increased by RPV administration.

Lastly, we also detected changes in *SERPINE1 mRNA*, which codes for Plasminogen Activator Inhibitor 1 (PAI-1). This serine protease is expressed in a wide variety of cells such as macrophages, adipocytes, vascular endothelial cells and fibroblasts, and increased levels of this protein have been associated with increased ECM deposition (Ghosk and Vaughan, 2012). Specifically, excessive PAI-1 contributes to increased collagen accumulation by reducing plasmin-dependent proteolytic activities in hepatic cells (Ghosk and Vaughan, 2012). Besides, PAI-I directly interacts with the α-3 proteasome subunit, preventing the degradation of the nuclear factor of inhibitor alpha (IκBα), a gene involved in inflammation (Ghosk and Vaughan, 2012). The concentration-dependent decrease in *SERPINE1* expression with RPV administration may suggest the ability of this drug to reduce immune system activation via the nuclear factor kappa B (NF-κB) signalling, as RPV has already been shown to interfere with NF-κB signalling in activated HSC (Gruevska, 2022). To conclude this first section, we confirmed the overall anti-inflammatory roles of RPV in two different ways: by inducing the expression of anti-inflammatory genes and by reducing that of pro-inflammatory mediators and chemokines.

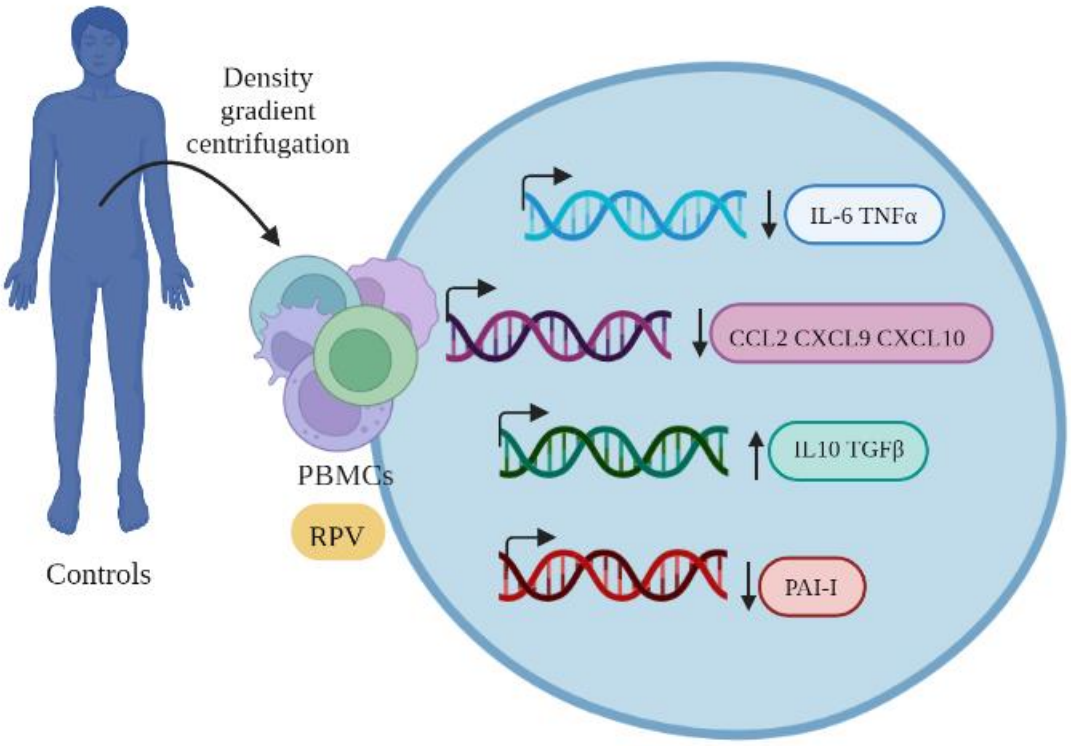


Figure 11. Overview of the findings from the study of inflammation markers in PBMCs treated with Rilpivirine *ex vivo*.

In the second section of the project, we analysed the molecular mechanisms by which RPV exerted its effects by using primary HSCs. Our firsts steps focused on the fact that, when HSCs are activated and differentiate into MF-like HSCs (in our case simply by being in contact with flask's plastic surface), they proliferate and posterior positive feedback loops increase ECM deposition, leading to LF and CLD progression.

To simulate CLD environmental conditions, TGF-β has widely been used in research to stimulate MF-like differentiation and proliferation in HSCs (Martí-Rodrigo *et al*., 2020). However, PDGF-ββ is, in fact, the most potent molecule for stimulating MF-like HSC growth (Pinzani, 2002). Anti-inflammatory and anti-fibrotic roles of RPV had only been studied in TGF-β-induced stimulated primary HSCs and,

despite that cellular proliferation was reduced when treating cells with RPV, cellular viability was reduced as well (Lucantoni *et al*., 2022). At that time, it could not be affirmed that RPV inhibited HSC proliferation since viability was also reduced. However, with the present results it is clear that RPV lowers HSC number in a concentration-dependent way and that viability among treatments remains unaltered, therefore confirming that RPV does inhibit HSC proliferation.

As a drawback for this experimental approach, it must be mentioned that viability measurements were not performed in the same assay: while Lucantoni and colleagues used the acid phosphatase assay, in this project we performed the experiment using Trypan Blue staining. To further confirm the inhibitory role of RPV, viability could be measured performing the phosphatase assay. In this way, both results could be completely comparable. However, to be completely sure about the inhibitory effect of RPV, a proper proliferation assay such as the MTS assay should be performed, as reported in other pharmacological studies regarding liver fibrosis (Zhang *et al*., 2012). In this assay, we would expect lower cell counts with increasing RPV concentrations.

Once we determined that proliferation capacity was altered by RPV, we focused our studies on the molecular elements that are responsible for these observed changed *in vitro*. Previous studies using PRV showed some effects over MAPK signalling elements in LX2 HSC cells (Gruevska, 2022). As PDGF-ββ has shown to activate MAPK signalling in HSCs (Marra *et al*., 1999) and it was used for the analysing HSC proliferation in the previous experiment, this same molecule was used to stimulate HSCs in this second experiment.

We have demonstrated how some elements of the MAPK signalling cascade are downregulated when treating primary HSCs that were stimulated with PDGF-ββ with RPV. We have confirmed the stimulatory role of PDGF-ββ on selected protein kinases pAkt, pJNK1/2 and pERK1/2, as the protein level significantly increased in all cases between Veh and Veh + PDGF-ββ treatments, in line with the results obtained in previous publications (Marra *et al*., 1999; Novo *et al*., 2012; Lin *et al*., 2016). Provided evidence of a decrease in JNK activation clearly follows the line of previous studies where pSTAT3 expression, a downstream element of the JNK signalling, was reduced with RPV treatment in HSCs in a concentration dependent manner (Martí-Rodrigo *et al*., 2020). This activated form of STAT3 favours the transcription of genes involved in cell survival, proliferation, and fibrogenesis, so that RPV provokes a decrease in the activation of the upstream element of the same signalling cascade should not be a surprise. However, the concentration-dependent decrease in other signalling cascade such as pAkt reveals that the molecular target of the ARV drug is not an exclusive element of the JNK signalling cascade as it is shared by both protein kinases. pAkt (or PKB) directly catalyses mTOR phosphorylation, activating this apoptosis inhibitor. This may be related to the previously published finding of RPV affecting autophagy in HSCs (Lucantoni *et al*., 2022), however, further analysis are needed to assess how mTOR is influenced by RPV in PDGF-ββ-stimulated primary HSCs.

In contrast with the mentioned kinases, the activation of pp38, a key kinase in one of the MAPK signalling routes was not reduced by RPV as expected. However, this effect might have some biological explanations. In the case of p38, unlike the rest of kinases, PDGF-ββ did not stimulate its phosphorylation. Previous studies analysed this protein activity in HSCs stimulated with PDGF-ββ with a different inducer exposure time: rather than exposing the cells to PDGF-ββ only for 10 min, other authors studied pp38 phosphorylation with an exposure time of 3 h (Zhang *et al*., 2012). Maybe, it is a matter of substrate preference, PDGF-ββ could preferably stimulate some of the MAPK signalling routes, rather than others. For example, PDGF-ββ could firstly activate pAkt and JNK branches, instead of pp38 cascade, causing that the phosphorylation activities of the first two enzymes could be studied in shorter time periods, while pp38 cascade should be studied upon longer periods of time.

RPV did not reduce pERK1/2 activation as expected. While it is true that enzymatic activity was increased with PDGF-ββ stimulation, confirming previous studies (Novo *et al*., 2012), increasing concentrations of RPV did not produce any change in enzyme phosphorylation and thus, in ERK activation. It has been described that pERK1/2 is the effector enzyme in which many of the MAPK

signalling branches converge (Figure 12) and it is directly in charge of enabling the expression of proliferative and pro-inflammatory genes by direct interaction with the DNA sequence, or by catalysing the activation or deactivation of transcription factors that act as cell cycle promoters or differentiation stimuli (e.g., cFos) (Foglia *et al*., 2019).

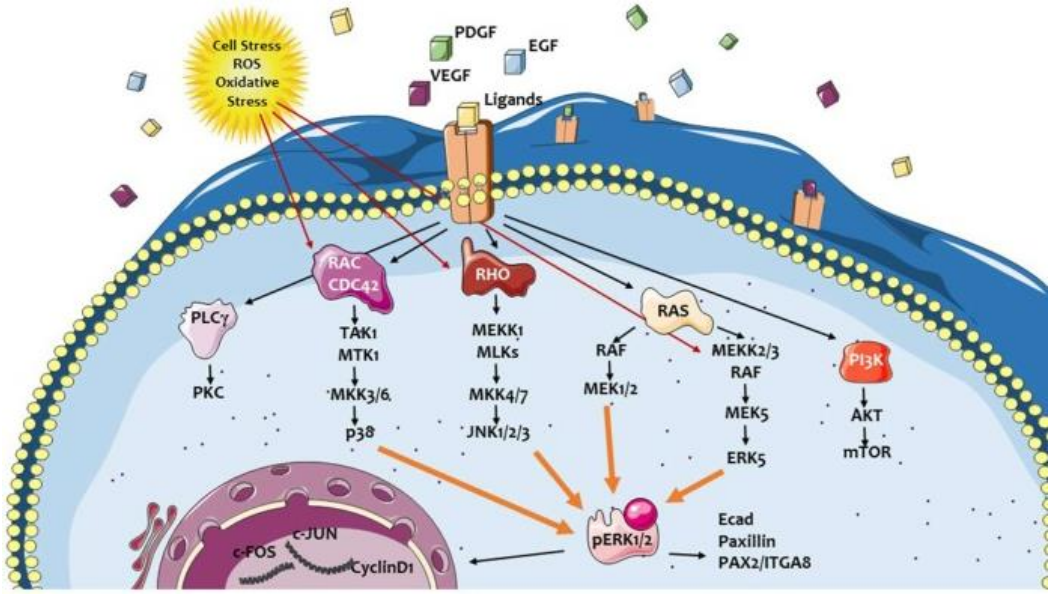


Figure 12. MAPK cascades in activated HSCs. (Foglia *et al*., 2019). At least four different MAPK signalling cascades have been described. In most cases, pERK1/2 is the effector protein in charge of enhancing transcriptional activity.

The fact that some of the kinases of MAPK signalling cascade are downregulated by RPV but that the effector enzyme ERK1/2 does not respond to this treatment opens a range of possibilities. Firstly, again, the experimental time frame may be inadequate to analyse MAPK signalling, since it has been evidenced that all the ERK upstream elements are not stimulated with 10 min of PDGF-ββ exposure. Secondly, MAPK signalling is complex, and some elements not present in the proposed map could be playing important roles as well. Thirdly, PDGF-ββ could stimulate more than one route, not only MAPK signalling cascade, and these alternative routes (not affected by RPV) could as well stimulate ERK or stimulate HSC differentiation. Fourthly, RPV could have target preference and higher drug concentrations may be needed to inhibit ERK upstream regulators and hence the absence of effect on ERK1/2 activation. Further studies are required to study the possible way in which RPV could influence alternative PDGF-ββ–induced pathways. For example, the effect of RPV on NF-κB and STAT1/3 signalling in PDGF-ββ–stimulated HSCs could be analysed as it has been done with the profibrotic molecule TGF-β (Martí-Rodrigo *et al*., 2020), expecting that RPV enhanced STAT1 and reduced STAT3 activation.

Finally, collagen α-1 was used as a positive control of RPV effect. The concentration-dependent reduction in collagen production confirmed the antifibrotic activity of RPV.

In the third experiment using primary HSCs, we analysed if RPV affected chemotaxis of activated HSCs. We have provided evidence of the stimulatory role of PDGF-ββ on activated HSC migration, as previously described (Novo *et al*., 2012). Besides, it could be partially affirmed that RPV decreases activated HSC migration without stimulation and when PDGF-ββ is used as chemoattractant. However, more independent experimental repetitions are needed to confirm the observed effect. Moreover, it should be noted that HSCs were isolated from two different patients subject to surgeries and interindividual variability is big between them. The fact that in one case a high number of HSCs have

migrated without PDGF-ββ stimulation could be explained by the presence of basal hepatic damage in one of the patients, since it is know that, when there is damage at the level of hepatic tissue, HSCs migration is stimulated towards damaged aeras, favouring the progression of CLD.

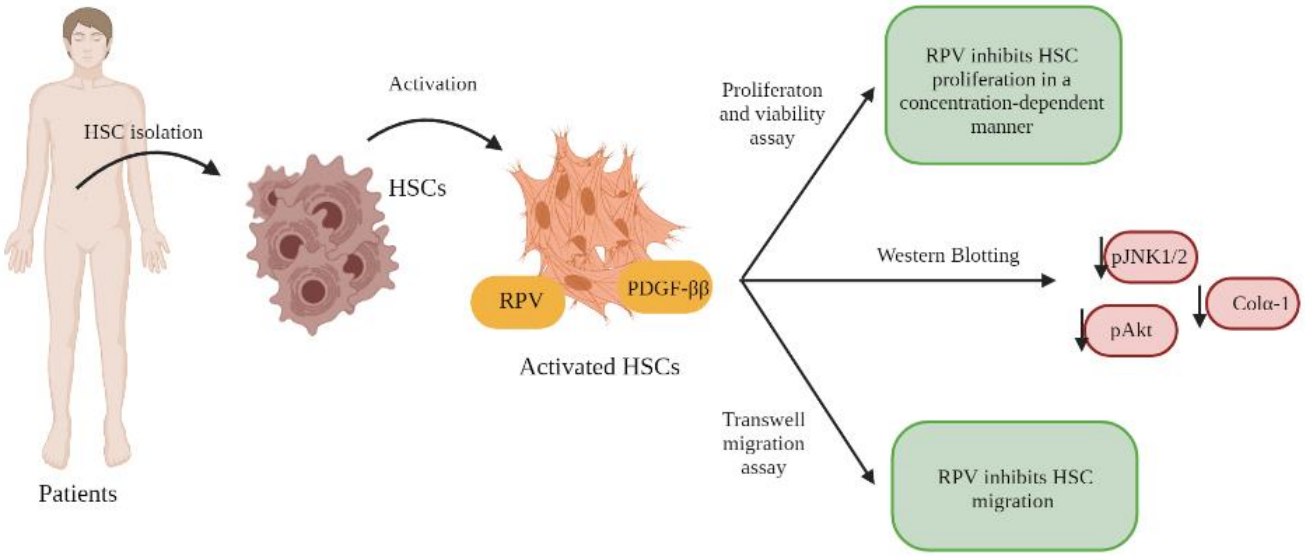


Figure 13. Overview of the findings from the study of primary HSCs treatments with RPV and PDGF-ββ.

To conclude, the results presented in this project support the anti-inflammatory role of RPV in the overall immune system using as a model PBMCs isolated from control individuals. Moreover, when it comes to hepatic tissue, we demonstrated that in HSCs stimulated with PDGF-ββ RPV inhibits proliferation and migration and interferes with MAPK signalling cascades. These findings could serve as a starting point for research to further delve into the molecular mechanisms involved in RPV's hepatoprotective action.

6. CONCLUSIONS

I. Rilpivirine exerts an anti-inflammatory effect in PBMCs isolated from healthy individuals and treated *ex vivo*, enhancing the expression of anti-inflammatory molecules and repressing the expression of chemokines and pro-inflammatory mediators.

II. Rilpivirine diminishes the proliferation of PDGF-ββ-stimulated primary human HSCs *in vitro*.

III. Rilpivirine reduces Akt and JNK activation in primary human HSCs treated with PDGF-ββ *in vitro*. Findings could be related with a pro-apoptotic and inhibitory effect of Rilpivirine in activated HSC.

IV. Rilpivirine diminishes chemotaxis of primary human HSCs *in vitro*.

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8. ANNEXES

Annex 1. Ethics committee approval certificate for the treatment of human liver samples

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PUBLICA

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 y la Dra. Nadezda Apostolova, 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*** (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

Annex 2. Controls informed consent

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"**

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 paciente

Fecha:

Por favor, proporcione la siguiente información

Edad: Sexo:

Peso: Altura:

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 .....

Medicamentos que toma habitualmente o que ha tomado en el último mes.

Indique cuales .....

Annex 3. Informed consent for the donation of liver tissue

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

DEPARTAMENT CLÍNIC MALVA-ROSA

Hospital Clínic Universitari

HOJA DE INFORMACIÓN AL PACIENTE SOMETIDO A CIRUGÍA PANCREÁTICA O BILIAR

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 acepta o no participar en este estudio. Lea esta hoja de información con atención y nosotros le aclararemos las 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 se va a someter a una cirugía pancreática o biliar. 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 su atención sanitaria. Así mismo, podrá retirarse del estudio en cualquier momento, sin tener que dar explicaciones.

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, especialmente debido 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 estima que en España una de cada cuatro personas padece esta enfermedad. Aunque en algunos pacientes se 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

Teniendo en cuenta que el número de pacientes con enfermedad hepática crónica de los que se podrá obtener muestras de su hígado va a ser bastante baja, se obtendrán también muestras hepáticas obtenidas de pacientes sin enfermedad hepática crónica. En este caso, se utilizarán

resecciones hepáticas de pacientes intervenidos de colecistectomía o cirugía pancreática. Se prevé la inclusión de 10 pacientes.

4. Actividades del estudio

Se prevé obtener el número total de muestras a lo largo de 1 año (10 pacientes). A partir de la última muestra recogida se añadirán 6 meses para el análisis y publicación de los datos. En un principio, no se prevé que su participación en el estudio conlleve la realización de más visitas y más pruebas de las que se realizarían 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 donación de una muestra de tejido hepático, el riesgo es la presencia de hemorragia, pero se trata de una reacción cuya frecuencia es muy baja. Además, la probabilidad de esta complicación es aún menor dado el tamaño tan reducido de la muestra extraída. No se prevén molestias de ningún otro tipo.

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 la enfermedad hepática crónica, 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 Orgánica 3/2018, 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 sólo 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 comprobar los 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 fines de 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 o proceso asistencial, pero se trata de un procedimiento fuera de la práctica clínica habitual.
Se le solicita permiso para utilizar con fines científicos una muestra de su hígado. Se le pedirá que done una pequeña muestra hepática durante la intervención quirúrgica que se le vaya a realizar como parte de proceso asistencial. No espera ningún efecto negativo de este procedimiento para Ud. y la única reacción indeseada sería hemorragia en la zona de extracción del tejido pero es un efecto muy poco frecuente.

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 obtenidos durante 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 razonables por 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 pueden ayudar 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 el promotor/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 generales del 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 la de 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 a cabo 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 material o 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 condiciones explicadas en esta hoja de información.

Uso de Muestras
Consiento al almacenamiento y uso de las muestras y de los datos asociados para esta investigación en 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 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 biológicas adicionales.

☐ 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 condiciones explicadas en esta hoja de información.

Uso de Muestras
Consiento al almacenamiento y uso de las muestras y de los datos asociados para esta investigación en 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 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 biológicas adicionales.

☐ 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: ____/____/____

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

DEPARTAMENT CLÍNIC MALVA-ROSA

Hospital Clínic Universitari

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	
CÓDIGO DEL ESTUDIO	
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 condiciones explicadas en esta hoja de información.

<u>Uso de Muestras</u>
Consiente al almacenamiento y uso de las muestras y de los datos asociados para esta investigación en 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 para mi 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 biológicas adicionales.

☐ 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: ____/____/____