Universitat Autònoma de Barcelona

New and Combined Treatments for Cirrhosis and Portal Hypertension: Effects on Hemodynamics and Hepatic Fibrosis in Experimental Animal Models

Sarai Rodríguez Navarro

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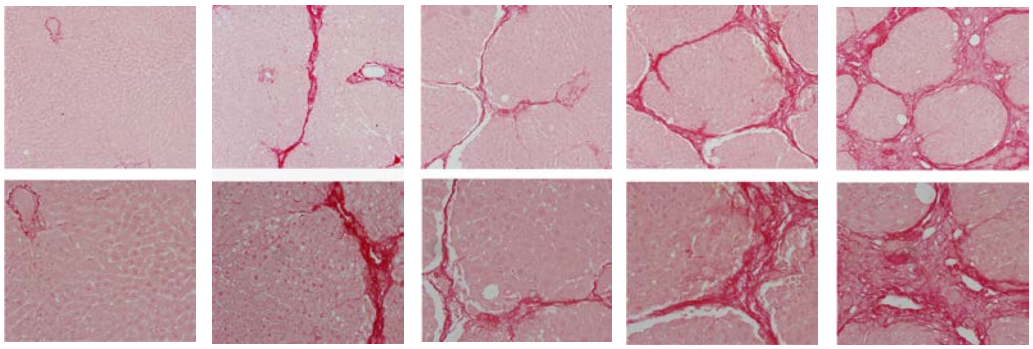

DOCTORAL THESIS

Sarai Rodríguez Navarro

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Barcelona, 2016

Laboratori de Malalties Hepàtiques
Institut de Recerca Vall d'Hebron (VHIR)

Departament de Medicina - Facultat de Medicina
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Tractaments nous i combinats per a la cirrosi i la hipertensió portal: efectes en l'hemodinàmica i la fibrosi hepàtica en models d'experimentació animal

Doctoral thesis presented by **Sarai Rodríguez Navarro** for the degree of PhD.

PhD program in Medicine - Universitat Autònoma de Barcelona

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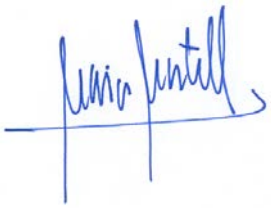

Work performed in the Laboratori de Malalties Hepàtiques
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«Life is like riding a bicycle. To keep your balance you must keep moving.»

Albert Einstein (1879-1955)

«Pour chaque fin il y a toujours un nouveau départ.»

Le Petit Prince - Antoine de Saint-Éxupéry (1900-1944)

A la meva família...

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LIST OF PUBLICATIONS

The present thesis is based on the following publications:

1. Coll M, **Rodríguez S**, Raurell I, Ezkurdia N, Brull A, Agustin S, Guardia J, Esteban R, Martell M, Genescà J. Droxidopa, an oral norepinephrine precursor, improves hemodynamic and renal alterations of portal hypertensive rats. *Hepatology*. 2012 Nov;56(5):1849-60.

2. **Rodríguez S**, Raurell I, Ezkurdia N, Augustin S, Esteban R, Genescà J, Martell M. The renal effects of droxidopa are maintained in propranolol treated cirrhotic rats. *Liver Int*. 2015 Feb;35(2):326-34.

3. **Rodríguez S**, Raurell I, Torres M, García-Lezana T, Genescà J, Martell M. A nitric oxide-donating statin decreases portal pressure with a better toxicity profile than conventional statins in cirrhotic rats. Manuscript submitted to *Scientific Reports* (April 2016).

Other works published by the author of the present thesis:

- Ezkurdia N, Coll M, Raurell I, **Rodríguez S**, Cuenca S, González A, Guardia J, Esteban R, Genescà J, Martell M. Blockage of the afferent sensitive pathway prevents sympathetic atrophy and hemodynamic alterations in rat portal hypertension. *Liver Int*. 2012 Sep;32(8):1295-305.

- Ezkurdia N, Raurell I, **Rodríguez S**, González A, Esteban R, Genescà J, Martell M. Inhibition of neuronal apoptosis and axonal regression ameliorates sympathetic atrophy and hemodynamic alterations in portal hypertensive rats. *PLoS One*. 2014 Jan 6;9(1):e84374.

SUMMARY

Cirrhosis, the most frequent cause of portal hypertension (PHT) in Western countries, is considered a multistage disease progressing from asymptomatic initial stages to decompensated cirrhosis with multiple clinical manifestations, which are a leading cause of death and liver transplantation worldwide. Therefore, therapies in liver cirrhosis should be adapted to each stage of the disease. Although many advances has been made in the last decades to understand the pathophysiology of PHT and develop new pharmacological approaches, up to now, non-selective beta-blockers (NSBB) still remain the mainstay of treatment in cirrhotic patient with PHT in order to reduce portal pressure (PP) and prevent variceal bleeding.

The present doctoral thesis focuses on the study of new therapeutic strategies for the management of liver cirrhosis at different stages of the disease. In particular, two potential oral new drugs were tested, alone or in combination with other conventional drugs, to see their efficacy in several experimental animal models of PHT: the portal vein ligation (PVL), the bile duct ligation (BDL), and the carbon tetrachloride (CCl_4) models.

Three studies constitute this thesis. In the first study, the pro-adrenergic drug droxidopa, already used in humans for other indications, was evaluated for the management of the hemodynamic and renal alterations associated with liver cirrhosis; in the second study, combinations of droxidopa with other PP-lowering drugs (NSBB or statins) were performed in order to achieve a synergistic effect and, in the third study, a comparison of conventional statins (simvastatin, atorvastatin) with the nitric oxide (NO)-donating atorvastatin NCX 6560, in terms of PP lowering effect and toxicity, was also carried out.

Droxidopa produced a marked diuretic and natriuretic effect, and improved the systemic and hemodynamic alterations of portal hypertensive rats by increasing mean arterial pressure and superior mesenteric artery resistance and by reducing superior mesenteric artery blood flow. A chronic treatment with propranolol plus droxidopa reduced PP, maintaining the increase in diuresis and natriuresis caused by droxidopa. Neither the acute combination of carvedilol plus droxidopa nor the combination with atorvastatin achieved a synergistic effect. The comparison among statins showed a magnified toxic effect of these drugs in a model that mimics a deteriorated liver function and cholestasis (especially with simvastatin treatment), and NCX 6560 improved PHT similarly to atorvastatin in two cirrhotic models (BDL and CCl_4), but with less toxicity and a better intrahepatic vasoprotective profile.

SUMMARY

Altogether, the results presented in this thesis point to a potential therapeutic use of droxidopa in the management of cirrhotic patients with refractory ascites and type-2 hepatorenal syndrome, even in those patients on propranolol therapy, and to a safer use of NCX 6560 in the potential long-term statin treatment of PHT.

RESUM

La cirrosi, la causa més freqüent d'hipertensió portal (HTP) en els països occidentals, és considerada una malaltia amb múltiples fases, que evoluciona des d'etapes inicials asimptomàtiques a un estat de cirrosi descompensada amb diverses manifestacions clíniques, les quals representen la principal causa de mort i de trasplantament hepàtic a tot el món. Per tant, caldria adaptar les diferents teràpies per a la cirrosi a cadascuna de les fases de la malaltia. Malgrat els avenços realitzats en les últimes dècades per entendre millor la fisiopatologia de la HTP i poder desenvolupar noves estratègies farmacològiques, fins al moment, els beta-bloquejants no selectius (BBNS) continuen sent la base del tractament dels pacients cirròtics amb HTP per a reduir la pressió portal (PP) i prevenir l'hemorràgia per varius.

Aquesta tesi doctoral se centra en l'estudi de noves estratègies terapèutiques per al tractament de la cirrosi hepàtica en diferents etapes de la malaltia. Concretament, hem provat dos nous potencials fàrmacs orals, sols o en combinació amb d'altres fàrmacs convencionals, per veure la seva eficàcia en diversos models d'experimentació animal d'HTP: el model de lligadura de la vena porta (LVP), el de lligadura del conducte biliar (LCB), i el model d'intoxicació per tetraclorur de carboni (CCl_4).

Tres estudis conformen aquesta tesi. En el primer estudi, es va avaluar la utilitat de la droxidopa, un fàrmac pro-adrenèrgic que ja s'utilitza en humans per a altres indicacions, en el tractament de les alteracions hemodinàmiques i renals associades a la cirrosi hepàtica; en el segon estudi, es van testar combinacions de droxidopa amb d'altres fàrmacs que disminueixen la PP (BBNS o estatines), per tal d'intentar aconseguir un efecte sinèrgic i, en el tercer estudi, es va dur a terme una comparació, pel que fa a la reducció de la PP i la toxicitat, de l'efecte de les estatines convencionals (simvastatina, atorvastatina) amb el NCX 6560, una atorvastatina alliberadora d'òxid nítric (ON). La droxidopa va produir un efecte diürètic i natriürètic destacat, i va millorar les alteracions sistèmiques i hemodinàmiques de les rates amb HTP mitjançant l'augment de la pressió arterial mitjana i la resistència de l'artèria mesentèrica superior conjuntament amb una reducció del flux sanguini en l'artèria mesentèrica superior. El tractament crònic amb propranolol més droxidopa va reduir la PP, tot mantenint l'increment en la diüresi i la natriüresi de la droxidopa. Ni la combinació aguda de carvedilol més droxidopa, ni la combinació amb atorvastatina, van aconseguir un efecte sinèrgic. La comparació entre les diferents estatines va mostrar un major efecte tòxic d'aquests fàrmacs en un model que reprodueix una funció hepàtica deteriorada i colèstasi (especialment amb el tractament amb simvastatina), i el NCX 6560 va millorar la HTP de manera similar a l'atorvastatina en dos models cirròtics (LCB i CCl_4), però amb menor toxicitat i un millor perfil vasoprotector intrahepàtic.

RESUM

En conjunt, els resultats d'aquesta tesi apunten a un potencial ús terapèutic de la droxidopa per al tractament dels pacients cirròtics amb ascites refractària i síndrome hepatorenal tipus 2, fins i tot en aquells pacients que estan en tractament amb propranolol, i a un ús més segur del NCX 6560 en el potencial tractament a llarg termini de la HTP amb estatines.

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ABBREVIATIONS

In alphabetical order:

Abbreviation:	**Meaning:**
%	percentage
&	and
α-SMA	alpha-smooth muscle actin
ΔP	portal pressure gradient
µg	microgram
µL	microliter
µm	micrometer
ACE	angiotensin-converting enzyme
ADH	antidiuretic hormone (also known as arginine vasopressin, AVP)
ADMA	asymmetric dimethylarginine
Akt	protein kinase B
ALD	alcoholic liver disease
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
BCA	bicinchonidic acid
BDL	bile duct ligation
BH_4	tetrahydrobiopterin
bid	*bis in die* (twice a day)
bpm	beats per minute
BSA	bovine serum albumin
BW	body weight
$C_{24}H_{39}NaO_4$	sodium deoxycholate
$C_6H_9Na_3O_9$	trisodium citrate dihydrate
CCl_3	trichloromethyl
CCl_4	carbon tetrachloride
CD31/PECAM-1	platelet/endothelial cell adhesion molecule-1
CD43	leukosialin
cGMP	cyclic guanosine monophosphate
CK	creatine kinase
cm^3	cubic centimeter
CO_2	carbon dioxide
CoA	coenzyme A

Abbreviation:	Meaning:
DAAM	Departament d'Agricultura, Ramaderia, Pesca i Alimentació (Generalitat de Catalunya)
Dbh	dopamine beta hydroxylase
Ddc	Aromatic-L-amino acid decarboxylase (DOPA decarboxylase)
dH_2O	distilled water
dil	dilution
dL	deciliter
DMN	dimethylnitrosamine
DMSO	dimethyl sulfoxide (C_2H_6OS)
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EEC	European Economic Community
EIA	enzyme immunoassay
eNOS	endothelial nitric oxide synthase
ET	endothelin
et al.	*et alii* (and others)
EU	European Union
FDA	Food and Drug Administration
Fig.	figure
FPP	farnesyl pyrophosphate
g	gram
G	gauge / goat antibody
g	gravitational or relative centrifugal force (rcf)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
GGPP	geranylgeranyl pyrophosphate
GRK2	G-protein-coupled receptor kinase-2
GTP	guanosine triphosphate
GTPCH-I	guanosine triphosphate-cyclohydrolase I
h	hour
H&E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA-R	3-hydroxy-3-methylglutaryl-coenzyme A reductase

Abbreviation:	Meaning:
HR	heart rate
HRS	hepatorenal syndrome
HSC	hepatic stellate cell(s)
HVPG	hepatic venous pressure gradient
IgG	immunoglobulin G
IHC	immunohistochemistry
IHVR	intrahepatic vascular resistance
IL	interleukin
im	intramuscular
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
IU	international unit
iv	intravenous
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium phosphate monobasic
KLF2	Krüppel-like factor 2
L	liter
LH	lithium heparine
M	mouse antibody
mA	milliamper
MAP	mean arterial pressure
mg	milligram
min	minute
MLCP	myosin light chain phosphatase
mm	millimeter
mM	millimolar
MW	molecular weight
N_2 (l)	liquid nitrogen
NA	noradrenaline (norepinephrine)
Na^+	sodium ion
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NaF	sodium fluoride
NAFLD	non-alcoholic fatty liver disease
NaPPi	sodium pyrophosphate tetrabasic ($Na_4P_2O_7$)

50 ABBREVIATIONS

Abbreviation:	Meaning:
NCX 6560	(βR,δR)-2(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1heptanoic acid 4-(nitrooxy)butyl ester
nm	nanometer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
NSAIDS	non-steroidal anti-inflammatory drugs
NSBB	non-selective beta-blocker(s)
O Ak	okadaic acid ($C_{44}H_{68}O_{13}$)
O/N	overnight
O_2^-	superoxide anion
ºC	centigrade degrees
$ONOO^-$	peroxynitrite
PBF	portal blood flow
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
p-eNOS	phosphorylated endothelial nitric oxide synthase
PHT	portal hypertension
PIM	protein inhibitor mix
p-moesin	phosphorylated moesin
PMSF	phenylmethylsulfonyl fluoride
PP	portal pressure / pyrophosphate
PVDF	polyvinylidene fluoride
PVL	portal vein ligation
Q	blood flow within the entire portal venous system
qd	*quaque die* (once a day)
R	portal venous system vascular resistance / rabbit antibody
RAAS	renin-angiotensin-aldosterone system
RABF	renal artery blood flow
RAR	renal artery resistance
ref.	reference
RIA	radioimmunoassay
Rock-2/RhoK/ROCK	Rho-associated protein kinase 2 / Rho-kinase

Abbreviation:	Meaning:
RT	room temperature
s	second
SBP	spontaneous bacterial peritonitis
sc	subcutaneous
SD OFA	Sprague-Dawley Oncins France Strain A
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
sGC	soluble guanylyl cyclase
SMA	superior mesenteric artery
SMABF	superior mesenteric artery blood flow
SMAR	superior mesenteric artery resistance
Snap-25	synaptosomal-associated protein-25
SNS	sympathetic nervous system
SOD	superoxide dismutase
SOV	sodium orthovanadate (Na_3VO_4)
TAA	thioacetamide
TBS	tris-buffered saline
TGF-β	transforming growth factor-beta
Th	tyrosine hydroxylase
TIPS	transjugular intrahepatic portosystemic shunt
TNF-α	tumor necrosis factor-alpha
Tris HCl	Trizma® hydrochloride
USA	United States
V	volt
VEGF	vascular endothelial growth factor
VHIR	Vall d'Hebron Institut de Recerca
WB	Western blot

Due to space restrictions, the following abbreviations are also used in the manuscript of the third study and/or in some tables in the methods section:

Abbreviation:	Meaning:
Ab	antibody
ATO	atorvastatin
CARB	carbidopa
CARV	carvedilol
CTL	control

52 ABBREVIATIONS

Abbreviation:	Meaning:
DRO	droxidopa
NCX	NCX 6560
PRO	propranolol
SIM	simvastatin
VEH	vehicle

1. INTRODUCTION

1.1. Pathophysiology of portal hypertension (PHT) and its complications

PHT is a major complication occurring in human liver disease, which results from several pathological conditions that increase the resistance to the portal blood flow (PBF) into the liver (1).

1.1.1. Definition of portal hypertension (PHT) and cirrhosis

PHT is defined as a pathological increment of the hepatic venous pressure gradient (HVPG), an indirect measure of portal pressure (PP), above 5 mmHg. Values between 5 and 9 mmHg correspond to preclinical PHT and when the HVPG is greater than 10 mmHg, PHT is considered clinically significant, since the relevant complications of the disease (see 1.1.3.) may occur, leading to death and liver transplantation worldwide (2,3).

Depending on where the impediment to PBF takes place, PHT can be classified as prehepatic PHT (involving the splenic, mesenteric or portal vein, as in portal vein thrombosis), intrahepatic PHT (liver diseases) or posthepatic PHT (diseases blocking the hepatic venous outflow, as in the Budd-Chiari syndrome). PHT also promotes the formation of collateral vessels (portosystemic collaterals), through which the blood is derived to the systemic circulation bypassing the liver.

The most frequent cause of PHT in Western countries is liver cirrhosis, responsible for 90 % of PHT cases. The main causes of cirrhosis are excessive alcohol intake, viral hepatitis infection (mainly chronic hepatitis B and C) and non-alcoholic fatty liver disease (NAFLD) associated with the metabolic syndrome and obesity (2,4). Cirrhosis is histologically characterized as the replacement of the normal anatomical structure of the liver by abnormal nodules of regenerating hepatocytes surrounded by fibrous bands (5). As a result of continued liver injury, the inflammatory response triggered by the liver in order to repair the damaged tissue fails and hepatocytes are substituted with abundant extracellular matrix (ECM) and scar. Additionally, the liver resident or recruited inflammatory cells release several cytokines -interleukin 1 (IL-1), platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-α) or transforming growth factor-beta (TGF-β), among others- that together with oxidative stress lead to hepatic stellate cell (HSC) activation, changing their phenotype from a quiescent state to a myofibroblast-like cell. These cells acquire proliferative, contractile, proinflammatory and profibrogenic properties that further stimulate ECM deposition, fibrogenesis and cirrhosis (6,7).

Cirrhosis is distinguished between compensated and decompensated stages, the latter defined by the appearance of the main complications of PHT (see 1.1.3.) (8). Besides, cirrhosis can be classified in different stages, with different likelihood of mortality, based on

clinical parameters: 1) compensated with no esophageal varices (1 % mortality per year), 2) compensated with varices (3-4 % mortality), 3) decompensated with ascites (20 % mortality), 4) decompensated with gastrointestinal bleeding (57 % mortality) and 5) decompensated with infections and renal failure (67 % mortality) (3).

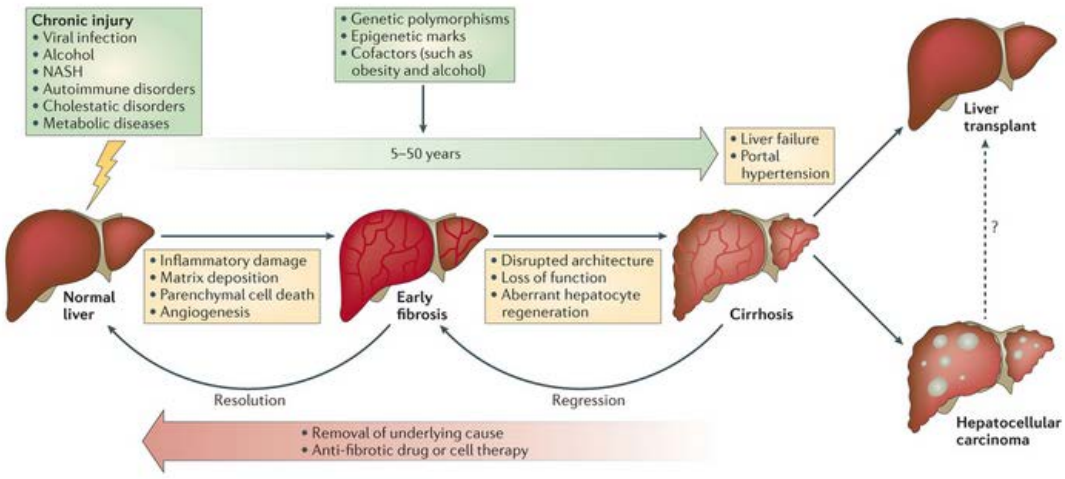


Fig. 1: **Natural history of chronic liver disease** (from Pellicoro A *et al., Nat Rev Immunol* 2014)**.**

1.1.2. Factors influencing portal hypertension (PHT)

As in any vascular system, the PP gradient is determined by the product of blood flow and the vascular resistance that opposes to the flow. Following Ohm's law this relationship is defined as:

$$\Delta P = Q \times R$$

in which ΔP is the PP gradient, Q is the blood flow within the entire portal venous system (including portosystemic collaterals) and R the portal venous system vascular resistance, which represents the sum of the resistance of the portal vein, the hepatic vascular bed, and the portosystemic collaterals (9).

This relationship shows that PP may be increased by an increment in PBF, in vascular resistance or a combination of both. Nevertheless, it is well established that the primary factor leading to PHT in cirrhosis is an increased resistance to PBF (10). As the disease progresses, an increment in the splanchnic blood flow leads to an hyperdynamic circulation state that contributes to maintain and aggravate PHT and its complications (see 1.1.2.4.) (11).

1.1.2.1. Increased vascular resistance to portal blood flow (PBF)

Increased resistance to PBF may occur at any site within the portal venous system (including the intrahepatic portal vein and sinusoids), but not in the intrahepatic hepatic vein (12).

1.1.2.1.1. Intrahepatic vascular resistance (IHVR)

The increase in IHVR in cirrhosis results from the combination of architectural abnormalities caused by fibrosis, scaring and vascular thrombosis, and functional abnormalities leading to endothelial dysfunction and increased hepatic vascular tone (3). This latter dynamic and reversible component represents up to 30-40 % of the total increased IHVR (13,14). It is mainly a consequence of an increased production and response to vasoconstrictors such as endothelin (ET) -mainly ET-1 responsible for HSC proliferation, contraction and increased ECM synthesis (15–17)-, adrenergic vasoconstrictors, angiotensin-II, prostaglandins, thromboxane A_2 and leukotrienes (18–21), and a deficit of both the production of vasodilators and the response to them, mainly nitric oxide (NO) (see **1.1.4.**). This impairment of the endothelium-dependent vasodilation is what is called endothelial or liver microvascular dysfunction (14,22,23), and it has been shown to precede inflammation and fibrosis in a model of NAFLD (24).

1.1.2.1.2. Portosystemic collateral resistance

In advanced liver disease, portosystemic collaterals can account for 90 % of the blood entering the portal system (25). Thus, vascular resistance of these vessels may also play a role in PP. Portosystemic collaterals formation involves neovascularization and opening of existing vessels, and therefore, interfering the molecular pathways involved in angiogenesis reduce their formation (see 1.1.2.3.).

1.1.2.2. Increased portal blood flow (PBF)

Splanchnic arteriolar vasodilation together with angiogenesis increases the volume of blood that reaches the portal vein. Splanchnic vasodilation is mainly attributed to an overproduction and increased response to circulating and local vasodilators and to a low vascular response to vasoconstrictors (hypocontractility), probably due to the presence of excessive vasodilator molecules (1,9,26).

1.1.2.2.1. Excessive vasodilators

Both an increased concentration of circulatory vasodilators and an enhanced production of local vasodilators contribute to splanchnic vasodilation (9). Early studies focused on the role of circulating vasodilators of splanchnic origin, accumulated as a consequence of reduced

hepatic metabolism due to poor function of the cirrhotic liver and increased portosystemic collateral vessels. Among these humoral vasodilators, glucagon was given strongest evidence (27,28) and its inhibition with somatostatin partially reduced portal venous inflow due to splanchnic vasoconstriction (29).

However, NO (see 1.1.4.) continues to be recognized as the most important vasodilator molecule that mediates excessive arterial vasodilation (30). The normalization of NO production corrected systemic hemodynamic alterations in cirrhotic rats (31,32), although its inhibition delayed but did not prevent the hyperdynamic circulation, suggesting that other factors could also be involved (33). Moreover, mice lacking endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) also developed hyperdynamic circulation, indicating that inhibition of NO was not enough to treat the hyperdynamic circulatory syndrome observed in PHT (34).

Actually, other vasodilator molecules such as carbon monoxide, prostacyclin, endothelium-derived hyperpolarizing factor, endocannabinoids, TNF-α, adrenomedullin or hydrogen sulfide are also increased in splanchnic vasodilation (9,11,35). However, none of these molecules is considered as the only factor responsible for splanchnic vasodilation, evidencing its multifactorial origin (36).

1.1.2.2.2. Hypocontractility

The decreased response to vasoconstrictors (hypocontractility) is a characteristic of the arterial splanchnic and systemic circulation in PHT. It explains why hyperdynamic circulation is exacerbated with the progression of the disease, despite the activation of some homeostatic vasoconstrictor systems, such as sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS) or the secretion of antidiuretic hormone (ADH) (9).

Apart from the excessive vasodilator molecules such as NO and endocannabinoids (1), the impaired activation of the contractile pathways also contributes to vascular hypocontractility, since neither the affinity nor the number of vasoconstrictor receptors are reduced in PHT (37). An example is the impaired vascular activation of RhoA that together with a downregulation of Rho-kinase (Rock-2) was suggested to contribute to vascular hypocontractility and vasodilation in cirrhotic rats (38).

Other factors that can also contribute to splanchnic vasodilation are the downregulation of proteins implicated in adrenergic neurotransmission, including neuropeptide Y (NPY) (39,40), the sympathetic nerve atrophy (or regression) observed in the mesenteric arteries (41,42) (see 1.1.5.1.), as well as the arterial wall thinning (1).

1.1.2.3. Angiogenesis

The formation of new vessels (angiogenesis) is also a key mechanism in the progression of PHT (43). It is mainly stimulated by vascular endothelial growth factor (VEGF) and PDGF and occurs within the damaged liver and in the splanchnic vascular bed, modulating HSC activation and fibrogenesis, splanchnic vasodilation and portosystemic collaterals formation (44). Therefore, the inhibition of these molecular pathways is effective in reducing PP, hyperdynamic circulation and portosystemic collateralization (45–48), as well as in attenuating liver fibrosis (49–51).

1.1.2.4. Hyperdynamic syndrome

In cirrhotic patients there is a marked reduction in systemic vascular resistance associated to peripheral vasodilation (11). This arterial vasodilation leads to central hypovolemia, which promotes an increment in cardiac output and in intravascular volume in order to compensate the low effective arterial blood volume. These hemodynamic alterations – arterial peripheral vasodilation, low mean arterial pressure (MAP) and systemic vascular resistance, increased cardiac output and blood volume expansion- lead to the hyperdynamic circulatory state in PHT, involving both the splanchnic and systemic circulation (52,53).

Despite the formation of portosystemic collaterals, the hyperdynamic syndrome in PHT further increases portal blood inflow, aggravating and perpetuating PHT, and fostering multiple organ dysfunction and the development of PHT major complications (see **1.1.3.**).

However, a new hypothesis -the systemic inflammation hypothesis- focus on the role of bacterial translocation and the spread of bacterial products as a primary event leading to systemic inflammation and arterial vasodilation (54). Following this theory, advanced cirrhosis and decompensation develop as a consequence of a progressive inflammatory process. This is supported by the fact that cirrhosis is associated with systemic inflammation and the grade of inflammation parallels the severity of liver, circulatory and renal dysfunction (55,56).

1.1.3. Complications of portal hypertension (PHT)

Cirrhotic PHT is a vascular disease that involves several systems and organs. Complications of PHT include variceal bleeding, ascites and hepatorenal syndrome (HRS), spontaneous bacterial peritonitis (SBP), hepatic encephalopathy, hepatopulmonary syndrome and blood clotting disorders (2).

When PP is high, portosystemic collaterals or varices that have formed in order to bypass the liver become distended because of an increased PBF. These vessels cannot resist high

pressures and are likely to rupture, causing the main complication of PHT: variceal bleeding. Gastroesophageal varices are present in about 50% of cirrhotic patients (57) and recurrent variceal hemorrhage occurs when HVPG is above 12 mmHg (8).

As a consequence of the hyperdynamic circulation, water and sodium retention are induced and fluid (ascites) is accumulated in the peritoneal cavity. Once formed, it can get infected by bacterial flora which translocate from the bowel, causing SBP. In the end-stage of the disease, renal failure usually occurs because of marked intrarenal vasoconstriction and systemic vasodilation (see 1.1.3.1.).

On the other hand, the neuropsychiatric manifestation of chronic liver diseases, hepatic encephalopathy, is caused by the presence of toxic molecules (mainly ammonia) in the systemic circulation, as a result of the portosystemic shunts and the inability of the damaged liver to detoxify toxic substances (58).

Thrombocytopenia, leucopenia and anemia are also frequent in portal hypertensive patients, and some patients, due to vasodilation in the lungs, can also develop hepatopulmonary syndrome, characterized by marked hypoxemia (59).

Patients with chronic liver disease and PHT are also at risk of developing hepatocellular carcinoma (HCC), being the presence of PHT a contraindication for liver resection (60).

1.1.3.1. Hepatorenal syndrome (HRS)

The HRS is a type of kidney failure that occurs in advanced cirrhosis, characterized by functional impairment of the kidneys due to vasoconstriction of the renal arteries, preserving tubular function and lacking histological abnormalities (61,62). It is clinically apparent by ascites refractory to diuretic therapy and usually develops after a precipitating factor, particularly a bacterial infection, although in some cases it may also occur spontaneously. There are 2 forms of HRS: type 1, characterized by an acute progressive decrease in kidney function (with a rapid increase in serum creatinine levels) and very short survival without treatment, and type 2, with longer survival and less severe and stable kidney failure (62–64).

Renal vasoconstriction in HRS occurs as a consequence of severe splanchnic vasodilation associated with PHT. As stated above, progressive splanchnic arterial vasodilation causes an increase in portal venous inflow aggravating PHT, and a decrease in MAP due to arterial underfilling and central hypovolemia, which leads to the PHT hyperdynamic circulation. This, in turn, activates vasoconstrictor and antinatriuretic mechanisms in order to maintain blood pressure. In preascitic (compensated) cirrhosis, PHT and splanchnic vasodilation is moderate, and arterial underfilling is compensated with an increment in plasma volume, consequence of the renal water and sodium retention, and in cardiac output. However, as

the disease progresses, the increase in cardiac output and in plasma volume is not sufficient to compensate arterial underfilling, and the systemic vasoconstrictor systems (RAAS, SNS and ADH) are continuously activated. This produces a constant retention of renal sodium and water, which leads to the accumulation of ascites in the peritoneal cavity, edema, and renal failure due to intrarenal vasoconstriction, reduction in renal blood flow and glomerular filtration rate (GFR) (11,64–66).

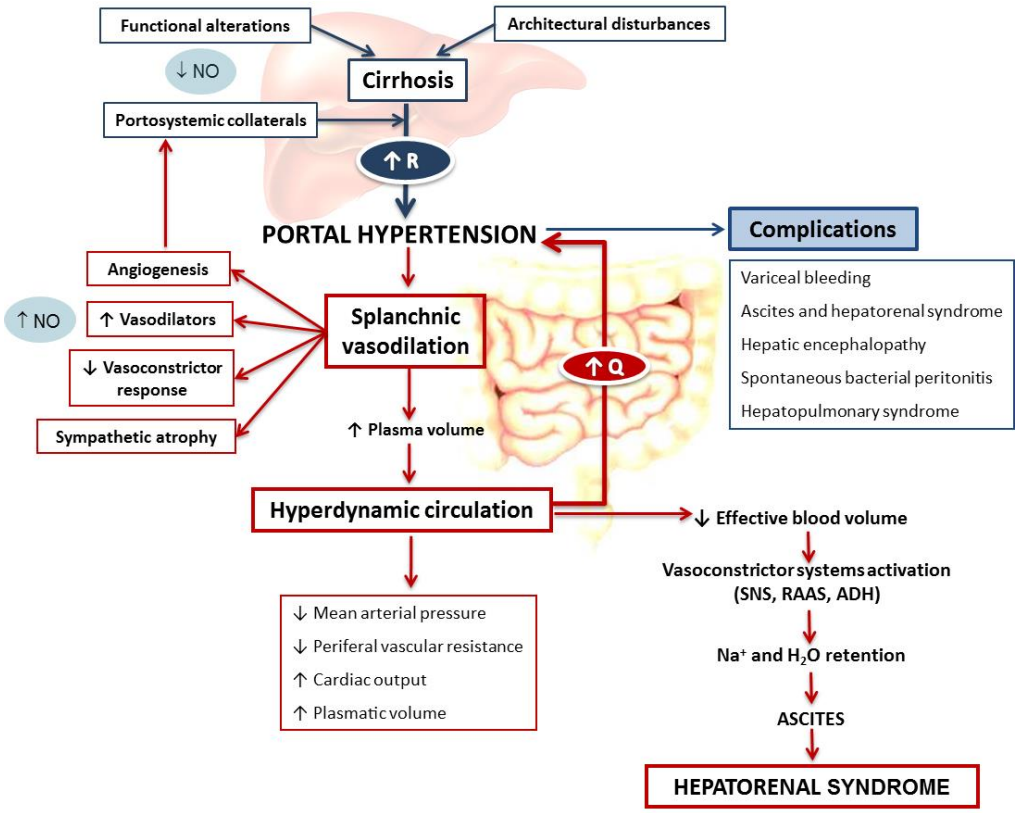


Fig. 2: Physiopathology of portal hypertension (PHT) and hepatorenal syndrome (HRS).

ADH: antidiuretic hormone, NO: nitric oxide, Q: portal blood flow, R: portal vascular resistance, RAAS: renin-angiotensin-aldosterone system, SNS: sympathetic nervous system.

In addition, the systemic inflammation hypothesis proposes that circulatory dysfunction in PHT is closely related to splanchnic and systemic inflammation. Renal inflammation could explain an impaired synthesis of renal prostaglandins leading to an imbalance between intrarenal vasoconstrictors and vasodilators, which results in intense renal sodium and water retention, hyponatremia, intrarenal vasoconstriction and HRS (54).

Liver transplant is the preferred treatment for HRS, since its prognosis is quite poor (57,62). The current management of ascites and edema is based on dietary salt restriction and administration of diuretics to increase renal sodium excretion (67), whereas reversal of HRS is achieved with pharmacologic treatment with vasoconstrictor drugs aiming at counteracting splanchnic vasodilation (64) (see 1.2.3. for further details about HRS treatment).

1.1.4. The role of nitric oxide (NO) in portal hypertension (PHT)

The endothelial-derived relaxing factor NO is a gaseous signaling molecule involved in many biological processes and is a key molecule in the pathophysiology of PHT, in which it plays a dual role: on the one hand, liver cirrhosis is associated with endothelial dysfunction and deficiency of endothelial NO release (23), and on the other hand, arterial vasodilation in the splanchnic and systemic circulation exhibit marked endothelial NO overproduction (31,68).

NO is synthesized as a byproduct of the transformation of L-arginine into L-citrulline by a family of 3 NO synthases (NOS): neuronal NOS (nNOS) and eNOS, that are constitutively expressed, and iNOS, which is induced under proinflammatory stimuli (endotoxin lipopolysaccharides and inflammatory cytokines). NO has a short life and is quickly oxidized to the stable and inactive end-products nitrite and nitrate (9,35). The finding of increased serum and urinary nitrite and nitrate levels in patients with cirrhosis, associated with high plasma renin activity, high aldosterone and ADH levels and low Na^+ urinary excretion, also supports the important role of NO in the genesis of hyperdynamic circulation in PHT (69). NO causes vasodilation through stimulation of soluble guanylyl cyclase (sGC) to generate cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells and contributes to increase splanchnic blood flow facilitating the collateralization of the portal system (70). eNOS has been shown to be the major source of NO overproduction in the splanchnic arterial circulation (30,71,72). Nevertheless, nNOS-derived NO production is also involved (73) and the contribution of iNOS has also been reported in cirrhotic rats with ascites (74). In the mesenteric circulation, the eNOS signaling pathway is activated through several endothelial cell stimuli such as VEGF (75), angiotensin (1-7) through a MAS receptor (76), shear stress (77) and inflammatory cytokines (78,79), and can be regulated through different mechanisms, including increases of the cofactor tetrahydrobiopterin (BH_4) by bacterial translocation-mediated upregulation of guanosine triphosphate-cyclohydrolase I (GTPCH-I) (80), binding of eNOS to the molecular chaperone heat shock protein 90 (Hsp90) (81) or Akt-dependent eNOS phosphorylation (82).

On the other hand, NO decreased bioavailability in cirrhotic livers results mainly from a decreased eNOS translation efficiency and enzymatic activity, together with an increased oxidative stress that leads to NO scavenging (14). Basically, increased superoxide radicals

(O_2^-) spontaneously react with NO to form peroxynitrite ($ONOO^-$), decreasing NO availability (83). Thus, antioxidant molecules such as vitamin C or superoxide dismutase (SOD) mimetics have shown an amelioration of IHVR and PHT (84,85). The reduced eNOS activity in cirrhotic livers is attributed to several post-translational alterations, including impaired eNOS phosphorylation and activation due to abnormal Akt signaling (86), eNOS binding to the inhibitory protein caveolin-1 or to asymmetric dimethylarginine (ADMA) (87,88), increased expression of the eNOS inhibitor G-protein-coupled receptor kinase-2 (GRK2) (89), low levels of the eNOS cofactor BH_4 (90) and eNOS negative regulation through the RhoA/Rock-2 pathway (91).

Considering the deficient NO release in cirrhotic livers, strategies aiming at increasing NO availability at the liver circulation to decrease the vascular tone, are a rational approach to PHT treatment (43,92), since NO supplementation in cirrhosis can not only restore the hepatic decreased vasodilator content, but also attenuate the enhanced production of vasoconstrictors (93). However, due to the complex dual role of NO in PHT, this strategy can cause a more detrimental effect in the splanchnic and systemic circulation than a beneficial effect in the intrahepatic circulation (94). The use of NO donors was hampered for this reason, since their vasodilatory action was not limited to the hepatic circulation, and they caused arterial hypotension and activated endogenous vasoactive systems, which led to sodium and water retention, and renal failure (92,95). Liver-selective NO donors such as NCX 1000 (a NO-releasing derivative of ursodeoxycholic acid) and V- PYRRO/NO (a NO donor prodrug), both selectively metabolized in the liver, were thought to overcome this problem when tested in cirrhotic rats (96–99). However, when tested in other species (humans and mice) they showed widespread vascular effects and NCX 1000 even failed to lower PP (100,101). For this reason, statins have become a promising alternative to increase liver NO levels, improving IHVR and portal hemodynamics, without deleterious effects on systemic hemodynamics (102,103) (see 1.2.2.).

1.1.5. The nervous system in portal hypertension (PHT)

As stated above, the SNS in one of the potent vasoconstrictors systems activated as a consequence of splanchnic arterial vasodilation and central hypovolemia (9) and is responsible for the development of HRS (104). High levels of circulating catecholamines were described in patients with cirrhosis and PHT (105–107), and a positive correlation between circulating noradrenaline (NA), adrenaline and the worsening of PHT was also reported (108). Actually, patients with advanced liver disease, more pronounced PHT and HRS, exhibit the highest NA and adrenaline plasma levels. Thus, plasma NA levels were considered an independent prognostic factor in cirrhosis, since they correlated with the severity of the disease and patient survival (109).

Although a general activation of the SNS is seen both in humans and animal models of PHT (110), regional divergences in SNS activity within cirrhosis were also proposed (111). Actually, previous findings from our group showed a downregulation of genes related to the adrenergic system in the superior mesenteric artery (SMA) of portal hypertensive rats, together with a reduction in the protein expresion of tyrosine hydroxylase (Th), dopamine beta hydroxylase (Dbh) and synaptosomal-associated protein-25 (Snap-25), proteins associated with NA synthesis and the release of neurotransmiters from neurons into the synaptic space (39). This contradicted the traditional concept about the overactivation of the adrenergic system in cirrhosis and PHT, and suggested that there may be vascular areas localized especially in the splanchnic area where the sympathetic tone is abolished. That is, a local adrenergic inhibition (mesenteric) whithin a global context of general adrenergic activation (in kidney, esqueletic muscle and other organs).

1.1.5.1. Sympathetic atrophy

The SMA adrenergic dysfunction was accompanied by morphological alterations in the nerves surrounding the SMA: a reduction in the number of nervous structures, and in the total and Th-immunostained nervous area (41). Therefore, PHT seemed to be associated with a regression of the sympathetic innervation in the proximal parts of SMA (sympathetic atrophy) that could be contributing to the splanchnic vasodilation associated to PHT.

This sympathetic nerve atrophy probably represents a late event in the pathogenesis of mesenteric vasodilation, since 5 days after the ligation of the portal vein, when the hyperdynamic circulation is fully established (112), adrenergic downregulation was not yet observed (39).

A decrease in Th, Dbh and Snap-25 protein expression in mesenteric resistance arteries was also observed in portal hypertensive rats compared with sham rats. Interestingly, nervous structures and protein expression in renal arteries showed no differences compared with control animals, indicating that the sympathetic nerve atrophy is a specific phenomenon of the mesenteric vascular bed, possibly as a local consequence of PHT (41).

In summary, apart from the humoral factors involved in splanchnic vasodilation, the nervous system also plays an important role in the genesis of PHT circulatory abnormalities (39,41,42,113). The neuronal theory proposes that PHT, via baroreceptors in the mesenteric area that detect pressure changes, activates the central nervous system through the afferent nerves. This activation, in turn, via efferent pathways, results in an overexpression of neuromodulators (nerve growth factor, its precursor, semaphorin 3A and p75 neurotrophin receptor) in superior mesenteric ganglia, that activates signaling pathways related to axonal regression and apoptosis, and leads to the sympathetic atrophy

associated with splanchnic vasodilation (42,113,114). Thus, correcting sympathetic dysfunction in the mesenteric bed could decrease PHT and ameliorate hyperdynamic circulation.

Intravenous (iv) injection of NPY, a sympathetic neurotransmitter co-stored and co-released with NA that increments α1-adrenergic vasoconstriction, showed an improvement in PHT and hyperdynamic circulation in cirrhotic rats via an amelioration of splanchnic vasodilation (115). Moreover, the administration of gambogic amide, an agonist of the tyrosine kinase receptor A, leading to the activation of survival and axonal growth pathways and inhibition of neuronal apoptosis and axonal regression ones, ameliorated sympathetic atrophy and hemodynamic alterations in rats with PHT (113), hence supporting the contribution of sympathetic atrophy to splanchnic vasodilation.

1.2. Therapy of portal hypertension (PHT) in liver cirrhosis

Most efforts for the management of cirrhotic patients concentrate on preventing the appearance of PHT complications while the patients are still in the compensated phase. In this phase, patients are classified in those with clinically significant PHT and those with subclinical PHT. The goal of treatment for the last group is to prevent the advent of clinically significant PHT, whereas the treatment in the first group is focused on preventing decompensation (116).

The advances made in the past to both prevent and treat the common complications of PHT are summarized in Table 1 (117).

Pharmacological treatment of PHT has been mainly devoted to gastroesophageal varices related events to avoid the first bleeding episode, stop acting bleeding or prevent bleeding recurrence (118). Due to the complex physiopathology of PHT, caution must be taken in any treatment since they should decrease PP but without adverse events on the systemic circulation or on liver function. Non-selective beta-blockers (NSBB), such as propranolol or nadolol, are the mainstay oral chronic treatment for PHT to reduce PP and prevent variceal bleeding (see 1.2.1.), whereas other splanchnic vasoconstrictors (terlipressin and somatostatin or its analogues) are used parenterally in acute variceal bleeding and in HRS (119). Shunt procedures are also an effective measure to reduce PP by decreasing portal venous outflow resistance (120).

Table 1: **Prevention and treatment for the complications of portal hypertension (PHT)** (from Schuppan D *et al.*, *Lancet* 2008).

	Prevention	Treatment
Variceal bleeding[72–75]	Non-selective β blockers* Variceal band ligation	Acute: Resuscitation Vasocontrictors† Sclerotherapy Band ligation TIPS Surgical shunts Chronic: Variceal obliteration TIPS Surgical shunts
Ascites[72,76]	Low sodium diet	Low sodium diet Diuretics Large volume paracentesis TIPSS (LeVeen/Denver shunts)
Renal failure[77]	Avoid hypovolaemia	Discontinue diuretics Rehydration Albumin infusion Hepatorenal syndrome: add terlipressin or midodrine (noradrenaline) and somatostatin (octreotide)
Encephalopathy[78]	Avoid precipitants	Treat precipitating factors: Infection Bleeding Electrolyte imbalance Sedatives High protein intake Lactulose Neomycin, metronidazole, rifaximin
Spontaneous bacterial peritonitis[72]	Treat ascites	Early diagnosic paracentesis: >250 neutrophils per mL, intravenous antibiotics (plus albumin) Secondary prophlaxis with oral antibiotics such as levofloxacin

TIPSS=transjugular intrahepatic portosystemic shunt. *Nadolol, propranolol. †Vasopressin, octreotide/somatostatin, terlipressin.

Currently, there is no indication to use NSBB to prevent the formation of varices (121), whereas patients with small varices with signs of increased risk of bleeding should be treated with them to prevent the first variceal bleeding. Patients with medium/large varices can be treated either with NSBB or endoscopic band ligation. For the management of the acute bleeding episode, antibiotic prophylaxis is recommended and vasoactive drugs (terlipressin, somatostatin, octreotide) should be started as soon as possible and used in combination with endoscopic band ligation (116). Transjugular intrahepatic portosystemic shunt (TIPS) is reserved for those patients who fail standard therapy or for patients who are likely to fail (early TIPS), and the prevention of recurrent bleeding consists of the combination of NSBB and endoscopic band ligation (122) (see Fig. 3).

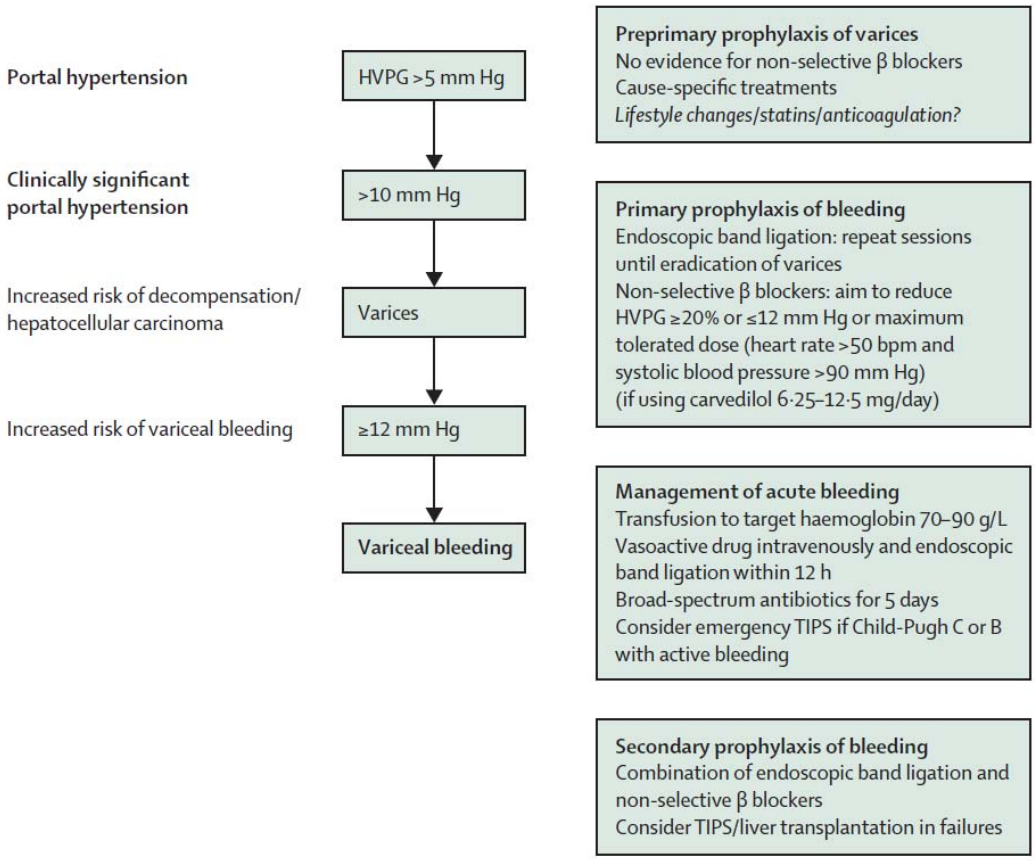


Fig. 3: Prevention and treatment of portal hypertension (PHT) and varices at various degrees of severity (from Tsochatzis EA *et al.*, *Lancet* 2014)**.**

bpm: beats per minute, HVPG: hepatic venous pressure gradient, TIPS: transjugular intrahepatic portosystemic shunt.

In the end-stage of cirrhosis, however, liver transplantation is the most effective option with a 70 % survival after 5 years (117). Nevertheless, the low availability of organs, the long waiting list for transplantation, histocompatibility issues and recurrence of the disease in some cases (especially in viral hepatitis C) urge the need of new therapeutic strategies.

Before the appearance of PHT complications, the etiological treatment of the underlying liver disease is the chosen therapeutic option, since it may improve liver structure and function, reduce PHT and prevent complications. Therefore, current treatments for cirrhosis and PHT aim to interrupt or eliminate the source responsible for the disease: antiviral drugs for hepatitis B and C infection, alcohol abstinence for alcoholic liver disease (ALD) or lifestyle modification for NAFLD (120). It has been shown that suppression or interruption of viremia (in chronic hepatitis B and C, respectively) allows the regression of cirrhosis, hence preventing PHT complications (123,124) and that alcohol abstinence reduces the risk of complications and improves survival in alcoholic cirrhosis (125). Besides, a lifestyle modification with diet and moderate exercise also decreases body weight (BW) and PP in cirrhosis with obesity (126), and bariatric surgery has also been shown to be effective in improving hepatic steatosis (127) and may play a role in the very early pre-primary prophylaxis of PHT (120).

If the chronic liver injury leading to fibrogenesis and PHT cannot be interrupted, other strategies aimed at modulating the factors influencing PHT -reducing IHVR, PBF, portosystemic collaterals or hyperdynamic circulation- can be used.

As mentioned above, only the correction of the increased PBF by means of splanchnic vasoconstrictors such as NSBB, vasopressin and its derivatives, and somatostatin and its analogues has demonstrated efficacy in decreasing PP up to now (14).

Modulation of the pathways that increase IHVR, angiogenesis or hyperdynamic circulation, although encouraging, have mainly been tested in experimental animal models. Drugs currently under investigation to decrease IHVR include statins, antioxidants, RAAS inhibitors as well as other antifibrotic strategies (119).

A detailed explanation about statins can be found in section 1.2.2.. Regarding the reduction of hepatic oxidative stress with antioxidants, many strategies including administration of exogenous recombinant human manganese SOD (128) or SOD mimetics (85), N-acetylcysteine (129), as well as vitamin C and E (84,130), resveratrol (131) or dark chocolate (132) have shown to ameliorate IHVR and PHT, and some of them were successful even in cirrhotic patients (84,132).

Inhibition of leukotrienes (with montelukast), blockage of the thromboxane A_2 receptor (with terutroban) or of the leptin receptor (with an anti-leptin receptor antibody), HSC-specific inhibition of Rock-2 or modulation of myofibroblasts with relaxin also showed to

decrease PP in cirrhotic animals (133–137). In addition to that, other approaches to reduce IHVR and PP in experimental cirrhotic animals include the administration of nitroflurbiprofen, amiloride, spinorolactone, obeticholic acid or metformin, among others (138–142).

Finally, as mentioned before, angiogenesis inhibition also induces an improvement in PHT. Besides the inhibitors of VEGF and PDGF (45–47), the multikinase inhibitors sunitinib and sorafenib also ameliorated PHT in animal models (49,50,143,144) and sorafenib even did it in half of the patients in a pilot study (145). Recently, green tea extract with anti-angiogenic properties has also shown beneficial effects on liver fibrosis, portosystemic shunting and mesenteric angiogenesis (146) and the ectopic overexpression of the angioinhibitor vasohibin-1 also seems to be a promising therapeutic strategy (147).

In summary, due to the multiple factors involved in PHT, a rational therapy should target several pathophysiological mechanisms (multitarget therapy) (148).

1.2.1. Non-selective beta-blockers (NSBB)

NSBB (propranolol, nadolol) are the only drugs recommended for prophylaxis against variceal bleeding in cirrhotic patients, being propranolol the most commonly used (116). They decrease the splanchnic blood inflow by reducing the cardiac output (via β1 receptor blockage) and also by inhibiting splanchnic vasodilation in mesenteric arterioles (via β2 receptor blockage), resulting in unopposed α1-adrenergic-mediated vasoconstriction and, therefore, in a further decrease in portal inflow (122). Carvedilol, a new and increasingly used NSBB with intrinsic anti-α1-adrenergic activity, was found to be more effective in reducing PP than propranolol (149,150). It is more potent than propranolol as a β-receptor antagonist and through its mild anti-α1-adrenergic activity, it decreases the hepatic vascular tone and hepatic resistance, resulting in a further decrease in PP (151). It has also shown to achieve a hemodynamic response in 56 % of patients non-responders to propranolol (152). Furthermore, it has antioxidant, anti-inflammatory and antifibrotic properties and the ability of inhibiting vascular smooth muscle cell proliferation (153–157). An attenuation of liver lesions induced by angiotensin-II infusion and a reduction in liver fibrosis markers have also been described in rats treated with carvedilol (158,159).

In addition to their protective role for variceal bleeding, NSBB might also be beneficial for other complications of PHT: ascites, SBP, HRS, hepatic encephalopathy and survival (160). Their advantages also include their low cost and ease of administration (oral administration). However, not all patients respond to NSBB, around 30-50 % of patients are non-responders. An acute response to iv propranolol, specifically a 10 % decrease in HVPG or to ≤ 12 mmHg, may be used to identify NSBB responders, which is also associated with a

significant reduction in the risk of variceal bleeding and decompensation (116). Contraindications for NSBB treatment are reported in about 15 % of patients and, due to their common side-effects (fatigue, weakness, shortness of breath), dose-reduction or discontinuation of the treatment may be required in another 15 % of patients (122,160). Moreover, the safety of NSBB in patients with end-stage liver disease, especially in those with refractory ascites and SBP, has also been questioned (161–163). However, further studies have shown that NSBB do not increase the mortality of cirrhotic patients with ascites (164–166). Therefore, NSBB can routinely be maintained in patients with ascites but close monitoring of blood pressure, serum sodium and serum creatinine is necessary to consider dose reduction or discontinuation in those patients who develop low blood pressure, hyponatremia and impairment in renal function (116).

1.2.2. Statins

Statins are a family of drugs commonly prescribed as lipid-lowering agents in metabolic syndrome and cardiovascular diseases that also exert beneficial lipid-independent pleiotropic effects through the inhibition of the limiting enzyme in cholesterol synthesis, the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R). Its inhibition leads to a reduction in mevalonate production and in the synthesis of isoprenoids, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are needed for the isoprenylation of small guanosine triphosphate (GTP)-binding proteins. These signaling proteins, such as the small Ras, Rac, and Rho GTPases, need the lipophilic attachments for their anchoring to the cell membrane where they become activated by GTP and transduce signals to their effectors. They are mainly involved in cell proliferation, cytoskeletal organization, and activation of transcription factors (167–169) (see Fig. 4).

Therefore, the pleiotropic effects of statins are due to the inhibition of these GTPases proteins and include anti-inflammatory and antioxidant actions and an improvement in the endothelial function. Statins have shown to suppress the activity of prooxidant enzymes and pro-inflammatory transcriptional pathways in the endothelium, enhance eNOS expression and activity, while improving its enzymatic coupling, leading also to an increase in NO bioavailability (168).

Statins are selectively distributed in the liver; only a small proportion of the drug binds to plasmatic proteins and is systemically distributed. They are generally well tolerated and their most important adverse events include liver and muscle toxicity (167).

Recent evidences from epidemiological studies seem to indicate that these drugs could also be useful in the treatment of chronic liver disease. Indeed, statin use is associated with a decreased risk of fibrosis progression, cirrhosis, hepatic decompensation, HCC and death in

patients with chronic liver disease, especially in those with hepatitis C virus infection (170–174).

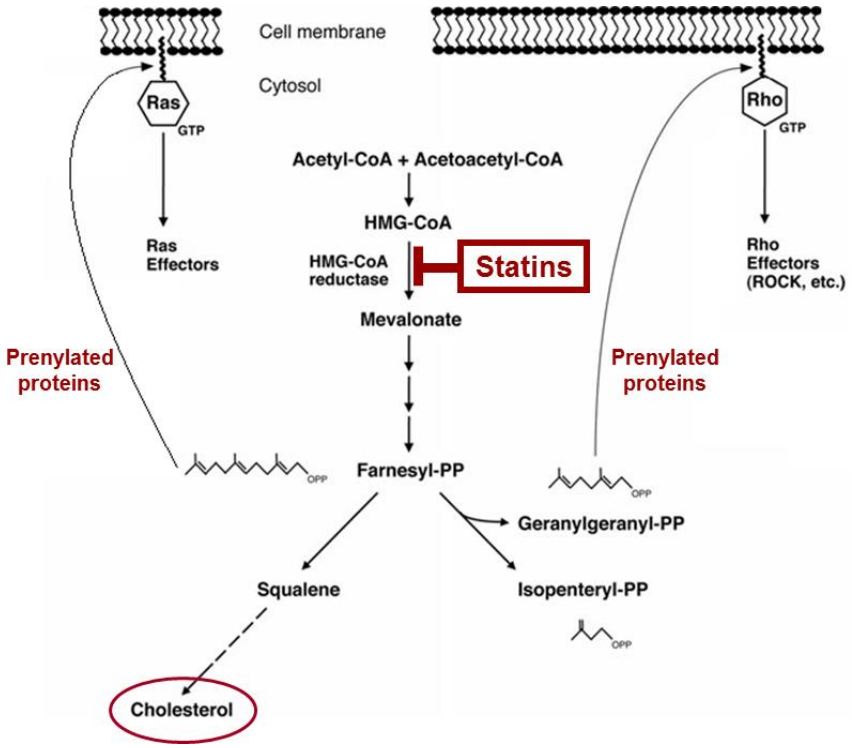


Fig. 4: **Cholesterol biosynthesis pathway and isoprenoids as modulators of guanosine triphosphate (GTP)-binding proteins** (adapted from Sawada N *et al*. *Antioxid Redox Signal* 2014)**.**

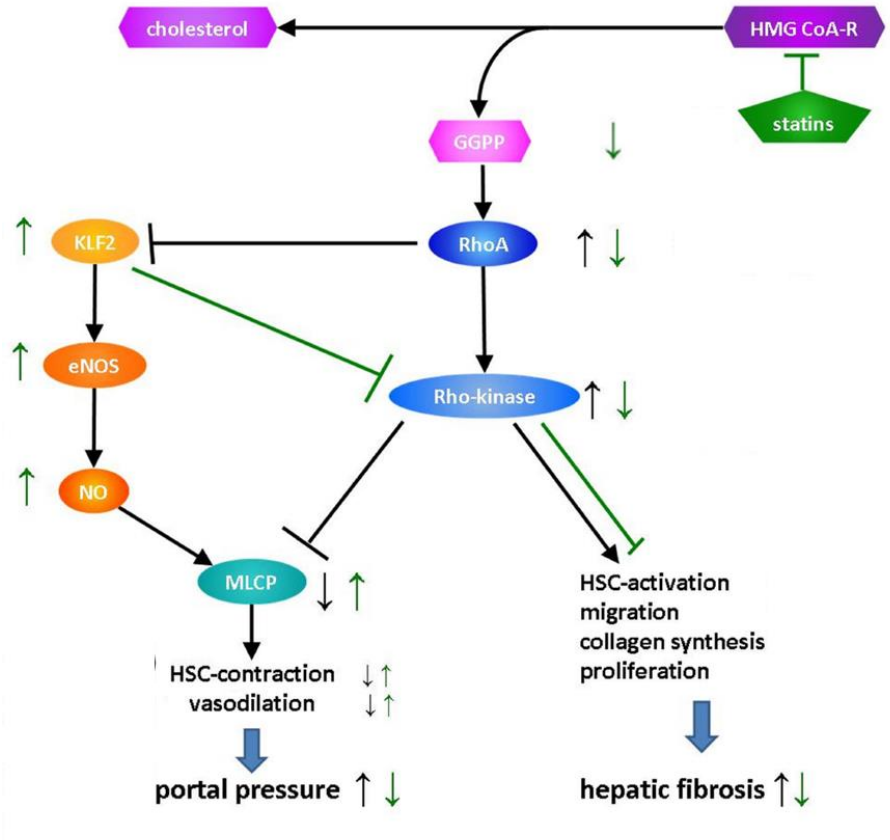
PP: pyrophosphate, CoA: coenzyme A, GTP: guanosine triphosphate, HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A, ROCK: Rho-associated protein kinase 2.

Among statins, simvastatin has shown to lower IHVR and PP in clinical trials (102,103) and even improve survival of decompensated cirrhotic patients after variceal bleeding (175). In cirrhotic rats though, atorvastatin showed a greater PP lowering effect together with an attenuation of fibrosis and HSC activation (176,177) and a reduction in neo-angiogenesis in the splanchnic circulation (178).

A summary of the mechanisms involved in the beneficial intrahepatic effects of statins is represented in Fig. 5 (179). Mainly, they include improvement in microvascular dysfunction through increasing NO availability and cGMP liver content via an increase in eNOS activity -through Akt-dependent eNOS phosphorylation- (180), and inhibition of RhoA/Rock-2 (176). Statins also attenuate liver angiotensin-II-induced inflammatory actions (181) and hepatic

fibrosis via decreased turnover of HSC and downregulation of profibrotic cytokine expression (177,182). An overexpression of the transcription factor Krüppel-like factor 2 (KLF2) that orchestrates a variety of vasoprotective pathways induced by statins has also shown to confer hepatic endothelial vasoprotection and promote HSC deactivation (183,184). Actually, exogenous hepatic KLF2 upregulation improved liver fibrosis, endothelial dysfunction and PHT in cirrhotic rats (184).

Fig. 5: **Schematic overview of the mechanisms by which statins (in green) attenuate liver fibrosis and reduce portal pressure (PP)** (adapted from Trebicka J *et al*. *Gut* 2015)**.**



eNOS: endothelial nitric oxide synthase, GGPP: geranylgeranyl pyrophosphate, HMG-CoA-R: 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HSC: hepatic stellate cell, KLF2: Krüppel-like factor 2, NO: nitric oxide, MLCP: myosin light chain phosphatase.

1.2.3. Treatment of ascites and hepatorenal syndrome (HRS)

Treatment with NSBB to ameliorate PHT and prevent variceal bleeding showed to reduce the risk of developing ascites and HRS when HVPG was lowered by 10 % or more (185). However, once ascites has developed, the initial management consists of limiting dietary sodium and administering diuretics to increase renal sodium excretion. Aldosterone antagonists (spironolactone) are the diuretics of choice and their combined administration with loop diuretics (furosemide), may be helpful in patients with recurrent ascites (67). With refractory ascites -not responding to diuretics-, large-volume paracentesis (ascites drainage) with iv albumin administration to reduce the risk of post-paracentesis circulatory syndrome, or TIPS, are commonly used as a first and second-line treatment, respectively (186,187). Recently, the alfapump® system, an implanted pump for the automated and continued removal of ascites from the peritoneal cavity into the bladder (to be eliminated through normal urination), also showed to be an effective method for the management of ascites (188).

Other potential therapies to decrease the need of large-volume paracentesis include midodrine, endocannabinoids antagonists, albumin or clonidine administration (64).

Both clonidine and midodrine interact with the SNS activity, but may have opposite roles: clonidine is a sympatholytic α2-adrenergic agonist that inhibits the SNS activity by reducing the release of NA, whereas midodrine is an α1-adrenergic agonist that improves circulatory dysfunction by increasing MAP and suppressing the activity of the homeostatic vasoconstrictor systems (189). Nevertheless, both drugs may have a beneficial role in the management of ascites. The additional administration of clonidine to diuretics was associated with lower ascites recurrence and fewer diuretic requirements (190), probably due to the suppression of the afferents sympathetic activity to the renal nerves, involved in renal sodium and water retention (191). On the other hand, in a randomized pilot study evaluating the long-term administration of midodrine, this drug also showed to be superior to standard treatment alone in the control of refractory or recurrent ascites and it also improved systemic hemodynamics without any renal or hepatic dysfunction (192). Besides, the combined therapy of both drugs was also assessed, but was not superior to midodrine or clonidine alone (193). A recent study comparing both drugs in cirrhotic rats with ascites showed that clonidine at low doses has a better effect on diuresis and natriuresis than midodrine (194). However, only midodrine is currently recommended for refractory ascites in guidelines (195) and clonidine is still considered an experimental option under evaluation.

Since SBP is associated with ascites accumulation, primary prophylaxis of SBP with norfloxacin, by decreasing bacterial translocation and systemic inflammation, reduces the incidence of SBP, and also delays the development of HRS, improving survival in advanced cirrhosis (196). Treatment with pentoxyfiline, a TNF-α inhibitor, in patients with severe alcoholic hepatitis also showed to prevent the development of HRS (197,198).

Once the renal failure is established, pharmacologic treatment with vasoconstrictors to reverse splanchnic vasodilation together with volume expanders such as albumin, are effective in 40-50 % of patients with type-1 HRS and also improve survival (62). The ADH analogue terlipressin (administered iv in boluses or by continuous infusion) is the vasoconstrictor of choice and was also associated with an improvement in renal function in type-2 HRS (199). However, a recent study showed no benefits in terlipressin plus albumin treatment in patients with type-2 HRS neither in pre-transplantation nor in post-transplantation outcomes (200). Other vasoconstrictor drugs such as the α1-adrenergic agonists (iv NA or oral midodrine plus subcutaneous (sc) octreotide) administered with albumin have also been recommended for the treatment of HRS (67,201).

Finally, all patients with HRS should be considered for liver transplantation unless they have contraindications, since the 3 year-survival rate is approximately 60 %, higher than that expected by the natural course of the disease (64). The extracorporeal liver support systems MARS® and Prometheus®, for albumin dialysis and fractioned plasma separation and adsorption, respectively, have also shown to be effective in the management of HRS and are considered a bridge option to liver transplantation (64,202).

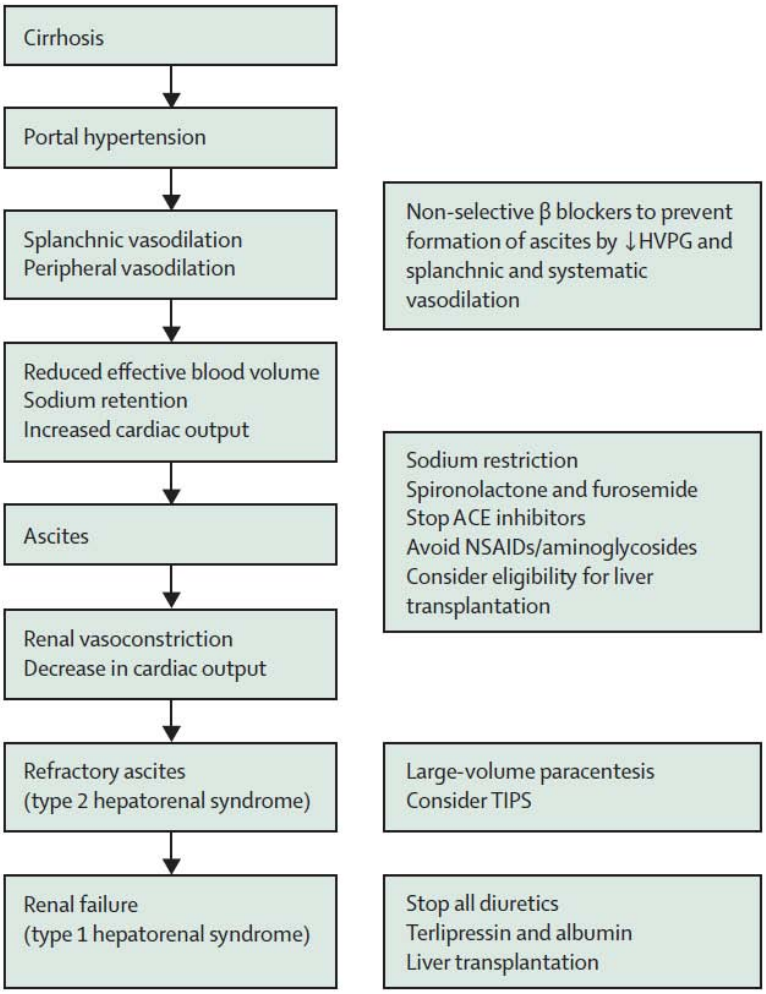


Fig. 6: **Prevention and treatment of ascites at various degrees of severity** (from Tsochatzis EA *et al*., *Lancet* 2014)**.**

ACE: angiotensin-converting enzyme, HVPG: hepatic venous pressure gradient, NSAIDS: non-steroidal anti-inflammatory drugs, TIPS: transjugular intrahepatic portosystemic shunt.

1.2.4. Potential new drugs for portal hypertension (PHT) and its complications

Two potential oral new drugs for the management of PHT and its complications have been tested in this thesis: droxidopa, a pro-adrenergic drug already used for other indications, and NCX 6560, a new molecular entity consisting of a NO-releasing derivative of atorvastatin.

1.2.4.1. Droxidopa

Droxidopa or L-threo-3,4-dihydroxyphenylserine (L-threo-DOPS) is a synthetic catecholamine acid analogue, gradually converted to NA by aromatic-L-amino acid decarboxylase (DOPA decarboxylase or Ddc) in vivo (see Fig. 7), with long-acting pressor effects also when orally given (203). The Ddc enzyme is widely expressed throughout the body, allowing the conversion of droxidopa into NA outside neurons and enabling the release of NA into the blood stream, where it could also act as a circulating hormone (204).

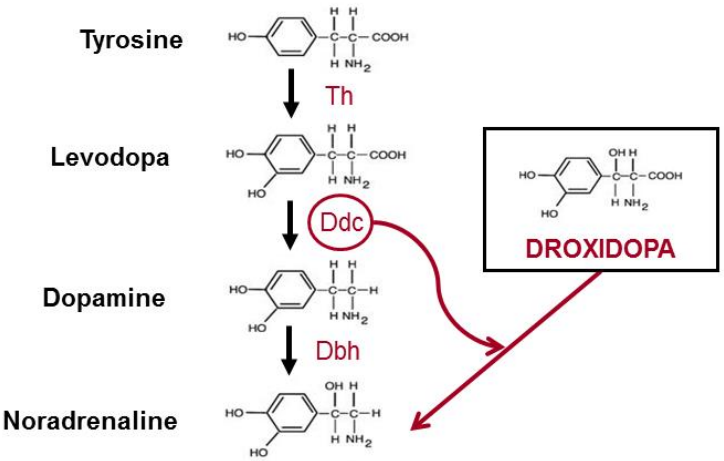


Fig. 7: Noradrenaline (NA) synthesis pathway (classical and through droxidopa).

Dbh: dopamine beta hydroxilase, Ddc: aromatic-L-amino acid decarboxylase (DOPA decarboxylase), Th: tyrosina hydroxylase.

Thus, droxidopa is believed to exert its pharmacological effect through NA and not through the parent molecule or other metabolites, acting then as a NA prodrug. NA is distributed throughout the body and thought to increase blood pressure through binding and activation of adrenergic receptors, inducing peripheral arterial and venous vasoconstriction (205). In addition, small and transient rises in plasma NA have also been shown in humans after droxidopa administration (206). Droxidopa also showed to increase, in a dose-dependent manner, the urinary volume and sodium and chloride ions in mice and rats (207)

and its diuretic effect was shown to be dependent on the NA pressor effect via peripheral α-adrenergic receptors activation (208). It crosses the blood-brain barrier and may affect the central as well as the peripheral autonomic nervous system (209). However, treatment with carbidopa, a Ddc inhibitor that blocks the enzyme outside the brain, abolished the droxidopa pressor effect and since pure autonomic failure (one of the conditions for which the drug is indicated) entails loss of postganglionic sympathetic nerves, the pressor effect of droxidopa seems to result from the conversion of droxidopa to NA outside the central nervous system, both in sympathetic nerves and in non-neuronal cells (206).

Droxidopa was first developed by Dianippon Sumitomo Pharma Co., Ltd. and initially commercialized in Japan in 1989 for the treatment of symptomatic neurogenic orthostatic hypotension (210). Then, in 2006, it was licensed to Chelsea Therapeutics Inc. which continued with the drug clinical development program, including 10 clinical studies that enrolled a total of 666 unique patients with symptomatic neurogenic orthostatic hypotension, 631 of whom were treated with droxidopa (209). In January 2007, droxidopa received an orphan drug designation for the treatment of rare diseases or conditions (209), and in February 2014 it was finally approved by the Food and Drug Administration (FDA) under the accelerated approval program (211). In June of the same year, Lundbeck, a global pharmaceutical company specializing in psychiatric and neurological disorders, completed the acquisition of Chelsea Therapeutics Inc., allowing the availability of the drug in the US from September 2014 as Northera™ (210).

Northera™ is currently indicated for the treatment of orthostatic dizziness, lightheadedness, or the “feeling that you are about to black out” in adult patients with symptomatic neurogenic orthostatic hypotension caused by primary autonomic failure (Parkinson’s disease, multiple system atrophy, pure autonomic failure), dopamine beta-hydroxylase deficiency, or non-diabetic autonomic neuropathy (205). It has been shown to be a safe drug both in preclinical and in clinical studies, being headache, dizziness, nausea, hypertension and fatigue the most common adverse events reported (205,211). Droxidopa and its metabolites are primarily cleared renally and the drug showed to be safe in patients with mild or moderate renal impairment (GFR greater than 30 mL/min) (205). The effectiveness beyond two weeks of treatment has not been demonstrated yet and Lundbeck is committed to the further study of the drug including a long-term, phase IV, multi-center, placebo-controlled, randomized study (210).

1.2.4.2. NCX 6560

NCX 6560 is a new molecular entity developed by NicOx, an international company focused on the research of NO-donating molecules, consisting of a NO-donating atorvastatin (see Fig. 8). It has a double mechanism of action: on the one hand, as an atorvastatin, it inhibits the HMG-CoA-R resulting in the multiple favorable pleiotropic effects of statins and, on the other hand, it also provides some beneficial physiological properties thanks to the NO released from the molecule.

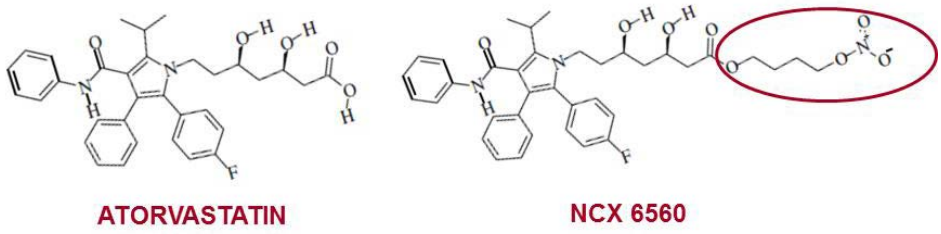


Fig. 8: **Chemical structure of atorvastatin and NCX 6560.**

NCX 6560: (βR,δR) – 2(4 – fluorophenyl) – β, δ –dihydroxy – 5- (1 -methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1heptanoic acid 4-(nitrooxy)butyl ester.

It was first developed in 2006 for its potential benefit in cardiovascular diseases as a lipid-lowering agent (212). In several preclinical studies has shown to exert greater lipid-lowering, antithrombotic and anti-inflammatory effects than atorvastatin and even prevent statin-induced myopathy in mice (213–216). The extra benefits of NCX 6560 are associated to a large extent to the release of bioactive NO (217), which *in vivo* showed to be a slow and sustained process reaching a peak of plasmatic nitrites and nitrates levels 3 h after NCX 6560 oral administration and persisting over 6 h (213). Treatment with NCX 6560 prevented the increase in creatine kinase (CK) levels observed with atorvastatin treatment in mice (214,215), reduced vascular and systemic peroxidation more than atorvastatin in a mouse model of accelerated atherosclerosis, suggesting that the NO donation potentiates the *in vivo* antioxidant action of atorvastatin (214), and it also reduced neutrophil recruitment impairing their activation (216). However, the extra anti-inflammatory effect of NCX 6560 showed to be independent from the cGMP pathway in lipopolysaccharide-stimulated cells, since they remained unaltered in the presence of the sGC inhibitor ODQ (213).

A successful phase 1b clinical study in subjects with high low density lipoprotein-cholesterol was also completed, and showed that up to 144 mg/day, NCX 6560 was well tolerated and produced marked lipid-lowering effect 2 weeks after the beginning of treatment (218–220). Unfortunately, further clinical studies were discontinued in July 2012 (221).

1.3. Experimental animal models in portal hypertension (PHT)

Experimental animal models have been an useful tool to study the pathophysiology of PHT and its complications, allowing an in-depth study of some parameters difficult to study in humans.

There are several models and are classified according to the origin of PHT in prehepatic, intrahepatic or posthepatic PHT models, aiming at reproducing portal vein thrombosis, liver cirrhosis and Budd-Chiari syndrome, respectively (222). Rats and rabbit were the most commonly used species due to their size and ease of manipulation in contraposition to dogs and pigs that were abandoned due to their high cost. More recently, because of advances in molecular biology and the need of genetically modified animals (knock-out and transgenic mice), the methodology for hemodynamic studies has been implemented to mice and the models have been well characterized (223).

1.3.1. Prehepatic portal hypertension (PHT) models

The portal vein ligation (PVL) model generates a calibrated stenosis of the portal vein. It has been widely used in rodents and although it does not develop ascites, it allowed the characterization of hyperdynamic circulation in PHT (224) and the validation of the peripheral arterial vasodilation hypothesis as the trigger for sodium and water retention in cirrhosis (112,225,226). The conventional rat PVL is obtained ligating the portal vein around a 20 G blunt-tipped needle and reproduces all the hemodynamic alterations of the hyperdynamic syndrome seen in patients with PHT: increment in PP and PBF, splanchnic vasodilation with increased SMA blood flow (SMABF), low MAP and augmented cardiac output and portosystemic shunting. The alterations appear much faster than in patients: one week after the ligation, the percentage of portosystemic shunting -the amount of portal blood inflow diverted to collaterals- approaches 100 %. However, the main limitation of the model is that PHT develops acutely. The degree of PHT is maximal at 24 h and decreases afterwards due to the development of portosystemic collaterals, contrary to what is observed in the clinical practice (222).

By using needles of greater caliber (16 or 18 G), mild stenosis and thus less severe degrees of PHT are induced (75,227).

1.3.2. Intrahepatic portal hypertension (PHT) models

Within the intrahepatic PHT models, the sinusoidal intrahepatic PHT models are the most commonly used. These can be classified according to the type of injury in cholestatic or toxic models.

Surgical bile duct ligation (BDL) is the most common model used to induce obstructive cholestatic injury in mice and rats (228). It is especially appropriate for rats since they lack of gallbladder. The model consists of a double ligature and dissection of the common bile duct (229) to develop biliary fibrosis-cirrhosis after 4-6 weeks. At 4 weeks the animal shows severe PHT, hyperdynamic circulation and 30-60 % of portosystemic shunting. Mortality after the 5^{th} week is high (around 20 %), and approximately 60 % of the rats develop ascites. This model also has some limitations: a biliary cyst can be formed in some rats which may compress the portal vein and increase PP, the architectural disturbances typical of liver cirrhosis are not commonly observed and the model is not suitable to perform pharmacologic studies with drugs that are eliminated through biliary excretion (222). However, it is especially useful to reproduce the hepatopulmonary syndrome (230).

The toxic models induce liver inflammation, fibrogenesis and cirrhosis and are obtained by administration of several hepatotoxins: carbon tetrachloride (CCl_4), thioacetamide (TAA) or dimethylnitrosamine (DMN) (228). However, their main limitation is the wide heterogeneity of cirrhosis that they produce.

The most commonly used approach is the periodic administration of CCl_4 in mice or rats that causes a chronic liver injury leading to cirrhosis. CCl_4 is metabolized by hepatocytes through members of the cytochrome P450 family of oxidizing enzymes, giving rise to reactive trichloromethyl (CCl_3) radicals, which mediate lipid peroxidation, cytotoxic effects and eventually lead to massive centrilobular liver necrosis (231). CCl_4 can be administered through different routes: sc, intramuscular (im), intraperitoneal (ip), orally, or through inhalation, and usually phenobarbital is added to drinking water to induce the cytochrome P450 enzymatic system and accelerate the generation of CCl_3. The cirrhosis yield and time needed varies among protocols. Sc route is associated with low mortality rates, but it is not recommended due to its low yield of cirrhosis and prolonged period of exposure (222,231). Im and ip routes are related to high mortality rates (50 %) due to necrosis of the tissue, and oral CCl_4 administration is associated with frequent early mortality (231). Proctor *et al*. (232) proposed to individualize the CCl_4 dose according to weight gain/loss of the animal in response to the last dose, which also allowed to obtain a more homogeneous response to CCl_4. The classical inhalation protocol is performed twice a week and mortality prior the development of ascites is less than 25 % and it increases to 40 % in rats with permanent ascites (231). In mice, when testing sc, ip and different inhalation protocols, the best results regarding mortality and the degree of PHT were obtained with short-cycles, thrice-weekly of inhalation (233). Usually, after 12-15 weeks of CCl_4 administration rats develop micronodular cirrhosis, PHT, portosystemic shunting (30-60 %) and hyperdynamic circulation. When CCl_4 is maintained for 16-20 weeks, most rats also develop ascites. The

main limitations of this model are the difficulty to obtain a homogeneous group both in cirrhosis severity and in time needed to develop it, since animals have different sensitivity to CCl_4 (222), the potential damage to other organs (especially the kidneys) by CCl_4 and the fact that CCl_4 is toxic for the investigator (231). Moreover, CCl_4-induced liver cirrhosis together with ascites rapidly regresses within 7 to 10 days after the withdrawal of the hepatotoxin, and compared with the BDL model, the systemic hemodynamic alterations are more moderate in this model (228).

The TAA model develops macronodular cirrhosis and PHT in 12 weeks (234). TAA is administered in drinking water or ip and contrary to the CCl_4 model, cirrhosis is maintained after the withdrawal of the hepatotoxin. However, to fully obtain the hyperdynamic syndrome, administration for 18 weeks is needed (235). Around 40 % of the rats develop ascites and after 22 weeks the rats also develop cholangiocarcinoma (236).

The DMNA model induces hepatocellular necrosis. DMNA is usually administered ip and rats develop fibrosis with PHT, cirrhosis and accumulation of ascites after 24 weeks of treatment (237). This model is used to study the progression from fibrosis to cancer (228) and its main limitation is the high carcinogenic potential of DMNA for the investigator (222).

Other intrahepatic PHT models include the experimental infection with *Schistosoma mansoni* injected in the abdominal wall (presinusoidal intrahepatic PHT model), which is rarely used, the oral administration of monocrotaline (postsinusoidal intrahepatic PHT model), which has not been hemodynamically characterized, and fat enriched dietary models that induce liver steatosis (sinusoidal intrahepatic PHT), with increasing interest in the liver community, but not well-characterized from the hemodynamic point of view either (222).

1.3.3. Posthepatic portal hypertension (PHT) models

The model was developed in dogs (238) and adapted to rats (239) and involve the placement of an ameroid in the hepatic veins (dog) or in the suprahepatic inferior cava vein (rat), inducing a progressive occlusion of the venous outflow. Posthepatic PHT models have been seldom used (222).

2. HYPOTHESIS AND AIMS

As explained in the introduction chapter, cirrhosis is no longer seen as a unique stage of advanced liver disease, but as a multistage disease, reflecting the progression, reversibility and prognosis of the disease, and allowing linking of all these parameters to relevant outcomes and therapeutic strategies. Therefore, therapies in liver cirrhosis should be adapted to each stage of the disease. Moreover, treatment combinations must be tested to achieve synergistic or complementary effects, without increasing the adverse events.

The general objective of this thesis was to investigate new therapeutic strategies for the management of liver cirrhosis at different stages of the disease. Concretely, two potential oral new drugs, alone or in combination with other conventional drugs, were tested to see their efficacy: droxidopa, a pro-adrenergic drug already used for other indications, and NCX 6560, a new molecular entity consisting of a NO-releasing derivative of atorvastatin.

The specific objectives are outlined in each study.

2.1. First study: New indication for droxidopa as a treatment for cirrhosis and portal hypertension (PHT)

2.1.1. Hypothesis

The administration of a NA precursor drug (droxidopa) increases the sympathetic mesenteric vascular tone, improving splanchnic vasodilation and consequently, the systemic hemodynamic alterations associated with liver cirrhosis.

2.1.2. Aims

1) To evaluate the effect of an acute administration of droxidopa on hemodynamic parameters and diuresis volume of sham and portal hypertensive rats (PVL and BDL).

2) To assess the effect of a chronic droxidopa treatment on portal and systemic hemodynamics and biochemical and renal parameters of cirrhotic rats (BDL and CCl_4).

3) To study the molecular mechanism through which droxidopa exerts splanchnic vasoconstriction in SMA.

2.2. Second study: Combined treatments with droxidopa

2.2.1. Hypothesis

The combination of droxidopa with other drugs that either decrease the IHVR or further reduce the SMABF causes a decrease in PP while maintaining the beneficial systemic properties (hemodynamic and renal) of droxidopa.

2.2.2. Aims

1) To assess the acute effects of droxidopa when combined with 3 other drugs (carvedilol, propranolol or atorvastatin) on hemodynamic parameters and diuresis volume of BDL rats.

2) To determine the best combined therapy and establish the appropriate range of doses in the acute studies.

3) To assess the chosen combination as a chronic treatment in order to evaluate the effects on portal and systemic hemodynamics and biochemical and renal parameters in CCl_4-cirrhotic rats.

2.3. Third study: NCX 6560 as a treatment for cirrhosis and portal hypertension (PHT). Comparison with other conventional statins

2.3.1. Hypothesis

NCX 6560, a NO-releasing derivative of atorvastatin, is superior to conventional statins (simvastatin, atorvastatin) in improving portal hemodynamics and intrahepatic vascular alterations in liver cirrhosis, while decreasing the potential side effects of statins.

2.3.2. Aims

1) To compare 3 statins (simvastatin, atorvastatin and NCX 6560) in their efficacy in lowering PP in a decompensated cirrhotic model (BDL).

2) To evaluate the hepatic and muscular toxicity following statin treatment in two models of cirrhosis (BDL and CCl_4).

3) To study the beneficial intrahepatic effects of statin treatment in both cirrhotic models (BDL and CCl_4): assessment of liver fibrosis, and intrahepatic markers of liver inflammation, endothelial dysfunction, vasoconstriction and HSC activation.

3. FIRST STUDY

New indication for droxidopa as a treatment for cirrhosis and portal hypertension (PHT)

3.1. Summary of the study

Previous findings from our group showed that a downregulation of genes related to the adrenergic system, together with a sympathetic atrophy in the SMA of portal hypertensive rats (39,41), could be contributing to the splanchnic vasodilation found in PHT. Therefore, our objective was to increase the sympathetic mesenteric vascular tone via the administration of a pro-adrenergic drug (droxidopa). Droxidopa can be converted to NA even under this situation of sympathetic deficiency, in which the enzymes responsible for NA synthesis are inhibited, since the conversion of droxidopa to NA also takes place in non-neuronal cells -as already described in pure autonomic failure, which entails loss of postganglionic sympathetic nerves (206)-. Moreover, the fact that iv NA administration improves HRS (201), further supported our hypothesis.

In this study we aimed to evaluate the effects of droxidopa on the hemodynamic and renal alterations of portal hypertensive rats and we present evidences for the potential use of droxidopa in the management of PHT, since it is capable of improving systemic and splanchnic circulatory dysfunction and renal function.

Droxidopa was tested in sham, PVL and BDL rats as an oral single dose (25-50 mg/kg, acute treatment) and in BDL and CCl_4 rats as a chronic treatment (15 mg/kg/day, twice a day (*bid*), 5 days) (see further details in 10.1.2.1.).

The acute administration of droxidopa in the PVL and BDL models caused a marked increase in the 2 h diuresis in both models, and a significant and maintained increase in MAP and SMA resistance (SMAR), together with a significant decrease in SMABF and PBF, without affecting PP and renal artery blood flow (RABF).

The chronic droxidopa administration in BDL and CCl_4 rats showed similar improvements in hemodynamic alterations, and again a significant increase in the 24 h diuresis volume, without affecting liver function parameters. Besides, in the CCl_4 model, 24 h sodium excretion was also significantly increased and was accompanied by an improvement in the creatinine clearance.

We also studied by Western blot (WB) the levels of proteins involved in the signaling pathway leading to vasoconstriction or vasodilation in the SMA from BDL rats treated chronically with droxidopa. What we observed was that part of the droxidopa hemodynamic effect could be mediated by a direct NA dependent activation of the RhoA/Rock-2 pathway, which indirectly inhibits the Akt-eNOS vasodilatory pathway (240), since Rock-2 activity (assessed as an increase in the phosphorylated moesin (p-moesin)/moesin ratio) was increased, and there was a decrease in the phosphorylated Akt

(p-Akt)/Akt and phosphorylated eNOS (p-eNOS)/eNOS ratios, that could explain the reduction in SMABF.

In summary, the acute and chronic administration of the NA precursor droxidopa is able to improve the systemic and hemodynamic alterations of cirrhotic rats by increasing MAP and SMAR and reducing SMABF and PBF. Additionally, droxidopa also produces a marked diuretic and natriuretic effect. Due to the advantage of its oral administration and the fact that it has demonstrated to be safe in other indications in humans, these results suggest that droxidopa might be an effective therapeutic agent for the hemodynamic and renal alterations of liver cirrhosis and should be tested in cirrhotic patients.

3.2. Manuscript I

Coll M *et al*. *Hepatology* 2012 Nov;56(5):1849-60. doi: 10.1002/hep.25845. Epub 2012 Oct 14.

Available at: http://onlinelibrary.wiley.com/doi/10.1002/hep.25845/pdf

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LIVER FAILURE/CIRRHOSIS/PORTAL HYPERTENSION

Droxidopa, an Oral Norepinephrine Precursor, Improves Hemodynamic and Renal Alterations of Portal Hypertensive Rats

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4. SECOND STUDY

Combined treatments with droxidopa

4.1. Summary of the study

In our previous study we showed that droxidopa, despite showing no changes in PP, could be an effective therapeutic agent for the management of the hemodynamic and renal alterations of liver cirrhosis given that it is able to improve systemic and splanchnic hemodynamics (increase MAP and SMAR and decrease SMABF) and to substantially increase diuresis and natriuresis of cirrhotic rats (241). Here, we wanted to find strategies to minimize the intrahepatic effects derived from the chronic adrenergic stimulation, achieving a net reduction in PP, while maintaining droxidopa hemodynamic and renal benefits. We attempted to achieve this goal by means of combined therapies with drugs with proven efficacy in decreasing PP through a reduction either in SMABF or in IHVR, such as statins (simvastatin, atorvastatin) or NSBB (carvedilol, propranolol). Thus, our aim in this study was to evaluate the effects of combined treatments with droxidopa on the hemodynamic and renal alterations of cirrhotic rats, and we present evidences for the potential use of droxidopa in cirrhotic patients with renal alterations that are on propranolol therapy, since the combination of both drugs decreases PP, maintains droxidopa diuretic effect and balance the adverse events of each treatment in cirrhotic rats.

Three acute drug combination protocols in which a single droxidopa oral dose (20-25 mg/kg) was given to BDL rats pretreated with different doses of carvedilol, propranolol or atorvastatin, and a chronic study (5 days) combining droxidopa (20 mg/kg/day, *bid*) with propranolol (25 mg/kg/day) in the CCl_4 model were performed (see 10.1.2.2. for further details).

Droxidopa treatment alone reproduced our previous results: significant increases in MAP and SMAR, together with decreases in SMABF. Portal pressure, in turn, remained unchanged in two of the acute protocols but it was significantly increased compared to vehicles in the acute atorvastatin study; evidencing the importance of a droxidopa combined treatment in order to prevent an increase in portal pressure.

Both the combination of droxidopa with carvedilol and with atorvastatin, despite being very promising due to the effect of both drugs in decreasing IHVR (see **Fig. 9**), failed to decrease PP. However, in the atorvastatin-droxidopa combination, the PP in the combined protocol was much lower than in the droxidopa group and the beneficial increase in MAP and SMAR and decrease in SMABF caused by droxidopa was maintained. In the carvedilol-droxidopa combination, though, the SMABF and SMAR were similar to animals treated only with carvedilol or with droxidopa alone, respectively. Besides, treatment with carvedilol or atorvastatin alone did not exert any diuretic effect. By contrast, the propranolol-droxidopa combination significantly reduced PP maintaining a mild increase in MAP and improving, in

an additive way, the decrease in SMABF and increase in SMAR caused by droxidopa. This combination also preserved droxidopa diuretic effect and, therefore, it was chosen for the chronic study in the CCl_4 model.

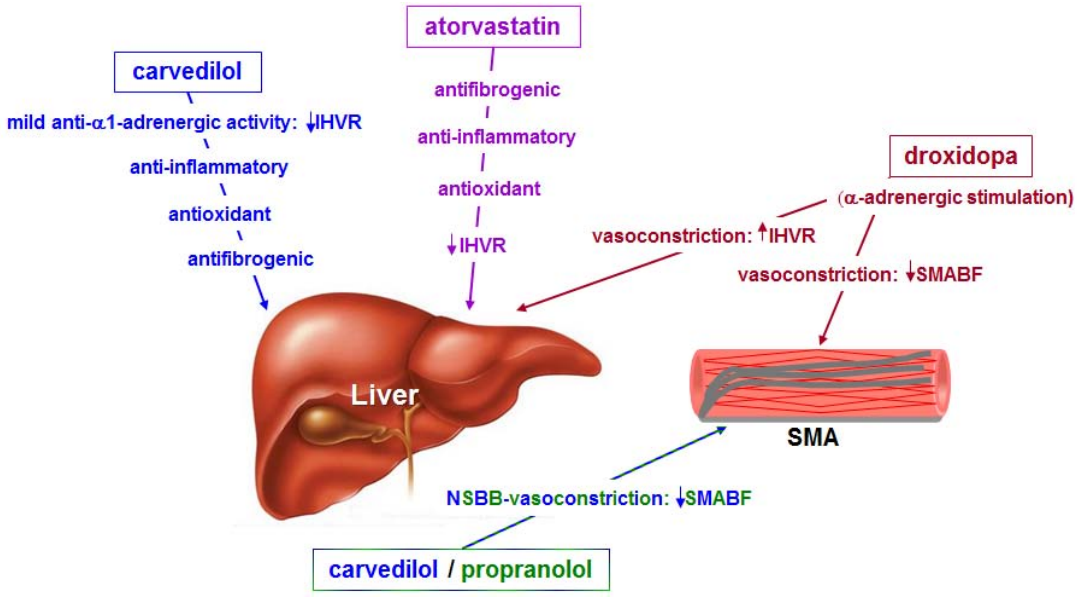

Fig. 9: **Drugs used in the combination studies and summary of their properties.**

IHVR: intrahepatic vascular resistance, NSBB: non-selective β-blocker(s), SMA: superior mesenteric artery, SMABF: superior mesenteric artery blood flow.

The chronic administration of propranolol and droxidopa in CCl_4-cirrhotic rats maintained most of the beneficial hemodynamic effects shown in the acute study: a decrease in PP along with a significant reduction in SMABF and an increase in SMAR. Moreover, the combination did not alter liver function parameters and a mild increase in total diuresis volume accompanied by a significant increment in sodium and fractional sodium excretion and an improvement in free water clearance was observed.

In summary, the results presented in this study are consistent with previous data indicating that droxidopa might be an effective therapeutic agent for the hemodynamic and renal alterations of liver cirrhosis. In addition, chronic treatment with propranolol plus droxidopa decreases portal pressure, maintaining the increase in diuresis and natriuresis caused by droxidopa in cirrhotic rats. Thus, these results suggest that droxidopa could be an effective therapy for the renal alterations of cirrhotic patients on propranolol therapy and that this combined treatment may balance the possible adverse effects of each drug.

4.2. Manuscript II

Rodríguez S *et al*. *Liver Int* 2015 Feb;35(2):326-34. doi: 10.1111/liv.12472. Epub 2014 Feb 12.

Available at: http://onlinelibrary.wiley.com/doi/10.1111/liv.12472/pdf

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CIRRHOSIS AND LIVER FAILURE

The renal effects of droxidopa are maintained in propranolol treated cirrhotic rats

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5. THIRD STUDY

NCX 6560 as a treatment for cirrhosis and portal hypertension (PHT). Comparison with other conventional statins

5.1. Summary of the study

Recent evidences indicate that statins could be useful in the treatment of chronic liver disease (242), but there are still some concerns about their safety in cirrhotic patients, especially regarding hepatotoxicity and the risk of developing myopathy (175,243–245).

In this study, we aimed to evaluate the hepatic effect of a NO-releasing atorvastatin (NCX 6560) in comparison to conventional statins (atorvastatin and simvastatin) and we present evidences for the potential more toxic effect of simvastatin in BDL rats, the beneficial effect of atorvastatin and NCX 6560 in liver cirrhosis, and more importantly, the greatly reduced toxicity of NCX 6560.

Statins were evaluated in two models: in four-week BDL rats, a cirrhotic model that simulates a decompensated chronic liver disease, and in thirteen-week CCl_4-intoxicated rats, a model of early/compensated cirrhosis. Hemodynamic and serum biochemical parameters were analyzed, liver cGMP measurements performed and the liver protein expression of Rock-2, p-moesin, alpha-smooth muscle actin (α-SMA), eNOS, p-eNOS, KLF2 and the platelet/endothelial cell adhesion molecule-1 (CD31/PECAM-1) was determined by WB. Histological assessment of liver fibrosis and inflammation was also completed. Hepatic and muscular toxicity due to statin treatment was defined based on alanine aminotransferase (ALT) and CK levels in vehicle rats from both models. Thus, we defined hepatic toxicity as an increment in ALT levels superior to 200 IU/L (BDL model) and to 500 IU/L (CCl_4 model), whereas muscular toxicity was considered when CK levels were above 1000 IU/L (BDL model) and 6000 IU/L (CCl_4 model).

BDL rats received 2 different oral doses of statins or vehicle for 7 days: simvastatin 25 mg/kg/day (n=10) and 10 mg/kg/day (n=11), atorvastatin 15 mg/kg/day (n=14) and 10 mg/kg/day (n=15), NCX 6560 17.5 mg/kg/day (n=11) and 11.7 mg/kg/day (n=15). These doses were chosen according to previous literature (176,177,180,246) and using an NCX 6560 dose equivalent to the atorvastatin dose on the basis of their molecular weight (MW) divergence. An additional dose of NCX 6560 35.1 mg/kg/day (n=15) was also assessed.

We could not complete our first aim due to the adverse events found in the animals treated with simvastatin (high mortality rate with the highest simvastatin dose and hepatic and muscular toxicity even reducing the dose). Thus, these animals were not considered for further hemodynamic or sample analysis. CCl_4-intoxicated rats were also treated with atorvastatin 15 mg/kg/day (n=13), NCX 6560 17.5 mg/kg/day (n=13) or vehicle (n=8) for a longer period (10 days). A control group (n=8), only receiving phenobarbital in drinking water, was also included.

Atorvastatin and NCX 6560 similarly reduced PP in both models (there was a significant reduction in the PP in the BDL model and a trend towards a reduction in the CCl_4 model), without changing systemic hemodynamics. Unlike traditional NO donors, which due to their extrahepatic effects worsened splanchnic vasodilation and renal function (92,95), treatment with NCX 6560 showed to maintain urinary volume (whereas atorvastatin equivalent doses decreased it).

Likewise, at equivalent doses, treatment with NCX 6560 greatly reduced muscular toxicity and eliminated hepatic toxicity caused by atorvastatin in the decompensated BDL model. Considering the two equivalent doses of each statin, the accumulated toxicity (hepatic and/or muscular toxicity) was significantly lower with NCX 6560 treatment (37.9 % with atorvastatin vs 11.5 % with NCX 6560, p=0.02). In the early/compensated cirrhotic CCl_4 model, toxicity was minimal with atorvastatin and nonexistent with NCX 6560 treatment.

Atorvastatin and NCX 6560 also caused a mild decrease in liver fibrosis and inflammation, a decrease in liver α-SMA expression and Rock-2 activity (decreased phosphorylation of its substrate moesin), and a significant increase in liver cGMP levels.

NCX 6560 treatment also induced a higher intrahepatic vasoprotective profile (increased KLF2, p-eNOS and decreased CD31), especially in the CCl_4 model, and even significantly increased albumin levels compared with atorvastatin-treated rats in this model, hence reinforcing the idea of a major benefit of statins when given earlier in the development of cirrhosis and for longer periods (177).

In summary, conventional statins ameliorate PHT, but their adverse events are magnified in a model that mimics a deteriorated liver function and cholestasis. By contrast, NCX 6560 improves PHT similarly in the two cirrhotic models with a safer toxicity profile compared with conventional statins. Furthermore, due to its liver NO release, it induces a higher intrahepatic vasoprotective profile that might have a more long-term beneficial effect on the intrahepatic vascular alterations of PHT than conventional statins. Thus, these results suggest that NCX 6560 could be a safer option for long-term statin treatment of PHT in cirrhotic patients.

5.2. Manuscript III

Rodríguez S *et al*. 2016. Manuscript submitted to *Scientific Reports* (April 2016)

(see **11. APPENDIX 2: MANUSCRIPT III**)

SREP-16-13019 Receipt of New Paper by Scientific Reports 13 de Abril 2016 14:53

Dear Ms Rodriguez,

Please note that a Scientific Reports manuscript tracking system account has been created for you. This is because you are listed as a co-author on the manuscript "A NITRIC OXIDE-DONATING STATIN DECREASES PORTAL PRESSURE WITH A BETTER TOXICITY PROFILE THAN CONVENTIONAL STATINS IN CIRRHOTIC RATS" (reference number: SREP-16-13019), which was recently submitted to Scientific Reports.

6. DISCUSSION

Cirrhosis is the most frequent cause of PHT in Western countries and its complications are a leading cause of death and liver transplantation worldwide. In the past years, the concept that cirrhosis is a dynamic and bidirectional disease consisting of multiple stages -ranging from asymptomatic initial stages to decompensated cirrhosis with multiple clinical manifestations- and that patients in different stages of cirrhosis have different risks of developing complications and of dying has been extensively accepted (8,116). Therefore, treatments must be targeted to each stage of the disease and, whenever possible, individualized to each patient, based on their genome, transcriptome, metabolome or microbiome analysis (120).

Traditionally, treatment of PHT has been based on drugs aimed at correcting the increased splanchnic blood flow that contributes to maintain and aggravate PHT (old paradigm), but progress in our knowledge of the mechanisms involved in the increased resistance to PBF, the formation of portosystemic collaterals and the angiogenic process that also contributes to splanchnic vasodilation has opened new perspectives for developing more effective treatment strategies (new paradigm) (148). However, despite all the advances made in the last decades to understand the pathophysiology of PHT and to develop new pharmacological approaches, up to now, NSBB still remain the mainstay of treatment in cirrhotic patients with PHT in order to ameliorate PP and prevent variceal bleeding. Nitrates and angiotensin-II receptor antagonists, such as losartan, were also tested in patients with cirrhosis but were not successful because they potentiated systemic vasodilation (nitrates) or failed to reduce the HVPG (losartan), while other proposed potential therapies -spironolactone, pentoxifylline, prazosin or ET receptor antagonists, among others- have not moved to the clinic (247). Thus, new treatments to improve the efficacy of the current ones or combined therapies to achieve synergistic or complementary effects, without increasing the adverse events, are still needed.

In the present thesis we aimed to study new therapeutic strategies for the management of liver cirrhosis at different stages of the disease using both the old and new treatment paradigms. In particular, we tested the pro-adrenergic drug droxidopa to correct splanchnic vasodilation and improve systemic hemodynamics, ascites and HRS in decompensated cirrhosis, and we also focused on the intrahepatic component of PHT, evaluating the effect of different statins in both an early and a decompensated cirrhotic model, following the new treatment trends in PHT.

As explained in the introduction chapter of this thesis, statins, originally used as lipid-lowering agents, are becoming very popular as a potential therapy in chronic liver disease (242). They seem to have beneficial effects on both sinusoidal endothelial cells and HSC within the liver (247) and have shown to improve endothelial dysfunction, PP and liver

fibrosis in experimental cirrhotic models (176,177,180). Besides, they are also beneficial in cirrhotic patients with PHT (102,103) and a recent clinical trial also showed an increased in survival in decompensated patients receiving simvastatin (175). However, differences among statins and concerns about their safety, in terms of hepatic toxicity and rhabdomyolysis, still exist, especially in decompensated cirrhotic patients (175,243–245). Actually, in experimental animal models they have shown to have a major benefit when given earlier in the development of cirrhosis and for longer periods, attenuating liver fibrosis and HSC activation (177). The results of our third study, in which we compared the effect of conventional statins (atorvastatin and simvastatin) with the NO-donating atorvastatin NCX 6560, are in line with these observations. In fact, we showed that the adverse events of this family of drugs are magnified in the BDL model that mimics a deteriorated liver function and cholestasis (decompensated liver disease). Surprisingly, simvastatin was the most toxic statin: BDL rats treated with simvastatin showed a higher mortality rate (80 % at the higher dose) compared with the other treatment groups and the remaining animals presented both muscular and hepatic toxicity. A reduction of the dose to 10 mg/kg/day still maintained high toxicity levels, hindering the evaluation of systemic and portal hemodynamics with simvastatin treatment, whereas lower simvastatin doses were not tested since previous studies reported lack of therapeutic benefits in BDL rats (248). Atorvastatin treatment also showed some muscular and hepatic toxicity in the BDL model and much lower toxicity rates in the CCl_4 model. The differences in toxicity in the two models are probably due to the distinct severity of the disease: with the CCl_4 model we aimed to reproduce an early cirrhotic state, while the BDL model mimicked a severely deteriorated liver function since it causes a more rapid and aggressive injury and impairs biliary clearance of drugs. On the whole, these results reinforce the idea that caution is required when prescribing statins, especially in those patients with deteriorated liver function (Child-Pugh class C), who might develop rhabdomyolysis at lower doses than the general population (175).

NCX 6560 could be, therefore, a safer alternative for the treatment of cirrhotic patients with PHT and severe liver damage, since our results in BDL and CCl_4 rats demonstrated that this drug, having a better toxicity profile, achieves an equivalent atorvastatin PP lowering effect without affecting systemic hemodynamics. NCX 6560 did not only improve statin-induced myopathy (215), but also prevented hepatic toxicity caused by equivalent doses of atorvastatin, probably due to its greater anti-inflammatory and antioxidant properties (213,214,216). In addition, NCX 6560 effects seemed to be liver-selective, preventing impairment of systemic hemodynamics and the risk of renal failure, the main drawback of NO donors (95). In fact, NCX 6560 did not enhance splanchnic vasodilation, there were no

changes in MAP, and diuresis and serum creatinine levels from NCX 6560-treated cirrhotic rats were maintained compared with vehicles, while diuresis was increased compared with equivalent doses of atorvastatin in both models. Moreover, it is also worth to be noted that this drug has been previously tested in clinical trials and showed to be well tolerated (218–220).

Regarding the beneficial intrahepatic effects in the two models, both atorvastatin and NCX 6560 had similar effects: they slightly reduced the liver fibrotic area, lowered the liver α-SMA and p-moesin expression and significantly increased p-eNOS, compared with vehicles, confirming previous results with atorvastatin (176). This was also associated with a similar increase in hepatic cGMP, the second messenger of NO, indicating an improvement in NO availability, probably due to a decrease in oxidative stress related to statin therapy (168). The fact that cGMP levels were not higher with NCX 6560 treatment compared with atorvastatin suggests that the improvement in toxicity observed with the NO-donating drug might be independent from the cGMP signaling pathway. This goes along with previous findings indicating that NO donors also exert direct antifibrogenic action -inhibition of proliferation, motility, and contractility of HSC-, through a mechanism not related to the activation of the classical sGC/cGMP pathway (249) and with the fact that the greater anti-inflammatory effect of NCX 6560 was also shown to be independent from the cGMP signaling pathway, since the presence of a sGC inhibitor maintained the inhibition of inflammation markers in lipopolysaccharide-stimulated cells (213).

In our study, NCX 6560 also tended to have a slightly better anti-inflammatory effect that could partially explain its lower hepatic toxicity. Additionally, probably due to its liver NO release, NCX 6560 induced a greater beneficial intrahepatic effect: it significantly increased KLF2 and eNOS protein expression compared with the atorvastatin group in the BDL model, and the p-eNOS/eNOS ratio was much higher than in the atorvastatin group in the CCl_4 model. These effects could also be contributing to its lower hepatic toxicity, improving the endothelial phenotype -as seen by the decrease of CD31- and therefore, to hepatocytes viability, and might provide a more long-term beneficial effect on the intrahepatic vascular alterations of PHT than conventional statins.

Overall, the intrahepatic improvements of statin treatment were greater in the CCl_4 model than in the BDL model, supporting, again, the idea that statin therapy should be given early in the development of cirrhosis (177).

As the disease progresses, once cirrhosis and PHT are fully established, the hyperdynamic circulation, developed as a consequence of the splanchnic arterial vasodilation, contributes to aggravate PHT and the main complications of the disease may occur (11). At that stage, treatment of decompensated cirrhotic patients is focused on the management of these

complications. Apart from variceal bleeding, one of the most common complications of cirrhosis is the accumulation of ascites in the peritoneal cavity, as a consequence of the continuous activation of the systemic vasoconstrictor systems (RAAS, SNS and ADH). Although these systems have positive effects on the maintenance of arterial pressure, they have a negative influence on kidney function, leading to marked intrarenal vasoconstriction and the development of HRS in late stages of the disease (64–66).

The first two studies of the present thesis focus on this late stage of the disease. Following previous findings from our group in which a downregulation of genes related to the adrenergic system and a sympathetic atrophy in the SMA of portal hypertensive rats were proposed as another factor contributing to the splanchnic vasodilation associated with PHT (39,41), we thought of reverting this sympathetic deficit and increasing the mesenteric vascular tone, via the administration of the pro-adrenergic drug droxidopa, that can be directly converted to NA by the Ddc enzyme, which is widely expressed throughout the body.

With that aim, in our first study, we evaluated the potential effect of droxidopa, a drug already used for other indications, on the management of the hemodynamic and renal alterations associated with liver cirrhosis, since as a vasoconstrictor drug it should correct the splanchnic vasodilation (old treatment paradigm) and probably ameliorate systemic hemodynamics, ascites and HRS in decompensated cirrhosis. As stated in previous chapters, the fact that the iv administration of NA and other vasoconstrictors improve renal function and HRS (64,201), further supported our hypothesis.

The results of the oral acute and chronic administration of droxidopa in portal-hypertensive rats showed an improvement in systemic and splanchnic circulatory dysfunction, with an increased MAP and SMAR and a reduced SMABF, together with an amelioration of renal function shown by an increment in diuresis and natriuresis, thus evidencing the potential therapeutic role of this drug in the management of refractory ascites and HRS.

Droxidopa can exert its pressor effect by two different mechanisms of action: it is converted to NA in peripheral sympathetic neurons, but it can also act as a circulating hormone and be decarboxylated outside neurons, allowing the release of NA into the blood stream (204,206). Considering the sympathetic mesenteric atrophy described in portal hypertensive rats (41) the increment in NA plasma levels seen after the acute administration of droxidopa in PVL rats should be derived mainly from non-neuronal cells. However, the NA release in the sympathetic terminals not affected by this atrophy could also contribute to increase the mesenteric vascular tone. We showed that the vasoconstrictive effect of droxidopa was especially important in the splanchnic circulation, lowering the SMABF of portal-hypertensive rats to levels similar to sham animals and

greatly increasing SMAR, while it was less pronounced in renal arteries, suggesting that the improvement in systemic hemodynamics caused by droxidopa is mainly due to the correction of the splanchnic vasodilation and consequently, to the amelioration of the severe arterial underfilling. As shown in the WB analysis of SMA from BDL rats treated chronically with droxidopa, part of its hemodynamic effect on the splanchnic circulation could be mediated by a direct NA dependent activation of the RhoA/Rock-2 signaling pathway inducing vasoconstriction, which indirectly inhibits the Akt-eNOS vasodilatory pathway (240), further reducing the SMABF.

Droxidopa itself does not seem to have any renal direct effect (208). Therefore, the reason why it increased diuresis and natriuresis in cirrhotic rats, despite the renal vasoconstrictive environment derived from the high NA levels, and the fact that RABF, at least in the BDL model, was not increased, could be through the predominant systemic hemodynamic effect of droxidopa-derived NA via activation of the peripheral α-adrenergic receptors. Therefore, as a consequence of the amelioration of the systemic hemodynamics and the subsequent improvement in the arterial vascular underfilling, the pressure diuresis would increase and, at the same time, the release of systemic and endogenous renal vasoconstrictors (ADH, RAAS, ET) would be inhibited, leading to an improvement in the intrarenal perfusion, and diuresis and natriuresis. In this sense, the fact that plasma aldosterone levels were decreased with droxidopa treatment favor this hypothesis. Other possible mechanisms contributing to the diuretic effect of droxidopa would be the activation of renal α2-adrenergic receptors by NA that might lead to the inhibition of renin release (250) and of renal vasopressin action (251).

Despite the droxidopa benefits in systemic hemodynamics and renal function, the vasoconstrictor effect of droxidopa was not enough to decrease PP and a half-dose reduction was even necessary in the acute studies to avoid an increase in PP. This is probably due to the fact that the decrease in PBF was compensated by an increment in IHVR secondary to a direct intrahepatic effect of NA (252). However, this intrahepatic vasoconstriction caused by droxidopa did not significantly alter liver function parameters in cirrhotic rats, but it is a consequence that should be kept in mind for long-term therapies with droxidopa, since there are also some evidences supporting the activation of HSC and the promotion of hepatic fibrosis through α-adrenergic stimulation (253). Therefore, in our second study we assessed combinations of droxidopa with other PP-lowering drugs in order to improve droxidopa profile and achieve a synergistic effect. We thought of combined therapies with drugs with proven beneficial intrahepatic effects such as statins, which as stated above, are gaining credibility as a treatment for PHT (247), and at the same time, we also wanted to explore the effects of a probable clinical situation, given that in case of using

droxidopa in decompensated cirrhosis, it would probably be concomitantly administered with NSBB and information about possible interactions is very relevant. Besides, NSBB could also have an additive effect on splanchnic vasoconstriction, allowing a decrease in PP.

Neither the combination of carvedilol plus droxidopa nor the combination of atorvastatin plus droxidopa achieved a reduction in PP while maintaining droxidopa beneficial properties in BDL rats. The blockage of the systemic and renal effects of droxidopa with carvedilol treatment was in accordance with previous clinical observations, in which carvedilol showed to cause hypotension in advanced cirrhosis (254). By contrast, atorvastatin did not alter the beneficial systemic, splanchnic and diuretic effects of droxidopa and, despite not significantly reducing PP compared with vehicles, it was able to block the increase in PP caused by the adrenergic drug. Thus, in a possible clinical scenario of cirrhotic patients in long-term treatment with statins who might receive droxidopa, the statin would have a hepatic protective role without compromising the beneficial effects of droxidopa. Finally, the combination of propranolol plus droxidopa showed the best hemodynamic profile with a significant reduction in PP and an additive effect in splanchnic hemodynamics. Moreover, given that propranolol alone also exerted a diuretic effect in our BDL rats, probably due to the suppression of renin release in the kidney (255–257), this combination maintained the renal effects of droxidopa and it was chosen for the chronic study in the CCl_4 model.

The chronic administration of propranolol and droxidopa in CCl_4-cirrhotic rats maintained most of the beneficial hemodynamic effects shown in the acute study: a decrease in PP along with a significant reduction in SMABF and an increase in SMAR. Additionally, the combination did not alter liver function parameters and a mild increase in total diuresis volume accompanied by a significant increment in sodium and fractional sodium excretion and an improvement in free water clearance was also observed. Although droxidopa diuretic effect was more evident in our first study (241), in the acute studies in which not only droxidopa alone, but also in combination with propranolol or atorvastatin, increased the two-hour-diuresis, the diuretic effect was widely demonstrated. Perhaps droxidopa diuretic effect is more immediate and remains masked in the 24-hour diuresis, or maybe its diuretic effect is more evident during the first days of treatment, favoring ascites elimination, which would be in line with the fact that treated cirrhotic rats showed lower ascites volume than vehicles at the end of treatment.

Taken together the data from the two first studies with droxidopa, we can support its evaluation for the management of the hemodynamic and renal alterations in cirrhotic patients, given that the renal abnormalities in those patients are well-defined and the drug is already used in humans. Additionally, although there are still some concerns about the

safety of NSBB in patients with end-stage liver disease (161–163), recent studies indicate that they are not harmful and do not increase mortality (164–166). Therefore, those patients with type-2 HRS on propranolol therapy could benefit from droxidopa administration, since the combination of both drugs would balance the possible adverse effects of each one and improve the hemodynamic and renal alterations of these patients with advanced liver cirrhosis.

Overall, the studies included in this doctoral thesis propose new treatments for the management of PHT and its complications: a safer use of NCX 6560 in the potential long-term statin treatment of PHT, and a potential therapeutic use of droxidopa in the management of cirrhotic patients with refractory ascites and type-2 HRS, even in those patients on propranolol therapy.

Especially in the two first studies, that were the continuation of previous investigations from our group, it is worth to remark the translational aspect (from bench to bedside) of the research line carried out, and see its evolution in the last decade: going from basic research (with the first microarrays studies), to preclinical studies in different PHT animal models with combinations that we will probably encounter in a clinical setting, and hopefully, to clinical trials in patients very soon.

7. SUMMARY OF THE RESULTS

7.1. First study: New indication for droxidopa as a treatment for cirrhosis and portal hypertension (PHT)

- The acute administration of droxidopa (a single 25 mg/kg dose) in PVL and BDL rats was able to improve hemodynamic alterations in portal hypertensive rats: increased MAP and SMAR and decreased SMABF and PBF.
- The acute treatment with droxidopa also showed a marked diuretic effect in both models (PVL and BDL).
- The chronic administration of droxidopa in BDL and CCl_4 rats showed a similar hemodynamic response to the acute treatment, without affecting liver function: increase in MAP and SMAR and decrease in SMABF.
- Chronic droxidopa treatment was associated with a significant increase in diuresis in both models (BDL and CCl_4), and a significant increase in natriuresis and an improvement in the creatinine clearance was also observed in the CCl_4 model.
- The beneficial hemodynamic effects of droxidopa on splanchnic circulation seem to be due to an increase in the Rock-2 activity and a decreased activation of the vasodilatory pathway Akt-eNOS in SMA.

7.2. Second study: Combined treatments with droxidopa

- The acute carvedilol-droxidopa combination in BDL rats did not show any additive beneficial effect: MAP was similar to vehicles, PP was not decreased, and SMABF and SMAR were also similar to animals treated only with carvedilol or with droxidopa alone, respectively.
- The acute atorvastatin-droxidopa combination in BDL rats maintained droxidopa systemic effects (increase in MAP and SMAR and decrease in SMABF) but failed to reduce PP compared with the vehicle group, although PP levels were much lower than in the droxidopa group.
- The acute propranolol-droxidopa combination in BDL rats showed a partial synergistic effect: MAP was slightly increased and PP was significantly reduced compared to vehicles, and SMABF and SMAR decreased and increased, respectively, in an additive way.
- Carvedilol pretreatment blunted droxidopa diuretic effect, atorvastatin did not exert any diuretic effect but diuresis was significantly increased in the combined treatment, whereas propranolol alone also increased urinary volume and the combination did maintain and even improved droxidopa diuretic effect in BDL rats.

- The chronic combined propranolol-droxidopa treatment in CCl_4 rats maintained most of the beneficial hemodynamic effects shown in the acute study: a reduction in PP together with a significant reduction in SMABF and an increase in SMAR. However, the mild increase in MAP was not preserved
- The chronic propranolol-droxidopa treatment did not significantly alter liver function parameters, maintained droxidopa diuretic and natriuretic effect, and even improved free water clearance in CCl_4-cirrhotic rats.

7.3. Third study: NCX 6560 as a treatment for cirrhosis and portal hypertension (PHT). Comparison with other conventional statins

- The adverse events associated with statin treatment were exacerbated in the BDL model that mimics a deteriorated liver function and cholestasis (decompensated liver disease):
  - Animals experienced a significant weight loss with statin treatment
  - Mortality and hepatic and muscular toxicity were associated with statin treatment
- Simvastatin treated BDL rats showed a higher mortality rate (80 % at the higher dose) compared with the other treatment groups and the remaining animals presented both muscular and hepatic toxicity.
- At equivalent doses, treatment with NCX 6560 reduced and eliminated muscular and hepatic toxicity caused by atorvastatin, respectively (BDL model). The toxicity was minimal in the CCl_4 model (early/compensated cirrhosis) and non-existent with NCX 6560 treatment.
- Both atorvastatin and NCX 6560 treatment similarly ameliorated PHT without changing systemic hemodynamics in both models (the PP was significantly reduced in the BDL model and a mild decrease was observed in the CCl_4 model).
- Treatment with NCX 6560 increased diuresis compared with equivalent doses of atorvastatin in both models.
- Both atorvastatin and NCX 6560 treatment similarly improved liver fibrosis and inflammation, increased liver cGMP levels and reduced liver α-SMA protein expression and Rock-2 activity in both models. By contrast, treatment with NCX 6560 induced a higher intrahepatic vasoprotective profile (an increase in KLF2 and p-eNOS and a decrease in CD31 protein levels) than atorvastatin.

8. CONCLUSIONS

The main conclusions obtained as a result of the studies presented in this thesis are:

1. Droxidopa administration is capable of improving the systemic and splanchnic circulatory dysfunction of portal hypertensive rats (PVL, BDL and CCl_4 models), and it also increases diuresis and natriuresis.
2. Droxidopa could be an effective therapeutic agent for the management of the hemodynamic and renal alterations of liver cirrhosis and should be tested in cirrhotic patients.
3. Neither the combination of carvedilol plus droxidopa nor the combination of atorvastatin plus droxidopa achieves a reduction in PP while maintaining droxidopa beneficial properties in BDL rats.
4. Only the combination of propranolol plus droxidopa has a synergistic effect: it decreases PP while maintaining and improving droxidopa hemodynamic and renal properties (BDL and CCl_4 models).
5. Droxidopa could be an effective therapeutic agent for the management of type-2 HRS in patients on propranolol therapy. The addition of droxidopa to propranolol treatment could balance the possible adverse effects of each drug and improve the hemodynamic and renal alterations of these patients with advanced liver cirrhosis.
6. Conventional statins ameliorate PHT, but their adverse events are magnified in a model that mimics a deteriorated liver function and cholestasis.
7. NCX 6560, a NO-donating atorvastatin, improves PHT with a better toxicity profile than conventional statins in two cirrhotic models (BDL and CCl_4).
8. Due to its liver NO release, NCX 6560 induces a higher intrahepatic vasoprotective profile that might have a more long-term beneficial effect on the intrahepatic vascular alterations of PHT than conventional statins.
9. NCX 6560 could be a safer option for long-term statin treatment of PHT in cirrhotic patients.

9. FUTURE PERSPECTIVES

As already mentioned, the studies included in this doctoral thesis, based on experimental animal models, propose new treatments for the management of PHT and its complications: a potential therapeutic use of droxidopa in the management of cirrhotic patients with refractory ascites and type-2 HRS, even in those patients on propranolol therapy, and a safer use of NCX 6560 in the potential long-term statin treatment of PHT. However, they raise some new questions and open the opportunity to further explore these potential therapies.

Regarding droxidopa studies, the following step will be to demonstrate its effects in cirrhotic patients with refractory ascites: an initial establishment of the optimal dose in those patients and the evaluation of its safety will be needed and, additionally, the assessment of its efficacy, compared with placebo, in reducing the volume of ascites evacuated and in increasing MAP, diuresis and creatinine clearance should also be performed.

Concerning statins and NCX 6560, some questions still need to be solved before translating the results into the clinic: a long-term treatment comparison of NCX 6560 with lower simvastatin doses in a non-cholestatic model of chronic liver disease will offer us more information about the toxicity of statins and the potential safer use of NCX 6560. Alternatively, further studies with NCX 6560 and conventional statins in an experimental model of NAFLD, will also provide useful information for the treatment of this future most prevalent liver disease.

Hopefully, the knowledge gained with these and further studies will, someday, contribute to improve the treatment of PHT and patients quality of life.

10. APPENDIX 1: METHODS

10.1. Animal experiments

All the animal studies were approved by the Animal Care Committee of the Vall d'Hebron Institut de Recerca (VHIR, Barcelona, Spain) (DAAM Permission No.: 6105, 6259 and 7834) and conducted in the animal facilities of VHIR. All animals received humane care in compliance with institutional guidelines from the European Commission on the protection of animals used for scientific purposes (Directive 86/609/EEC and Directive 2010/63/EU).

10.1.1. Experimental animal models of portal hypertension (PHT)

There are several well-characterized models to study PHT and its complications. In this thesis we mainly used three models for hemodynamic studies: the PVL model, the BDL model and the CCl_4 model. The BDL and CCl_4 models were also used to study hepatic fibrosis and cirrhosis, ascites and the renal complications of cirrhosis.

Both adult male Sprague-Dawley Oncins France Strain A (SD OFA) and Wistar rats (Charles River Laboratories, L'Arbresle, France) were used in the different experiments. The use of Wistar rats in the CCl_4 model was preferred due to its major predisposition to develop ascites compared with SD OFA rats. All rats were kept under constant temperature and humidity in a 12 h controlled dark/light cycle and fed *ad libitum* with a grain-based chow (Teklad 2014, Harlan Laboratories, Indianapolis, IN, USA) containing a fixed formula of ingredients with 0.1 % Na^+.

10.1.1.1. Portal vein ligation (PVL) model

Prehepatic PHT was induced by PVL in male SD OFA rats weighing 200-220 g as previously described (224). Rats were anesthetized with inhaled isoflurane (5 % induction and 2 % maintenance). After shaving the abdomen fur and scrubbing with povidone-iodine (Betadine®, Meda, Solna, Sweden) or 70 % ethanol, a midline abdominal incision (laparotomy) was performed and the portal vein was freed from the surrounding tissue. A 2-knot ligature (Silkam* 3-0 suture thread, non-absorbable (B. Braun, Melsungen, Germany) was placed around a 20 G (0.889 mm) blunt-tipped needle (BD Microlance ™, Franklin Lakes, NJ, USA) lying along the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein (see**Fig. 10**).

Reestablishment of the blood flow in the portal vein was controlled and after rinsing with saline, the laparotomy was closed with continuous suture (Vicryl* 4-0, absorbable (Ethicon Inc., Somerville, NJ, USA)) for the muscular layer and skin. To prevent surgical-wound

infections, nitrofural 0.2 % ointment (Furacin®, Seid Labs., Barcelona) was topically applied on the muscular layer before suturing the skin, and povidone-iodine was also gently applied into the skin. In control animals (sham-operated rats), the portal vein was isolated and manipulated in the same manner, but not ligated. Analgesia (buprenorphine hydrochloride 0.05 mg/kg BW (Buprex®, Rb Pharmaceuticals Inc., Richmond, VA, USA)) was administered sc and repeated if indicated to all animals. After surgery, the animals were returned to their cages where they were kept for 14 days before hemodynamic measurements.

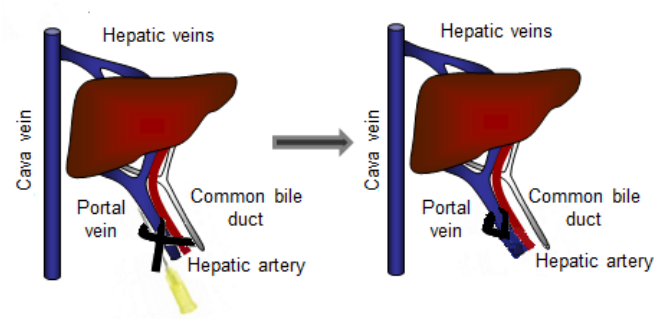

Fig. 10: **Schematic representation of the portal vein ligation (PVL) surgery.**

10.1.1.2. Bile duct ligation (BDL) model

Intrahepatic PHT caused by secondary biliary cirrhosis was induced by common BDL in male SD OFA rats weighing 200-220 g as previously described (229). Animals were anesthetized with inhaled isoflurane (5 % induction and 2 % maintenance) and the abdomen was shaved and scrubbed as stated above. After a laparotomy, the common bile duct was freed from the surrounding tissue and occluded by double ligature (3 knots each one) with a Mersilk* 4-0, non-absorbable suture thread (Ethicon Inc., Somerville, NJ, USA). The bile duct was then dissected between the two ligatures (see Fig. 11).

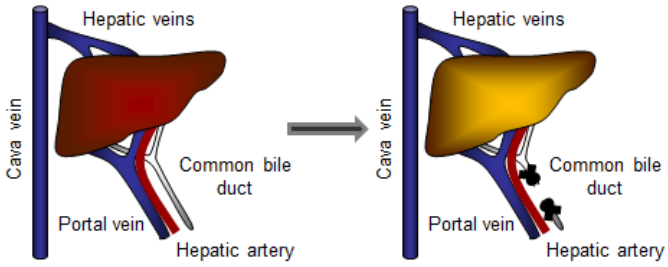

Fig. 11: **Schematic representation of the bile duct ligation (BDL) surgery.**

After rinsing with saline, the laparotomy was closed with continuous suture for the muscular layer and skin, and nitrofural 0.2 % and povidone-iodine were gently applied as stated above. Analgesia (buprenorphine hydrochloride 0.05 mg/kg BW) and prophylactic antibiotic treatment (ampicillin 10 mg/kg BW (Gobemicin®, Normon Labs., Madrid, Spain)) were administered intramuscularly. Animals also received weekly im vitamin k1 (fitomenadione 8 mg/kg BW (Konakion®, Roche, Basel, Switzerland)) to decrease mortality from bleeding (258). In sham-operated rats, the common bile duct was isolated but not occluded. These animals also received analgesia, antibiotic treatment and weekly vit k1.

After the surgery, animals were returned to their cages where they were kept before hemodynamic measurements. Most experiments were performed in 4-week BDL rats. After four weeks, rats develop jaundice and a strong fibrotic reaction due to the cholestatic injury. Only when testing droxidopa as a chronic treatment, did the animals reach the fifth week after BDL. The mortality rate at this stage was less than 10 % and between 20-30 % of the animals presented ascites.

10.1.1.3. Carbon tetrachloride (CCl_4) model

Intrahepatic PHT was induced by continuous administration of the hepatotoxin CCl_4 (Sigma-Aldrich, Saint Louis, MO, USA) both orally and inhaled. Before the first CCl_4 administration, rats received phenobarbital 0.3 g/L (Luminal®, Kern Pharma, Terrassa, Spain) in drinking water (*ad libitum*) to increase the yield of cirrhosis. Control rats received phenobarbital in drinking water but did not follow the CCl_4 administration protocol.

10.1.1.3.1. Oral administration

This protocol was followed to test chronic droxidopa treatment in cirrhotic rats with HRS. CCl_4 was administered weekly by oral gavage to SD OFA rats weighing 100-120 g, individualizing the dose according to weight gain/loss of the animal in response to the previous doses, as proposed by Proctor and colleagues (232). In the 100-200 g period (usually between 1-2 weeks) the animals received only phenobarbital in drinking water. When animals reached 200 g BW, CCl_4 administration started. The first dose per rat was 20 µL CCl_4 a week. The rats were weighed twice weekly (the day of CCl_4 administration, usually on Mondays, and 48 h later, usually on Wednesdays) and the next CCl_4 doses were calculated according to weight changes 48 h after the last CCl_4 dose (following **Table 2**).

Table 2: Carbon tetrachloride (CCl_4) dose estimation.

Weight changes 48 h after the last CCl_4 dose *	≤ 6 weeks CCl_4 administration	> 6 weeks CCl_4 administration
weight stable or weight gain	dose increment in 60 µL	dose increment in 80 µL
2-6 % weight loss	dose increment in 40 µL	dose increment in 60 µL
6.1-10 % weight loss	dose increment in 20 µL	dose increment in 40 µL
10.1-15 % weight loss	same dose	same dose
> 15 % weight loss	dose reduction in 40 µL	dose reduction in 40 µL

* Weight change from Monday to Wednesday.

All CCl_4 doses were mixed with 1000 µL water (due to the higher density of CCl_4 a higher density, it remains at the bottom and its inhalation is avoided). Before initiating droxidopa treatment (on Mondays), instead of the estimated corresponding dose, the rats received a reminder CCl_4 dose according to the previous one (see Table 3). Phenobarbital in drinking water was kept until hemodynamic measurements were performed.

Table 3: Carbon tetrachloride (CCl_4) reminder doses.

Previous CCl_4 dose (µL)	Reminder CCl_4 dose (µL)
< 500	50
500-1000	100
>1000	150

SD OFA rats with cirrhosis induced by CCl_4 develop SBP in a high percentage of animals. Following this protocol, mortality rate was around 30 %. Only 35% of the rats developed ascites, at 10-30 weeks after the initiation of CCl_4, showing low ascites volumes (0.5-10 mL). Presence of ascites was determined by checking morphological abdominal distension (lifting the rat by the tail) and also by pressing the abdomen (in rats with ascites, testicles bulged easily).

10.1.1.3.2. Inhalation protocol

Cirrhosis was induced by repetitive CCl_4 inhalation as previously described (231). The rats were placed in a methacrylate gas chamber (60 x 40 x 21 cm^3) and air was bubbled (1 L/min) through a flask containing CCl_4. The CCl_4 inhalation protocol was repeated twice a week (Monday and Friday) starting with exposures of 0.5 min of bubbled air plus 0.5 min of gas atmosphere (air bubbling is closed and the animals remain in the shut chamber) during 3 sessions, continuing with 1 min of bubbled air plus 1 min of gas atmosphere (3 more sessions) and increasing the CCl_4 exposure times (one more min each 3 sessions) up to 5

min of bubbled air plus 5 min of gas atmosphere per session. The exposure time was then maintained at 5 min of bubbled air plus 5 min of gas atmosphere until the animals developed ascites. Since CCl_4 at high doses has anesthetic properties, the animals were continuously checked to prevent respiratory arrest (hitting the gas chamber when necessary). 4-5 rats were included in each CCl_4 inhalation session and at least 2 min of chamber ventilation was needed between one group of rats and the following one. Control rats received phenobarbital in drinking water but did not follow the inhalation protocol (phenobarbital is stopped in these rats at equivalent times of those from cirrhotic rats).

Two different protocols were followed:

1) To obtain rats with HRS and high ascites volumes for the chronic propranolol-droxidopa treatment, Wistar rats weighing 200-220 g received phenobarbital in drinking water (*ad libitum*) and after one week with phenobarbital, the CCl_4 inhalation protocol began. The protocol was then stopped once the animals showed presence of ascites (checked as mentioned above). Phenobarbital was kept in drinking water until the last CCl_4 inhalation session, which was anticipated one day (on Thursdays instead of Fridays) to prevent increments in transaminases levels during treatment (3 days before treatment was enough time to recover transaminases levels (259)). Following this protocol, mortality rate was less than 5 %. 60 % of the rats developed ascites, at 19-33 weeks after the first CCl_4 inhalation session, showing high ascites volumes (5-40 mL). To prevent pronounced reductions in MAP, in these animals with huge ascites volumes, ascites was emptied at different stages before the laparotomy needed for the hemodynamic measurements.

2) To obtain an early cirrhosis model, to evaluate statins beneficial intrahepatic effects, male Wistar rats weighing 100-120 g followed the CCl_4 inhalation protocol but only for 13 weeks. In this case, phenobarbital, in drinking water (*ad libitum*) and started one week before the beginning of the inhalation protocol, was stopped 5 days before the initiation of statin treatment. CCl_4 inhalation was kept during treatment to mimic a continuous and chronic liver injury, but last inhalation session was at least 3 days before ending treatment, also to prevent increments in transaminases levels. The days when the inhalation protocol was required, treatment by oral gavage was given before, otherwise the animals became very nervous. With this protocol, there was no mortality rate, all the rats exhibited fibrosis, but none presented ascites.

10.1.2. Drug administration

All treatments in the different studies were administered by oral gavage except carbidopa (Sigma-Aldrich, Saint Louis, MO, USA) that was administered ip with a 25 G needle. Before the administration, all solutions were vortexed until complete homogenization. The rats were weighed daily during chronic treatments.

10.1.2.1. FIRST STUDY: New indication for droxidopa as a treatment for cirrhosis and portal hypertension (PHT)

Droxidopa (Chelsea Therapeutics Inc., Charlotte, NC, USA) acute hemodynamic effects (oral single dose) were tested in PVL, BDL and sham-operated rats. The rats were randomly assigned to receive droxidopa or vehicle (1 % carboxymetylcellulose solution and 0.2 % Tween 80 emulsifier (both from Sigma-Aldrich, Saint Louis, MO, USA)) 14 and 28 days after the ligation of the portal vein and common bile duct, respectively. Sham-operated rats received droxidopa 50 mg/kg (n=5) or its vehicle (n=5), whereas PVL rats received droxidopa 50 mg/kg (n=7), 25 mg/kg (n=7) or vehicle (n=9). Other 4 PVL rats received 30 mg/kg carbidopa, a Ddc inhibitor, ip 30 min before droxidopa (50 mg/kg) administration. To measure NA and aldosterone plasma levels (40 min after droxidopa or vehicle treatment), an extra set of PVL rats receiving droxidopa 50 mg/kg (n=5), carbidopa 30 mg/kg plus droxidopa 50 mg/kg (n=4) or vehicle (n=5) was used. Likewise, droxidopa 25 mg/kg (n=8) or vehicle (n=6) was administered to BDL rats. A second group of BDL rats was used to measure renal hemodynamics after droxidopa 25 mg/kg (n=6) or vehicle (n=6) treatment. Finally, a third set of BDL rats was also used to evaluate the administration of propranolol (AstraZeneca, Reims, France) 25 mg/kg (n=7) plus droxidopa 25 mg/kg given 2 h later. Comparisons were made with the corresponding BDL animals treated with propranolol vehicle (water) plus droxidopa 25 mg/kg (n=7), propranolol 25 mg/kg plus droxidopa vehicle (n=7), or both vehicles (n=6).

Droxidopa hemodynamic effects after a chronic treatment (*bid*, 5 days) were also tested in BDL and CCl_4-intoxicated rats. Droxidopa 15 mg/kg/day (n=15) or vehicle (n=15) was randomly administered to BDL rats 28 days after bile duct dissection to measure blood and urine biochemical parameters (the hemodynamic measurements were done in 8 rats from each group). CCl_4-induced cirrhotic rats also received droxidopa 15 mg/kg/day (n=6) or vehicle (n=6) once the rats presented ascites, 14 to 20 weeks after the initiation of CCl_4 intoxication. Sham-operated rats (n=6) were administered droxidopa vehicle (*bid*, 5 days) 28 days after surgery.

Carbidopa in powder was kept at -20 ºC and administered as a solution of 10 mg of carbidopa per mL of water in 50 mM HCl (the mL of water was previously heated at 100 ºC

for 25 min and 50 µL HCl 1 M were gently added to the solution). Droxidopa in powder was kept at RT and administered as a solution of 13 mg of droxidopa per mL of vehicle (for both the 50 mg/kg and the 25 mg/kg doses) or 10 mg of droxidopa per mL of vehicle (for the 15 mg/kg dose). In order to completely solubilize droxidopa, its vehicle was heated at 100 ºC for 25 min before adding the drug and, once droxidopa was added, continued to be heated at 100 ºC while being vortexed for 30 more min before its administration (the solution acquired a brown color at that point and was left 5-10 min at RT before being given to the rats). Powder from propranolol was obtained from crushed tablets, kept at RT and administered as a solution of 40 mg of the active ingredient per mL of water.

10.1.2.2. SECOND STUDY: Combined treatments with droxidopa

Three acute studies in which droxidopa (oral single dose) was administered to 4-week BDL rats previously treated with carvedilol (LKT Laboratories Inc., Saint Paul, MN, USA), propranolol or atorvastatin (Almirall, Barcelona, Spain) were performed to evaluate the acute hemodynamic and diuretic effects of the combinations, as well as determine the best combination protocol and establish an approximate range of doses. Starting dosage was chosen considering previous literature (144,176,177,260,261).

1) *Carvedilol-droxidopa*: droxidopa 20 mg/kg or vehicle were administered to BDL rats treated with carvedilol 2.5 mg/kg or vehicle (same as droxidopa's one) 1 h before (droxidopa, n=7; carvedilol, n=6; carvedilol plus droxidopa, n=7; vehicles, n=7). Other combinations with carvedilol 5 mg/kg and 2 mg/kg plus droxidopa 20 mg/kg were also assessed (n=6 and n=9, respectively).
2) *Propranolol-droxidopa*: droxidopa 25 mg/kg or vehicle were administered to BDL rats treated with propranolol 25 mg/kg or vehicle (water) 2 h before (droxidopa, n=8; propranolol, n=7; propranolol plus droxidopa, n=7; vehicles, n=8). Other combinations of propranolol 50 mg/kg plus droxidopa 25 mg/kg and propranolol 25 mg/kg plus droxidopa 20 mg/kg were also assessed (n=6 and n=7, respectively).
3) *Atorvastatin-droxidopa*: BDL rats receiving a 5-day treatment with atorvastatin 15 mg/kg/day or vehicle (water) received a single dose of droxidopa 25 mg/kg or vehicle 2 h after the last dose of atorvastatin or vehicle (droxidopa, n=9; atorvastatin, n=7; atorvastatin plus droxidopa, n=7; vehicles, n=9).

To evaluate the effects of the best combination (*propranolol-droxidopa*) on the hemodynamics and renal alterations of cirrhotic rats, a combined chronic study was performed in CCl_4-intoxicated rats. Treatment began once the animals presented ascites (70% of the rats) after 19 to 33 weeks of CCl_4 inhalation, or once 36 weeks of CCl_4 inhalation

were reached, established as a limit (30% of the animals). The animals were randomly assigned to receive droxidopa 20 mg/kg or vehicle twice a day and propranolol 25 mg/kg or vehicle once a day (2 h before the first dose of droxidopa), for 5 days (vehicles, n=8; droxidopa, n=8; propranolol plus droxidopa, n=8). Cirrhosis was present in all the rats that followed the CCl_4 inhalation protocol and non-ascitic cirrhotic rats were equally distributed among the different treatments. Control rats (n=8) did not receive any treatment.

In all acute combinations two intakes were given (two drugs, a drug plus the other drug vehicle or two vehicles). In the chronic combination three treatments were given each day (propranolol plus twice droxidopa, propranolol vehicle plus twice droxidopa or propranolol vehicle plus twice droxidopa vehicle). Droxidopa in powder was kept, solubilized and administered as indicated above for the 25 mg/kg dose, and was administered as a solution of 12 mg of droxidopa per mL of vehicle for the 20 mg/kg dose. Carvedilol in powder was kept at 4 ºC and administered as a solution of 2.5 mg per mL of vehicle (for the 3 doses used). Propranolol 50 mg/kg was also administered as a solution of 40 mg/mL (as previously indicated above). Powder from atorvastatin was also obtained from crushed tablets and kept at RT. Atorvastatin was administered as a solution of 20 mg of the active ingredient per mL of water.

10.1.2.3. THIRD STUDY: NCX 6560 as a treatment for cirrhosis and portal hypertension (PHT). Comparison with other conventional statins

To evaluate the effects of statins in a decompensated cirrhosis model and compare the efficacy and toxicity among statins, 4-week BDL rats received daily (*qd*) oral doses of statins or vehicle for 7 days. Two doses of each statin were evaluated: simvastatin (Ratiopharm, Ulm, Germany) 25 mg/kg/day (n=10) and 10 mg/kg/day (n=11), atorvastatin 15 mg/kg/day (n=14) and 10 mg/kg/day (n=15) and NCX 6560 ((βR,δR)-2(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1heptanoic acid 4-(nitrooxy)butyl ester) (NicOx S.A., Sophia Antipolis, France) 17.5 mg/kg/day (n=11) and 11.7 mg/kg/day (n=15). An additional dose of NCX 6560 at 35.1 mg/kg/day (n=15) was also tested and polyethylene glycol (PEG) (Sigma-Aldrich, Saint Louis, MO, USA) 70 % was used as a vehicle (n=12). Starting dosage was chosen according to previous literature (176,177,180,246) and the equivalent doses of NCX 6560 and atorvastatin used were calculated on the basis of their different MW (MW NCX 6560/ MW atorvastatin: ratio 1.17) and contained the same amount of atorvastatin: 15 and 10 mg of atorvastatin are equivalent to 17.5 and 10 mg of NCX 6560, respectively.

To evaluate the effects of statins in an early cirrhosis model, 13-week CCl_4-intoxicated rats received daily (*qd*) oral doses of statins or vehicle for 10 days: atorvastatin 15 mg/kg/day

(n=13), NCX 6560 17.5 mg/kg/day (n=13) and vehicle (PEG 70 %) (n=8). Control rats (n=8) did not receive any treatment.

NCX 6560 in powder was kept at 4 ºC and administered as a solution of 10 mg of NCX 6560 per mL of PEG 70% (for 17.5 mg/kg and 11.7 mg/kg doses) or 20 mg of NCX 6560 per mL of PEG 70% (for the 35.1 mg/kg dose). Powder from atorvastatin and simvastatin was obtained from crushed tablets and kept at RT. Both statins were administered as a solution of 20 mg of the active ingredient per mL of water (for both doses used in each case).

10.1.3. Hemodynamic measurements

The rats fasted overnight (O/N) to avoid digestive influences on splanchnic hemodynamics (262) were anesthetized with 100 mg/kg ketamine hydrochloride (Ketolar®, Pfizer, New York City, NY, USA) plus 5 mg/kg midazolam (B. Braun, Melsungen, Germany), ip, five minutes after droxidopa (or its vehicle) treatment or 90 min after statin (or vehicle) treatment. Dose adjustments in anesthesia were made depending on the experimental animal model, the animal's condition and the treatment received: non-ascitic cirrhotic animals received 2/3 of the anesthesia needed for PVL and sham-operated rats, cirrhotic animals with ascites and those treated with NSBB or simvastatin received even less anesthesia or the same amount but administered in various boluses, to prevent breathlessness and bradycardia. Control and non-ascitic cirrhotic animals for the CCl_4 model usually needed some additional doses for complete anesthesia (see Table 4).

After shaving the fur from the surgical regions (inner leg and abdomen) and scrubbing with 70 % ethanol, rats were placed on their back. Animals were maintained at 37ºC throughout the study by a rectal temperature probe and the parameters were recorded with the data acquisition system PowerLab (Harvard Apparatus, Holliston, MA, USA), comprising the software Chart 5.0.

To determine MAP (mmHg) and heart rate (HR, bpm), an incision in the left inner leg was performed and the connective tissue was dissected until the femoral triangle comprising the femoral vein, artery and nerve was exposed. Once the femoral artery was freed from the nerve, vein and connective tissue, a small incision in the artery with a 23 G needle was done. A polyethylene PE-catheter (PE50) (BD Intramedic™ Polyethylene Tubing (Non-Sterile), Franklin Lakes, NJ, USA), which was connected to a highly sensitive pressure transducer (Harvard Apparatus, Holliston, MA, USA), was introduced into the artery and immobilized with two knots (Silkam* 3-0 suture thread).

Table 4: **Anesthesia dose adjustments.**

Animal model	Treatment	Anesthesia (recommended dose)
Sham-operated rats	VEH/DRO	100 mg/kg ketamine hydrochloride + 5 mg/kg midazolam
PVL rats	VEH/DRO/CARB	same as sham-operated rats
BDL-cirrhotic rats *	VEH/DRO/ATO/NCX	2/3 anesthesia sham-operated rats
	CARV	1/2 anesthesia sham-operated rats
	PRO	1/4 anesthesia sham-operated rats
	SIM	1/4 anesthesia sham-operated rats
CCl_4-control rats	none	same as sham-operated rats + reminder dose **
non-ascitic CCl_4-cirrhotic rats	VEH/DRO/ATO/NCX	2/3 anesthesia sham-operated rats + reminder dose **
	PRO	2/3 anesthesia sham-operated rats *
ascitic CCl_4-cirrhotic rats	VEH/DRO	1/3 anesthesia sham-operated rats
	PRO	1/4 anesthesia sham-operated rats

ATO: atorvastatin, CARB: carbidopa, CARV: carvedilol, DRO: droxidopa, NCX: NCX 6560, PRO: propranolol, SIM: simvastatin, VEH: vehicle.

* Non-ascitic CCl_4 rats treated with propranolol and all ascitic BDL rats received the anesthesia in various boluses.

** Successive additional doses (between 0.1-0.2 mL of a 50 % ketamine + 50 % midazolam mixture) were administered to some rats until they were fully anesthetized.

After a midline abdominal incision and exposing the mesentery, another PE50 catheter, also connected to a highly sensitive pressure transducer (Harvard Apparatus, Holliston, MA, USA), was introduced into the ileocolic vein for PP measurement (mmHg). One drop of cyanoacrylate glue was applied over the insertion point in order to avoid slipping of the catheter.

The SMA was isolated from connective tissue and a Doppler perivascular ultrasonic transit-time flow probe (1 mm diameter, Transonic Systems Inc., Ithaca, NY, USA), which contained ultrasound gel to facilitate signal transmission, was placed around the artery to continuously measure SMABF (mL/[min.100 g]). The same flow probe (with ultrasound gel) was placed on the left renal artery freed from the surrounding fat tissue to measure RABF (mL/[min.100 g]). Finally, PBF (mL/[min.100 g]) was measured with a Doppler perivascular ultrasonic transit-time flow probe (2 mm diameter, Transonic Systems Inc., Ithaca, NY, USA), also containing ultrasound gel, placed on the dissected portal vein.

In the acute studies with droxidopa in the BDL model, SMABF and PBF were measured together with MAP, HR and PP, alternating the determination of both blood flows every 10 min, while RABF was measured in an extra set of rats, together with MAP and HR. By contrast, in the CCl_4 model in statins studies, PBF was measured after having measured SMABF.

SMAR (mmHg/mL.min.100 g) was calculated as (MAP-PP)/SMABF, renal artery resistance (RAR) as MAP/RABF, and IHVR as PP/PBF.

Ascites volume was determined and BW, to normalize the parameters, was calculated as rat weight - ascites volume, considering 1 mL = 1 g.

For the acute first studies with droxidopa (alone or in combination), the hemodynamic parameters were continuously registered for 2 h whereas for the chronic study with droxidopa in BDL, hemodynamic measures were registered for 90 min after the last dose of droxidopa or vehicle. In CCl_4-induced cirrhotic rats for droxidopa studies and in both BDL and CCL_4 studies with statins, hemodynamic parameters were registered for 50-60 min and measurements were obtained as a single determination at the end of the register once the parameters were allowed to equilibrate. Each determination represented the average of 30 s of register.

Animals were euthanized by exsanguination under deep anesthesia.

10.1.4. Sample harvesting

Urine, blood and tissue samples were collected in the different studies.

In the chronic studies with droxidopa in BDL and CCl_4–induced cirrhotic rats, the urine was collected the 4th day of treatment by housing the rats in individual metabolic cages for 24 h. In the acute studies with droxidopa and in statin studies in BDL and CCl_4-induced cirrhotic rats, the diuresis volume was determined collecting the urine from the bladder after hemodynamic measurements, obtaining the urinary volume expressed as mL/2 h and mL/h, respectively.

Blood venous samples were obtained from the cava vein after the hemodynamic measurements and collected in lithium heparin tubes (green cap) (LH PST™ II BD Vacutainer®, Franklin Lakes, NJ, USA) or in plastic tubes without anticoagulant (red cap) (no additive (Z) Plus BD Vacutainer®, Franklin Lakes, NJ, USA), for subsequent plasma or serum analysis, respectively. Both plasma and serum samples were analyzed in the *Laboratoris de Bioquímica Clínica* from Vall d'Hebron University Hospital.

For the extra set of PVL rats used to measure NA and aldosterone plasma levels, blood venous samples were obtained from the cava vein 40 min after droxidopa or vehicle treatment, collected in EDTA tubes (light purple cap) (K_2EDTA 5.4 mg, BD Vacutainer®,

Franklin Lakes, NJ, USA) and centrifuged at 7000 *g* for 10 min at 4 ºC. The supernatant (plasma) was then transferred to a clean Eppendorf tube and kept at -20 ºC until subsequent analysis in the *Laboratoris de Bioquímica Clínica* from Vall d'Hebron University Hospital.

10.1.4.1. Biochemical analysis of blood and urine samples

Biochemical analysis of blood samples was performed in all chronic studies with droxidopa and in statins studies. For droxidopa chronic studies urine samples were also analyzed.

Serum levels of total bilirubin, albumin, ALT and aspartate aminotransferase (AST), alkaline phosphatase (ALP), and serum and urinary levels of sodium, potassium, creatinine and osmolality were determined using an automatic analyzer (Olympus AV5400, Olympus Europe GmbH, Hamburg, Germany). In the statins studies CK and cholesterol levels were also determined using the same automatic analyzer. In these studies, ALT and CK levels in vehicle rats from BDL and CCl_4 models were used to define hepatic and muscular toxicity due to statin treatment. Thus, we defined hepatic toxicity as an increment in ALT levels superior to 200 IU/L (BDL model) and to 500 IU/L (CCl_4 model), whereas muscular toxicity was considered when CK levels were above 1000 IU/L (BDL model) and 6000 IU/L (CCl_4 model). Animals with hepatic toxicity due to statin treatment were discarded from the study and were not used for sample and data analysis.

The NA and aldosterone plasma levels in PVL rats from the droxidopa study were determined by radioimmunoassay (RIA) (KatCombi RIA, IBL, Hamburg, Germany and Aldoctk-2, DiaSorin, Saluggia, Italy, respectively).

For the renal parameters in droxidopa chronic studies the following formulas were used: osmolal clearance (mL/min) was calculated as $[\text{osmolality}]_{urine} \cdot V / [\text{osmolality}]_{serum}$, creatinine clearance (mL/min) as $[\text{creatinine}]_{urine} \cdot V / [\text{creatinine}]_{serum}$, and free water clearance (mL/min) as V-osmolal clearance, where V is the urine flow rate (mL/min). Finally, sodium (mmol/24 h), potassium (mmol/24h) and creatinine excretion (mg/24 h) were determined as [solute]·urinary volume in 24 h and fractional sodium excretion (%) was calculated as $[Na^+]_{urine} \cdot [\text{creatinine}]_{serum} \cdot 100 / [Na^+]_{serum} \cdot [\text{creatinine}]_{urine}$.

10.1.4.2. Tissue harvesting

In the droxidopa chronic study in BDL rats, SMA samples were harvested from the aortic origin, placed in liquid nitrogen (N_2 (l)) and kept at -80 ºC until processed.

In statins studies, liver from cirrhotic rats was perfused with saline for exsanguination and cut into fragments (less than 0.5 cm thick). Liver samples were either snap-frozen N_2 (l) and

stored at -80ºC or fixed in 4 % paraformaldehyde solution (in embedding cassettes) for 24 h and changed to 50 % ethanol solution before paraffin embedding.

10.1.4.2.1. Paraffin embedding

Tissues in embedding cassettes were treated with a battery of alcohols (incremental alcohol %) and xylene in order to be dehydrated and allow paraffin penetration. Finally, they were submerged into liquid paraffin at 65 ºC (pre-warmed 24 h in advanced) and included in blocks (see Table 5).

Table 5: Paraffin embedding protocol.

Solution	Time (min)
ethanol 70 %	45
ethanol 96 % (1)	30
ethanol 96 % (2)	30
ethanol 100 % (1)	15
ethanol 100 % (2)	45
xylene (1)	15
xylene (2)	15
paraffin (1)	60
paraffin (2)	60

(1) and (2) indicate changing of the samples to a new solution (identical to the previous one).

Paraffin-embedded blocks were cut into 4 µm-thick sections with a microtome (Leica RM2235, Leica Biosystems, Wetzlar, Germany) and placed into Poly-L-lysine treated slides (Polysine™, Menzel-Gläser, Braunschweig, Germany) for subsequent histological techniques (histological staining and immunohistochemistry (IHC)).

10.2. Histological staining

The histological stainings were carried out on 4 µm paraffin sections. Sections were deparaffinized at 60 ºC for at least 1 h and then subjected to a battery of solutions in order to be hydrated (see Table 6).

Table 6: Hydration process for paraffin sections.

Solution	Time (min)
citrosol	20
ethanol 100 %	5
ethanol 95 %	5
ethanol 70 %	5
dH_2O	5

Citrosol is a xylene substitute. dH_2O: distilled water.

The hydration process was performed in Coplin jars or staining racks, at RT and without agitation.

10.2.1. Hematoxylin and eosin (H&E) staining

H&E staining was performed on 4 µm hydrated paraffin sections, incubating the samples for 5 min at RT in Mayer's hematoxylin solution (Sigma-Aldrich, Saint Louis, MO, USA), rinsing twice with warm tap water, and incubating for 50 s in eosin solution (Sigma-Aldrich, Saint Louis, MO, USA). The samples were then washed 5 times with warm tap water and treated with a battery of solutions to be dehydrated (see Table 7).

Table 7: **Dehydration process for paraffin sections.**

Solution	Time (min)
ethanol 70 %	0.25
ethanol 95 %	0.25
ethanol 100 %	0.25
xylene	5

Slides were dried of excess xylene and a drop of DPX mounting medium (Sigma-Aldrich, Saint Louis, MO, USA) was placed on top of the slide using a plastic Pasteur pipette. A coverslip was gently let fall onto the slide and pressed with tweezers to allow the DPX to spread beneath the coverslip, covering all the tissue. Slides were dried O/N in the hood.

10.2.2. Sirius red staining

(See 10.4.1. **Sirius red staining** for buffers and reagents)

Sirius red staining to detect collagen fibers was also performed on 4 µm hydrated paraffin sections. The samples were stained with 0.1% Picro-sirius red for 1h at RT with gentle agitation, washed twice with acidified water for 5 min and dried at RT. The samples were then dehydrated and mounted with DPX as detailed above. The fibrotic area was assessed using image analysis techniques. Ten fields (10x magnification) from each sirius red stained section were randomly obtained using an optical microscope Olympus BX61 (Olympus, Hamburg, Germany) equipped with a digital camera (large bile ducts and vessels excluded). The proportion of red-stained area per total area was measured using ImageJ 1.38 free software (National Institute of Health, Bethesda, MD, USA) and expressed as fibrotic rate (%). Briefly, RGB color images were decomposed in three channels (red, green and blue) and after stacking the 3 channels, all the analysis was done in the green color stack. A threshold (between 0-120) was set up and adapted manually if necessary and the area fraction (%) for each image was measured.

10.3. Immunological techniques

Protein expression in liver and SMA samples was determined by WB or IHC and cGMP levels in liver samples were determined by enzyme immunoassay (EIA).

(See **10.4.2. Western blot (WB)** and **10.4.3. Immunohistochemistry (IHC)** for buffers and reagents)

10.3.1. Western blot (WB)

For protein analysis by WB, samples were kept at -80ºC after being snap-frozen in N_2 (l). Two different protein extraction protocols were followed depending on the type of sample.

10.3.1.1. Superior mesenteric artery (SMA) samples

SMA samples from BDL rats treated chronically (*bid*, 5 days) with droxidopa (n=9) or vehicle (n=10) were used. The SMAs were homogenized in 250 µL RIPA lysis buffer using the Fast Prep System with Matrix Lysing tubes (type D, green cap) (MP Biomedicals, Santa Ana, CA, USA) that contain 1.4 mm ceramic spheres. For SMA samples, 3 x 25-s cycles at maximum speed (6.5) were needed for proper homogenization (samples were kept on ice between cycles). Then, to favor complete cellular lysis, the samples followed cycles of 10 s vortexing plus 1 min on ice for 15 min and were kept 1 h at -80 ºC afterwards. Finally, they were centrifuged at 16000 *g* for 30 min at 4 ºC. The supernatant was then transferred to a new clean Eppendorf tube and kept at -20 ºC for subsequent protein estimation.

10.3.1.2. Liver samples

Liver samples from BDL and CCl_4 rats from the statins studies were used. In the BDL model all samples were analyzed: vehicle (n=8), atorvastatin (n=9) and NCX 6560 (n=9), whereas in the CCl_4 study 7 samples from each group were analyzed: control (n=7), vehicle (n=7), atorvastatin (n=7) and NCX 6560 (n=7). The frozen liver samples were crushed to a powder form whilst still frozen and subsequently homogenized in Triton-lysis buffer. Thereafter, they were vortexed, sonicated (3 x 10 s), left on ice for 10 min, and centrifuged at 18,000 *g* for 10 min at 4 ºC. The supernatant was also transferred to a new clean Eppendorf tube and kept at -20 ºC for subsequent protein estimation.

In both types of samples (SMA and liver), supernatant protein concentration was quantified with Pierce™ bicinchonidic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), following kit's instructions. For SMA samples both 1/2 and 1/5 dilutions were needed whereas 1/20 and 1/50 dilutions were used in liver samples. BSA known concentrations were used as standard curve. The total protein concentration was

determined by a colorimetric reaction (color change of the sample solution from green to purple) reading at 652 nm with Nanodrop (Thermo Fisher Scientific, Rockford, IL, USA).

Equal amounts of protein were run on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For SMA samples, 20 µg of protein extracts per lane were used while in liver samples, 30 µg were used for all proteins except for eNOS and p-eNOS that needed 50 µg for proper detection.

11 µL of sample solution and MW marker were loaded to each well. Samples solutions were prepared as indicated in Table 8.

Table 8: Sample preparation for NUPAGE® precast electrophoresis gel loading.

Reagent	Volume (µL)
protein extract	x *
dH_2O	7.8-x
NuPAGE® LDS sample buffer (4X)	3
NuPAGE® reducing agent (10X)	1.2

Depending on protein concentration needed. dH_2O: distilled water.

Total volume = 12 µL (11 µL/well were loaded).

NuPAGE® reagents were from Thermo Fisher Scientific (Rockford, IL, USA).

Two protein ladders were used: SeeBlue® Pre-stained Protein Standard (Thermo Fisher Scientific, Rockford, IL, USA) and Precision Plus Protein™ Dual Color Standards (Bio-Rad. Hercules, CA, USA). The latter was preferred for detecting high MW proteins. The SeeBlue® ladder needed to be prepared equivalently to the sample's solutions (see Table 9). The samples and the SeeBlue® ladder were boiled for 10 min at 95 ºC before loading the gel. The Bio-Rad ladder was ready-to-use and 5 µL were directly loaded into the gel well.

Table 9: SeeBlue® Pre-stained Protein Standard preparation.

Reagent	Volume (µL)
protein ladder	3.6
dH_2O	4.2
NuPAGE® LDS sample buffer (4X)	3
NuPAGE® reducing agent (10X)	1.2

dH_2O: distilled water. Total volume = 12 µL (11 µL/well were loaded).

NuPAGE® reagents were from Thermo Fisher Scientific (Rockford, IL, USA).

All proteins were detected in precast 4-12 % gradient or 10 % NuPAGE® Bis-Tris Precast Gels (NOVEX®, Thermo Fisher Scientific, Rockford, IL, USA) depending on the protein MW (see Table 10), and using the XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific, Rockford, IL, USA).

Table 10: Primary antibodies for Western blot (WB).

Protein	MW (kDa)	Ab specie	Manufacturer (code)	Dilution
α-SMA	42	R	Abcam (ab5694)	1/200
Akt	60	R	Cell Signaling (9272S)	1/1000
p-Akt	60	R	Cell Signaling (9271S)	1/1000
CD31	130	R	Santa Cruz (sc-1506)	1/200
GAPDH	36	M	Ambion (AM4300)	1/5000
KLF2	37	G	Santa Cruz (sc-18690)	1/200
eNOS	140	M	BD Transduction Labs. (610296)	1/500
p-eNOS	140	R	Cell Signaling (9571S)	1/250
moesin	77	M	Santa Cruz (sc-13122)	1/300
p-moesin	77	R	Santa Cruz (sc-12895-R)	1/200
Rock-2	160	R	Santa Cruz (sc-5561)	1/200

Ab: antibody, G: goat antibody, M: mouse antibody, MW: molecular weight, R: rabbit antibody.

For proteins higher than 100 kDa (CD31, eNOS, p-eNOS and Rock-2), the 10 % gels and O/N transfer was preferred. Rock-2 could also be detected with 4-12 % gels and transfer 1h at RT.

All primary antibodies were diluted in TTBS1X except p-Akt that was diluted in PhosphoBLOCKER™ 5 % in TTBS 1X, and p-eNOS and Akt which were diluted in BSA 5 % in TTBS1X. All were incubated 1h at RT or O/N at 4 ºC except Akt, p-Akt and p-eNOS that needed to be incubated O/N at 4 ºC.

GAPDH was used as a loading control. When loading more than 50 µg of protein extract, GAPDH was diluted 1/10000 in TTBS1X.

The gels were washed with dH_2O and the wells cleaned with running buffer before use. The upper (cathode) buffer chamber was filled with running buffer (upper), which contained 500 µL NuPAGE® antioxidant (Thermo Fisher Scientific, Rockford, IL, USA), when performing protein gel electrophoresis under reducing conditions.

Gels were run at 200 V, 120 mA for about 2 h, until the pink band (22 kDa in the SeeBlue® ladder and 25 kDa in the Bio-Rad ladder) was gone, to be able to detect GAPDH (loading control).

Proteins separated by SDS-PAGE were blotted onto a polyvinylidene fluoride (PVDF) membrane with 0.45 µm pore size (Thermo Fisher Scientific, Rockford, IL, USA), previously hydrated with methanol (30 s), dH_2O (5 min) and transfer buffer (5 min). Blotting pads and Whatman papers were also kept in transfer buffer until the sandwich XCell II™ Blot Module

(Thermo Fisher Scientific, Rockford, IL, USA) was assembled in the following order: 2 blotting pads, 1 Whatman paper, the polyacrylamide gel, the PVDF membrane, 1 Whatman paper and 2 blotting pads (bubbles were avoided when assembling the sandwich). Transfer was done at 30 V and 400 mA for 1 h at RT, or O/N at 4 ºC, 15 V and 400 mV (see Table 10).

Once the transfer was done, membranes were stained with Ponceau S solution (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min to ensure that proteins were properly transferred. All incubations and washes were always done with constant agitation. Membranes were washed with TTBS1X three times for 10 min, and blocked either 1 h at RT or O/N at 4ºC with skim milk 5 % in TTBS1X, except when detecting Akt and eNOS that membranes were blocked with bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA) 5 %, and with phosphorylated proteins (p-Akt, p-eNOS and p-moesin), that PhosphoBLOCKER™ (Cell Biolabs, Inc., San Diego, CA, USA) 5 % was used, instead.

After blocking, membranes were washed for 10 min with TTBS1X, incubated with primary antibodies (see Table 10), washed three times with TTBS1X for 10 min, incubated thereafter with the corresponding secondary peroxidase-coupled antibody for 1 h at RT (see Table 11) and washed 3 more times with TTBS1X for 10min before developing.

Table 11: Secondary antibodies for Western blot (WB).

Antibody	Manufacturer (code)	Dilution
anti-goat IgG	Santa Cruz (sc-2020)	1/30000
anti-mouse IgG	GE Healthcare (NA931VS)	1/30000
anti-mouse IgG	Calbiochem (401215)	1/30000
anti-rabbit IgG	Cell Signaling (7074P2)	1/30000

Anti-mouse IgG from Calbiochem was needed when detecting moesin.

Dilutions of secondary antibodies were prepared in TTBS1X, except for p-Akt and p-moesin, which were prepared in PhosphoBLOCKER™ 5 % in TTBS1X.

Blots were developed with enhanced chemioluminiscence (Amersham ECL Prime, GE Healthcare, Uppsala, Sweden), incubating for 1 min with a mixture containing the peroxidase substrate (500 µL solution A-luminol + 500 µL solution B-peroxide), and images were captured with a CCD camera using a LAS-3000 Imaging System (Fujifilm, Tokyo, Japan). Protein expression was finally determined by densitometric analysis of bands using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). In each case, the intensity of the bands was normalized for the corresponding GAPDH, used as loading control.

When detecting the phosphorylated and the total protein in the same membrane (for Akt, eNOS and moesin), the detection of the phosphorylated protein was done first. Membranes were then stripped incubating them with Restore™ WB Stripping Buffer (Thermo Fisher

Scientific, Rockford, IL, USA) for 30 min at 55 ºC. Thereafter, they were washed thrice with TTBS1X 10 min, blocked again and incubated with the primary and secondary antibody as described above to detect total protein levels. In this case, the ratio between the intensities of the phosphorylated and the total protein (p-Akt/Akt, p-eNOS/eNOS or p-moesin/moesin), were calculated without requiring GAPDH normalization.

10.3.2. Immunohistochemistry (IHC)

IHC was used to detect CD43 (a pan-leukocyte marker) on the liver tissue. Antigens masked during routine fixation were retrieved incubating the 4 µm hydrated paraffin sections (see 10.2.) in citrate buffer for 20 min at 95 ºC and left at RT for 25 more min in the same buffer. After washing 3 times with PBS 1X with agitation, endogenous peroxidase activity was blocked incubating for 10 min with a 3 % H_2O_2 solution (also with agitation). Then the sections were washed twice, 5 min with dH_2O and 5 more min with PBS 1X. All the following incubations were performed in a humidity chamber. In order to reduce unspecific binding, the sections were blocked with 200 µL of normal goat serum (Sigma-Aldrich, Saint Louis, MO, USA) in a 1/20 dilution in PBS 1X for 30 min at RT. Then, they were incubated O/N at 4 ºC with 200 µL of primary antibody against CD43 (W3/13, dil. 1/500, AbD Serotec Ltd, Oxford, UK). Primary antibody dilution was prepared with Antibody diluent (Dako, Glostrup, Denmark) and, in the negative control, samples were incubated O/N with PBS 1X instead. After washing thrice with PBS 1X 5 min to eliminate the excess of primary antibody, the samples were incubated with a few drops of ENVISION-HRP anti-mouse secondary antibody (Dako, Glostrup, Denmark) 30 min at RT and washed 3 more times with PBS 1X 5 min to also eliminate the excess of secondary antibody. To visualize the protein, the VIP substrate kit for peroxidase enzyme (Vector. Burlingame, CA, USA), that produces a purple precipitate, was used. The samples were observed under the microscope or a magnifying glass and the substrate was left until the precipitate appeared, usually at 7 min. The reaction was then stopped with dH_2O first and with tap water afterwards. The samples were counterstained with Mayer's hematoxylin solution for 10 s, washed with abundant tap water and left in dH_2O for 5 min. Then, they were dehydrated (see Table 7) and mounted in DPX as also described above. Ten fields per section were randomly captured at 10x magnification using the optical Olympus BX61 microscope equipped with a digital camera, and images were quantified with the manual cell counter of Image J 1.38 free software obtaining the number of CD43 positive cells per field.

10.3.3. Enzyme immunoassay (EIA)

Measurements of cGMP, a marker of NO bioavailability, were performed in liver homogenates. Samples of frozen tissue were crushed to a powder form and aliquots from each sample containing 100-200 mg of tissue were dropped into 5 volumes of 5 % trichloroacetic acid (Sigma-Aldrich, Saint Louis, MO, USA) and homogenized on ice using a Polytron-type homogenizer (Heidolph DIAX 600 homogenizer, Schwabach, Germany). The precipitate was removed by centrifugation at 1500 *g* for 10 min at 4 ºC and the supernatant transferred to a clean test tube, washed five times with five volumes of water-saturated diethyl ether and the aqueous phase extract lyophilized O/N (Telstar LyoQuest laboratory freeze dryer, Terrassa, Spain). The dried extract was dissolved in ultrapure water (Spi-Bio, Massy, France) and cGMP levels were determined by EIA (Cayman Chemical Co., Ann Arbor, MI, USA) following the kit's instructions for acetylated samples. Results were read in the ELx800 Absorbance Reader (BioTek, Winooski, VT, USA) comprising the software Gene 5.1. and were expressed as pmol/(mL.100 mg).

10.4. Buffers and reagents

All reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), except the WB reagents (running and transfer buffers) that were from Invitrogen™ (Thermo Fisher Scientific, Rockford, IL, USA) and okadaic acid that was purchased from Calbiochem (Merck Millipore, Billerica MA USA).

10.4.1. Sirius red staining

Picro-sirius red 0.1 % (0.5L)

0.36 mM Direct Red 80: 0.5 g

Mix and dissolve in 500 mL saturated (1.3 % in H_2O) aqueous solution of picric acid. Keep at RT (highly explosive, can be re-used several times and kept for 3 years).

Acidified water (1L)

Mix 5 mL acetic acid glacial in 995 mL dH_2O.

10.4.2. Western blot (WB)

RIPA-lysis buffer

For SMA protein extraction

1 % sodium deoxycholate ($C_{24}H_{39}NaO_4$)

2 % (v/v) Protease Inhibitor Cocktail (ready-to-use solution in dimethyl sulfoxide (DMSO))

Mix in RIPA Buffer

For each SMA sample mix 2.5 mg sodium deoxycholate with 5 µL protease inhibitor cocktail solution and 250 µL RIPA buffer.

Triton-lysis buffer

For liver protein extraction

For each sample use 400 µL of Triton-lysis buffer. To prepare 1 mL Triton-lysis buffer mix:

200 µL TBS5X

50 µL NaPPi 0.2 M (sodium pyrophosphate tetrabasic, $Na_4P_2O_7$)

40 µL NaF 0.5 M (sodium fluoride)

100 µL Tritox™ X-100

10 µL SOV (sodium orthovanadate, Na_3VO_4)

10 µL A Ok (okadaic acid, $C_{44}H_{68}O_{13}$)

12 µL PIM (protein inhibitors mix)

4 µL PMSF (phenylmethylsulfonyl fluoride)

Mix in 574 µL dH_2O.

- To prepare **TBS5X** mix 5 mL TBS10X (see below) with 5 mL dH_2O.

- To prepare **NaPPi 0.2M** mix 1.78 g NaPPi in 20 mL dH_2O. Heat at 37 ºC to dissolve and adjust pH to 9.8 with approximately 60 µL HCl 1N. Keep at -20 ºC in 1000 µL aliquots. Expires in 5 months.

- To prepare **NaF 0.5 M** mix 0.42 g NaF in 20 mL dH_2O. Keep at -20 ºC in 1000 µL aliquots. Expires in 5 months.

- To prepare **SOV** mix 0.037 g SOV in 1 mL dH_2O (must be fresh prepared).

- To prepare **A Ok** mix 25 µg A Ok in 31 mL dH_2O. Keep at -20 ºC in 1000 µL aliquots (opaque Eppendord tubes). Expires in 5 months.

- To prepare **PIM** mix:

· 1 mg antipain dihydrochloride (ref. A6191) in 1000 µL HEPES buffer 15 mM

· 1 mg aprotinin (ref. A1153) in 1000 µL HEPES buffer 15 mM

· 1 mg chymostatin (ref. C7268) in 800 µL HEPES buffer 15 mM + 200 µL DMSO

· 1 mg leupeptin (ref. L2884) in 800 µL HEPES buffer 15 mM + 200 µL DMSO

· 1 mg pepstatin A (ref. P4265) in 800 µL HEPES buffer 15 mM + 200 µL DMSO

· 1 mg trypsin inhibitor type III-O (ref. T2011) in 800 µL HEPES buffer 15 mM + 200 µL DMSO

· **HEPES** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) **buffer 300 mM** is prepared mixing 15 mg HEPES in 50 mL dH_2O. Adjust pH to 7.4 with approximately 30 µL HCl 1N.

· To prepare **HEPES buffer 15 mM** mix 1 mL HEPES buffer 300 mM in 19 mL dH_2O.

Keep PIM (total volume = 6 mL) at -20 ºC in 100 µL aliquots. Expires in 5 months.

- To prepare **PMSF** mix 10 mg PMSF in 1 mL absolute ethanol. Keep at -20 ºC. Expires in 5 months.

Running buffer (1L)

Mix 50 mL NuPAGE® MES SDS Running Buffer (20X) in 950 mL dH_2O.

Keep at 4 ºC.

To prepare **running buffer (upper)** add 500 µL NuPAGE® antioxidant to 200 mL running buffer and mix.

Transfer buffer (1L)

50 mL NuPAGE® Transfer Buffer (20X)

1 mL NuPAGE® antioxidant

100 mL methanol*

Mix in 849 mL dH_2O. Keep at 4 ºC.

*when transferring 2 gels at the same time use 200 mL methanol and 749 mL dH_2O.

Tris-buffered saline (TBS) 10X (1L)

1.4 M sodium chloride (NaCl): 80 g

27 mM potassium chloride (KCl): 2 g

254 mM Trizma® hydrochloride (Tris HCl): 40 g

Mix and dissolve in 800 mL dH_2O. Adjust pH to 7.6 and the volume to 1 L with additional dH_2O. Keep at RT.

To prepare **TTBS1X** mix 100 mL TBS 10X in 900 mL dH_2O and add 1 mL Tween-20 (while in agitation).

10.4.3. Immunohistochemistry (IHC)

Citrate buffer (1L)

10 mM trisodium citrate dihydrate ($C_6H_9Na_3O_9$): 2.94 g

Mix and dissolve in 800 mL dH_2O. Adjust pH to 6.0 with HCl 1 N and the volume to 1 L with additional dH_2O. Keep at 4 ºC.

Phosphate-buffered saline (PBS) 10X (1L)

1.4 M sodium chloride (NaCl): 80 g

27 mM potassium chloride (KCl): 2 g

100 mM sodium phosphate dibasic (Na_2HPO_4): 14.4 g

18 mM potassium phosphate monobasic (KH_2PO_4): 2.4 g

Mix and dissolve in 800 mL dH_2O. Adjust pH to 7.4 and the volume to 1 L with additional dH_2O. Keep at RT.

To prepare **PBS 1X** mix 100 mL PBS 10X in 900 mL dH_2O.

10.5. Statistical analysis

Statistical analysis was carried out using SigmaStat 3.0 and all values were expressed as mean ± standard error of the mean (SEM). Comparisons between two groups using Student's *t* test and multiple comparisons with one-way analysis of variance (ANOVA) test, followed by a *post hoc* test, were performed. For the toxicity analysis of statins, Fisher Exact test for contingency tables was used. Statistical significance was established at $p \leq 0.05$.

11. APPENDIX 2: MANUSCRIPT III

Rodríguez S *et al*. 2016. Manuscript submitted to *Scientific Reports* (April 2016)

TITLE PAGE

Title: A NITRIC OXIDE-DONATING STATIN DECREASES PORTAL PRESSURE WITH A BETTER TOXICITY PROFILE THAN CONVENTIONAL STATINS IN CIRRHOTIC RATS

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ABSTRACT

Statins present many beneficial effects in chronic liver disease, but concerns about safety exist. We evaluated the hepatic effects of a nitric oxide-releasing atorvastatin (NCX 6560) compared to conventional statins. Simvastatin, atorvastatin and NCX 6560 were evaluated in four-week bile duct-ligated rats (BDL) simulating decompensated cirrhosis and in thirteen-week carbon tetrachloride (CCl_4) intoxicated rats, a model of early cirrhosis. In the BDL model, simvastatin treated rats showed high mortality and the remaining animals presented muscular and hepatic toxicity. At equivalent doses, NCX 6560 greatly reduced muscular and hepatic toxicity caused by atorvastatin in the more advanced BDL model; toxicity was minimal in the CCl_4 model. Atorvastatin and NCX 6560 similarly reduced portal pressure without changing systemic hemodynamics in both models. Atorvastatin and NCX 6560 caused a mild decrease in liver fibrosis and inflammation and a significant increase in intrahepatic cyclic guanosine monophosphate. NCX 6560 induced a higher intrahepatic vasoprotective profile (increased Krüppel-like factor-2, activated endothelial nitric oxide synthase and decreased platelet/endothelial cell adhesion molecule-1), especially in the CCl_4 model, suggesting a higher benefit in early cirrhosis. In conclusion, NCX 6560 improves the liver profile and portal hypertension of cirrhotic rats similarly to conventional statins, but with a much better safety profile.

INTRODUCTION

Portal hypertension of cirrhosis is initiated by an increased hepatic resistance to portal blood flow and maintained and aggravated by an augmented portal inflow due to splanchnic arterial vasodilation and hyperdynamic syndrome. The increase in intrahepatic vascular resistance (IHVR) is mainly due to architectural distortion (fibrosis, nodules) and imbalance between vasoconstrictors and vasodilators leading to endothelial dysfunction and an increment in intrahepatic vascular tone [1-3].

Many therapeutic strategies aimed at decreasing IHVR (e.g.: antioxidants, nitric oxide (NO) donors, statins) have been developed [3-5]. NO donors such as isosorbide-5-mononitrate have been shown to decrease portal pressure (PP) [6], but their use has been limited due to systemic vasodilatory effects [7, 8]. Statins have also been shown to ameliorate portal hypertension and liver function [9-12]. Although statin therapy is commonly regarded as well tolerated in patients with mild liver disease [13-15], serious adverse effects have also been reported [16-18].

Statins exert beneficial lipid-independent pleiotropic effects through 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibition, reducing mevalonate production and therefore, the synthesis of isoprenoids, farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These compounds, in turn, serve as lipophilic attachments for various signaling proteins, such as the small Ras, Rac, and Rho GTPases, which are involved in cell proliferation, cytoskeletal organization, and activation of transcription factors [19-21].

Mechanisms involved in the beneficial intrahepatic effects of statins include improvement of endothelial dysfunction through increasing endothelial nitric oxide synthase (eNOS) activity (by an increment in eNOS phosphorylation at serine 1176

[1177 in the human sequence]) [10] and inhibition of RhoA/Rho-kinase [11]. Statins also cause attenuation of both liver angiotensin II-induced inflammatory actions [22] and hepatic fibrosis via decreased turnover of hepatic stellate cells and downregulation of profibrotic cytokine expression [23, 24]. Recently, an overexpression of the transcription factor Krüppel-like factor 2 (KLF2) that orchestrates a variety of vasoprotective pathways induced by statins has also shown to confer hepatic endothelial vasoprotection and stellate cells deactivation[25, 26].

Among statins, simvastatin has been tested in clinical trials and has shown to lower portal pressure and hepatic resistance [9, 12] and even improved survival after variceal bleeding in patients with cirrhosis [27]. In cirrhotic rats though, atorvastatin showed a greater PP lowering effect together with an attenuation of fibrosis and hepatic stellate cell activation [11, 23] and a reduction in neo-angiogenesis in the splanchnic circulation [28]. NCX 6560, a NO-releasing derivative of atorvastatin, exerts greater lipid-lowering, antithrombotic and anti-inflammatory effects than atorvastatin and prevents statin-induced myopathy [29-32].

Thus, the aim of this study was to evaluate whether NCX 6560 is superior to conventional statins (simvastatin, atorvastatin) in improving portal hemodynamics and intrahepatic vascular alterations in liver cirrhosis, while decreasing the potential side effects of statins.

RESULTS

In the bile duct-ligation (BDL) model, cirrhosis was present in all rats, 30 % of the animals presented ascites and some animals died before completing statin treatment (see mortality rates in Table 1), whereas in the carbon tetrachloride (CCl_4) cirrhotic model, all rats that followed the 13-week CCl_4 inhalation protocol exhibited extensive fibrosis, but none presented ascites.

Adverse events associated with statin treatment in cirrhotic rats

As seen in Table 1, statin treatment in BDL-cirrhotic rats was associated with hepatic and muscular toxicity. Furthermore, statin treated BDL rats experienced a significant weight loss compared with vehicles during treatment (Suppl. table 1). Simvastatin treated BDL rats showed a higher mortality rate (80 % at SIM-25) compared with the other treatment groups and the remaining animals presented both muscular and hepatic toxicity (creatine kinase (CK) > 1000 IU/L and alanine aminotransferase (ALT) > 200 IU/L, respectively). Reduction in simvastatin dose to 10 mg/kg/day decreased mortality rate, but high hepatic and muscular toxicity rates were maintained. Although atorvastatin and NCX 6560 treatment did not produce an increment in mortality rates, BDL rats treated with atorvastatin (both ATO-10 and ATO-15) and with the highest dose of NCX 6560 (NCX-35.1) showed some hepatic and muscular toxicity. However, at equivalent doses of NCX 6560 (NCX-11.7 and NCX-17.5), hepatic toxicity was eliminated and muscular toxicity reduced. Considering the two equivalent doses (ATO-15, NCX-17.5 and ATO-10, NCX-11.7) the accumulated toxicity (hepatic and/or muscular toxicity) was significantly lower with NCX 6560 treatment, compared with atorvastatin treatment (ATO 37.9 % *vs* NCX 11.5 %; $p = 0.02$) (Table 1).

Regarding the CCl_4 model that mimics a compensated cirrhosis, no mortality events were reported with the different treatments and only animals treated with atorvastatin showed a higher weight loss and hepatic or muscular toxicity (ALT > 500 IU/L and CK > 6000 IU/L, respectively) (Table 2, Suppl. table 2).

Hemodynamic and biochemical changes due to statin treatment in cirrhotic rats

Due to the high mortality and hepatic toxicity rates related to simvastatin treatment in the BDL model, the remaining simvastatin treated rats were not considered in the study and the analysis of their samples and data was not performed.

In this animal model, atorvastatin and NCX 6560 treatment at equivalent doses significantly reduced PP levels without changing systemic hemodynamics, compared with vehicles; no significant differences in the PP lowering effect among the different groups were seen. Doubling NCX 6560 dose up to 35.1 mg/kg/day caused an increase in toxicity, without achieving a significant reduction in PP (Table 3). A similar scenario was observed in CCl_4-early cirrhotic rats treated with equivalent doses of atorvastatin and NCX-6560 (Table 4).

Suppl. table 1 and Suppl. table 2 show the biochemical parameters of blood samples from each group in the two models. Regardless of the treatment group, statin treated cirrhotic rats showed no changes in cholesterol levels. In the BDL model, alkaline phosphatase levels were lower in the treated groups than in vehicle (especially with ATO-10 and NCX-35.1), ATO-15 caused a significant increment in total bilirubin levels and treatment with both doses of atorvastatin produced a significant increase in serum creatinine levels with a decrease in urinary volume that was prevented with NCX 6560

treatment. Concerning the CCl_4 model, rats treated with NCX-17.5 showed significantly increased albumin levels compared to ATO-15.

Protein pathway analysis by Western blot in liver samples from statin treated cirrhotic rats

ATO-15 and the equivalent dose of NCX 6560 caused no changes in Rho-associated protein kinase 2 (Rock-2) protein expression in BDL cirrhotic rats compared with vehicle, but Rho-kinase activity, assessed as the phosphorylation of the endogenous Rho-kinase substrate, moesin, at Thr-558, was slightly lower, although not significantly. In CCl_4-cirrhotic rats both the expression of Rock-2 and its activity decreased with ATO-15 and NCX-17.5 treatment. In addition, a reduction in alpha-smooth muscle actin (α-SMA) protein expression with statin treatment was also observed in both models, showing a more prominent and significant decrease in the CCl_4 model (Fig 1).

Both treatments with atorvastatin and NCX 6560 in the BDL model not only caused a significant increment in p-eNOS, but also in total eNOS. This increase in eNOS protein levels was much higher in the NCX 6560 group, being significantly different from atorvastatin total eNOS levels. By contrast, in the CCl_4 model an increase in the p-eNOS/eNOS ratio was observed with both treatments, being much higher in the NCX 6560 group (Fig. 2). A mild non-significant decrease in the expression of the platelet/endothelial cell adhesion molecule-1 (CD31/PECAM-1) with NCX-17.5 treatment was also observed in the BDL model, while in the CCl_4 model this reduction was significant, suggesting an improvement in the endothelial phenotype. Regarding the expression of KLF2, we also observed a significant increment in its expression in BDL rats treated with NCX-17.5 compared with VEH and ATO-15 treatment (Fig. 3A).

Liver fibrosis, inflammation and liver cyclic guanosine monophosphate (cGMP) levels in statin treated cirrhotic rats

Sirius Red stained liver sections from 4-week BDL rats and 13-week CCl_4 and control rats treated with VEH, ATO-15 and NCX-17.5 are shown in Fig. 4A. Although not significant, both statin treatments caused a reduction in the red-stained area per total area, suggesting a trend in the decrease of the fibrotic rate in both models (Fig. 4B). In addition, statin treatment also increased liver tissue cGMP content, a marker of NO bioavailability in the BDL cirrhotic liver. Hepatic cGMP levels in animals treated with ATO-15 (1.91 ± 0.33 pmol/(mL.100 mg)) and with NCX-17.5 (1.42 ± 0.25 pmol/(mL.100 mg)) were significantly higher than in VEH (0.74 ± 0.05 pmol/(mL.100 mg); $p = 0.002$ and $p = 0.016$, respectively) and no significant differences between ATO-15 and NCX-17.5 were seen ($p = 0.259$). Moreover, there was a non-significant decrease in the number of leukosialin (CD43) immunostained cells in liver sections from animals treated with ATO-15 and NCX-17.5 in both models, suggesting a lower hepatic inflammatory state (Fig. 4C).

DISCUSSION

Statins are progressively becoming a focus of attention as a potential new therapy for chronic liver disease [33]. Indirect evidences from epidemiological studies indicate that statin use is associated with a decreased risk of fibrosis progression, cirrhosis, hepatic decompensation, hepatocellular carcinoma and death in patients with chronic liver disease, especially with hepatitis C virus infection [34-38] . More direct evidences from clinical trials point to a decreased mortality in decompensated cirrhotic patients receiving statins [27]. However, the mechanisms involved in these effects are not well known, differences among statins and doses have not been assessed and more importantly, concerns about the safety of statins in decompensated cirrhotic patients exist.

Our results in cirrhotic rats confirm previous findings: a trend (although not significant) towards a reduction in PP after statin treatment in CCl_4-intoxicated rats [10, 28] and a significant decrease in PP in BDL rats [11, 39], without changing systemic hemodynamics. Unfortunately, we were not able to evaluate systemic and portal hemodynamics with simvastatin treatment, not even after reducing the statin dose to 10 mg/kg/day, due to its high mortality and hepatic toxicity in the BDL model. Almost two decades ago, the first experiments with simvastatin were performed by Oberti and colleagues [40]. BDL rats treated with simvastatin 2.5mg/kg/day over a 4-week period from the beginning showed neither hepatic toxicity nor therapeutic benefit on hemodynamics and liver fibrosis. Subsequently, two more groups described effects in PP with 3-day simvastatin 25 mg/kg/day treatment in CCl_4-cirrhotic rats and a decrease in portal-systemic collateral vascular resistance and PP in partially portal vein-ligated rats receiving simvastatin 20 mg/kg/day for 9 days, but no data on liver function tests were given [10,

[41]. Differences among the results in these studies might be attributed to different treatments and animal models. However, it is clear that at high doses (10-25 mg/kg/day) and for 7 or more days, the rat BDL model is not suitable to study simvastatin effects on liver cirrhosis, probably due to the accumulation of active metabolites in the liver unable to be cleared through biliary excretion [42].

Although not in the same magnitude, atorvastatin treatment is also associated with some muscular and hepatic toxicity in the BDL model and with lower toxicity rates in the CCl_4 model. Specifically in our study, we ascribe the differences in toxicity to the contrast between the two models: while the effect of statins in an early cirrhotic state is tested with the CCl_4 model, the BDL model causes a more aggressive, cholestatic injury that together with the fact that the model itself impairs drug clearance mimics a severely deteriorated liver function. Thus, the higher toxicity rates observed with statin treatment in the BDL model reinforce the idea that despite their beneficial effects in liver cirrhosis, caution when prescribing statins is required, in patients with deteriorated liver function, who might develop rhabdomyolysis at lower doses than the general population [27].

Both simvastatin and atorvastatin induce a similar adaptative response in cells, their actions are qualitatively and mechanistically identical and the main difference between them is their pharmacokinetics [43]. Considering that the equipotent dose of simvastatin and atorvastatin in humans is about 2:1, since plasma is cleared of atorvastatin more slowly than it is of simvastatin [44, 45], and that both drugs are mainly eliminated through biliary excretion [42], apparently there is no clear explanation for the high differences in toxicity that we observed in BDL cirrhotic rats. However, a study of the mechanisms involved in statins cytotoxicity, mainly through oxidative stress, in freshly isolated rat

hepatocytes showed that simvastatin was the most cytotoxic statin [46]. Besides, statin drug-induced liver injury in humans is rare, but can be associated with severe outcomes [16-18], and safety data from statin clinical trials should be interpreted with caution given that they normally exclude patients with advanced liver failure, although current evidences indicate that tolerability is good even in patients with liver cirrhosis [27].

For all these reasons, NCX 6560, a NO-donating atorvastatin, could be a safer alternative to treating cirrhotic patients with portal hypertension. Our results in BDL and CCl_4 rats prove that this drug achieves an equivalent atorvastatin PP lowering effect without affecting systemic hemodynamics. NCX 6560 not only improves statin-induced myopathy [31], but also prevents hepatic toxicity caused by equivalent doses of atorvastatin, probably due to its greater anti-inflammatory and antioxidant properties [29, 30, 32]. However, higher doses of NCX 6560 (35.1 mg/kg/day) were not associated with any significant additional PP lowering effect, while toxicity increased moderately. By measuring SMABF, a surrogate of portal blood inflow, we show that NCX 6560 does not enhance splanchnic vasodilation of cirrhosis, which together with the lack of changes in mean arterial pressure (MAP), suggests that NCX 6560 effects are liver-selective. Moreover, impairment of systemic hemodynamics and the risk of renal failure, the main drawback of NO donors [7], seems to be prevented, since diuresis from NCX 6560 treated cirrhotic rats was not decreased and serum creatinine levels were maintained compared with vehicles, whereas atorvastatin treatment reduced urinary volume in both models and even significantly increased serum creatinine levels in the BDL model, compared with vehicles. Given that serum creatinine levels in atorvastatin treated animals with muscular toxicity were significantly higher than in animals

without it (data not shown), the higher incidence of muscular toxicity due to atorvastatin treatment could be related to kidney failure. In addition, NCX 6560 also improved albumin levels in the CCl_4 model, compared with the atorvastatin group.

No changes in serum cholesterol levels were seen among the different groups in the two models. Therefore, the advantages of atorvastatin and NCX 6560 in liver hemodynamics must be caused by the so-called pleiotropic effects of statins [19-21]. According to hemodynamic results, both atorvastatin and NCX 6560 seemed to have similar beneficial intrahepatic effects in the two models: they slightly reduced the liver fibrotic area, lowered the phosphorylated moesin (p-moesin) expression and significantly increased p-eNOS, compared with vehicles, confirming previous results with atorvastatin [11]. This was associated with an increase in hepatic cGMP, the second messenger of NO, indicating an improvement in NO availability, probably due to a decrease in oxidative stress related to statin therapy [20]. Although we expected higher cGMP levels in rats treated with NCX 6560, no significant differences between ATO-15 and NCX-17.5 were observed, which is in line with the fact that NCX 6560 neither exerted a greater PP lowering effect nor a higher reduction in Rho-kinase activity than atorvastatin. This suggests that the improvement in toxicity seen in our model with the NO-donating drug might be independent from the cGMP signaling pathway.

Additionally, NCX 6560 tended to have a slightly better anti-inflammatory effect in the two models, which could partially explain its lower hepatic toxicity. Moreover, NCX 6560 seemed to have a more pronounced beneficial intrahepatic effect because it significantly increased KLF2 and eNOS protein expression compared with the atorvastatin group in the BDL model, and the p-eNOS/eNOS ratio was much higher than in the atorvastatin group in the CCl_4 model. These effects could also be

contributing to its lower hepatic toxicity, improving the endothelial phenotype, as seen by the decrease of CD31, especially in the CCl_4 model, and therefore, to hepatocytes viability.

Another important factor contributing to increase IHVR in cirrhosis is hepatic stellate cell activation. The transdifferentiation process of hepatic stellate cells into myofibroblasts with contractile, pro-inflammatory and fibrogenic properties, involves expression of α-SMA and cytoskeleton reorganization with loss of lipid droplets [47]. We also observed a decrease in α-SMA protein expression, more prominent in the CCl_4 model, in livers from statin treated rats compared with vehicles. Apart from conferring hepatic vasoprotection, KLF2 also promotes stellate cells deactivation through a KLF2-NO-guanylate cyclase paracrine mechanism [26, 48]. The high KLF2 expression seen in livers from NCX 6560 treated BDL rats could contribute to the significant increment in eNOS in this treatment group, without a significant decrease in the vasoconstrictive pathway (RhoA, Rho-kinase) responsible for the inhibition of eNOS [25, 49]. Neither differences in KLF2 expression in livers from atorvastatin treated BDL rats compared with vehicles, nor significant increments with statin treatment in the CCl_4 model were observed. Marrone and colleagues [26] showed that atorvastatin was the less effective, among the statins tested, in inducing KLF2 mRNA expression in sinusoidal endothelial cells, and although KLF2 was shown to be induced early during the progression of cirrhosis [25], the disparity of the results could be attributed to differences in the induction of cirrhosis and the stage of liver disease. In general terms, the intrahepatic improvements of statin treatment were greater in the CCl_4 model, reinforcing a major benefit of statins when given earlier in the development of cirrhosis and for longer periods [23].

In summary, conventional statins ameliorate portal hypertension, but their adverse events are magnified in a model that mimics a deteriorated liver function. By contrast, NCX 6560 has similar effects in the two cirrhotic models with a safer toxicity profile compared with conventional statins. Additionally, due to its liver NO release, it induces a higher intrahepatic vasoprotective profile that might have a more long-term beneficial effect in the intrahepatic vascular alterations of portal hypertension than conventional statins. Thus, these results suggest that NCX 6560 could be a safer option for long-term statin treatment of portal hypertension in cirrhotic patients.

MATERIAL AND METHODS

Experimental design

Two different approaches were designed in which statins were evaluated:

(i) Four-week BDL rats: a cirrhotic model that simulates a decompensated chronic liver disease in order to compare the efficacy and toxicity among statins (7 days) and establish the appropriate dose.

(ii) Thirteen-week CCl_4-treated rats: a model of early cirrhosis in order to evaluate their beneficial intrahepatic effects after a longer treatment period (10 days).

Experimental models of cirrhosis

In the decompensated chronic liver disease model, cirrhosis was induced by BDL. Male Sprague-Dawley OFA rats (Charles River Laboratories, L'Arbresle, France) weighing 200-220 g were anaesthetized with inhaled isoflurane and the common bile duct was occluded by double ligature with a 4-0 silk thread. The bile duct was then resected between the two ligatures. Animals received weekly intramuscular vitamin k1 to decrease mortality from bleeding [50].

To obtain an early cirrhosis model, male Wistar rats (Charles River Laboratories, L'Arbresle, France) weighing 100-120 g followed a CCl_4 inhalation protocol [39] for 13 weeks.

All rats were kept under constant temperature and humidity in a 12 h controlled dark/light cycle and fed *ad libitum* with a grain-based chow (Teklad 2014, Harlan Laboratories, Indianapolis, IN, USA) containing a fixed formula of ingredients with 0.1 % Na^+.

Drug administration

Four-week BDL rats received daily (q.d.) oral doses of statins or vehicle for 7 days. Two doses of each statin were evaluated: simvastatin (Ratiopharm, Madrid, Spain) 25 mg/kg/day (SIM-25, n = 10) and 10 mg/kg/day (SIM-10, n = 11), atorvastatin (Almirall, Barcelona, Spain) 15 mg/kg/day (ATO-15, n = 14) and 10 mg/kg/day (ATO-10, n = 15) and NCX 6560 ((βR,δR)-2(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1heptanoic acid 4-(nitrooxy)butyl ester) (NicOx S.A., Sophia Antipolis, France) 17.5 mg/kg/day (NCX-17.5, n = 11) and 11.7 mg/kg/day (NCX-11.7, n = 15). An additional dose of NCX 6560 at 35.1 mg/kg/day (NCX-35.1, n = 15) was also tested and polyethylene glycol (PEG) 70 % was used as a vehicle (VEH, n = 12). Starting dosage was chosen according to previous literature [10, 11, 23, 39] and the equivalent doses of NCX 6560 and atorvastatin used were calculated on the basis of their different molecular weights (MW NCX 6560 / MW atorvastatin: ratio 1.17) and contained the same amount of atorvastatin: ATO-15 equals to NCX-17.5 and ATO-10 to NCX-11.7.

In the CCl_4-cirrhotic model, the treatments (ATO-15, n = 13, NCX-17.5, n = 13 and VEH, n = 8) were given orally (q.d.) for 10 days. CCl_4 inhalation was kept during treatment to mimic a continuous and chronic liver injury, but last inhalation session was at least 3 days before ending treatment. Control rats (CTL, n = 8) received phenobarbital in drinking water, but neither followed the CCl_4 inhalation protocol nor received any treatment.

All treatments in the different studies were administered by gastric gavage. During treatment rats were weighed daily and experiments were performed ninety minutes after the last dose of statin or vehicle.

Hemodynamic measurements

Rats were anaesthetized for continuous measurement of MAP (mmHg), PP (mmHg), superior mesenteric artery (SMA) blood flow (SMABF, mL/[min.100 g]) and portal blood flow (PBF, mL/[min.100 g]). SMA resistance (SMAR, mmHg/mL.min.100 g) was calculated as ([MAP-PP]/SMABF) and IHVR as (PP/PBF) (see details in **supplementary material**).

Sample collection

For the determination of the diuresis volume, urine was collected from the bladder at the end of the 1-hour period of hemodynamic registration. Venous blood samples were obtained from the cava vein after the hemodynamic measurements. Liver from cirrhotic rats was perfused with saline for exsanguination and cut into fragments. Liver samples were either snap-frozen in liquid nitrogen and stored at -80 ºC or fixed in 4 % formaldehyde solution for 24 h and changed to 50 % ethanol solution before paraffin embedding.

Biochemical parameters

Serum levels of sodium, potassium, osmolality, creatinine, total bilirubin, ALT and aspartate aminotransferase (AST), alkaline phosphatase (ALP), CK, albumin and cholesterol were determined using an automatic analyzer (Olympus AV5400, Olympus Europe GmbH, Hamburg, Germany).

Hepatic and muscular toxicity due to statin treatment was defined based on ALT and CK levels in vehicle rats from both models. Thus, we defined hepatic toxicity as an increment in ALT levels superior to 200 IU/L (BDL model) and to 500 IU/L (CCl_4 model),

whereas muscular toxicity was considered when CK levels were above 1000 IU/L (BDL model) and 6000 IU/L (CCl_4 model). Animals with hepatic toxicity due to statin treatment were discarded from the study and were not used for sample and data analysis.

Western blot analysis

Protein extraction from liver samples and immunoblotting was performed as previously described [10] (see details in **supplementary material**). The following primary antibodies were used: α-SMA (dil. 1/200) (Abcam, Cambridge, UK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, dil. 1/5000) (Ambion, Austin, TX, USA), eNOS (dil. 1/500) (BD Transduction Laboratories, Lexington, KY, USA), phosphorylated eNOS (p-eNOS, Ser1177, dil. 1/250), KLF2 (N-13, dil. 1/200), CD31/PECAM-1 (M-20, dil. 1/200), p-moesin (Thr 558, dil. 1/200) and Rock-2 (H-85, dil. 1/200) (Santa Cruz Biotechnology, Dallas, TX, USA).

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) and compared with vehicles using Student's *t* test (SigmaStat 3.0) or ANOVA test, when indicated. For the toxicity analysis, Fisher Exact test for contingency tables were used. Statistical significance was established at $p \leq 0.05$.

Ethic statement

All animals received humane care in accordance with institutional guidelines from the European Commission on the protection of animals used for experimental and other

scientific purposes (Directive 2010/63/EU). All experiments were approved by the Animal Care Committee of the Vall d'Hebron Institut de Recerca (VHIR, Barcelona, Spain) (DAAM Permission No.: 7834) and conducted in the animal facilities of VHIR.

See **supplementary material** for details on Sirius Red staining, immunohistochemistry and cGMP determination.

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Author's contribution:

S.R: Study concept and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; *I.R., M.T. and T.G-L.:* Acquisition and analysis and interpretation of data, and administrative, technical, or material support; *J.G. and M.M.:* Study concept and design, analysis and interpretation of data, draft and final revision of the manuscript, obtained funding, and study supervision; All authors reviewed the manuscript.

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All authors declare to have nothing to disclose.

Table 1. Adverse events after one week treatment in 4-week bile duct-ligated rats.

	Dose (mg/kg/day)	n	Mortality rate (%)	Hepatic toxicity rate (%)	Muscular toxicity rate (%)
Vehicle		12	16.7	0	0
Simvastatin	25	10	80	100	100
	10	11	18.2	66.7	77.8
Atorvastatin	15	14	0	14.3	35.7
	10	15	6.7	6.7	33.3
NCX 6560	35.1	15	6.7	20	33.3
	17.5	11	0	0	9.1
	11.7	15	0	0	13.3

n, number of rats that initiated the treatment protocol; Mortality rate, % of animals that died during the experimental protocol (through treatment or anesthesia); Hepatic toxicity rate, % of animals with serum ALT levels > 200 IU/L; Muscular toxicity rate, % of animals with serum CK levels > 1000 IU/L. Equivalent doses: NCX 6560 17.5 mg/kg/day equals to atorvastatin 15 mg/kg/day and NCX 6560 11.7 mg/kg/day equals to atorvastatin 10mg/kg/day.

Table 2. Adverse events in control rats and in 13-week CCl_4-induced cirrhotic rats after a 10-day treatment.

	Dose (mg/kg/day)	n	Mortality rate (%)	Hepatic toxicity rate (%)	Muscular toxicity rate (%)
CCl_4-vehicle		8	0	0	0
CCl_4-atorvastatin	15	13	0	7.69	7.69
CCl_4-NCX 6560	17.5	13	0	0	0
Control		8	0	0	0

CCl_4, cirrhotic rats induced by carbon tetrachloride; n, number of rats that initiated the treatment protocol; Mortality rate, % of animals that died during the experimental protocol (through treatment or anesthesia); Hepatic toxicity rate, % of animals with serum ALT levels > 500 IU/L; Muscular toxicity rate, % of animals with serum CK levels > 6000 IU/L. Equivalent doses: NCX 6560 17.5 mg/kg/day equals to atorvastatin 15 mg/kg/day.

Table 3. Hemodynamic measurements in 4-week bile duct-ligated rats after one-week treatment (values taken 2 h 30 min after the last dose of treatment).

	Dose (mg/kg/day)	n	MAP (mmHg)	PP (mmHg)	SMABF (mL/[min·100 g])	SMAR (mmHg/mL·min·100 g)	Heart rate (bpm)
Vehicle		8	96.39 ± 6.84	18.53 ± 0.56	4.42 ± 0.31	17.99 ± 1.73	318.10 ± 12.88
Atorvastatin	15	9	82.46 ± 4.44	16.27 ± 0.67 *	4.98 ± 0.41	13.95 ± 1.34	314.82 ± 9.96
	10	11	84.11 ± 4.22	15.77 ± 0.59 **	3.63 ± 0.26	19.60 ± 1.59	314.65 ± 12.75
NCX 6560	35.1	9	105.83 ± 5.11	17.75 ± 0.64	4.30 ± 0.41	22.10 ± 2.43	327.77 ± 15.92
	17.5	9	85.35 ± 5.95	16.25 ± 0.86 *	3.88 ± 0.37	19.24 ± 2.22	310.84 ± 13.40
	11.7	9	95.67 ± 6.42	16.43 ± 0.63 *	3.91 ± 0.41	22.17 ± 3.18	330.51 ± 9.05

Values are expressed as mean ± SEM. n, number of rats; MAP, mean arterial pressure; PP, portal pressure; SMABF, superior mesenteric artery blood flow; SMAR, superior mesenteric artery resistance. Equivalent doses: NCX 6560 17.5 mg/kg/day equals to atorvastatin 15 mg/kg/day and NCX 6560 11.7 mg/kg/day equals to atorvastatin 10 mg/kg/day. * $p \leq 0.05$, ** $p \leq 0.01$ compared with vehicle.

Table 4. Hemodynamic measurements in control rats and in 13-week CCl_4-induced cirrhotic rats after a 10-day treatment.

	CCl_4-vehicle	CCl_4-atorvastatin (15 mg/kg/day)	CCl_4-NCX 6560 (17.5 mg/kg/day)	Control
n	8	11	13	7
MAP (mmHg)	81,56 ± 6,62	85,52 ± 2,69	81,73 ± 3,65	86,46 ± 7,74
PP (mmHg)	9,39 ± 0,62	8,77 ± 0,42	8,49 ± 0,31	7,59 ± 0,44 *
SMABF (mL/[min·100 g])	2,85 ± 0,56	2,23 ± 0,34	2,32 ± 0,31	1,63 ± 0,23
SMAR (mmHg/mL·min·100 g)	28,51 ± 4,42	39,86 ± 4,26	36,28 ± 3,89	51,90 ± 6,61 *
Heart rate (bpm)	318,28 ± 18,21	360,31 ± 17,86	307,27 ± 12,50	318,16 ± 17,31
PBF (mL/[min·100 g])	1,95 ± 0,26	2,08 ± 0,35	2,10 ± 0,21	1,66 ± 0,27
IHVR (mmHg/mL·min·100 g)	5,61 ± 0,96	5,02 ± 0,66	4,32 ± 0,42	5,02 ± 0,74

Values are expressed as mean ± SEM. CCl_4, cirrhotic rats induced by carbon tetrachloride; n, number of rats; MAP, mean arterial pressure; PP, portal pressure; SMABF, superior mesenteric artery blood flow; SMAR, superior mesenteric artery resistance; PBF, portal blood flow; IHVR, intrahepatic vascular resistance. * $p \leq 0.05$ compared with CCl_4-vehicle.

FIGURE LEGENDS

Fig.1. Western blot analysis of intrahepatic markers involved in vasoconstriction and hepatic stellate cell activation. Bar diagrams showing protein quantification of Rho-associated protein kinase 2 (Rock-2), p-moesin and α-smooth muscle actin (α-SMA). Protein levels are expressed as mean ± SEM and normalized to vehicles. Representative Western blots are shown below. GAPDH was used as loading control. (A) 4-week bile duct-ligated (BDL) rats after one-week treatment. VEH: vehicle, n = 8; ATO: atorvastatin 15 mg/kg/day,n = 9; NCX: NCX 6560 17.5 mg/kg/day, n = 9. (B) Control and 13-week carbon tetrachloride (CCl_4) rats after a 10-day treatment. CTL: control, n = 7; VEH: vehicle, n = 7; ATO: atorvastatin 15 mg/kg/day, n = 7; NCX: NCX 6560 17.5 mg/kg/day, n = 7. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared with vehicle.

Fig.2. Western blot analysis of intrahepatic eNOS expression. Bar diagrams showing protein quantification of endothelial nitric oxide synthase (eNOS) and p-eNOS. Protein levels are expressed as mean ± SEM and normalized to vehicles. Representative Western blots are shown below. GAPDH was used as loading control. (A) 4-week bile duct-ligated (BDL) rats after one-week treatment. VEH: vehicle, n = 8; ATO: atorvastatin 15 mg/kg/day, n = 9; NCX: NCX 6560 17.5 mg/kg/day, n = 9. (B) Control and 13-week carbon tetrachloride (CCl_4) rats after a 10-day treatment. CTL: control, n = 2; VEH: vehicle, n = 7; ATO: atorvastatin 15 mg/kg/day, n = 7; NCX: NCX 6560 17.5 mg/kg/day, n = 7. * $p \leq 0.05$, *** $p \leq 0.001$ compared with vehicle. † $p \leq 0.05$, ††† $p \leq 0.001$ compared with the equivalent dose of atorvastatin.

Fig.3. Western blot analysis of intrahepatic markers involved in endothelial dysfunction. Bar diagrams showing protein quantification of CD31 and Krüppel-like factor 2 (KLF2). Protein levels are expressed as mean ± SEM and normalized to vehicles. Representative Western blots are shown below. GAPDH was used as loading control. (A) 4-week bile duct-ligated (BDL) rats after one-week treatment. VEH: vehicle, n = 8; ATO: atorvastatin 15 mg/kg/day, n = 9; NCX: NCX 6560 17.5 mg/kg/day, n = 9. (B) Control and 13-week carbon tetrachloride (CCl_4) rats after a 10-day treatment. CTL: control, n = 2; VEH: vehicle, n = 7; ATO: atorvastatin 15 mg/kg/day, n = 7; NCX: NCX 6560 17.5 mg/kg/day, n = 7. * $p \leq 0.05$, ** $p \leq 0.01$ compared with vehicle. † $p \leq 0.05$, compared with the equivalent dose of atorvastatin.

Fig.4. Histologic assessment of liver fibrosis and inflammation. (A) Representative images of liver sections stained by Sirius Red from 4-week bile duct-ligated (BDL) rats or 13-week carbon tetrachloride (CCl_4) rats treated with statins or vehicle and from control rats (original magnification: 4x [white scale bar=200 µm]). (B) Bar diagram showing Sirius Red quantification expressed as fibrotic rate (%). (C) Bar diagrams showing leukosialin (CD43) immunohistochemistry quantification in livers from 4-week BDL rats and 13-week CCl_4 rats treated with statins or vehicle and from control rats. VEH: vehicle (BDL: n = 8, CCl_4: n = 7); ATO: atorvastatin 15 mg/kg/day (BDL: n = 9, CCl_4: n = 7); NCX: NCX 6560 17.5 mg/kg/day (BDL: n = 9, CCl_4: n = 7); CTL: controls (n = 7). ** $p \leq 0.01$ compared with vehicle.

Figure 1

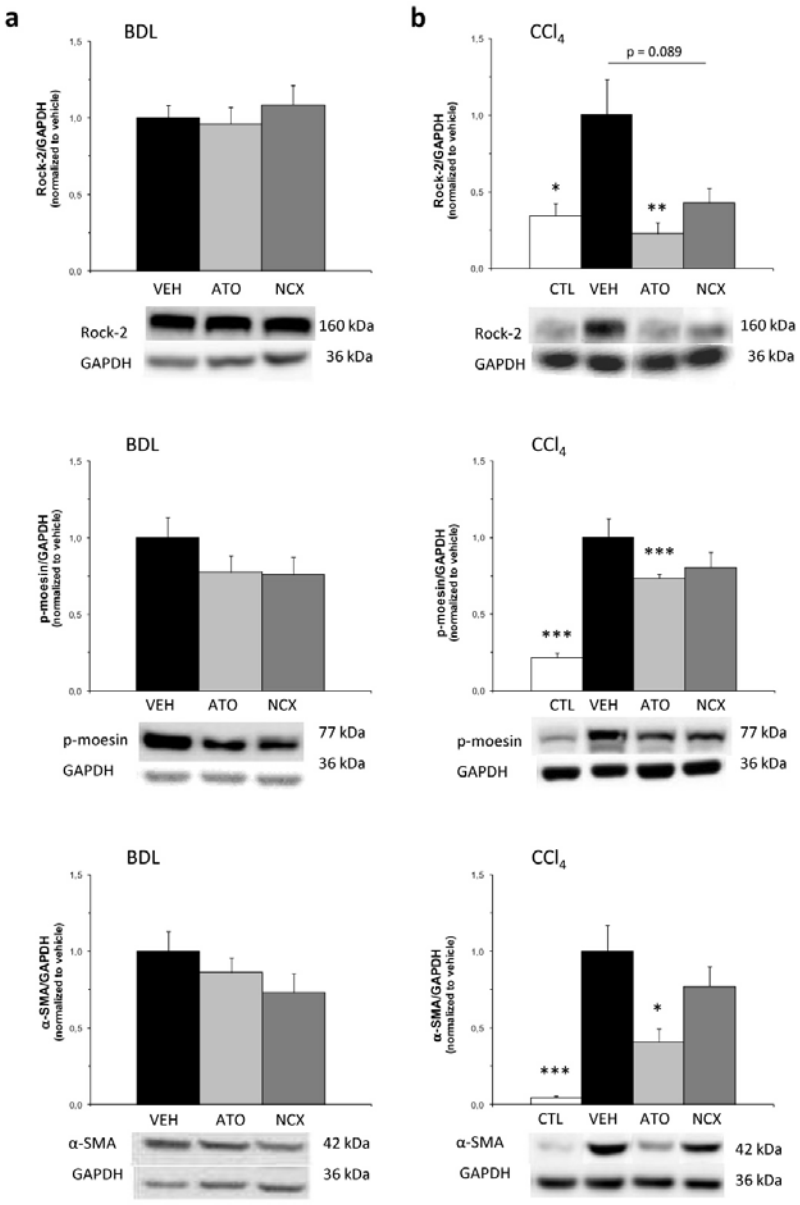

a
BDL
Rock-2/GAPDH (normalized to vehicle)
VEH
ATO
NCX
Rock-2
GAPDH
160 kDa
36 kDa
b
CCl4
p = 0.089
CTL
VEH
ATO
NCX
p-moesin/GAPDH (normalized to vehicle)
p-moesin
77 kDa
α-SMA/GAPDH (normalized to vehicle)
α-SMA
42 kDa

Figure 2

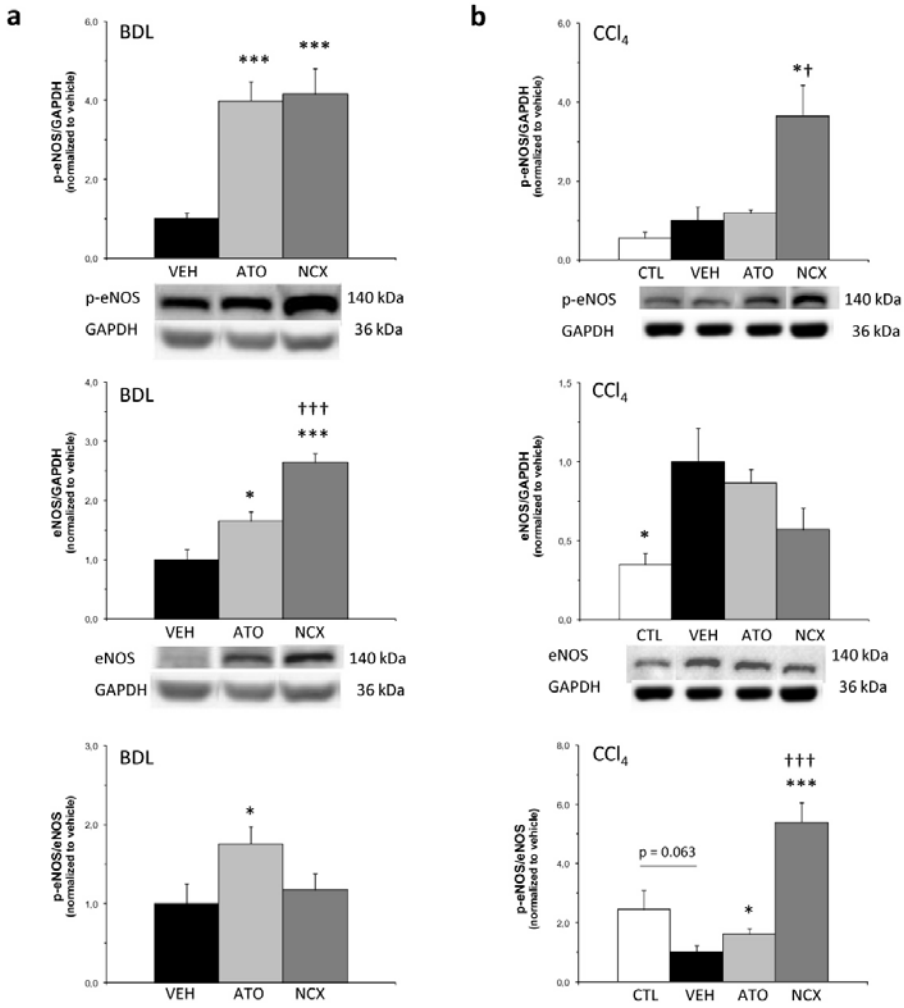

a
BDL
p-eNOS/GAPDH (normalized to vehicle)
***
***
VEH
ATO
NCX
p-eNOS
GAPDH
140 kDa
36 kDa
BDL
eNOS/GAPDH (normalized to vehicle)
†††
***
*
VEH
ATO
NCX
eNOS
GAPDH
140 kDa
36 kDa
BDL
p-eNOS/eNOS (normalized to vehicle)
*
VEH
ATO
NCX
b
CCl4
p-eNOS/GAPDH (normalized to vehicle)
*†
CTL
VEH
ATO
NCX
p-eNOS
GAPDH
140 kDa
36 kDa
CCl4
eNOS/GAPDH (normalized to vehicle)
*
CTL
VEH
ATO
NCX
eNOS
GAPDH
140 kDa
36 kDa
CCl4
p-eNOS/eNOS (normalized to vehicle)
p = 0.063
*
†††
***
CTL
VEH
ATO
NCX

Figure 3

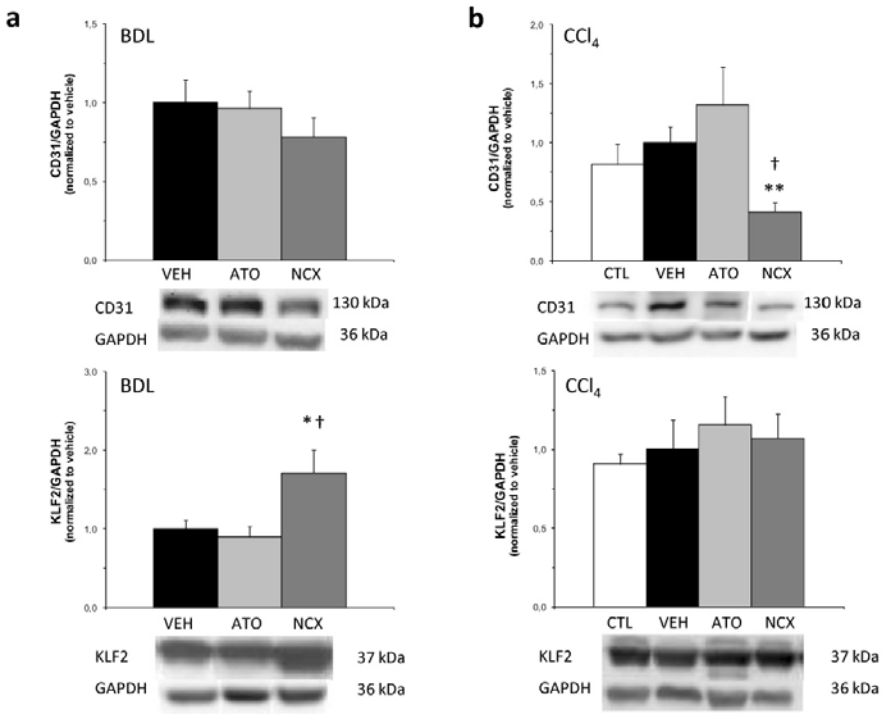
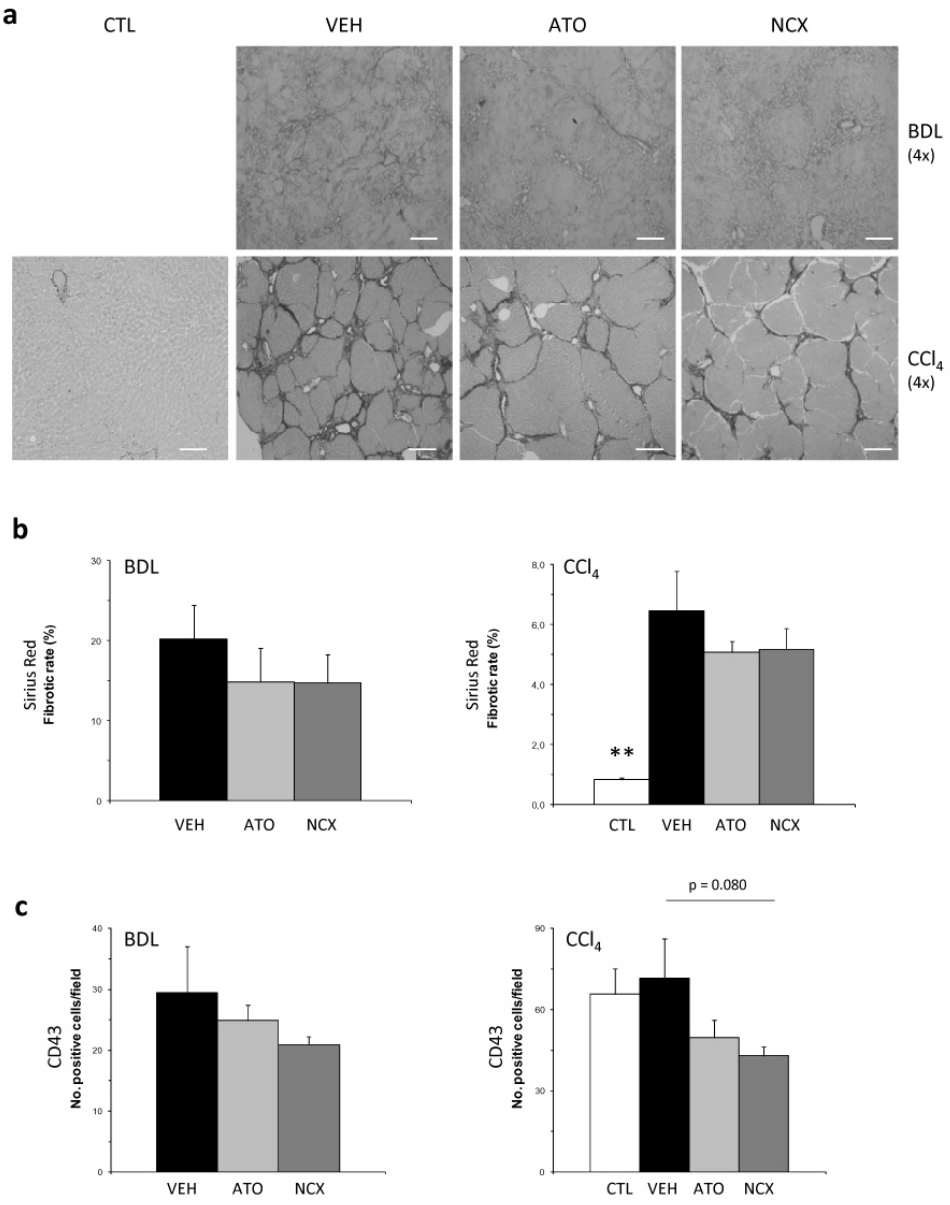

a
BDL
CD31/GAPDH (normalized to vehicle)
VEH
ATO
NCX
CD31
GAPDH
130 kDa
36 kDa
BDL
KLF2/GAPDH (normalized to vehicle)
*†
KLF2
GAPDH
37 kDa
36 kDa
b
CCl4
CD31/GAPDH (normalized to vehicle)
†
**
CTL
VEH
ATO
NCX
CD31
GAPDH
130 kDa
36 kDa
CCl4
KLF2/GAPDH (normalized to vehicle)
KLF2
GAPDH
37 kDa
36 kDa

Figure 4


a
CTL
VEH
ATO
NCX
BDL
(4x)
CCl4
(4x)
b
BDL
Sirius Red
Fibrotic rate (%)
VEH
ATO
NCX
CCl4
**
CTL
c
CD43
No. positive cells/field
p = 0.080

Supplementary material to:

A NITRIC OXIDE-DONATING STATIN DECREASES PORTAL PRESSURE WITH A BETTER TOXICITY PROFILE THAN CONVENTIONAL STATINS IN CIRRHOTIC RATS

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Methods

Hemodynamic measurements

Ninety minutes after statin or vehicle administration, 16-hour fasted rats were anaesthetized with ketamine hydrochloride (100 mg/kg) plus midazolam (5 mg/kg) intraperitoneally. Dose adjustments in anaesthesia were made depending on animal's condition and the treatment received. One polyethylene PE-catheter (PE50) was introduced into the femoral artery to MAP (mmHg) and after a midline abdominal incision, another catheter was introduced into the ileocolic vein for PP measurement (mmHg) using highly sensitive pressure transducers (Harvard Apparatus, Holliston, MA, USA). The SMA was isolated from connective tissue and a perivascular ultrasonic transit-time flowprobe (1mm diameter, Transonic Systems Inc., Ithaca, NY, USA) was placed around the artery to continuously measure the SMABF (mL/[min.100 g]). SMAR (mmHg/mL.min.100 g) was calculated as ([MAP-PP]/SMABF). The same flowprobe was placed on the dissected portal vein to measure portal blood flow (PBF, mL/[min.100 g]) and IHVR was calculated as (PP/PBF).

Ascites volume was determined and body weight calculated as (rat weight - ascites volume) considering 1 mL = 1 g. Animals were maintained at 37 ºC throughout the study by a rectal temperature probe.

Hemodynamic parameters were allowed to equilibrate and measures were obtained 2 h 30 min after the last dose of statin or vehicle (cirrhotic animals) or 1 h after manipulation (control rats), each value representing the average of 30 seconds. Animals were euthanized by exsanguination under anaesthesia.

Western blot analysis

For whole protein extraction samples of snap-frozen livers were crushed to powder while frozen and subsequently homogenized in Triton-lysis buffer (25.4 mM Tris/HCl pH 7.6, 137 mM NaCl, 2.7 mM KCl, 20 mM NaF, 10 mM $Na_4P_2O_7$, 10 nM okadaic acid, 2 mM Na_3VO_4, 2 μg/mL antipain, 2 μg/mL aprotinin, 2 μg/mL chymostatin, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 2 μg/mL trypsin inhibitor, 40 μg/mL phenylmethylsulfonylfluoride, and 10 % v/v Triton X-100). Thereafter they were sonicated (3 x 10 s), left on ice for 10 min, and centrifuged at 4 ºC and 18,000 *g* for 10 min. Supernatant protein concentration was assessed by BCA ™ Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein (30-70 μg of protein/lane) were run on a 4-12 % or 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) depending on the protein molecular weight. Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA USA) and membranes were blocked and incubated with primary antibodies and thereafter with corresponding secondary peroxidase-coupled antibody. GAPDH served as endogenous control. Blots were developed with enhanced chemioluminiscence (Amersham ECL Prime, GE Healthcare, Uppsala, Sweden) and protein expression was determined by densitometric analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Sirius Red staining

Liver fibrosis was assessed in 4μm sections from paraffin embedded liver samples. To detect collagen fibers, liver sections were deparaffinised, rehydrated, and stained with 0.1 % Picro-Sirius Red (Direct Red 80 in saturated aqueous picric acid [Sigma-Aldrich,

Saint Louis, MO, USA]). The fibrotic area was then assessed using image analysis techniques. Briefly, ten fields (10x magnification) from each Sirius Red stained section were randomly obtained using an optical microscope Olympus BX61 (Olympus, Hamburg, Germany) equipped with a digital camera (large bile ducts and vessels excluded) and the red-stained area per total area was measured using ImageJ 1.38 free software (National Institute of Health, Bethesda, MD, USA) and expressed as fibrotic rate (%).

Immunohistochemistry

Liver inflammation was assessed by anti-CD43 (a pan-leukocyte marker) immuhistochemistry in 4μm sections from paraffin embedded liver samples. Briefly, liver sections were deparaffinised, rehydrated, blocked with 1/20 dilution of goat serum and incubated overnight with primary antibody against CD43 (W3/13, dil. 1/500, AbD Serotec Ltd, Oxford, UK). Bound antibody was incubated with ENVISION-HRP anti-mouse secondary antibody (Dako, Glostrup, Denmark), visualized with the VIP substrate kit (Vector. Burlingame, CA, USA) that produces a purple precipitate and counterstained with hematoxylin. Ten fields per section were randomly captured at 10x magnification and images were quantified with the manual cell counter of Image J 1.38 free software (National Institute of Health, Bethesda, MD, USA) obtaining the number of CD43 positive cells per field.

Liver cyclic guanosine monophosphate (cGMP) concentration

Measurements of cGMP, a marker of NO bioavailability, were performed in liver homogenates from VEH, ATO-15 and NCX-17.5 treated animals without hepatic toxicity

(n=7, n=5 and n=6, respectively). Samples of frozen tissue were crushed to powder and aliquots from each sample containing 100-200 mg of tissue dropped into 5 volumes of 5 % trichloroacetic acid and homogenized on ice. The precipitate was removed by centrifugation at 1500 *g* for 10 min at 4 ºC and the supernatant transferred to a clean test tube, washed five times with five volumes of water-saturated diethyl ether and the aqueous phase extract lyophilized. The dried extract was dissolved in ultrapure water and cGMP levels were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI, USA). Results were expressed as pmol/(mL.100 mg).

Supplementary tables

Supplementary table 1. Characteristics and biochemical parameters of 4-week bile duct-ligated rats after one-week treatment.

	Vehicle	Atorvastatin (15 mg/kg/day)	Atorvastatin (10 mg/kg/day)	NCX 6560 (35.1 mg/kg/day)	NCX 6560 (17.5 mg/kg/day)	NCX 6560 (11.7 mg/kg/day)
n	8	9	11	9	9	9
Body weight (g)	331.10 ± 9.86	313.78 ± 20.55	278.21 ± 11.07 **	297.47 ± 12.10	300.99 ± 10.54	305.26 ± 9.25
Weight loss during treatment (g)	2.13 ± 3.54	41.59 ± 6.03 ***	34.93 ± 6.39 ***	42.30 ± 5.36 ***	25.70 ± 5.31 **	29.01 ± 4.45 ***
Urinary volume (mL/h)	0.71 ± 0.17	0.34 ± 0.08	0.21 ± 0.07 **	0.99 ± 0.15	0.76 ± 0.15 †	0.69 ± 0.15 ††
Serum Na^+ (mmol/L)	142.10 ± 1.44	140.81 ± 0.43	140.56 ± 0.70	141.34 ± 0.82	141.20 ± 0.74	141.30 ± 0.98
Serum K^+ (mmol/L)	4.72 ± 0.28	4.65 ± 0.20	4.57 ± 0.19	4.75 ± 0.21	4.84 ± 0.25	4.66 ± 0.13
Serum creatinine (mg/[dL.100 g])	0.12 ± 0.01	0.17 ± 0.02 *	0.19 ± 0.02 **	0.17 ± 0.02	0.14 ± 0.01	0.15 ± 0.01
Serum osmolality (mOsm/kg)	310.88 ± 2.36	304.44 ± 2.63	302.18 ± 2.34 *	316.11 ± 8.08	300.78 ± 1.61 **	304.50 ± 3.09
Total bilirubin (mg/dL)	8.15 ± 0.21	9.45 ± 0.44 *	7.62 ± 0.91	8.61 ± 0.50	8.27 ± 0.59	8.06 ± 0.57
AST (IU/L)	509.14 ± 76.10	615.75 ± 79.07	530.00 ± 88.61	1022.89 ± 253.93	462.11 ± 38.53	465.11 ± 26.15
ALT (IU/L)	68.38 ± 6.12	76.63 ± 9.57	80.00 ± 7.60	91.11 ± 10.26	72.78 ± 6.69	82.67 ± 7.98
Alkaline phosphatase (IU/L)	566.50 ± 62.92	438.38 ± 31.36	405.73 ± 26.83 *	376.67 ± 20.85 **	423.11 ± 36.53	410.89 ± 54.13
Creatine kinase (IU/L)	586.63 ± 72.03	640.22 ± 142.49	810.36 ± 209.00	830.50 ± 182.17	473.56 ± 85.25	428.00 ± 47.40
Serum cholesterol (mg/dL)	138.88 ± 13.87	153.67 ± 22.40	141.55 ± 11.35	138.56 ± 10.03	147.33 ± 15.56	130.11 ± 9.58
Serum albumin (g/dL)	2.37 ± 0.11	2.25 ± 0.11	2.23 ± 0.11	2.32 ± 0.06	2.26 ± 0.10	2.22 ± 0.15

Values are expressed as mean ± SEM. n, number of rats; AST, aspartate aminotransferase; ALT, alanine aminotransferase. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared with vehicle. † $p \leq 0.05$, †† $p \leq 0.01$ compared with the equivalent dose of atorvastatin (NCX 6560 17.5 mg/kg/day vs. atorvastatin 15 mg/kg/day and NCX 6560 11.7 mg/kg/day vs. atorvastatin 10 mg/kg/day).

Supplementary table 2. Characteristics and biochemical parameters of control and 13-week CCl_4-induced cirrhotic rats after a 10-day treatment.

	CCl_4-vehicle	CCl_4-atorvastatin (15 mg/kg/day)	CCl_4-NCX 6560 (17.5 mg/kg/day)	Control
n	8	12	13	8
Body weight (g)	354,45 ± 9,91	362,13 ± 8,85	344,88 ± 10,79	397,05 ± 12,55 *
Weight loss during treatment (g)	3,08 ± 1,37	10,15 ± 3,67	1,59 ± 2,50	N/A
Urinary volume (mL/h)	0,25 ± 0,05	0,21 ± 0,05	0,30 ± 0,05	0,32 ± 0,15
Serum Na^+ (mmol/L)	143,50 ± 0,59	141,89 ± 0,74	142,42 ± 0,65	141,93 ± 0,63
Serum K^+ (mmol/L)	5,00 ± 0,18	4,98 ± 0,21	5,34 ± 0,24	5,11 ± 0,20
Serum creatinine (mg/[dL.100 g])	0,25 ± 0,01	0,24 ± 0,02	0,24 ± 0,01	0,24 ± 0,01
Serum osmolality (mOsm/kg)	312,20 ± 1,72	311,67 ± 3,96	306,62 ± 1,74	311,00 ± 4,54
Total bilirubin (mg/dL)	0,08 ± 0,02	0,09 ± 0,02	0,11 ± 0,02	0,09 ± 0,02
AST (IU/L)	261,75 ± 47,94	210,50 ± 18,76	266,85 ± 34,62	147,88 ± 17,90 *
ALT (IU/L)	121,50 ± 48,40	120,83 ± 14,55	134,46 ± 27,64	67,00 ± 12,77
Alkaline phosphatase (IU/L)	94,25 ± 9,08	106,67 ± 4,84	116,15 ± 8,05	85,88 ± 6,49
Creatine kinase (IU/L)	1557,00 ± 229,85	1943,50 ± 592,66	1855,77 ± 328,62	1565,13 ± 279,49
Serum cholesterol (mg/dL)	64,20 ± 5,99	67,70 ± 3,40	74,23 ± 5,05	61,88 ± 2,74
Serum albumin (g/dL)	2,70 ± 0,05	2,71 ± 0,04	2,88 ± 0,07 †	2,94 ± 0,06 **

Values are expressed as mean ± SEM. CCl_4, cirrhotic rats induced by carbon tetrachloride; n, number of rats; N/A, not applicable; AST, aspartate aminotransferase; ALT, alanine aminotransferase. * $p \leq 0.05$, ** $p \leq 0.01$ compared with CCl_4-vehicle. † $p \leq 0.05$ compared with CCl_4-atorvastatin (15 mg/kg/day).

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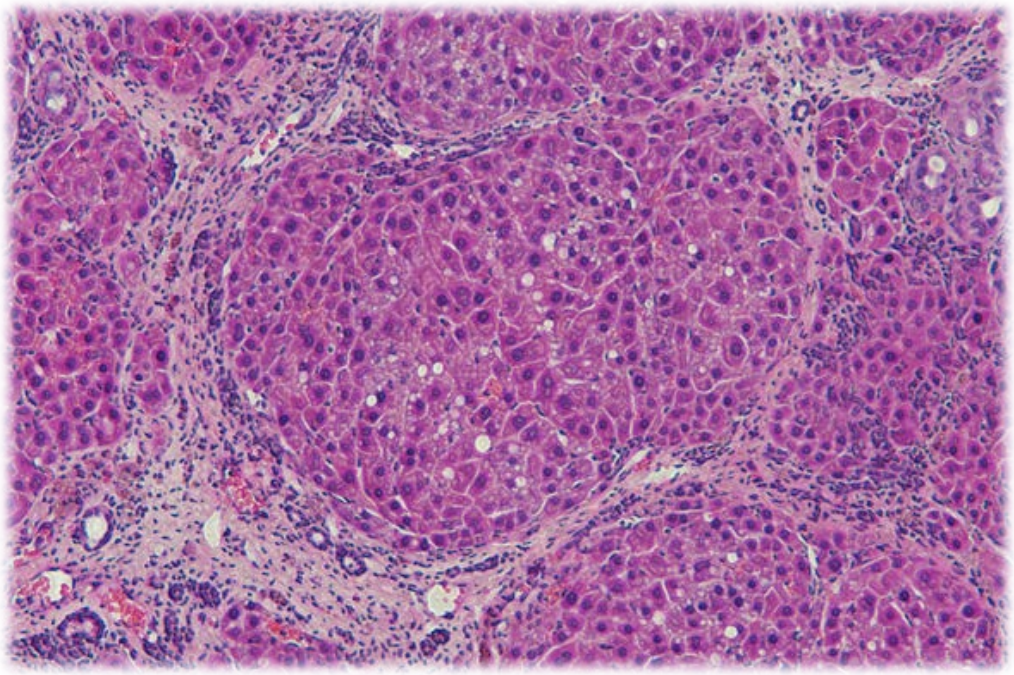

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