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Dipartimento di Medicina Interna e Terapia Medica

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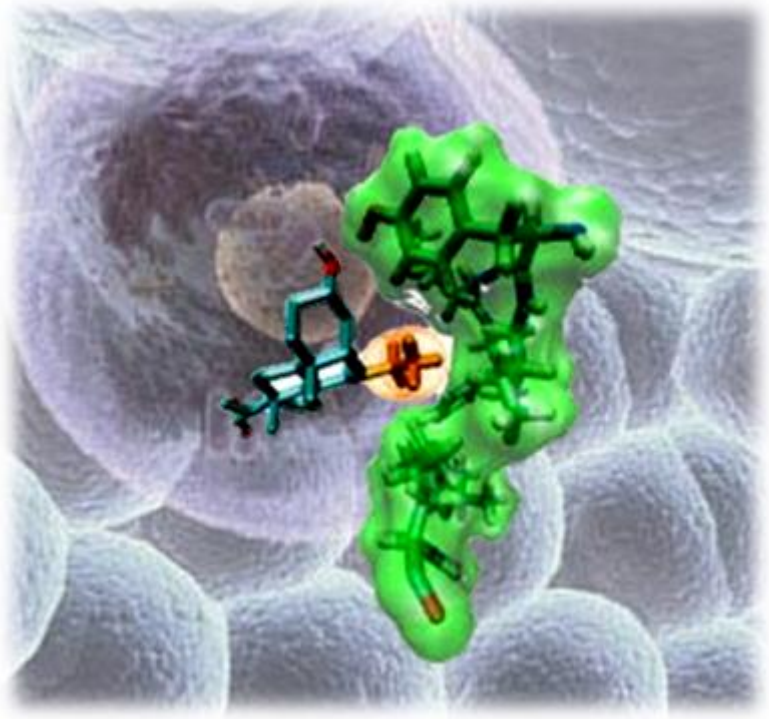

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Dottorato di Ricerca in Scienze Biomediche

XXIX Ciclo - A.A. 2013-2016

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Abstract

The Asymmetric Dimethylarginine (ADMA), a physiological compound naturally produced during protein methylation, is a unspecific, competitive inhibitor of Nitric Oxide Synthase (NOS) (Vallance et al. 1992). Since endothelial NO plays a crucial role in the protection against the onset and progression of many organ disorders, the ADMA-mediated inhibition of endothelial Nitric Oxide Synthase (eNOS) can exacerbate many pathological conditions.

ADMA is synthesized by Protein Arginine Methyltransferases (PRMTs), released as free amino acid after proteolysis and it is metabolized by Dimethylarginine Dimethylaminohydrolase (DDAH) enzyme, highly expressed in the liver. Finally, its import/export across cell membranes depends on Cationic Amino-acid Transporters (CATs) activity (Reade et al. 2002).

Increased ADMA concentration is also related to the degree of hepatic dysfunction in patients suffering from liver disorders, with varying aetiologies (Ferrigno, Laura G Di Pasqua, et al. 2015), including Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH), cholestatic hepatitis and Ischemia/Reperfusion (I/R) injury (Yagnik et al. 2002).

In these three types of hepatic disorders, an imbalance of ADMA/DDAH/NOS pathway occurs, leading to various consequences that worsen liver damage.

In the last few years, several factors that could affect the ADMA/DDAH/NOS pathway have been discovered. Recently, it has been shown that Farnesoid X Receptor (FXR) agonist, GW4064, increases hepatic DDAH-1 gene expression, with a concomitant decrease in plasma ADMA levels (Hu et al. 2006). Moreover, FXR agonists lead to upregulation of eNOS mRNA and protein expression, resulting in NO production increase (Laffitte et al. 2000). Lastly, recently published data revealed also that FXR agonists may positively affect CAT-1 expression both in liver and kidney (Li et al. 2009). Targeting the FXR to induce changes in ADMA levels may be considered as a potential strategy to reduce the effects of several hepatic diseases.

Thus, in this study we proposed several goals: first of all, how ADMA/DDAH/NOS axis changes in three different models of liver diseases, considering ADMA production, elimination after DDAH-1 metabolism and excretion.

Then, we wanted to assess any changes in hepatic ADMA uptake, considering its import/export across membranes, mediated by CATs.
Finally, we investigated how FXR agonist Obeticholic Acid (OCA) administration could induce changes in ADMA handling and its possible clinical implication in liver injury treatment.
In order to reach our goals, we used three different models of liver injury, a Methionine and Choline Deficient (MCD)-diet rat model, a Bile Duct Ligation (BDL)-rat model and a *in vivo* model of Ischemia/Reperfusion (I/R) injury.

We have documented, for the first time, that a diet deprived of methionine and choline induced a significant decrease in serum ADMA levels already after 4 weeks of treatment. We found, in our study, that MCD-fed animals showed decreased ADMA levels. This finding could be explained with low availability of methyl groups, since it was hypothesized that methyl groups of this dimethylarginine may be derived from the demethylation of methionine to homocyst(e)ine (Böger et al. 2000).
Moreover, mRNA and protein expression of PRMT-1, the enzyme involved in ADMA synthesis, increased in MCD animals, as a possible hepatic response to the decrease in serum or tissue ADMA levels.
For what concerns DDAH-1, the enzyme that metabolizes ADMA, we reported a significant increase in its mRNA expression after 8 weeks of diet, suggesting that ADMA decrease could be due to its greater degradation. Unfortunately, there were no changes in DDAH-1 protein expression and its activity was even significantly reduced in MCD rats, probably because of the oxidative stress, known to affect the catalytic site of DDAH. Subsequently, to investigate whether changes in ADMA levels were due to modifications in its transport, Cationic Amino-acid Transporters (CATs) were taken into account. In our study we observed an increase in CAT-1 mRNA expression, concomitantly with a decrease in serum ADMA. On the other hand, CAT-2A and CAT-2B mRNA, expressed particularly in the liver (Teerlink et al. 2009), decreased after 4 week-MCD diet, but an increase in CAT-2 protein content was detected, indicating that, in NASH, CAT-2 undergoes post-transcriptional modifications as predominant regulation mechanism. Significantly, multiple changes in ADMA transporters have been detected in the MCD dietary model of NASH (Teerlink 2005).

While serum ADMA levels decreased in a steatotic animal model, in a cholestatic model of 2-week Bile Duct Ligation (BDL), an increase in serum ADMA content has been found (Chang

et al. 2013; Yang et al. 2012). However, in our 72-hour BDL model no increase in serum ADMA concentration was observed, likely due to the short period of occlusion. Moreover, we documented for the first time that ADMA is differently distributed in the three hepatic lobes: in fact, it is significantly higher in the Right (RL) and Median Lobes (ML) in comparison to the Left Lobe (LL). Many publications reported on the different hepatic lobe functionality in response to a pathological situation, such as obstructive cholestasis. To explain the raised hepatic ADMA content in RL and ML, we evaluated the lobe heterogeneity of the enzymes involved in the synthesis and degradation of ADMA. A significant increase in PRMT-1 mRNA expression was observed in BDL group, compared with sham-operated animals, while no changes were detected in PRMT-1 mRNA expression among the three BDL lobes. As in our MCD model, we detected that DDAH activity showed no changes between the above mentioned groups. Probably, also in this case, the failure in DDAH activity is attributable to the high oxidative stress. Since we could not attribute tissue ADMA heterogeneity to a different level of DDAH activity, different ADMA distribution was likely associated with a decreased mRNA expression of CAT-2 transporters. The downregulation of CAT-2 was particularly evident in the right and median lobes, when compared with the respective sham-operated group and to the left lobe. Previous works demonstrated that a decrease in CAT-2 transporters related to an increase in tissue ADMA in a 2-week BDL model took place (Chang et al. 2013). Thus, in our BDL model, we confirmed the reduction in cationic transporters expression and we also documented that, immediately after the bile duct ligation, this event occurred in a lobe-specific manner. The mechanism involved in this inter-lobe variation is largely undiscovered yet, although factors such as portal streamlining of blood to the liver (DUCHEN 1961) and differences in the metabolic capacity of each lobe have been proposed for elucidating the heterogeneous liver lobe response (Lawson & Pound 1974).

By means of an *in vivo* model of Ischemia/Reperfusion (I/R) injury we documented, for the first time, that ADMA was significantly excreted by bile and a time-dependent increase was observed after 30 and 60 minutes of ischemia. Moreover, ischemia/reperfusion injury induced changes in biliary, serum and hepatic levels of ADMA, affecting *per se* the enzymes involved in ADMA synthesis and metabolism. Thus, this finding supports the hypothesis that ADMA/DDAH/NOS pathway may play a central role in acute hepatic I/R injury (Ferrigno et al. 2014). With regards to the increase in plasma ADMA content, is a widely accepted notion that several diseases, such as liver cirrhosis (Lluch et al. 2004), alcoholic hepatitis (Mookerjee, Malaki, et al. 2007) and acute liver failure are associated to the increase in

plasma ADMA concentration (Mookerjee, Dalton, et al. 2007), as well as in the first postoperative day after cardiopulmonary bypass due to the extensive ischemia/reperfusion damage, suggesting the use of ADMA as a reliable and feasible marker of early I/R injury (Siroen et al. 2004). Accordingly, we observed higher serum ADMA levels after 60 minutes of ischemia, when compared with control livers. At the same time, the hepatic ADMA content decreased significantly in the ischemic group, in comparison with the sham-operated one.
ADMA is already considered a marker for graft rejection, since it is present at high concentration in serum of patients rejecting the liver graft (Brenner et al. 2012). However, the increase of biliary excretion of ADMA, occurring already after 30 minutes of ischemia and preceding even its increased appearance in serum, could be considered a very early and new marker of dysfunction of liver graft in post-transplantation period (Ferrigno et al. 2014).

ADMA, being a potent inhibitor of all isoforms of NOS, plays a crucial role during ischemia/reperfusion injury. So, any agent able to affect ADMA/DDAH/NOS pathway in order to counteract the ADMA-mediated inhibition of eNOS, could be a strategy to prevent and treat this kind of occurrence. Thus, once obtained these innovative results about biliary excretion of ADMA, our next goal was to try to understand the role of FXR in controlling this mechanism. Hence, we conducted further investigations using the Obeticholic Acid (OCA), an FXR agonist, in our model of ischemia/reperfusion injury. In fact, recent data demonstrated that, under hypoxic conditions, the farnesoid X receptor could regulate the expression of various genes, among which those ones strictly related to ADMA metabolism and transport (Fujino et al. 2009).
We performed our experiments using the same *in vivo* model of I/R injury in rats, following 5-day OCA (10 mg/Kg/day) treatment.
Our results documented, for the first time, that the pharmacological administration of OCA increases significantly the biliary excretion of ADMA, during hepatic ischemia/reperfusion injury.
To clarify the molecular mechanism of this finding, we investigated the involvement of ADMA transporters, such as Multidrug and Toxin Extrusion Transport 1 (MATE-1) and Organic Cation Transporter 1 (OCT-1). It is known that MATE efflux transporters are proton-coupled antiporters, mostly expressed in the liver and kidney and positioned at apical membranes of renal tubular epithelia and bile *canaliculi* (Motohashi & Inui 2013). Since MATE-1 is the main responsible of elimination of organic cations across the apical membrane in the liver, its mRNA increase suggests its involvement in the further promotion of ADMA

biliary clearance, detected in animals subjected to ischemia and reperfusion and treated with FXR-agonist. Moreover, we consider MATE-1 the best candidate for ADMA efflux into bile, because this event occurred concomitantly with a downregulation of all other transporters, such as CAT-1, CAT-2A, CAT-2B and OCT-1. Thus, the MATE-1-mediated ADMA excretion by bile in the I/R group may represent an adaptive mechanism to limit the dangerous ADMA accumulation in the liver. In this study, the OCA treatment induced an impairment in the ADMA exchange by decreasing its transporters, CATs and OCT-1, and these events could clarify the additional increase in biliary ADMA observed in the I/R animals treated by FXR agonist.

Moreover, we reported that OCA did not affect DDAH-1 expression and activity and a possible explanation of our data could be provided by the comparison of the two experimental models, BDL and I/R. In the I/R model, ADMA is excreted by bile, contrarily BDL-rats are unable to eliminate ADMA by bile, producing the compensatory effect on increase in the expression of DDAH-1.

It is known that when ADMA levels increase, nitric oxide is not produced anymore, causing several complications to the systemic circulation. Published data demonstrated that FXR activation mediated by the physiological ligand Chenodeoxycholic Acid (CDCA) in hypertensive rats, upregulated the eNOS expression, reducing blood pressure (Li et al. 2015). Our results confirm again the OCA ability in increasing the eNOS protein and, interestingly, they demonstrate a decrease in the inducible isoform of NOS content, in particular during the hepatic I/R injury. Thus, both eNOS and iNOS are involved in the progression of hepatic I/R injury: while eNOS mediates protection, iNOS is involved in pro-inflammatory process. This event explained the decrease in the serum enzyme release in the I/R group treated with OCA.

In conclusion, the present study addresses several “hot” topics in liver pathophysiology and it provides noteworthy and original insights into the effect of obeticholic acid in increasing the biliary excretion of ADMA, by upregulating MATE-1 and downregulating OCT-1.

Even though the real consequence of these findings have to be further elucidated, in our work, we also demonstrated that, thanks to an eNOS increase and an iNOS decrease both mediated by OCA treatment, a reduction in the hepatic vasoconstriction was obtained, explaining the observed reduction in liver injury.

Our results support the idea that the advancement in the understanding of the molecular pathophysiology of liver injury might lead to the development of novel therapeutic alternatives, whose the FXR agonists represent a feasible and truthful example.

Abbreviations

ACC: acetyl-coA
AceCS: acetyl-coA synthase
ACT: actin
ADMA: asymmetric dimethylarginine
AE2: anion exchanger 2
AF: activation function domain
AGXT2: alanine-glycoxylate aminotransferase
ALF: acute liver failure
ALT: alanine aminotransferase
AP: alkaline phosphatase
APS: ammonium persulphate
ASBT: apical sodium-dependent bile salt transporter
AST: aspartate aminotransferase
ATP: adenosine triphosphate
BA: bile acid
BAAT: bile acid-coA amino acid N-acetyltransferase
BACS: bile acid-coA synthetase
BDL: bile duct ligation
BH_4: thetrahydrobiopterin
BSA: bovine serum albumin
BSEP: bile salt export pump
CA: cholic acid
CAD: coronary artery disease
cAMP: cyclic adenosine monophosphate
CAT: cationic amino-acid transporter
CCl_4: carbon tetrachloride
CDCA: chenodeoxycholic acid
ChREBP: carbohydrate response element binding protein
COX-2: cyclooxygenase-2
CREB: cyclic adenosine monophosphate regulatory element binding protein
CTRL: control
CYP: cytochrome P450
Cys: cysteine
DBD: DNA-binding domain
DCA: deoxycholic acid
DCF: dichlorofluorescein
DDAH: dimethylarginine dimethylaminohydrolase
DMA: Dimethylamine
DMSO: dimethyl sulfoxide
DR: direct repeat
DRIP-205: vitamin-D-receptor-interacting protein-205
6-ECDCA: 6α-ethyl-chenodeoxycholic acid
EDTA: ethylenediaminetetraacetic acid
eNOS: endothelial nitric oxide synthase
ER: everted repeat
ERK: extracellular signal–regulated kinase
ET-1: endothelin-1
FABP6: fatty acid-binding protein subclass 6
FAS: fatty acid synthase
FGF: fibroblast grow factor
FGFR4: fibroblast growth factor receptor-4
FXR: farnesoid X receptor
FXRE: farnesoid X receptor response element
G6Pase: glucose-6-phosphatase
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GCA: glycocholic acid
GCDCA: glycochenodeoxycholic acid
Glu: glutamate
GLUT2: glucose transporter 2
GPAT: glycerol-3-phosphate
GR: glucocorticoid receptor
GSH: reduced glutathione
GSSG: oxidized glutathione
HBV: hepatitis B virus

HCC: hepatocellular carcinoma
HDL: high-density lipoprotein
His: histidine
HNF-4α: hepatocyte nuclear factor-4α
hnRNPs: heterogeneous nuclear Ribonuclear Proteins
HRS: hepatorenal syndrome
HSCs: hepatic stellate cells
I/R: ischemia/reperfusion
IBABP: intestinal bile acid-binding protein
ICAM1: intercellular adhesion molecule 1
ICU: intensive care unit
IL: interleukin
iNOS: inducible nitric oxide synthase
IR: inverted repeat
JNK: c-Jun N-terminal kinase
KLF11: krueppel-like factor 11
L-Arg: L-arginine
LBD: ligand binding domain
LCA: lithocholic acid
L-Cit: L-citrulline
LDH: lactic dehydrogenase
LDL: Low-Density Lipoprotein
LL: left lobe
L-NMMA: N^G-monomethyl-L-arginine
LPL: lipoprotein lipase
LRH-1: liver receptor homolog-1
MAPK: mitogen-activated protein kinase
MCD: methionine and choline deficiency
MCP1: macrophage chemotactic protein 1
MDA: malondialdehyde
MDR3: multidrug resistance protein 3
ML: median lobe
MOF: multiple organ failure
MRP: multidrug related/resistance protein
NAFLD: non-alcoholic fatty liver disease
NASH: non-alcoholic steatohepatitis
NCor: nuclear co-repressor
NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells
NO: nitric oxide
NR: nuclear receptor
NRE: nuclear responsive element
NTCP: Na^+/taurocholate co-transporting polypeptide
OCA: obeticholic acid
OCT: organic cation transporter
OST: organic solute transporter
PBC: primary biliary cholangitis
PBS: phosphate-buffered saline
PDK-4: pyruvate dehydrogenase kinase-4
PEP: phosphoenolpyruvate
PEPCK: phosphoenolpyruvate carboxykinase
PGC-1α: peroxisome-proliferator-receptor (PPAR-)-γ co-activator-1α
PKB: protein kinase B
PLPT: phospholipid transfer protein
PNF: primary non function
PPAR: peroxisome-proliferator-receptor
PRMT: protein arginine methyltransferase
PSC: primary sclerosing cholangitis
PVDF: polyvinylidene difluoride
PXR: pregnane X receptor
RA: retinoid acid
RE: responsive element
RL: right lobe
RNS: reactive nitrogen species
ROS: reactive species of oxygen
RS9: ribosomal protein 9

RT: room temperature
RT-PCR: real-time polymerase chain reaction
RXR: retinoid X receptor
SDMA: ω-N^{G},N'^{G}-symmetric dimethylarginine
SDS: sodium dodecyl sulphate
SHP: small heterodimer partner
SMA: symmetric dimethylarginine
SOD: superoxide dismutase
SRB1: scavenger receptor B1
SRC-1: steroid receptor coactivator-1
SREBP-1c: sterol-regulatory-element-binding-protein-1c
SREBPs: sterol regulatory element-binding proteins
STAT3: signal transducer and activator of transcription 3
SULT2A1: sulfotransferase family 2A member-1
SUMO: small ubiquitin-like modifiers
T2DM: type 2 diabetes mellitus
TBARS: thiobarbituric acid reactive species
TBS: tris-buffered saline
TCA: taurocholic acid
TCDCA: tauroglycochenodeoxycholic acid
TGFβ: transforming growth factor β
TGs: triglycerides
TMB: tetramethylbenzidine
TNF-α: tumour necrosis factor-α
TUB: tubulin
TZDs: thiazolidinediones
UBC: ubiquitin C
UDCA: ursodeoxycholic acid
UGT2B4: uridin 5'-diphosphoate glucuronosyltransferase 2B4
VCAM1: vascular cell adhesion molecule 1
VDR: vitamin D receptor
VEGF: vascular endothelial cell growth factor
VLDL: very low density lipoprotein
VSMCs: vascular smooth muscle cells
γ-GT: γ-glutamyltranspeptidase

Introduction

The Asymmetric Dimethylarginine

Phosphorylation is the most common and largely studied protein post-translational modification, however other processes, such as arginine methylation, have an important role in regulating cellular function, such as signal trasduction, protein subcellular localization or other similar processes (McBride & Silver 2001).

Until today, it is known that the only products of protein post-translational modification, that can play a biological role, are asymmetric methylated arginines (Leiper & Nandi 2011). As a result of this process, one or two methyl groups are added to the guanidine nitrogen atoms of arginine residues so that the proteolysis of these modified proteins releases free methylarginines into the cytosol (Gary & Clarke 1998). Subsequently, these residues can leave the cell and enter the plasmatic circulation.

In eukaryotes, three forms of methylated arginine are found: N^G-Monomethyl-L-Arginine (L-NMMA), Asymmetric Dimethylarginine (ADMA) and ω-N^G,N'^G-Symmetric Dimethylarginine (SDMA) (Kakimoto & Akazawa 1970). L-NMMA and ADMA are both capable to inhibit Nitric Oxide Synthase (NOS) isoenzymes because they compete with L-arginine active binding site of each of the three isoforms of NOS, endothelial (eNOS) - neuronal (nNOS) - inducible (iNOS), blocking the synthesis of Nitric Oxide (NO).

NO, a gaseous compound, is the most important molecule among the mediators of endothelium-mediated vasodilation, and plays an important role in the maintenance of cardiovascular system functionality (Andrew & Mayer 1999). Despite its central role in supporting the smooth functioning of the cardiovascular system, NO is also able to cover a broad spectrum of functions, from pulmonary inflammation promotion (Hinder et al. 1999), to protection against oxidative cellular damage (Wink et al. 1995) and tumour cell invasion (Siegert et al. 2002). Dysfunction of the arginine-NO pathway is a common mechanism by which several cardiovascular events, *e.g.* platelet aggregation increase and cell adhesion to the endothelium and vascular muscle proliferation, affect the vascular wall (Siroen et al. 2006).

The ADMA levels in the bloodstream are much higher than those of L-NMMA and, for this reason, it is considered the principal inhibitor of NOS activity (Vallance et al. 1992) and an important marker of endothelial dysfunction (Sibal et al. 2010).

All three isoforms of methylated arginine, L-NMMA, ADMA and SDMA, instead, are inhibitors of arginine transport at super-physiological concentration, although the

physiological relevance of this inhibition remains unclear (Closs, Basha, et al. 1997; Tsikas et al. 2000).

The ADMA-mediated inhibition of NO production is determined by the concentration of intracellular and extracellular ADMA. ADMA is synthesized by Protein Arginine Methyltransferases (PRMTs) followed by proteolytic degradation. It is metabolized to citrulline and dimethylamine, by Dimethylarginine Dimethylaminohydrolase (DDAH-1), and enters cells through Cationic Amino-acid Transporters (CATs) (Reade et al. 2002).
ADMA is partially excreted by the kidney, but the most important way of eliminating ADMA is through DDAH-1 metabolism, which is highly expressed in liver and kidney (Teerlink 2005). Accordingly, ADMA concentration is positively related to the degree of hepatic dysfunction (Ferrigno, Laura G Di Pasqua, et al. 2015).

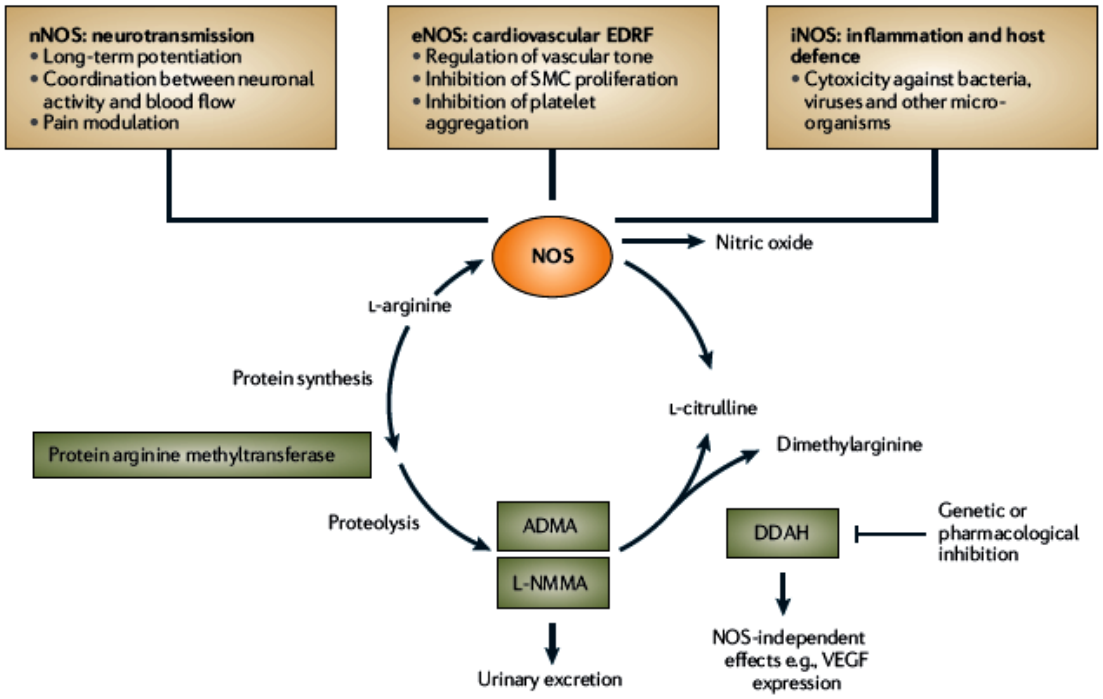


Figure 1. The ADMA/DDAH/NOS pathway. In mammals, three different isoforms of Nitric Oxide Synthase (NOS), producing Nitric Oxide (NO), are present: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). L-Arginine is the shared substrate for all three isoforms of NOS. Proteolysis of L-Arginine residues, methylated by Protein Arginine Methyltransferases (PRMTs), releases free methylarginines: the Asymmetric Dimethylarginine (ADMA) and ω-N^G,N'^G-Symmetric Dimethylarginine (SDMA), both competitive inhibitors of all NOS isoforms. ADMA is mostly metabolized by Dimethylarginine Dimethylaminohydrolase (DDAH) enzymes, and to a lesser extent is eliminated by renal excretion. By NOS-independent pathways, DDAH can perform physiological functions, such as regulation of Vascular Endothelial Growth Factor (VEGF) expression (Leiper & Nandi 2011).

1.1 Synthesis of ADMA

The methylation of protein arginine residues is catalysed by a family of intracellular enzymes termed Protein Arginine Methyltransferases (PRMTs) (Aletta et al. 1998). These enzymes catalyse the addiction of methyl groups to the guanidine nitrogen atoms of arginine side chain (Gary & Clarke 1998), forming first Monomethylarginine (MMA). It results in an increase of bulkiness and hydrophobicity of arginine, with no changes in the positive charge of arginine (Boisvert et al. 2005).

In mammalian cells, PRMTs have been classified into type I (PRMT-1, 3, 4, 6, and 8) and type II (PRMT-5, 7, and FBXO11), depending on their specific catalytic activity. Both types of PRMT, however, catalyse post-translational modification utilizing *S*-adenosylmethionine as methyl group donor, transforming it in *S*-adenosylomocysteine, and forming MMA from L-Arginine (L-Arg). In a second step, type I PRMT produces Asymmetric Dimethylarginine (ADMA), by the addition of a second methyl group to the already methylated guanidine nitrogen atom, while type II PRMT forms Symmetric Dimethylarginine (SDMA) (Gary & Clarke 1998). Approximately 65% of total cellular ADMA is contained in heterogeneous nuclear Ribonuclear Proteins (hnRNPs), whose activity is regulated by methylation.

Until today, it was assumed that arginine methylation process was irreversible and the only known mechanism for reversing it was protein turnover, culminating in the generation of free mono- and dimethylated arginines into the cytosol. However, recent studies have shown that specific enzymes, termed peptidyl-arginine deiminases, are capable to modify methylated arginines by the conversion of the peptidyl-methylarginine residues to peptidyl-citrulline (Wang et al. 2004; Cuthbert et al. 2004). Moreover, the “true” demethylation process depends on the activity of a Jumonji C domain-containing iron- and 2-oxoglutarate-dependent dioxygenase (JMJD6), that functions as a histone demethylase and catalyses the conversion of monomethylarginine back to arginine (Chang et al. 2007). Unfortunately, the physiological significance of JMJD6-mediated peptidyl-arginine demethylation has not been clarified yet, but it is known that most of these peptidyl-methylarginine residues avoids demethylation before proteins turnover, so, significant concentrations of free methylarginines are found in cells and biological fluids. The free fraction of ADMA and MMA are able to inhibit NOS, while SDMA has no effect on this enzyme (Tran et al. 2003).

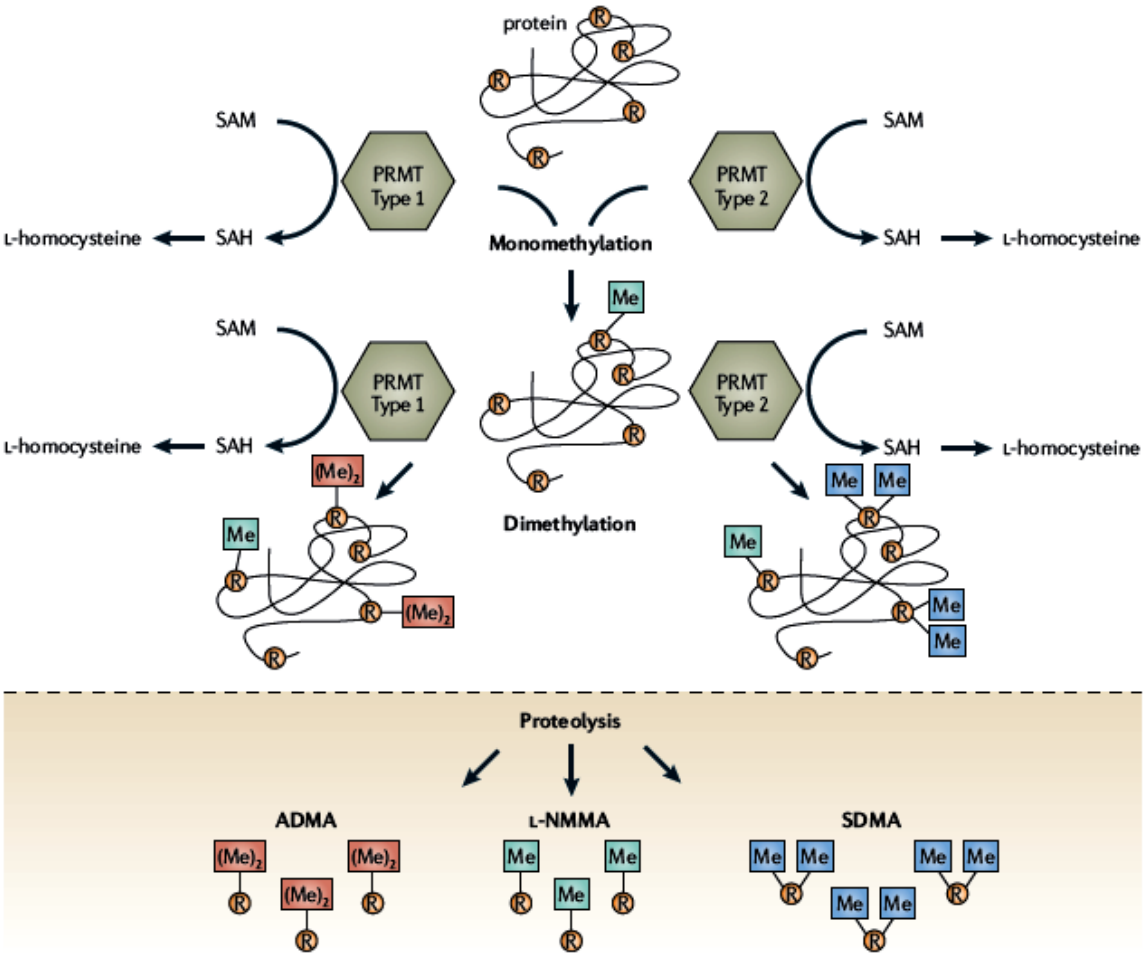


Figure 2. Endogenous synthesis of methylarginines. PRMTs catalyse methylation of Arginine residues (R), using *S*-Adenosyl-l-Methionine (SAM) as a methyl donor and producing *S*-Adenosyl-l-Homocysteine (SAH) as a by-product. Likely, protein arginine methylation takes place as a multi-step process. First, PRMTs catalyse a monomethylation reaction, then they catalyses a second methylation reaction, which results in the formation of either ADMA or SDMA. Type I PRMT produces ADMA, while type II PRMT forms SDMA. In the figure, 'Me' indicates monomethylation, whereas '$(Me)_2$' indicates asymmetric dimethylation. l-NMMA: *NG*-Monomethyl-l-Arginine (Leiper & Nandi 2011).

1.2 Metabolism of ADMA

Although it was originally assumed that both ADMA and SDMA are excreted only by urinary tract, it is now well known that there are at least two catabolic pathways useful for dimethylarginines metabolism (Kakimoto & Akazawa 1970). Intracellular ADMA, in fact, is metabolized to Citrulline (L-Citrulline) and Dimethylamine (DMA), a reaction catalysed by Dimethylarginine Dimethylaminohydrolase (DDAH), which has high activity in the liver and kidney. The second minor pathway uses both ADMA and SDMA as substrate for the Alanine-Glycoxylate Aminotransferase (AGXT2) (Ogawa et al. 1990). From the approximately 300 μmol of ADMA, which are generated daily in humans, about 250 μmol (>80%) are metabolized by DDAH (Achan et al. 2003). Two isoforms of DDAH have been identified, that are widely expressed in human tissues. DDAH-1 is the most important isoform for the regulation of circulating ADMA and it is mainly found in peripheral tissues such as liver, kidney, adrenal gland, and testis (Tran et al., 2000). DDAH-2 has an important role in the regulation of nitric oxide responses and nitric oxide activity, and it is mainly found in tissues expressing eNOS and iNOS, such as the heart and the aorta (Leiper et al. 1999).

The amino acidic sequence of DDAH isoforms is highly conserved, particularly in domains involved in substrate binding and hydrolysis. Among species, DDAH isoforms are highly conserved with homology in the murine, bovine and human gene sequences of DDAH-1 (92%) and DDAH-2 (95%).

Since DDAH catalyses ADMA metabolism (Ogawa et al. 1989), many investigations on this topic have demonstrated that the concentration of DDAH is greater than intracellular concentration of ADMA, which suggests that the DDAH enzyme active site is never fully saturated, allowing ADMA metabolism to be proportional to its concentration (Hong & Fast 2007). Although the DDAH activity is strongly implicated in the intracellular regulation of ADMA levels, alterations of its activity and/or expression lead to changes in ADMA levels (Dayoub et al. 2003). Dayoub and collaborators showed that the increase of DDAH mRNA expression in transgenic mice resulted in lower plasma ADMA levels and higher NO concentration in bloodstream (Dayoub et al. 2003). Recently, several factors have been found that could increase the concentration or activity of DDAH. One of them is the Farnesoid X Receptor (FXR) agonist, GW4064, which increases hepatic DDAH-1 gene expression with a concomitant decrease in plasma ADMA levels. No changes were found in the mRNA expression of the DDAH-2 isoform (Hu et al. 2006).

Although the increase in DDAH mRNA expression and activity is usually a desired effect in cardiovascular diseases, because of its effects on NO synthesis, it could be detrimental in tumours. In fact, tumours that show high level of DDAH grow faster than those one with lower DDAH expression. This occurrence takes place because, without its inhibitor, NOS produces more NO: this enhances Vascular Endothelial cell Growth Factor (VEGF) expression, leading to angiogenesis and faster tumour progression (Smith et al. 2003).

As there are many factors that can increase DDAH activity or expression, many others are able to decrease it, as well as Tumour Necrosis Factor-α (TNF-α), oxidized Low-Density Lipoprotein (ox-LDL), hypercholesterolemia, hyperglycemia, and hyperhomocysteinemia (Dayal & Lentz 2005).

Recent studies demonstrate that ADMA is also able to induce eNOS-derived superoxide production, when Thetrahydrobiopterin (BH_4) depletion, or absence of its substrate L-Arginine, occurs. These conditions bring the eNOS to became "uncoupled". During this state, the electron flow is diverted to molecular oxygen rather than to L-Arginine, resulting in production of superoxide rather than NO (Vásquez-Vivar et al. 1998; Xia et al. 1998). This situation can create a vicious circle: the increase of Reactive Oxygen Species (ROS) enhances intracellular ADMA levels and it further promotes the rise in ROS production, making a potential positive feedback (Druhan et al. 2008; Cardounel et al. 2005). This fatal loop plays an important role in the ADMA/DDAH/NOS pathway regulation, because the overproduction of ROS induces ADMA accumulation by inhibiting DDAH activity (Y. L. Tain et al. 2010). Increasing evidence shows that both oxidative and nitrosative stress can adversely affect DDAH activity (Leiper et al. 2002). The crystal structure of a microbial DDAH has identified a Cys-His-Glu catalytic triad (Cys-249, His-162, and Glu-114) in the active site of DDAH (Murray-Rust et al. 2001), that is highly sensitive and easily altered by ROS such as superoxide and nitrosative stress. The presence of a reactive cysteine residue in the active site of DDAH ensures that DDAH activity can be altered in conditions of oxidative or nitrosative stress (Leiper et al. 2002). Therefore, oxidative stress accompanies many of the inhibiting stimuli on DDAH, whereas many of the activating stimuli are antioxidants. In fact, probucol, a potent antioxidant, and taurine, a semi-essential amino acid with antioxidant properties, positively regulate DDAH activity (Jiang et al. 2006; Tan et al. 2007).

It is therefore clear that the DDAH plays a central role in the metabolism of ADMA and its sensitivity to inhibiting and inducing factors could be a possible mechanism to control of its activity. Because DDAH is largely expressed at hepatic level, it is possible to assume that the

liver is an important organ in the regulation of both circulating ADMA concentration and NO synthesis (Richir et al. 2009; Ferrigno, Laura G Di Pasqua, et al. 2015).

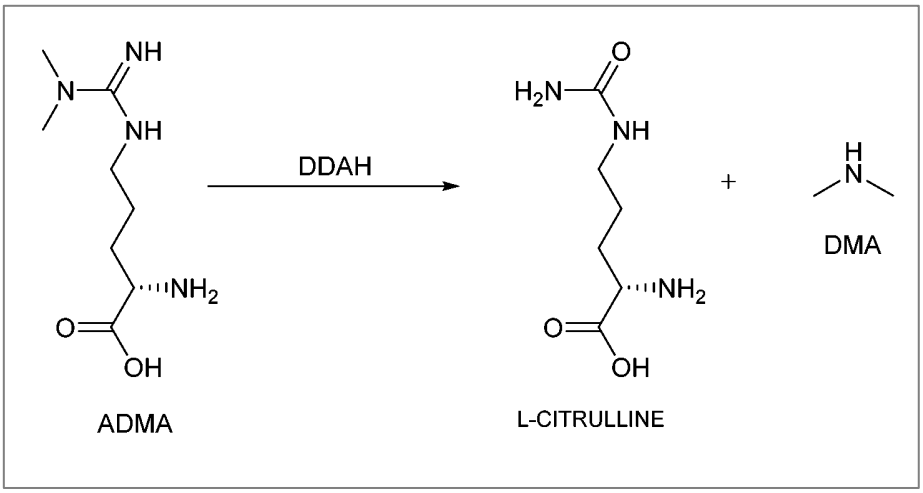


Figure 3. ADMA metabolism. Dimethylarginine Dimethylaminohydrolase (DDAH) metabolizes ADMA, producing citrulline and Dimethylamine (DMA).

1.3 Excretion of ADMA

To date, it is well known the role of kidney in the excretion of methylarginines, in fact, ADMA and SDMA are both excreted into the urine (Al Banchaabouchi et al. 2000). Despite the kidney is also capable to generate and metabolize ADMA, however it is overall considered an ADMA clearing organ in healthy humans. Nijveldt and colleagues evaluated the renal uptake of ADMA from bloodstream, in both humans and rats, by measuring the differences in arterio-venous concentration (Nijveldt et al. 2002). Kidney-mediated ADMA clearance is a combine of ADMA degradation by DDAH, that produces L-Citrulline and DMA, and urinary excretion. About 80% of 300 µmol of daily-produced ADMA is metabolized by DDAH, conversely the remaining 20% is excreted in the urine. In contrast, DDAH has no activity on SDMA, thus the renal excretion is the most important eliminatory pathway of disposal to remove it from the organism (Achan et al. 2003).

In a recent study, it was demonstrated that the intravenous injection of ADMA increased at a significant rate the urinary excretion of DMA, compared to placebo. The finding of a very close negative correlation between ADMA and creatinine urine concentration in patients, in fact, suggests that this fraction of ADMA depends on renal function.

Interestingly, patients affected by renal dysfunction show that plasma ADMA basal level correlates in a significant way with serum creatinine and glomerular filtration rate (Fliser et al. 2005).

Furthermore, elevated plasma ADMA levels are well known risk factor and predictor of Coronary Artery Disease (CAD) and cardiovascular mortality (Sydow & Münzel 2003). Cardiac output shows a decrease after infusion of pathophysiological relevant concentrations of ADMA to volunteers, but, in this case, the high amount of ADMA in plasma CAD patients correlates with lower urinary ADMA/creatinine ratios, as compared with patients without CAD (Maas et al. 2007; Cavusoglu et al. 2010).

The DMA/ADMA ratio in the urine was significantly higher in patients with 3-vessel disease (CAD 3) compared to patients without CAD. This finding suggests that a positive feedback between elevated plasma ADMA levels and DDAH, responsible for ADMA metabolism, occurs. These data indicate increased DDAH activity in patients suffering for CAD, thus the metabolic breakdown product of ADMA, DMA, is mostly eliminated in human urine (Achan et al. 2003).

Thus, in the end, the DMA/ADMA molar ratio in the urine may be useful as a biomarker for whole body DDAH activity, particularly related to ADMA synthesis (Wolf et al. 2012).

Another interesting finding of Vallance and collaborators shows that a marked increase in plasma ADMA level in patients with terminal renal failure worsens the damage, probably due to the reduced NOS activity (Vallance et al. 1992). Moreover, the renal failure adversely affects DDAH activity and the discovery that in glomerulus endothelial cells and in renal tubular cells both DDAH and NOS enzymes are co-localized gives value to the hypothesis that the intracellular ADMA concentration is widely regulated in NO-generating endothelial cells, within the kidney as well (Tojo et al. 1997).

In conclusion, when DDAH-rich renal tissue destruction occurs, an impairment in ADMA metabolism takes place. As further evidence, metabolic balance studies in healthy subjects report that the kidney is a major extraction site for ADMA from the bloodstream. Overall, these data may provide a link between renal dysfunction and inexorably ADMA plasma accumulation (Fliser et al. 2005).

1.4 Transport of ADMA

All amino acids are subjected to a huge exchange among various organs in whole body.

The massive and fast exchange of amino acids between the cytosol and the bloodstream is mediated by specific transport systems and also ADMA undergoes the same fate (Mann et al. 2003). Both the generation and degradation of free ADMA are cytosolic processes. It is thought that most of the ADMA is fast metabolized in the intracellular compart of the same cell where it was synthetized, but a small part of it may avoid degradation and enter the circulation (Teerlink 2005). In addition to its direct inhibition of the three isoforms of NOS, ADMA is also able to inhibit NO synthesis by competing with L-Arginine, the physiological forerunner of NO, for cellular transport across specific transporters, termed Cationic Amino-acid Transporters (CATs) (Reade et al. 2002).

Positive charged amino acids, for example lysine, ornithine and arginine, can pass across the cell membrane by means of functionally distinct transport systems: y^+ and y+L. The main transport system expressed in NO producing cells is the cationic amino acid system y^+ and, therefore, it is likely to be specialized in providing L-Arginine as substrate for NOS (Reade et al. 2002).

1.4.1 System y^+ transporters

System y^+, a Na^+-independent amino acid transport system, belongs to a family of CAT and exclusively transports cationic amino acids (White 1985), meanwhile both cationic and neutral amino acids are transported by system y+L (Mann et al. 2003). CATs transporter system was the first mammalian amino acid carrier system to be identified in mammalian cells. They are part of the subfamily of the Solute Carrier 7 (SLC7), that is composed by several members, among which CAT-1, CAT-2A and CAT-2B are the most significant for arginine and ADMA transmembrane transport. In the early 1990s, studies conducted in *Xenopus* oocytes revealed that the ecotropic MuLV receptor is involved in Na^+-independent transport of cationic amino acids, influencing its activity (Kim et al. 1991). Later, this receptor was renamed mCAT-1, that stood for “mouse cationic amino acid transporter”, and was classified as an integral membrane protein, formed by 14 putative transmembrane domains and intracellular N-terminal and C-terminal domains. CAT-1-mediated transport of basic amino acids is independent from the pH value, but it is saturable at circulating plasma concentrations (~0.1–0.2 mM). Moreover, since CAT-1 activity is largely affected by the concentration of cationic amino acids on the opposite side of the cell membrane, it has been

demonstrated that it is sensitive to a process termed *trans*-stimulation, inducing a huge exchange of arginine between neighbouring cells (Closs et al. 2006). This condition is closely related to another feature of CAT-1: it is sensitive to changes in membrane potential. In fact, during cell membrane hyperpolarization, as after administration of vasoactive agonists, the driving force of CAT-1 increases in different kind of cells. This process results in a cellular uptake of arginine, more than the physiological one, that can stimulate NOS activity (Mann et al. 2003).

Interestingly, both eNOS and CAT-1 co-localize in the same *caveolae* of the plasma membrane (McDonald et al. 1997). In this way CAT-1 can directly provide plasma arginine to NOS, but at the same time, it is responsible for the ADMA entry and NOS inhibition. Garcia-Cardeña and colleagues have demonstrated a protein-protein interaction between eNOS and caveolin, a caveolar coat protein expressed in endothelial cells. Probably, this eNOS-caveolin interaction is responsible for the eNOS positioning adjacent to CAT-1, also localized in the *caveolae*, forming a highly efficient signal transduction cascade and optimizing the production of NO (García-Cardeña et al. 1996).

CAT-1 has been reported to be present in many tissues (Humphrey et al. 2004). To be notice that CAT-1, CAT-2A and CAT-2B have many glycosylated amino acid residues, suggesting that these carriers are located in the cytoplasmic membrane.

CAT-2A has a central role in the liver and it is weakly expressed in skeletal muscle and pancreas, whereas pro-inflammatory cytokines and bacterial polysaccharide induce the activity of CAT-2B in a great variety of tissues (Closs et al. 2006). CAT-2A and CAT-2B are two splice variants differing only in a stretch of 42 amino acids (Closs, Gräf, et al. 1997).

CAT-1 has high affinity for cationic amino acids, which show a *Km* of 100 to 250 μM for it and slightly higher values for CAT-2B. On the contrary, CAT-2A is a low-affinity carrier for cationic amino acids and its affinity to them is up to 100-fold lower then CAT-1 and CAT-2B.

Hosokawa et al. succeeded in isolation of isoform 3 of CATs in mouse and rat brain. CAT-3 is a Na^+-independent carrier of cationic amino acids, although its substrate specificity differs from other CAT isoforms and its *Km* is 100-fold lower than that for CAT-1 (Hosokawa et al. 1997). In rodents, CAT-3-mediated transport of L-Arginine is inhibited by other cationic amino acids, such as L-Citrulline and L-Methionine. Human CAT-3 is not neuron specific, as is the case of rats and mice (Hosokawa et al. 1997; Ito & Groudine 1997). In addition, human CAT-3 shows high affinity for cationic amino acids, but its transport activity does not contribute to the transfer of amino acids such as L-Citrulline, L-Methionine, L-Cysteine or L-

Glutamate (Vékony et al. 2001). Emerging data have shown another CAT isoform, in human placenta, named CAT-4 (Sperandeo et al. 1998). It shows 41-42% sequence homology to members of CAT family, although recent studies conducted in *Xenopus* oocytes or glioblastoma cells (U373 MG cell line) overexpressing CAT-4, indicate a lack of cationic amino acids transport activity (Wolf et al. 2002). It remains to be investigated whether CAT-3 and CAT-4 play a functional role in endothelial and/or smooth muscle in mediating cell cationic amino acids transport.

1.4.2 System y+L transporters

Devés and collaborators described for the first time in 1992 the y+L transport system (Devés et al. 1992). While system y^+ is ubiquitously expressed, system y+L is more restricted to blood cells like erythrocytes, platelets, lymphocytes and then, it is also present in kidney and placenta (Deves & Boyd 1998). Moreover, these carriers display much higher affinity for cationic amino acids, with a *Km* for lysine ~10 μM, when compared to system y^+. Furthermore, this transport system is stereo-selective, electroneutral, and sensitive to *trans*-stimulation. Noteworthy, these carriers show Na^+-independent cationic amino acid transport mechanism, but it is sufficient a substitution of Na^+ by K^+ to reduce, in a detrimental way, their affinity for neutral amino acids. Despite the system sensitivity for neutral amino acids, it shows higher affinity to L-Leucine, L-Methionine, L-Isoleucine and L-Glutamine then L-Alanine, L-Serine or L-Cysteine (Devés & Boyd 2000).

Estévez *et al.* demonstrated, by studies in *Xenopus* oocytes, that the ubiquitous transmembrane protein 4F2hc, also known as CD98, induces amino acids transport activity, resembling system y+L. Besides, these studies confirmed the association of 4F2hc with a membrane oocyte protein, that it is essential for the expression of system y+L transport activity (Estévez et al. 1998).

1.4.3 Further potential candidates for ADMA transport

In addition to the carriers so far elucidated, ADMA transport may be mediated by other ones that belong to the family of organic cation transporters, like Organic Cation Transporter 1 and 2 (OCT-1 and OCT-2, also known as SLC22A1 and SLC22A2) and to the Solute Carrier Family (SLC-family), as well as Multidrug and Toxin Extrusion Transporter-1 (MATE-1) (Strobel et al. 2013).

The most relevant carrier of the organic cation transporter family is OCT-1, which can carry in or out of hepatocytes some positively charged endogenous compounds, as well as ADMA, or drugs. OCT-1 is highly expressed in normal human hepatocytes, in particular in sinusoidal membranes of hepatocytes around the central veins of hepatic *lobuli* and its gene shows an extensive variability among individuals; however it has negligible expression in other tissues (Jonker & Schinkel 2004). The encoding OCT-1 transporter gene (SLC22A1 gene) regulation is quite difficult and several regulatory factors contribute to its unique hepatocyte-specific expression pattern. Interestingly, recent findings have been elucidated the involvement of Farnesoid X Receptor (FXR) in downregulating this transport gene expression. This data point out to potential novel mechanisms of xenobiotic-transporting and drug-metabolizing proteins regulation in the human liver (Hyrsova et al. 2016).

The OCT-2 transporter is positioned in the basal membrane of renal proximal tubular cells and in neuronal cells too (Koepsell et al. 2007). Given its localization, OCT-2 plays a crucial role in the excretion of several organic cations, exogenous drugs and, reasonably, it has affinity also for ADMA (Pietig et al. 2001). OCT-2 carrier mediates a bidirectional transport, that is independent of pH or sodium, but it is subjected to the ionization degree of certain substrates (Barendt & Wright 2002; Winter et al. 2011).

In 2005, Otsuka and colleagues discovered two different human MATE genes, MATE-1 and MATE-2, both located in tandem on chromosome 17. Furthermore, they also localized MATE-proteins at organ level. MATE-1 is placed in the apical membrane of renal tubule cells and bile *canaliculi* (Otsuka et al. 2005). The MATE-1 transporter performs its function especially as efflux protein, in particular it effluxes organic cations using a proton-coupled electroneutral antiporter. This data suggest that both urine and bile cations export are mediated by MATE-1 (Yokoo et al. 2007; Tsuda et al. 2007).

Noteworthy, MATE-1 and OCT-2 share several substrates, especially those ones showing a similar structure to L-Arginine and ADMA. Thus, it has been suggested that OCT-2 and MATE-1 coordinate activity is responsible for uptake from the blood and export into the urine by renal tubular secretion (Schwabedissen et al., 2010).

The activity of CATs, as well as other transporter categories, determine the distribution of cationic amino acids across cells, as well as regulating the intracellular ratio of arginine to ADMA concentration. Accordingly, it is important to consider the distribution and regulation of all kind of amino acids transporters to well understand how intracellular arginine and methylarginine concentrations are maintained.

Previous reports confirmed that the liver plays a central role in the metabolism of ADMA: first of all, the liver takes up large amounts of it from the systemic circulation by means of CATs; then, it degrades the ADMA by means of DDAH-1 (Ferrigno, Laura G Di Pasqua, et al. 2015). By keeping this in mind, any agent or factor targeted at enhancing liver ADMA uptake and metabolism could be a possible strategy to prevent or treat cardiovascular diseases, where a greater NO supply is required. Brand new data revealed that the Farnesoid X Receptor (FXR) may positively affect DDAH-1 and CAT-1 expression both in liver and kidney, when this receptor is pharmacologically activated (Li et al., 2009). This finding could provide new strategies of treatment for several diseases where ADMA-mediated inhibition of NOS activity occurs.

ADMA In Healthy & Unhealthy Conditions

ADMA is an amino acid which, competitively inhibiting NOS, decreases the synthesis of NO (Vallance et al. 1992). In healthy people, ADMA can be detected since birth. As demonstrated by Vida *et al.*, venous cord blood presents elevated ADMA levels (~1.06 μM), although after postnatal day 2 they fall significantly to almost the basal adult levels (~0.66 μM) (Vida et al. 2007). In children, plasma ADMA levels are higher than those observed in adults. A gradual decrease of ADMA levels occurs from birth up to 25 years of age, with a mean diminish of 15 nM *per* year (Tsikas 2008). In healthy adults are produced approximately 300 μmol (~60 mg) of ADMA *per* day. Bode-Böger and collaborators detected a noteworthy rise in ADMA plasma levels in people older than 70 years (Bode-Böger et al. 2003; Bode-Böger et al. 2007). By reducing NO synthesis, ADMA causes several endothelial dysfunctions such as vasoconstriction, blood pressure elevation and atherosclerosis (Cardounel et al. 2007). Moreover, increasing ADMA is also associated with many diseases, such as peripheral arterial disease, coronary artery disease, preeclampsia, hypertension, stroke, heart failure, chronic kidney disease, portal hypertension in cirrhosis, diabetes mellitus and insulin resistance in essential hypertension patients (Vallance & Leiper 2004; Böger 2006a).

ADMA is increasingly recognised as a pivotal factor in hypertension. Correlations between ADMA plasma levels and arterial pressure have been elucidated in patients that did not manifest any vascular diseases, but only a simple initial hypertension (Miyazaki et al. 1999; D. Wang et al. 2009). Other evidence comes from different researches by Surdacki and Wang (Surdacki et al. 1999; D. Wang et al. 2009). They reported that in untreated hypertensive subjects, in which ADMA levels have been risen, NO production diminishes. Similar symptoms were found in hypertensive children and in elderly hypertensive people (Goonasekera et al. 1997; Kielstein et al. 2003). Interestingly, it seems that, treating hypertension with *telmisartan*, the angiotensin AT_1-receptor blocker, reduces the circulating levels of ADMA in tissue slices from rat kidney, incubated with angiotensin II. Probably, ADMA decrease was due to the enhanced DDAH-1 protein expression (Onozato et al. 2008). Increasing evidences demonstrate that elevated plasma ADMA levels occur also in patients suffering from Diabetes Mellitus (DM) Type I and II (Tarnow et al. 2004; Altinova et al. 2007; Hanai et al. 2009). In fact, studies conducted in insulinopenic diabetic rats have

revealed an increase of 50% in renal angiotensin II concentration. This condition is associated with increased plasma ADMA levels. Also in this case, the producing and metabolizing ADMA pathway in kidney is affected by administration of *telmisartan*, resulting in diminishing of PRMT-1 and increasing of DDAH-1 activities (Onozato et al. 2008).

One of the leading cause of end-stage renal failure is Diabetic Nephropathy (DN), that occurs in patients with diabetes, determining high disabilities and mortality (Ziyadeh et al. 2000). DN is characterized by functional and structural modifications in the glomerulus, accompanied by proximal tubular cell atrophy and tubulointerstitial fibrosis, much more serious symptoms in terms of renal prognosis (Fine et al. 1998; Nangaku 2006). Moreover, emerging data suggest that chronic renal hypoxia may play a pivotal role in the progression of tubulointerstitial fibrosis in Chronic Kidney Disease (CKD), including DN (Fine et al. 1998; Rosenberger et al. 2008). Several factors could elicit the chronic renal hypoxia, as well as loss of peritubular capillaries, or reduced NO production and/or its disposal (Fine et al. 1998; Nangaku 2006). Furthermore, Kang and colleagues elucidated that the inhibition of NOS exacerbates renal injury (Kang et al. 2002; Kang et al. 2001). Therefore, the increase in plasma ADMA levels in these diabetic patients is accompanied by endothelial dysfunction, which leads to atherosclerosis and to the worsening of kidney conditions (Lin et al. 2002; Kielstein et al. 2001).

Experimental studies in animal models as well as evidence in humans suggested that ADMA increase occurs when endothelium dysfunction has not yet become clinically evident. It is well known that several cardiovascular risk factors, such as metabolic diseases and systemic or local inflammation, cause endothelial dysfunction, that may precedes evident coronary artery disease. Low NO level, whose role is of primary importance in maintaining endothelial homeostasis, is associated with impaired endothelial function (Sibal et al. 2010). So, ADMA, as an inhibitor of NOS, has been shown to be the strongest risk predictor, beyond traditional risk factors, of cardiovascular events and all-cause of cardiovascular mortality in people with coronary artery disease. Interventions, such as treatment with L-Arginine, have been shown to improve endothelium-mediated vasodilation in people with high ADMA levels, thus slowing down the progress of the cardiovascular disease.

2.1 Hepatic dysfunctions affect ADMA handling

As described above, ADMA handling is completed by liver and kidney concerted action. The potential role of the liver in the metabolism of dimethylarginines was elucidated by Canergie and collaborators, reporting a decreased urinary excretion ratio SDMA/ADMA in patients suffering from various liver diseases, such as chronic active hepatitis, due to an increased output of ADMA (Carnegie et al. 1977). Later, the role of the liver in ADMA management was strengthened by the discovery at hepatic level of high Cationic Amino-acid Transporters (CATs) expression (Hattori et al. 1999). These y^+ transport systems are able to catch ADMA from circulation and to lead it in the hepatic cells (Closs, Basha, et al. 1997), where large amounts of DDAH-1 can metabolize ADMA in citrulline and dimethylamine (Ogawa et al. 1989; Kimoto et al. 1993). Evidence of these occurrences came from Nijveldt and co-workers, which confirmed in a rat model that the liver takes up great quantity of ADMA from bloodstream, but this hepatic action does not affect the SDMA handling (Nijveldt, Teerlink, Siroen, et al. 2003). Similar studies in humans were conducted by Siroen *et al.* in patients undergoing partial liver resection. By measuring organ fluxes and fractional extraction rate, they demonstrated the role of the liver in the uptake of ADMA, together with the kidney (Siroen et al. 2005).

Therefore, it seems clear that, since the liver plays a central role in regulating circulating ADMA, any hepatic dysfunction may affect the correct metabolism of dimethylaginines.

2.1.1 Acute Liver Failure (ALF)

Acute Liver Failure (ALF) is a condition in which a massive destruction of hepatic cells occurs, affecting all liver functions. This state has as consequence a series of further complications such as icterus, encephalopathy, coagulopathy, renal insufficiency and finally Multiple Organ Failure (MOF).

Occasionally, ALF can occur concomitantly with a severe inflammatory process. Thus, Mookerjee and colleagues, hypothesizing that acute liver failure may be associated with increased plasma ADMA levels, found marked increase of its concentration in ALF patients compared with controls. Further, they confirmed that increased circulating ADMA was related to the degree of inflammation and with the release of pro-inflammatory cytokines TNF-α, Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) (Mookerjee, Dalton, et al. 2007). The rise of ADMA, during this specific kind of illness, is probably due to the inactivation of DDAH-1, caused by inflammation. In fact, in patients undergoing liver transplantation, it has

been observed a restoration of DDAH-1 activity, inflammatory cytokines reduction and, at the end, a plasma ADMA content decrease (Ito et al. 1999).

2.1.2 Chronic Liver Failure

Chronic liver disease is characterized by alteration of liver architecture, replacement of hepatic normal tissue by fibrotic one and formation of regenerative nodules. The next stage is characterized by the occurrence of cirrhosis. Cirrhosis is a disabling condition that can lead to severe complications as recurrent ascites, hepatic encephalopathy, bleeding from oesophageal varices and reduced life expectancy. It may be improved or even reverted with specific treatments in the early stages, but in advanced stages, the only feasible treatment is liver transplantation.

Lluch *et al.* observed in their studies that patients suffering from decompensated alcoholic cirrhosis showed higher plasma ADMA and NO levels, compared with compensated alcoholic cirrhosis patients and control group. Moreover, the researchers found a positive correlation between plasma ADMA concentration and the severity of liver injury, because of the inhibiting NOS activity of ADMA (Lluch et al. 2004).

In addition, in end-stage liver diseases, like decompensated cirrhosis or hepatorenal syndrome, increased plasma ADMA levels are accompanied by high concentration of oxidative stress markers, such as 15(s)-8-iso-PGF2_{α}, an oxidative stress product formed by lipid peroxidation (Tsikas et al. 2003).

A further complication of cirrhosis is portal hypertension due to an increased intrahepatic vascular resistance that, in the long run, could lead to compromised portal venous blood flow. In their study, Laleman and colleagues investigated the role of ADMA in the pathophysiology of portal hypertension and they found that decreased eNOS and DDAH-1 activity occurred, worsening the liver damage. These data suggest that ADMA plays a pivotal role in the progression of the disease (Laleman et al. 2005).

2.1.3 Liver surgery

After major liver surgery, elevated plasma ADMA levels have been also described (Nijveldt et al. 2004). Moreover, the pivotal role of the liver in eliminating ADMA from bloodstream was also demonstrated in patients undergoing liver transplantation, that showed, preoperatively, significantly higher plasma ADMA levels, compared with control volunteers (Mookerjee, Dalton, et al. 2007; Siroen et al. 2004).

One day after transplantation, plasma ADMA levels dropped in patients, up to reach control levels, but in patients who rejected the liver graft, a clear increase in ADMA concentration preceded the first episode of rejection. So, after this evaluation, it is reasonable to affirm that elevated ADMA levels could be a potential marker of dysfunction of the liver graft, when it occurs in the post-transplantation period.

It is useful to note, besides, that during orthotopic liver transplantation, the organ undergoes a period of cold ischemia, during which NOS is inhibited by several inhibitors produced by liver, as just ADMA. Moreover, their production is directly related to the length of the cold ischemia; thus, long periods of ischemia are more harmful than short ones, predicting better graft outcome. For this reason, monitoring ADMA levels in the preservation solution could be a useful way to predict early liver graft function (Martín-Sanz et al. 2003).

2.1.4 Hepatorenal Syndrome (HRS)

Hepatorenal syndrome is an acute renal failure, occurring in patients with advanced liver disease. HRS is a life-threatening condition with poor prognosis. Moreover, the pathophysiology of this syndrome is still not completely understood. However, recent studies have provided newer treatment strategies with improved prognosis. In fact, nowadays patients, suffering from HRS, can survive if the disease is rapidly diagnosed, medical treatment is readily administered and liver graft is available for the transplantation (Lluch et al. 2006; Ng et al. 2007).

Patients suffering from HRS show elevated ADMA, SDMA and NO levels compared with healthy subjects. The main HRS symptom is severe renal vasoconstriction, probably due to the loss of liver ADMA uptake activity from circulation. The retention of SDMA is positively correlated with plasma creatinine levels, demonstrating that the kidney plays an important role in the clearance of SDMA, considered a marker of renal dysfunction in cirrhosis. On the contrary, there are no evidence of correlation between creatinine and ADMA. This finding, instead, demonstrates that the reduced renal clearance is not responsible for the accumulation of ADMA, but it is probably due to the liver impairment (Lluch et al. 2004; Ng et al. 2007).

2.1.5 Multiple Organ Failure (MOF)

Multiple Organ Failure (MOF) takes place when the concomitant deterioration of several organs occurs. Patients with MOF show higher mortality, ranging from 30%-80% depending on the number of failed organs and, for this reason, it is considered the most challenging

problem in Intensive Care Unit (ICU) patients (Baue et al. 1998). The most predisposing factors for the development of MOF are sepsis and severe trauma, both surgical and nonsurgical. In the progression of MOF, a relevant role is attributable to liver and kidneys. In fact, Davids and colleagues, by means of a rabbit model of critical illness, elucidated how plasma ADMA levels were significantly related with ADMA levels in the liver (Davids et al. 2012). Later, Nijveldt *et al.* suggested the so-called "ADMA-Multiple Organ Failure hypothesis", reporting that, in critically ill patients, hepatic function parameters independently correlated with ADMA concentration. This finding provides further evidence for the theorized role of the liver and, thus, the ADMA-MOF hypothesis could be a possible explanation for the association between high plasma ADMA concentration and adverse outcome (Nijveldt, Teerlink & van Leeuwen 2003). The MOF-hypothesis was elaborated after the evaluation of a Phase III trial, where, using the unspecific NOS inhibitor N^G-monomethylarginine, mortality rates, in patients suffering from septic shock, increased significantly (López et al. 2004).

The rationale of the treatment was that NO could have a role in the deterioration of septic patients when it is produced in excessive amount: Inducible NOS isoform (iNOS), induced in macrophages in response to septic shock, overproduces NO (Nathan & Hibbs 1991), and the NO overproduction, when released in the wrong site, may affect healthy cells. However, the unspecific blockade of all NOS isoforms, impaired the good functioning of circulating system. eNOS, the endothelial isoform of NOS, is a homeostatic regulator of blood pressure and blood flow, vascular smooth-muscle proliferation, platelet aggregation and leukocyte adhesion (Moncada & Higgs 1993).

In the mid-90s, Huang *et al.* observed that the pharmacological administration of an arginine analogue, in order to reduce the overproduction of NO, could concurrently affect different NOS isoforms. More relevant was the following step. Interestingly, they demonstrated, in mutant mice lacking the eNOS gene, that increased hypertension occurred because of the absence of NO (Huang et al. 1995).

Moreover, Avontuur *et al.* proved that, the L-Arginine infusion, during *ex vivo* experiments on rat heart, reverted local ischemia (Avontuur et al. 1995). These data propose that the organ exposure to NO in critical illness is of vital importance. Advisedly, worsening conditions in critically ill patients are frequently related to increased serum ADMA levels. At last, recent data have been shown that the arginine/ADMA ratio is a more powerful predictor of organ failure with respect to ADMA alone. In fact, the arginine/ADMA ratio in ICU patients is

linked with circulatory and organ failure as well as mortality in septic patients (Visser et al. 2010; Böger 2006b).

ADMA in NAFLD/NASH, Cholestasis & Ischemia: Experimental Models of Liver Diseases

Animal models may be an helpful as well as essential tool for studies of disease pathogenesis. At best, animal models should reproduce the established pathological symptoms of the human diseases, and, in addition, they should also summarize the context of development of a particular disease. Animal models rarely reproduce a pathology in all its aspects, however, with their limits, animal models are the only strategy to deal with *in vivo* studies (Larter 2007).

3.1 NAFLD/NASH and MCD nutritional model

Non-Alcoholic Fatty Liver Disease (NAFLD) is a widespread pathological condition, which is characterized by relevant lipid intracellular deposition in the hepatic parenchyma. It is a chronic condition affecting approximately one-third of the US population and it is often associated with a wide spectrum of metabolic abnormalities, including dyslipidemia, hypertension, insulin resistance and obesity; symptoms that are collectively known as ‘‘metabolic syndrome’’ (Barshop et al. 2008). The growing incidence of this disease in Western society makes the NAFLD the leading cause of recovery into hepatology clinics in US. Fortunately, only about 10% of patients suffering from NAFLD progresses to Non-Alcoholic Steatohepatitis (NASH), a more serious pathological condition. The latter one is distinguishable because it involves liver inflammation and apoptotic cell death and, ultimately, it can result in cirrhosis and/or liver failure (Reddy & Rao 2006).

In the early ‘80s, Ludwig and colleagues coined the term Non-alcoholic Steatohepatitis, “NASH,” to describe, at morphologic level, the severity of liver injury in 20 patients monitored at the Mayo Clinic over a 10-year period (Ludwig et al. 1980). These patients showed the same hallmarks present in alcoholic hepatitis, such as steatosis and lobular inflammation, but they have no history of alcohol abuse. Approximately the 90% of them, however, was obese and they showed symptoms of hyperlipidemia and diabetes mellitus type II. During the years, many other terms have been used to indicate this clinical problem, counting, for example, pseudo-alcoholic liver disease, alcohol-like hepatitis, diabetic hepatitis, non-alcoholic Laennec’s disease and steatonecrosis (Sheth et al. 1997). However,

since the disease counts a wide-range of pathology, the umbrella term "NAFLD" introduced for the first time in 1986, became the preferred one (Schaffner & Thaler 1986).

NAFLD is asymptomatic for the 48–100% of cases. In fact, patients often discover to be affected incidentally, during routine laboratory examination, because the hepatic panel reveals an increased serum Alanine Aminotransferase (ALT) level (Ludwig et al. 1980).
Even though the exact pathogenesis is still unclear, the more accredited hypothesis is that a lot of insults, called "hits", are involved in the onset and progression of liver damage (Day & James 1998). The initial hit results in macro-vesicular steatosis; in this phase, insulin resistance (Tominaga et al. 1995) probably plays an important role in the net lipids retention, in particular triglycerides, that accumulates within the hepatocytes. It seems probable that this occurrence results from decreased catabolism of fatty acids, due to compromised mitochondrial β-oxidation (Reid 2001).
Oxidative stress is usually considered the second hit. It causes membrane lipid peroxidation (Day & James 1998), cytokine production and Fas ligand induction (Pessayre et al. 2001); furthermore, it seems responsible for the progression from simple steatosis to NASH and subsequently to cirrhosis and hepatocellular carcinoma. Other putative agents triggering the progression to fibrosis and cancer are the presence of bacterial toxins (Yang et al. 1997), overproduction of inflammatory cytokines as TNF-α, ATP stores depletion and cytochrome P450 Cyp2E1/Cyp4A enzyme activity dysregulation (Leclercq et al. 2000). Finally, it is difficult to understand the role of leptin in NASH. Only one study suggests that increased serum leptin may stimulate the onset of hepatic steatosis and its progression to steatohepatitis (Uygun et al. 2000), on the contrary, another study concludes that leptin levels correlate directly with severity of hepatic steatosis, but not with inflammation or fibrosis (Chitturi et al. 2002).

The milestone of treatment of patients suffering from NAFLD/NASH is weight loss (Chalasani et al. 2012). It is now well established that calories reduction, physical exercise and behaviour modification could have beneficial effects on NASH progression. In fact, patients who have started on a diet, with an average weight loss of 9.3% from baseline, display histologic improvement in NASH severity (Promrat et al. 2010). Unfortunately an earlier study of patients, showing the typical spectrum of symptoms of metabolic syndrome, suggests that 2 years after the start of the diet, most of them fails to sustain weight loss (Svetkey et al. 2008).

To date, *orlistat* is the only weight loss drug studied in NASH patients. Overweight NASH patients, randomized to be treated either whit *orlistat*, vitamin E and dietary modification or with vitamin E and dietary modification alone, showed improvements in both hepatic inflammation and insulin sensitivity when they achieved weight loss 9% or greater, but this trial did not show any relevant advantage of *orlistat* over vitamin E alone (Harrison et al. 2009).

Since NASH patients presents also insulin resistance, it is not surprising that an alternative hypothesis for NAFLD/NASH treatment is the treatment already accepted for management of diabetes. Biguanides and Thiazolidinediones (TZDs) are insulin sensitizers. In fact, the biguanide *metformin* improves hepatic insulin sensitivity through activation of AMP-activated kinase and following blockade of gluconeogenesis (Zhou et al. 2001). Unfortunately, a meta-analysis demonstrated that *metformin* does not give any benefits in NASH patients treatment, making it useless for treatment of NASH (Vernon et al. 2011).

Conversely, TZDs have been considered promising agents as for the NASH treatment. One of the approved and recommended drug for the management of biopsy-proven NASH in both diabetic and non-diabetic patients is the TZD *pioglitazone* (Chalasani et al. 2012). In NASH diabetic patients, *pioglitazone* significantly reduces steatohepatitis (Belfort et al. 2006; Aithal et al. 2008), displaying greater histologic improvement of the disease, compared with lifestyle interventions (Sanyal et al. 2010). Despite the hopeful results of *pioglitazone*, its use is limited in patients with NASH because of TZD-induced weight gain and other safety concerns in long-term administration.

Finally, therapeutic strategies that target oxidative stress and inflammation, such as vitamin E and vitamin C, have also been employed in NASH patients. Unfortunately, the undefined interaction of vitamin E with cardiac (Saremi & Arora 2010) and oncologic disease risk (Lippman et al. 2009), makes this treatment of limited utility in the general NASH population.

Since the encouraging results obtained by TZD administration, it is not hard to imagine that a category of drugs, able to improve insulin sensitivity and to reduce, at the same time, hepatic inflammation without the PPAR-y-associated side effect of weight gain, has become the “holy grail” of NASH therapeutics.

The latest candidates for NASH treatment are hydrophilic Bile Acids (BAs). They modulate both glucose and lipid homeostasis (Lefebvre et al. 2009) through two receptors, Farnesoid X Receptor (FXR) (Makishima et al. 1999; Wang et al. 1999) and TGR5 (Kawamata et al. 2003) and show a significant anti-inflammatory activity (Hollman et al. 2012).

Several agonists for both receptors have been produced, although the FXR agonist Obeticholic Acid (OCA) is the first in-class synthetic BA under analysis for NASH treatment (Reddy & Rao 2006).

Despite its incidence and serious complications, the molecular mechanisms regulating the onset and the progression of NAFLD to NASH continue to be unclear. Further studies are required to fill the gaps and to identify better therapies in order to prevent and treat this disorder (Feldstein & Gores 2004).

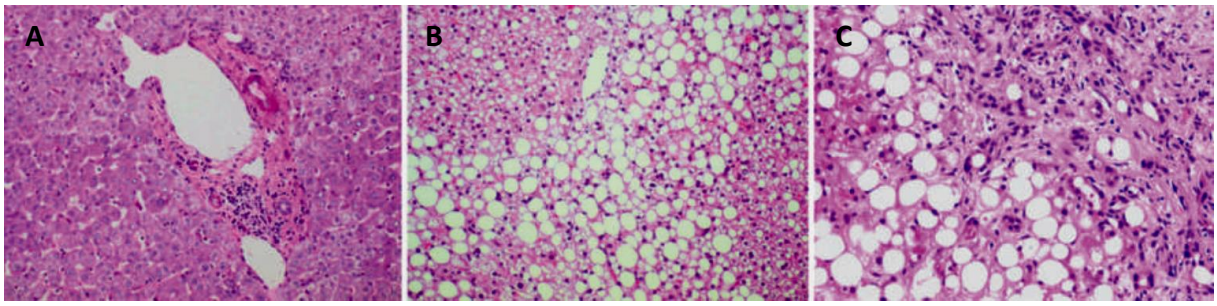


Figure 4. Histopathologic overview of healthy and steatotic human liver. A: Healthy liver. **B:** Non-Alcoholic Fatty Liver Disease (NAFLD); steatohepatitis is absent but prominent macro-vesicular steatosis can be seen. **C:** Non-Alcoholic Steatohepatitis (NASH) with notable ballooned hepatocytes, an elevated immune response and collagen deposition indicative of fibrosis (Hebbard & George 2011).

3.1.1 Animal model for the study of NAFLD/NASH

Since the studies of NAFLD/NASH in humans are limited by the shortness of human biopsies, combined to ethical reasons and to the long time needed for the onset of the disease, a valuable aid in the study of these diseases is provided by animal models, that can help to elucidate both pathogenesis of NASH and assessment of the therapeutic effects of different agents. Despite all their limitations, several animal models have been developed in an attempt to mimic the pathophysiology, biochemical changes, morphological findings and clinical features of human NAFLD/NASH. Currently, two different types of animal models are prevailing: the genetic and the nutritional models (Di Pasqua et al. 2016).

3.1.2 Genetic models of NAFLD/NASH

Zucker Rats. Zucker rats (*fa/fa*) are carriers of a spontaneous mutation in the leptin receptor (*fa* allele). It decreases the receptor affinity for leptin, and consequently, Zucker rats develop severe obesity and are hyperleptinemic, hyperphagic, inactive, obese and insulin resistant (Figure 5). In Zucker model, macro/micro-vesicular steatosis is extensively diffuse, mainly in the periportal area (Fellmann et al. 2013).

Ob/ob Mice. *Ob/ob* mice are leptin deficient, because they exhibit a spontaneous mutation in the leptin gene, so they are not able to produce leptin in their adipose tissue. In normal conditions, after its secretion by adipocytes, leptin reaches the receptor and regulates the feeding behaviour and energy bursts, promoting a reduced food intake and increasing energy metabolism. In the *ob/ob* mice, the lack of interaction between leptin and its receptor makes these mice hyperphagic, extremely obese, and inactive (Kucera & Cervinkova 2014). In addition, these animals have an altered metabolic profile and exhibit hyperglycemia, insulin resistance, hyperinsulinemia, and spontaneous development of fatty liver (Diehl 2005).

Db/db Mice. This mouse model presents a spontaneous mutation in the leptin receptor gene (*Ob-Rb*), so, even if they exhibit normal leptin levels, they are resistant to its effects. Thus, the *db/db* animals are obese, show insulin resistance or diabetes, and develop macro-vesicular hepatic steatosis (Wortham et al. 2008).

SREBP-1c Transgenic Mice. In mammals, a family of transcription factors known as Sterol Regulatory Element-Binding Proteins (SREBPs) controls intracellular levels of cholesterol and fatty acids and it works as a feedback regulatory system. Transgenic mice that overexpress the transcription factor SREBP-1c undergo a deregulation of adipocyte differentiation, which leads to insulin resistance and diabetes. Moreover, whereas the total amount of systemic fat tissue decreases, these animals reveal a significant hepatic lipid accumulation (Shimomura et al. 1998).

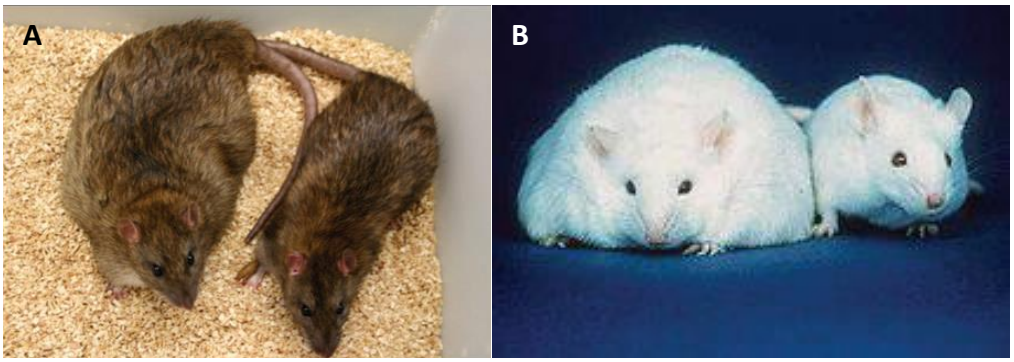


Figure 5: Genetic animal models used for the development of NAFLD/NASH. A: Zucker rats: the recessive (*fa/fa*) genotype develops obesity and liver steatosis (at left side), while the dominant (*lean*) genotype is phenotypically normal (at right side of the picture); **B:** *Ob/ob* mice: the mutant obese (*ob/ob*) mouse (at left side) may present three-fold higher body weight relative to the wild type mouse (at right side of the picture) (The Jackson Laboratory) (Sanches et al. 2015).

The major hindrance of genetic models is that they almost exclusively induce biochemical changes associated with NAFLD, but none of them exhibits peculiar tracts of NASH (Xin et al. 2014). On the contrary, nutritional models resulting in NASH show histopathological tracts similar to those found in humans suffering from NASH (Sanches et al. 2015).

3.1.3 MCD diet: rat nutritional model of NAFLD/NASH

The Methionine and Choline Deficient (MCD) diet has high sucrose and fat composition (40% sucrose, 10% fat), but lacks methionine and choline, crucial for the correct lipid β-oxidation and the production of Very Low Density Lipoprotein (VLDL) in the liver (Anstee & Goldin 2006). Direct consequence of the diet administration is accumulation of intrahepatic lipid and decreased VLDL synthesis.

MCD-animals exhibit body weight loss due to the loss of white adipose tissue (40% in 10 weeks), their liver decreases proportionally in size and develops steatosis, especially in peri-central zone, with consequent onset of necro-inflammation. Taken together, all these symptoms are very reminiscent of those ones of human NASH (Rinella et al. 2008; George et al. 2003). The severity of NASH in rodents fed with the MCD diet is strictly related to their gender and strain (Phung et al. 2009). MCD-fed mice display high levels of inflammation, due to the activation of macrophages that infiltrate liver tissue, the activation of Nuclear

Factor κB (NFκB), Interleukin-6 (IL-6), Transforming Growth Factor β (TGFβ) and Tumour Necrosis Factor-α (TNF-α) (Ip et al. 2004; Yu et al. 2006).

Moreover, other peculiar features found after the MCD diet administration are the increased expression of Intercellular Adhesion Molecule 1 (ICAM1), Vascular Cell Adhesion Molecule 1 (VCAM1) and Macrophage Chemotactic Protein 1 (MCP1), accompanied by raised macrophage phagocytic activity and increased leukocyte adherence (Leclercq et al. 2004; Dela Peña et al. 2005; McCuskey et al. 2004). Although MCD-animals show severe liver damage, as demonstrated by increased serum Alanine Aminotransferase (ALT) levels, a reduction in plasma triglyceride and cholesterol levels takes place. This finding is in contrast with what has been observed in overweight and obese individuals suffering from fatty liver disease (Weltman et al. 1996; George et al. 2003). A part from these findings, the major drawback of the MCD diet is that the metabolic profile appears to be the opposite of that seen in patients, with regards to insulin, leptin and glucose levels, whereas serum adiponectin is not affected by diet, and the animals are peripherally insulin sensitive (Larter et al. 2008; Nagasawa et al. 2006). Nonetheless, some study have recently demonstrated that the MCD diet can reduce hepatic insulin sensitivity by means of the activation of oxidative stress and c-Jun N-terminal kinase (JNK), in order to suppress insulin receptor activity (Schattenberg et al. 2005; Schattenberg et al. 2006). MCD-diet fed rats, within a period of 8 weeks, develop hepatic steatosis, hepatocyte injury, inflammation and ultimately fibrosis, a spectrum of changes that mimics the hepatic pathology of NASH (Veteläinen et al. 2007). As already mentioned, liver injury induced by an MCD diet is accompanied by elevated transaminases, protein cytokines, oxidative stress and changes in mitochondrial function (Simon et al. 2014).

Concluding, genetic models are more suitable for diabetes and NAFLD, while nutritional models, like MCD, are more suitable for studies on lipotoxicity, inflammation and on the pathogenesis of cirrhosis and Hepatocellular Carcinoma (HCC).

3.1.4 ADMA/DDAH/NOS pathway in MCD-rats

In this context already particularly compromised, it is not impossible that even the ADMA/DDAH/NOS pathway may be affected. Since ADMA competes with L-Arginine to bind all isoforms of NOS, inhibiting NO production and causing vasoconstriction, increased platelet aggregation, increased cell adhesion to the endothelium and increased vascular muscle cell proliferation (Richir et al. 2008); an alteration of its handling in NAFLD/NASH could induce further cardiovascular complications.

Indeed, cardiovascular disorders (whose ADMA is a sensible marker) are the major cause of death in NAFLD patients. Insulin resistance, hepatic dysfunction and chronic inflammation, that all occur in NAFLD/NASH, are also strictly linked to the increased incidence of cardiovascular events in NAFLD (Boga et al. 2015).

3.2 BDL-rats as a model for obstructive cholestasis

A common impairment in bile formation, occurring in a wide variety of human liver diseases is cholestasis (Miyoshi et al. 1999). Cholestasis can develop after a mechanical blockade in the duct system due to gallstone presence or it could be a metabolic impairment caused by genetic defects or acquired as a side effect of many medications. In the classical hereditary cholestasis, underlying progressive familial intrahepatic cholestasis type I-III, mutations in genes encoding for transporters involved in hepatocellular bile formation, such as ATP8B1, BSEP, and Multidrug Resistance Protein 3 (MDR3), occur (Hirschfield 2013). Mutations in bile acid synthesis enzymes or in gene encoding for the bile acids receptor Farnesoid X Receptor (FXR) are much more rare (Monte et al. 2009; Gomez-Ospina et al. 2016). Furthermore, intrahepatic cholestasis of pregnancy or drug-induced liver damage have been associated to mutations in the Notch signalling pathway, that often causes child cholestasis in Alagille Syndrome, to various polymorphisms in MDR3, Multidrug Resistance associated Protein 2 (MRP2) and, lastly, to FXR (Hirschfield 2013).

Remarkably, mutations of Sodium Taurocholate Co-transporting Polypeptide (NTCP) outcome in marked hypercholanemia without classical clinical features of cholestasis or impaired enterohepatic circulation (Vaz et al. 2015). Cystic Fibrosis Transmembrane conductance Regulator (CFTR) inherited mutations are the most common genetic contributors to cholestasis in cholangiocytes (Hirschfield 2013). Whereas, polymorphisms in Anion Exchanger 2 (AE2) show a role as disease modifiers in Primary Biliary Cholangitis (PBC) (Poupon et al. 2008). Finally, changes in tight junction proteins along the hepatocytes canalicular membrane and cholangiocytes result in genetic forms of cholestasis (Sambrotta et al. 2014).

Not hereditary cholestasis originates at the hepatocellular level, *e.g.* estrogen-induced mild cholestasis, sepsis-induced cholestasis, and drug-induced cholestasis, or at cholangiocytes and bile duct levels, *e.g.* PBC, Primary Sclerosing Cholangitis (PSC), secondary sclerosing cholangitis, and intraluminal/extraluminal bile duct obstructions, or it may be a combination of hepatocellular and cholangiocellular origins (Geier et al. 2006). The most shared hallmark

of different forms of cholestasis is the impairment of bile acids circulation along the enterohepatic flow, ensuing in the accumulation of potential toxic bile acids in the bloodstream and at intracellular level. The overall purpose to treat cholestasis, thus, is to decrease hepatic and systemic accumulation of bile acid and to reduce bile acid pool size.

The main clinical feature of cholestasis is displayed by toxic bile salts retention in the liver. This occurrence is accompanied by intracellular metabolic disorders, inflammation and reduced detoxification capacity (Shoda et al. 2001), which may enhance generation of reactive oxygen/nitrogen species and oxidative stress in hepatocytes (Portincasa et al. 2007). The accumulation of ROS species triggers many events responsible for lipid and protein oxidation (Muriel & Suarez 1994; Chojkier 1995).
However, both bile salts retention and oxidative stress cannot entirely explain the progression of cholestatic liver toward organ failure. Growing data suggest that other tissues may also be affected during prolonged cholestasis, but, at the same time, it seems that extrahepatic factors may take part in the determination of cholestatic liver injury (Assimakopoulos et al. 2006). This hypothesis is confirmed by the fact that extended interruption of enterohepatic bile salt circulation harms the intestinal permeability and favours endotoxemia at portal level, worsening the hepatic damage (Welsh et al. 1998; Parks et al. 2000). All these damaging events cause also a decrease in the reduced Glutathione (GSH) stores, producing the impairment of the detoxification defences (Vendemiale et al. 2002).
GSH is the most important intracellular detoxifying agent and it plays a crucial role in bile formation and biliary excretion of toxic compounds (Ballatori & Truong 1989; Grattagliano et al. 2005; T. K. Lee et al. 2001). Since GSH and its related enzymes exert important functions also in the intestinal mucosa, its levels are maintained by both intracellular *ex novo* synthesis and recycling of the biliary-derived GSH. The second aliquot may be dramatically affected by bile flow break down and may harm the intestinal GSH-dependent antioxidant defence.

3.2.1 BDL-rat model

The Bile Duct Ligation (BDL) rat-model has been extensively employed to study cholestatic liver injury in association with oxidative stress and fibrogenesis. Developing and adult BDL rats show high transaminase serum levels as well documented by concentration of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), γ-Glutamyltranspeptidase (γ-GT), bilirubin, Alkaline Phosphatase (AP), and Lactic Dehydrogenase (LDH) (Fursule & Patil 2010; Y.-L. Tain, Hsieh, et al. 2010). In rat, BDL-operation stimulates biliary epithelial cells

and hepatocyte progenitors proliferation, ensuing in increasing bile ductules, associated to fibrosis and portal inflammation. Conversely, cholangiocytes proliferation begins after BDL at the portal tract edge. Two weeks after the operation, obstructive jaundice occurs and it leads to cirrhosis in 4 to 6 weeks (Marques et al. 2012). In a time-course progression pattern, pathological changes, including fibrotic changes, are more serious at 4 weeks after BDL than at 2 weeks (Veidal et al. 2010). Finally, developing BDL rats also show a similar trend of liver pathology progression (Sheen et al. 2010).

3.2.2 ADMA in BDL-rat model

This rat model of cholestasis displays higher plasma ADMA, SDMA and L-Arginine levels in comparison with control rats (Sheen et al. 2010; Yang et al. 2012). In fact, BDL rats present an increased ADMA/L-Arginine ratio respect to sham-operated (Y.-L. Tain, Hsieh, et al. 2010; Huang et al. 2012). Furthermore, during the development of disease from 2 to 4 weeks in BDL-rats, progressive plasma ADMA accumulation occurs, whereas plasma L-Arginine levels diminish in a proportional way when compared with sham rats (Schwedhelm et al. 2008).

Since plasma ADMA concentration is tightly controlled by the liver activity, it is reasonable to suppose that hepatic dysfunction occurring in BDL rats may cause a dysregulation in ADMA handling. After BDL treatment, rats show peculiar symptoms, characterized by widespread damage to major organs, including liver, intestine, kidney, heart and also brain (Sheen et al. 2010; Ljubuncic et al. 2000; Grintzalis et al. 2009) (Figure 6). Multiple organ injury is strictly related to increased levels of ADMA, which being a pro-oxidant agent, enhances ROS generation (Y.-L. Tain, Hsieh, et al. 2010; Ljubuncic et al. 2000), resulting in elevated oxidative stress and production of circulatory toxins such as bile acids and Malondialdehyde (MDA). Therefore, it is a systemic phenomenon encompassing all tissue and organs (Assimakopoulos et al. 2006; Huang et al. 2009).

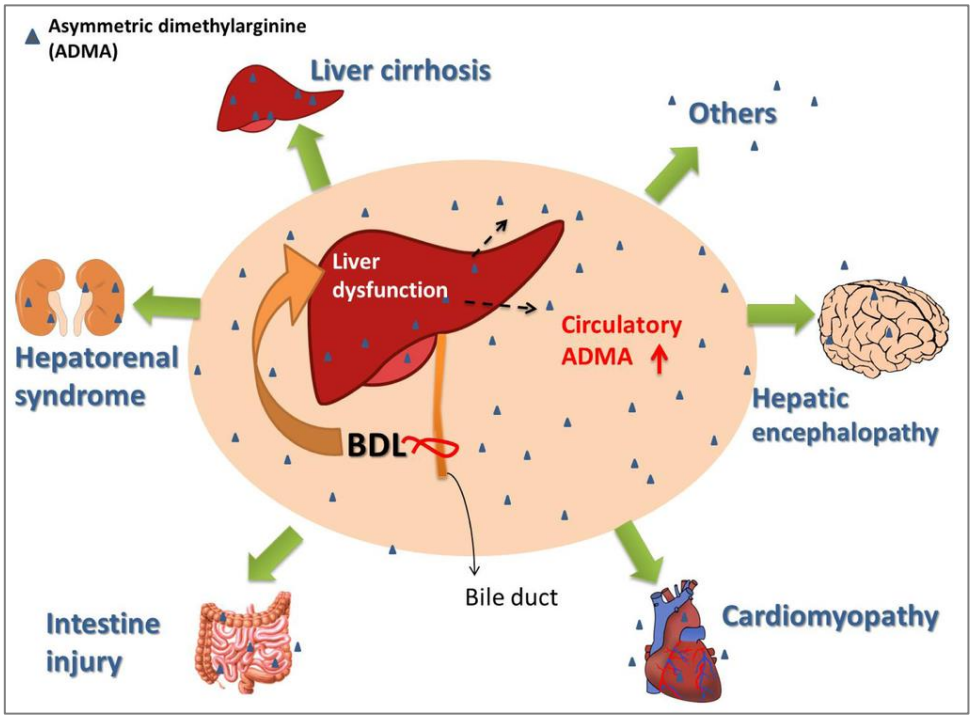


Figure 6. ADMA & MOF. The role of increased circulatory Asymmetric Dimethylarginine (ADMA) in multiple organ damages in the bile duct ligation rats.

A possible consequence of the long clinical course of all chronic liver diseases like cholestasis is, frequently, the development of cirrhosis. The main characterizing feature of this dysfunction is the transformation of normal liver architecture tissue into fibrotic one, formed by structurally abnormal nodules. The architectural distortion of the tissue leads to increased intrahepatic resistance and, subsequently, to portal hypertension and endothelial dysfunction (D'Amico et al. 2006; Iwakiri & Groszmann 2007). Latter ones may be attributable to a different regulation of eNOS in the affected liver tissue compared to the splanchnic vessels, where is downregulated and upregulated, respectively (Sarela et al. 1999). iNOS, instead, is intensively induced and it is highly expressed in hepatocytes from BDL rats (Wei et al. 2002), differently from human cirrhotic liver, in which iNOS is highly expressed in the inflammatory cells infiltrating the portal tracts, blood monocyte-like cells, hepatocytes, sinusoidal cells, and endothelial cells (Mohammed et al. 2003).

In rat with bile duct excision-induced biliary cirrhosis, ADMA mediates the decrease of NOS activity (Laleman et al. 2005). Moreover, it has been demonstrated by Serna *et al.* that NO release increases in rats with small mesenteric arteries secondary biliary cirrhosis, and that the

ADMA/DDAH/NOS pathway plays a role in the increased generation of endothelial NO. In mesenteric vessels, the increased DDAH-1 and DDAH-2 expression protect different isoforms of NOS from the increased plasma ADMA levels, due to cirrhosis (Serna et al. 2013).

Previous researches demonstrated that hepatic PRMT-1 expression increased in the BDL developing rat; on the contrary, the hepatic protein expression of DDAH-1 and DDAH-2, and their activity were not affected by cirrhosis (Y.-L. Tain, Hsieh, et al. 2010; Huang et al. 2012; Y. L. Tain et al. 2010).

Concerning ADMA transporters, the hepatic CAT-1 protein level was increased in the BDL rat (Tain et al. 2013), whereas the expression of CAT-2 was reduced (Chang et al. 2013). Since ADMA is metabolized in the whole body, some organs may utilizes CATs to export ADMA to the plasma compartment and other ones may serve as a bridge for ADMA influx.

Yang *et al.* administered vitamin E to decrease lipid peroxidation in the BDL adult rats and reported the suppression of hepatic MDA and PRMT-1, with a concomitant increase in DDAH-2, eNOS, phospho-eNOS and ADMA levels in the cirrhotic liver (Yang et al. 2012). Finally Tain *et al.* found that melatonin decreased liver injury in BDL rats by reducing the ADMA level and oxidative stress, enhancing DDAH activity (Y.-L. Tain, Hsieh, et al. 2010).

Until now, studies have been primarily focused on the cellular alterations taking place in an individual lobe, while there are no data on the changes occurring in all hepatic lobes, simultaneously. Recently, emerging evidences have been shown that it could be a functional heterogeneity among the individual liver lobes, that may help to clarify heterogeneous damage distribution within a single lobe or among different lobes during acute hepatic necrosis. Indeed, several studies demonstrated a marked lobe variation in the degree and distribution of tissue damage in chemical carcinogenesis (Richardson et al. 1986), copper distribution (Faa et al. 1995), cirrhosis (Regev et al. 2002), Ischemia/Reperfusion (I/R) (Palladini et al. 2012), and *acetaminophen* hepatotoxicity (Irwin et al. 2005). One possible explanation may be connected with the distribution of the liver vasculature. In fact, an incomplete mixing of blood coming from the gastrointestinal tract and spleen occurs, and it is cause of variation in the delivery of nutrients and toxins to the different liver lobes. For example, venous portal blood draining the stomach and the spleen is conveyed to the left side of the liver. Moreover, another potential mechanism that may explain hepatic variability is a different lobar gradient of gene expression profiles (Malarkey et al. 2005) as for

acetaminophen hepatotoxicity, *i.e* a condition in which the variability of lobar injury has been correlated to different regional gene expression (Ruepp et al. 2002).

Thus, it is likely that somewhat similar may occur also during cholestasis, affecting the modulation of ADMA/DDAH/NOS pathway too. It would be interesting to clarify this mechanism in order to provide feasible strategies for the treatment of cholestatic liver disorders.

3.3 Hepatic Ischemia/Reperfusion injury

Hepatic Ischemia/Reperfusion (I/R) injury represents a complication occasionally occurring during liver transplantation, resection surgery and hypovolemic shock. It is often connected with cholestasis, explained as a reduction in the expression of hepatobiliary transporter proteins (Tanaka et al., 2006).

Ischemia is an inadequate supply of blood to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism to keep tissues alive. Several factors contribute to the onset of this damage, potentially leading to cell death, either for apoptosis or necrosis, while the recipient could suffer from Primary Non Function (PNF). The extent of I/R injury depends on the length of preservation period and on organ conditions. So, it is reasonable that a short ischemia period makes organ injury reversible; on the contrary, when long ischemia period occurs, the injury becomes irreversible.

Reperfusion is necessary to re-establish the delivery of oxygen and nutrients to support cell metabolism and remove potentially damaging by-products of cellular metabolism, nonetheless it can elicit pathogenic processes, exacerbating injury caused by ischemia *per se* (Kalogeris et al. 2012).

During ischemia, hepatocytes show morphological changes of the plasmatic membrane, consisting mainly in blebs generating. Blebs contain cytosol and endoplasmic reticulum, while mitochondria and lysosomes are excluded. Cell volume increases about 30%-50%, and when blebs burst out, the release of enzymes and intracellular catabolites occurs, together with the collapse of the ionic and electrochemical gradients. Moreover, hypoxia causes mitochondrial impairment, cellular acidosis and Kupffer cells activation (Lemasters 1999).

During reperfusion, Reactive Oxygen Species (ROS) increase, triggering a further activation of Kupffer cells within 2 hours. After 48 hours post injury, the inflammatory process begins, causing neutrophils infiltration, cytokines releasing and proteases activation (Carden & Granger 2000).

The fundamental event of ischemic damage is the reduction of ATP synthesis, produced by mitochondria. Without oxygen, oxidative phosphorylation stops and the proton gradient between the intermembrane space and the inner mitochondria is abolished, interrupting ATP synthesis. This rapid fall in intracellular ATP concentration induces a cascade of events leading to reversible cell damage, that, over time, gradually becomes irreversible, causing cell death and destruction of the parenchymal tissue. The importance of ATP depletion is demonstrated by the ability of glycolytic substrates to rescue sinusoidal endothelial cells from lethal cell injury (Nishimura et al. 1998). Unfortunately, glucose does not protect hepatocytes against anoxic injury since hepatocytes lack hexokinase IV, which catalyses the first glycolysis reaction to obtain Glucose 6-Phosphate (G6-P) from glucose. However, hepatocytes are able to produce energy from glycogen that, once it is transformed in glucose 6-phosphate, can enter the glycolytic cycle. When glucose and glycogen are sold out, hepatocytes exploit fructose or, in addition, they can enhance gluconeogenesis (Nieminen et al. 1990).
Other consequences of ATP depletion are the release of free radicals and destruction of plasmatic membranes because Na^+/K^+ ATP-ases cannot control electrochemical gradient anymore, causing cellular oedema (Reddy et al. 2004).

Another process occurring during I/R injury is intracellular acidosis. This event is due to the increased production of protons, caused by metabolic modifications that quickly saturate the buffering capacity of the cell. Intracellular acidosis alters the physiological functioning of the cell by increasing intracellular Na^+ through the activation of Na^+- and Ca^{2+}-exchangers, increasing the production of free radicals, changing the affinity of proteins and their tertiary structures, inhibiting enzymes, and disrupting the function of sarcoplasmic pumps and carriers (Martin et al. 2006). The main source of protons during ischemia comes from the production of lactate from pyruvate by Lactate Dehydrogenase (LDH). The accumulation of extracellular lactate greatly reduces the effectiveness of the lactate/proton co-transporter, preventing the removal of protons. Additionally, the residual metabolic activity also contributes to acidosis, as the hydrolysis of an ATP molecule releases a proton (Gourdin & Dubois 2013).

During ischemia, the excessive amount of calcium triggers the activation of several enzymes: proteases, responsible for cytoskeleton damages and vesicles formation; phospholipases, causing deformation of plasmatic membrane; endonucleases, determining chromatin condensation; in mitochondria, ATP synthase, pyruvate dehydrogenase, isocitrate

dehydrogenase, that attempt to stop ATP depletion. An imbalance of calcium homeostasis leads to cell death of hepatocytes as well as endothelial and Kupffer cells (Schanne et al. 1979).

Kupffer cells are specialised macrophages within the liver, localised between sinusoid walls, that form part of reticulo-endothelial system, and blood vessels. Their task is the defence of the liver from bacteria, viruses and other exogenous compounds. They are critical in the earliest stages of Ischemia/Reperfusion injury. During I/R process, Kupffer cells are overproduced, and their products, such as cytokines, are consequently overexpressed. This leads to the exacerbation of injury. Among cytokines are included: Interleukine-1 (IL-1) and Interleukine-6 (IL-6), that increase the oxidative stress damage and induce apoptosis in hepatocytes; Tumour Necrosis Factor (TNF)-α, which stimulate chemokines release, that in turn recall other cells of immunity. Moreover, cytokines promote the secretion of selectines and integrines, that occlude sinusoids, prolonging hypoxia and triggering any further activation of Kupffer cells (Kobayashi et al. 2002).

After I/R process, Reactive Oxygen Species (ROS) are overproduced. When liver is reoxygenated during reperfusion, xanthine oxidase produces Superoxide (O_2^-) and Hydrogen Peroxide (H_2O_2), that are subsequently converted to Hydroxide (OH^-) mediated by Ferrous (Fe^{2+}) oxidation. Besides xanthine oxidase, mitochondria and other ROS-producing systems contribute to the O_2^- and H_2O_2 formation (Becker 2004).

In physiologic conditions, cells have specific endogenous systems, able to reduce ROS effect. Superoxide Dismutase (SOD), reduced Glutathione (GSH), vitamins A, E, C, some elements (selenium, zinc, magnesium) and other molecules, such as melatonin and bilirubin are involved in the counterbalancing ROS production and inactivation (Sauer et al. 2002). Nevertheless, at certain conditions, this equilibrium can shift in favour of free radicals: oxidative stress is so established.

Injury induced by reactive oxygen species occurs at several levels. ROS can produce mutations in DNA, once superoxide binds to guanine, forming 8-hydroxyguanosine. At plasmatic membrane level, lipid peroxidation takes place: long fatty acid chains of membrane phospholipids are converted into molecules characterised by lower molecular weight, such as fatty acids, alkenes, ketons, MDA, that in turn can oxidize membrane proteins. Moreover, primary structure of proteins, and consequently their functionality, can be modified by Reactive Nitrogen Species (RNS) (Walker et al. 2001).

ROS act also as signalling molecules that upregulate nuclear transcription factors like NF-κB and subsequently release TNF-α and IL-1 (Cursio et al. 1999).
Additionally, ROS activate inflammatory pathways that lead to neutrophil accumulation in the liver during reperfusion (Papadopoulos et al. 2013).

During liver I/R injury, NO reduction is associated with the worsening of the hepatic damage. In this particular condition, the NO steady state production decreases, due to a reduced eNOS activity. On the contrary, restoration of NO to more physiological levels diminishes the liver ischemic injury, enhancing hepatic oxygenation and sinusoidal microcirculation (Siriussawakul et al. 2010).
There is a still open controversy about the detrimental or beneficial effects of NO in I/R injury. In a rat model of hepatic I/R, Wang and co-workers detected an increased iNOS mRNA and protein expression on the first day following hepatic reperfusion. Upregulation of iNOS correlated with increased hepatic injury, as shown by elevated serum levels of ALT and AST, two markers for liver injury. Administration of a non-selective NOS inhibitor, significantly increased AST and ALT, while administration of a selective iNOS inhibitor significantly reduced transaminases levels. These data suggested to the authors that the harmful effects of the non-selective NOS inhibitor were due to inhibition of eNOS, while the protective effects of the selective inhibitor were caused by inhibition of iNOS (Wang et al. 1998).

3.3.1 ADMA inhibits NOS activity

As already mentioned, ADMA is an endogenous inhibitor of all isoforms of NOS because of its ability to compete with L-Arginine for each of these three enzymes and also for cellular transport across CATs.
During hepatic Ischemia/Reperfusion injury, ADMA increases because of the lacking activity of DDAH-1, that is highly affected by ROS presence. This condition triggers a fatal loop: more ADMA accumulates, more NOSs are inhibited and more damage increases (Ferrigno et al. 2014). Moreover, Martín-Sanz and colleagues, demonstrated that a positive correlation exists between liver function and survival after transplantation and handling of dimethylarginines (Martín-Sanz et al. 2003). Ever more, in fact, recent findings have been shown that iNOS is upregulated following hepatic I/R injury and this upregulation is preceded by NF-κB activation. Although iNOS appears to intensify the I/R damage and eNOS appears

to protect organs, as heart and kidney, from it, the relative contribution of each isoform of NOS in the liver injury following reperfusion remains unclear (Zhang et al. 2009).

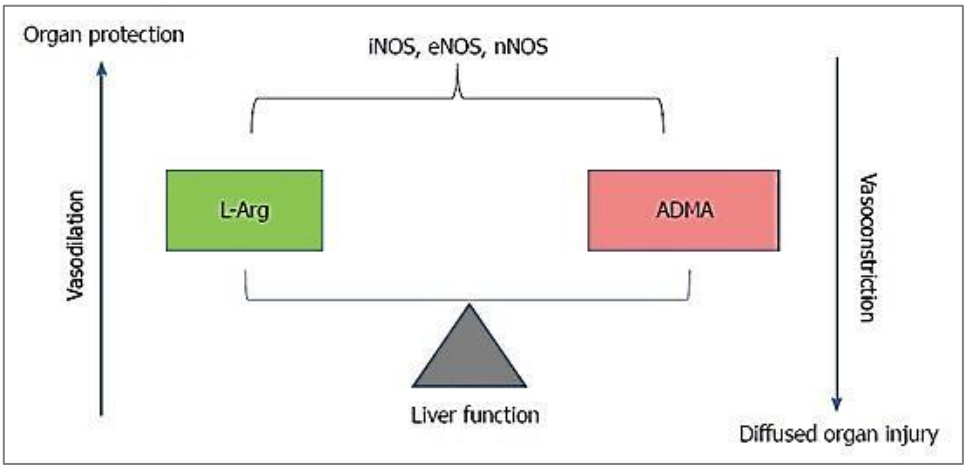


Figure 7. Role of L-Arg/ADMA ratio. ADMA accumulation blocks NOS and induces consequent endothelial dysfunction in the vasculature. On the contrary, high L-Arg levels, as substrate for NOS, induces vasodilation (Ferrigno, Laura G Di Pasqua, et al. 2015).

In a study on vascular Endothelial Cells (ECs), it has been verified that treatment with a nuclear FXR agonist, such as physiologic CDCA or semi-synthetic GW4064, leads to upregulation of eNOS mRNA and protein expression, resulting in NO production increase. FXR seems to be able to regulate eNOS expression at transcriptional level because eNOS promoter activity was significantly increased *via* pharmacological or genetic activation of FXR. Furthermore it was identified an Inverted Repeat separated by two nucleotides (IR2) as a novel FXR Responsive Element that appears to be involved in CDCA or GW4064-mediated upregulation of eNOS in vascular ECs (Laffitte et al. 2000).

Nevertheless, we are very far from understanding the real relationship between ADMA and hepatic I/R injury: the molecular mechanisms involved in Ischemia/Reperfusion injury are not completely understood. Only few works reported that hepatic I/R injury may affect the ADMA/DDAH/NOS pathway, but to clarify this mechanism could be an interesting potential strategy capable of reducing the effects of I/R.

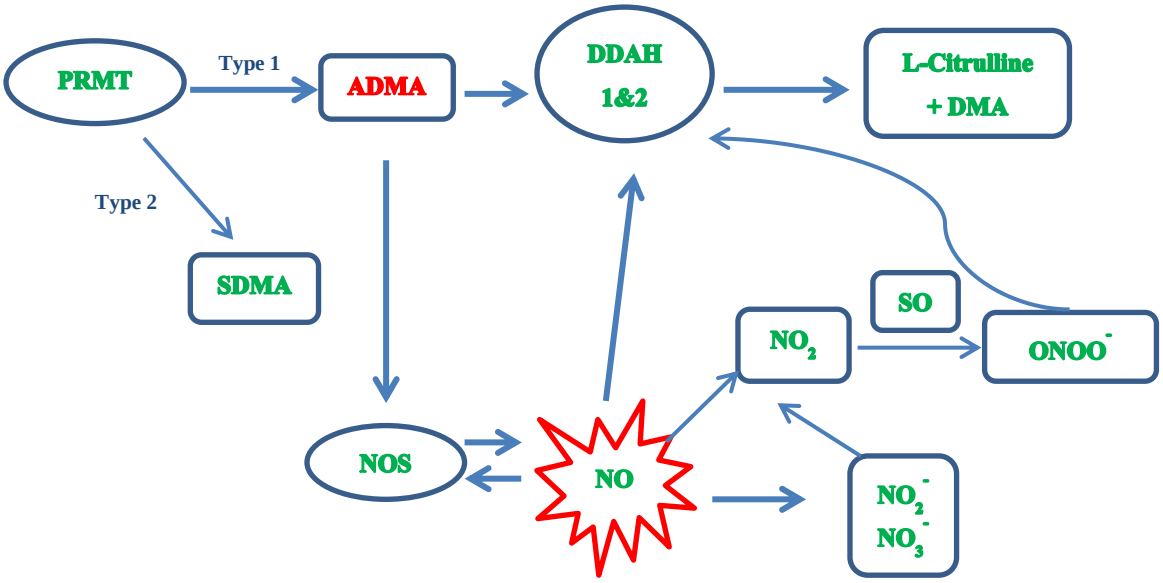


Figure 8. The DDAH/ADMA/NOS pathway. The methylation of protein incorporated arginine by PRMTs and subsequent proteolysis of arginine methylated proteins leads to a production of the methylarginines ADMA and SDMA. ADMA (but not SDMA) inhibits the enzyme NOS, which is essential for the production of NO. NO is converted to nitrite (NO_2^-) and nitrate (NO_3^-) and, under low oxygen conditions, can be converted to the superoxide (SO) to form the peroxynitrite ($ONOO^-$), leading to cellular damage and death and Dimethylamine (DMA).

The Farnesoid X Receptor

Farnesoid X Receptor (FXR) is as a member of the Nuclear Receptor (NR) superfamily. It is highly expressed in several tissues, such as liver, intestine, kidney, adrenal glands, whereas lower levels of its expression are described in adipose tissue and heart (Sepe et al. 2015; Fiorucci et al. 2007). NRs are able to act as transcription factors, hence they bind directly to the DNA sequence, regulating the expression of specific target genes, thereby they control organism development, homeostasis, and metabolism. Generally, the regulation of gene expression by nuclear receptors occurs when a specific ligand is present. More specifically, ligand binding to a nuclear receptor results in a conformational change in the receptor and its activation, leading to up- or downregulation of gene expression.

Farnesoid X Receptor (FXR) was identified at first as a rat orphan receptor, activated by high concentrations of farnesol, an isoprene intermediate in the mevalonate biosynthetic pathway, presumably as a consequence of its conversion to an unidentified metabolite with higher activity (Forman et al. 1995; Blumberg & Evans 1998). Some years later, Zavacki and colleagues discovered that FXR is also activated by Retinoid Acids (RAs) (Zavacki et al. 1997). Since this activation requires over-physiological concentrations of RA and it has been impossible to demonstrate direct binding of these compounds to the receptor, it is reasonable that they are unlikely to act as direct ligands. This raises the hypothesis that either an unidentified retinoid metabolite was the endogenous FXR ligand, or that both retinoids and farnesoids were mimicking the activity of an authentic ligand (Blumberg & Evans 1998). Subsequently, transfecting monkey kidney CV-1 cells and human hepatocellular carcinoma HepG2 cells with murine or human FXR expression plasmid, and treating the cells with a series of bile acid metabolites, it became clear that bile acids were the physiological ligands of FXR (Makishima et al. 1999).

4.1 Genetics and Structure of FXR

In mammals, two FXR genes are known: FXRα (NR1H4-nuclear receptor subfamily 1, group H, member 4) and FXRβ (NR1H5). In humans and primates, FXRβ is a *pseudogene*, while it constitutes a functional nuclear receptor protein in mice, rats, rabbits and dogs. It has been proposed to be a lanosterol sensor, even if its physiological function remains to be established (Otte et al. 2003). The FXRα gene is evolutionarily conserved, from fish to humans,

suggesting the essential role of this gene among the species. The human FXRα gene is mapped on chromosome 12q23.1, while the murine gene on chromosome 10c.2 and in both species, it contain 10 introns and 11 exons. FXRα encodes four transcriptional isoforms (FXRα1, α2, α3 and α4), as a result of alternative splicing of exon 5 and it uses two distinct promoters, which begin transcription from either exon 1 (5' promoter) or exon 3 (3' promoter). The 4 isoforms display tissue-specific expression and few target genes are isoform-dependent regulated (Huber et al. 2002; Zhang et al. 2003).

Since Farnesoid X Receptor α isoforms belong to NR superfamily, they share the common nuclear receptor structure. FXR is composed of a variably N-terminal domain, a central DNA Binding Domain (DBD), highly conserved, and a moderately conserved C-terminal Ligand Binding Domain (LBD). In the N-terminal and the C-terminal domains are present the ligand-independent Activation Function 1 domain (AF1) and the ligand-dependent Activation Function 2 domain (AF2), respectively. The latter mediates receptor activation/repression through binding to co-activator/co-repressor complexes. Two cysteine-coordinated Zn^{2+} finger motifs, directly involved in DNA binding, are located in the DBD region. The DNA Binding Domain is considered as an allosteric transmitter of information to other receptor regions. Conversely, LBD, implicated in co-regulator interaction, is constituted of a 12 α-helices bundle, forming a hydrophobic pocket, the cradle of endogenous and synthetic lipophilic ligands. Moreover, LBD is the first mediator of self-assembly reactions (such as dimerization or tetramerization), necessary for high-affinity DNA response element binding. The DBD and LBD are separated by a hinge region, a flexible amino acids linker between these two regions (Fiorucci et al. 2007; Modica et al. 2010; Bain et al. 2007) (Figure 9).

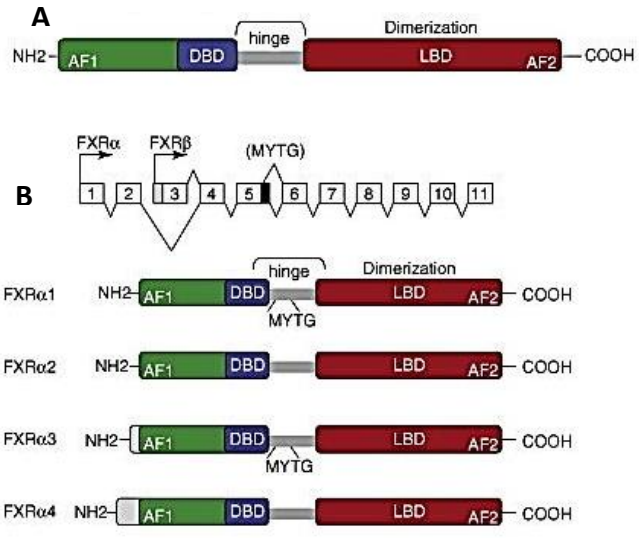


Figure 9. Molecular structure of FXR. A: Nuclear receptors share common structure. They are composed of NH_2-terminal domain, which contains AF1 *trans*-activation domain and DBD that recognizes specific DNA sequences, C-terminal domain, that contains AF2 domain and LBD domain, useful for dimerization or tetramerization. DBD and LBD are linked by a variable linker region, termed hinge domain. **B:** Schematic representation of exon-intron organization of FXRα gene and relative protein isoforms. Different isoforms are generated by alternative promoter usage, or alternative splicing mechanism. FXRα1 and FXRα3 present an insertion of four amino acids (MYTG) in the hinge-domain region (Fiorucci et al. 2007).

The Farnesoid X Receptor Response Element (FXRE) consists of Inverted Repeats (IRs) with the core sequence *AGGTCA* separated by one nucleotide (designated IR1), or Direct Repeats separated by four nucleotides (DR4), or Everted Repeats separated by eight nucleotides (ER8) (Kalaany & Mangelsdorf 2006).

Co-activator and co-repressor complexes are implicated in FXR activation and repression, respectively. When the ligand is absent, mono- or dimerized FXR is associated with co-repressor complex, such as Nuclear Co-repressor (NCor), which recruits histone-deacetylase activity. Deacetylation of histone tails makes the chromatin more compact, therefore suppresses transcription. Receptor activation, instead, causes the release of the co-repressor complex and the AF2-dependent recruitment of a co-activator complex, such as Steroid Receptor Coactivator-1 (SRC-1), PRMT-1, Peroxisome-Proliferator-Receptor (PPAR-)-γ, Co-activator-1α (PGC-1α) and vitamin-D-Receptor-Interacting Protein-205 (DRIP-205). These proteins are able to relax chromatin and activate gene transcription. However, the mechanisms by which FXR ligands recruit co-activators remain still unknown (Pellicciari et al. 2005; Rizzo et al. 2005) (Figure 10).

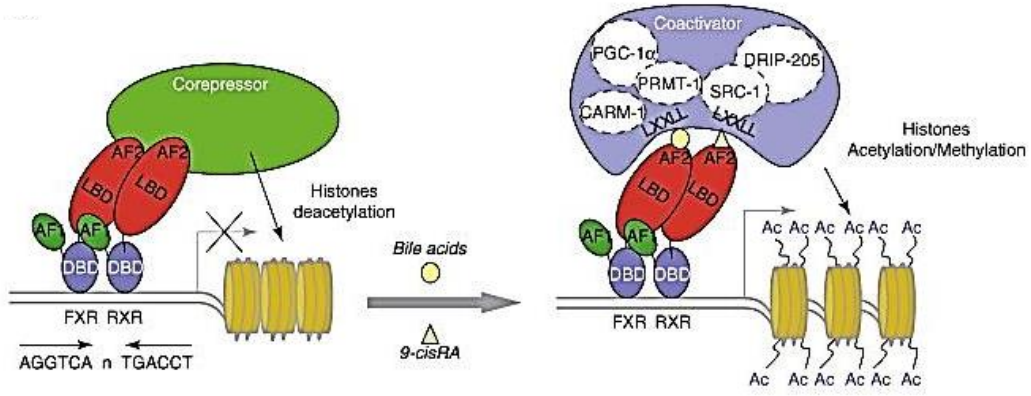


Figure 10. Co-activator and co-repressor complexes involved in FXR activation and repression, respectively. In the absence of ligand, the FXR heterodimer is associated with co-repressor complexes, that recruit histone deacetylase activity. The ligand bound determines the recruitment of AF2-dependent co-activator complexes, that allow chromatin decompactation and gene transcription (Fiorucci et al. 2007).

The effector function of nuclear receptors is accomplished through several distinct mechanisms, which include both *trans*-activation and *trans*-repression activities upon receptor-specific ligand binding. Nuclear receptors can also be the targets of other signalling

pathways that modify the receptors, or their transcriptional co-modulators, affecting their activity and functions (Pawlak et al. 2012).

Trans-activation can be accomplished through different mechanisms.

1. **Simple *trans*-activation.** It represents the most common mechanism. In this process, Farnesoid X Receptor, belonging to the metabolic receptor subfamily, heterodimerizes with Retinoid X Receptos (RXR) and binds directly to its FXR Responsive Element (FXRE). FXR can bind different REs with different affinity, modulating different target genes expression.
2. **Composite *trans*-activation.** Through this mechanism, FXR binds to an FXRE and synergizes with other DNA binding transcription factors. In fact, it has been shown that many FXREs contain another half NRE-site in close proximity (Chong et al. 2010). This could suggest that other nuclear receptors can modulate FXR function.
3. **Monomeric *trans*-activation.** FXR does not necessarily require the heterodimerization with RXR; in some cases, FXR has an RXR-independent transcriptional activity, precisely through monomeric *trans*-activation. For Uridin 5'-diphosphoateglucuronosyltransferase 2B4 (UGT2B4), FXR induces bile acid glucuronidation, binding to a NR half site regardless of RXR presence.
4. **Tethering *trans*-activation.** Gene transcription can be induced also without direct DBD binding. In this situation, the NR does not interact directly to DNA, but it acts *via* another DNA binding transcription factor. This mechanism has been studied thoroughly for Glucocorticoid Receptors (GRs), but not for FXR yet.

Nuclear Receptors can exert their activity also in a negative manner, repressing gene transcription. Similarly to *trans*-activation, *trans*-repression activity occurs through several ways.

1. **Tethering *trans*-repression.**
2. **Simple *trans*-repression.**
3. **Monomeric *trans*-repression.**
4. ***Trans*-repression by squelching.** Squelching is the competition between nuclear receptors and pro-inflammatory transcription factors for the same co-factors. It is the case of GRs and NF-κB, in which they inhibit the gene transcription of the other NR.
5. ***Trans*-repression by *trans*-activation.** Gene transcription can be blocked using a cytosolic inhibitor of NR, which tightly controls NR localization in the cytosol, preventing it from activating genes (Figure 11).

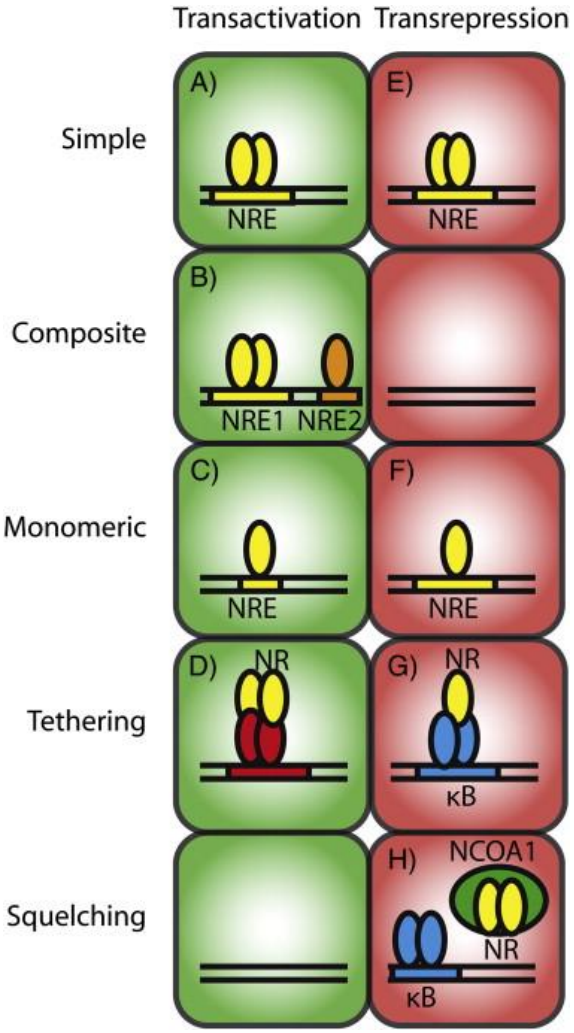


Figure 11. Positive and negative gene regulation mediated by Nuclear Receptor. Illustration of NR mechanisms of positive and negative regulation of gene transcription. **A)** Simple *trans*-activation of NR dimer on a Nuclear Responsive Element (NRE). **B)** Composite *trans*-activation of a NR dimer together with another NR on a NR half-site. **C)** Monomeric *trans*-activation of a NR on a NRE half site. **D)** Tethering *trans*-activation of a NR dimer binding to another transcription factor. **E)** Simple *trans*-repression by a NR dimer on a negative NRE. **F)** Monomeric *trans*-repression by a NR on a NRE. **G)** Tethering of a NR to NF-κB on a κB RE inhibits NF-κB activity. **H)** Co-factor squelching (Hollman et al. 2012).

Farnesoid X Receptor can be subjected to post-translational modifications, such as phosphorylation, acetylation and SUMOylation (Small Ubiquitin-like Modifiers). In the FXR DNA binding domain, two phosphorylation sites are present, both increase the *trans*-activation activity (Frankenberg et al. 2008). Two lysines, instead, are subjected to acetylation, which causes a decreased dimerization with RXR, reducing the binding to FXREs (Kemper et al. 2009). Another important modification is the addition of small ubiquitin-like modifiers in the consensus-sumo site. FXR SUMOylation is important for tethering *trans*-repression of NF-κB, an inflammatory marker (Hollman et al. 2012).

4.2 FXR ligands

Ligands identification for nuclear receptors significantly enabled their study. FXR was recently "deorphanized": it was originally proposed to be a receptor for farnesol (Forman et al. 1995) and now it is well known that bile acids are the endogenous ligands for this nuclear receptor (Makishima et al. 1999). Since FXR plays a crucial part in bile acid homeostasis, lipid and glucose metabolism, liver regeneration and in several pathologies, many agonists and antagonists, both natural and synthetic, have been studied to restore physiological conditions.

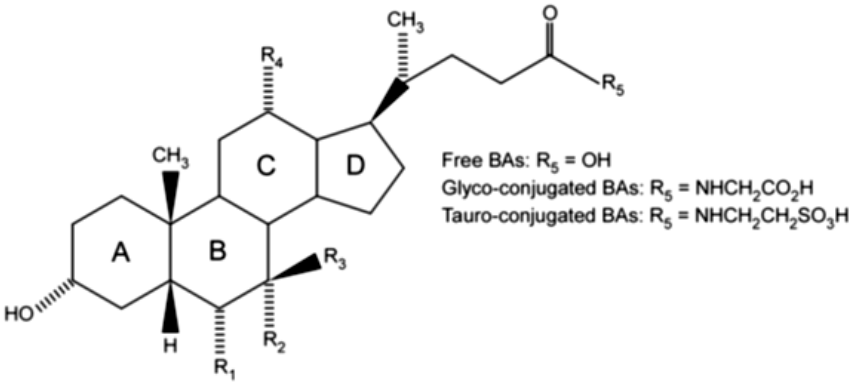
4.2.1 Agonists

Because FXR involvement in many physiological activities, FXR ligands have shown beneficial effects in the treatment of liver disorders, including various forms of cholestasis and fatty liver (steatosis) (Divers 2005). The most studied agonists are the endogenous ligands, that are bile acids, followed by semisynthetic and synthetic molecules.

4.2.2 Endogenous ligands of FXR: bile acids

Bile acids are cholesterol end-products, and their structure consists of a steroid nucleus with an acidic side chain. They are water-soluble amphipathic molecules characterized by a concave hydrophilic face (α face) and a common convex hydrophobic face (β face). The β face interacts with the hydrophobic pocket of the FXR LBD, while the α face contains several hydroxyl groups in the 3, 7 and/or 12α positions, influencing the affinity for Farnesoid X Receptor (Fujino et al. 2004). Greatest FXR activation through ligand binding needs the proper positioning of helix 3 *versus* helix 12, in order to generate the LXXLL docking groove. An essential part in the affinity is played by the 7α-hydroxyl group. In fact, its presence confers high capacity to Chenodeoxycholic Acid (CDCA) to activate FXR by inducing a highly stable interaction between helix 3 and helix 12, resulting in a strong agonist activity, while the absence of this 7α-hydroxyl group, such as the case of Deoxycholic Acid (DCA) and Lithocholic Acid (LCA), compromises stable co-activator recruitment, resulting in partial agonistic properties. Moreover, the affinity of Cholic Acid (CA) and DCA for FXR LBD is also reduced by the presence of a 12α-hydroxyl group, absent in CDCA. In this way, CA and DCA cannot accommodate easily into the pocket. The carboxyl extremity of bile acids is oriented towards the entry of the hydrophobic pocket of the LBD, suggesting why conjugated BAs conserve a high affinity for FXR and the ability to activate it (Pellicciari et al.

2006). Structural modifications of the carboxyl terminus of BAs may result in altered FXR activity (Figure 12).



Hydrophobicity

	$R_1^{(\alpha)}$	$R_2^{(\alpha)}$	$R_3^{(\beta)}$	$R_4^{(\alpha)}$
UDCA	H	OH	H	H
CA	H	OH	OH	OH
CDCA	H	OH	OH	H
DCA	H	H	H	OH
LCA	H	H	H	H

Figure 12. Structure and hydrophobic/hydrophilic profile of bile acids. Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA) are primary BAs, while Deoxycholic Acid (DCA) and Lithocholic Acid (LCA) are secondary BAs and, lastly, Ursodeoxycholic Acid (UDCA) is a primary BA predominantly detected in bears. Hydroxyl groups that are in α-orientation are located below the steroid nucleus and are axial to the plane of the steroid nucleus, on the contrary hydroxyl groups that are in β-orientation are located above the steroid nucleus and are equatorial to the plane of the steroid nucleus. The hydrophobicity increases as follows: UDCA, CA, CDCA, DCA, LCA (Modica et al. 2010).

4.2.3 OCA, a semisynthetic FXR ligand

The semisynthetic bile acid 6α-Ethyl-Chenodeoxycholic Acid (6-ECDCA), also called Obeticholic Acid (OCA) or INT-747, places the 6α-ethyl group into one such hydrophobic cavity, that exists among the side chains of Ile359, Phe363, and Tyr366 (Pellicciari et al. 2002). Thanks to the 6α-ethyl substitution, the obeticholic acid is an orally active synthetic FXR agonist, approximately 100-fold greater FXR agonistic activity than CDCA (K.K. & C.L. 2014) and does not activate other nuclear receptors (Mudaliar et al. 2013).

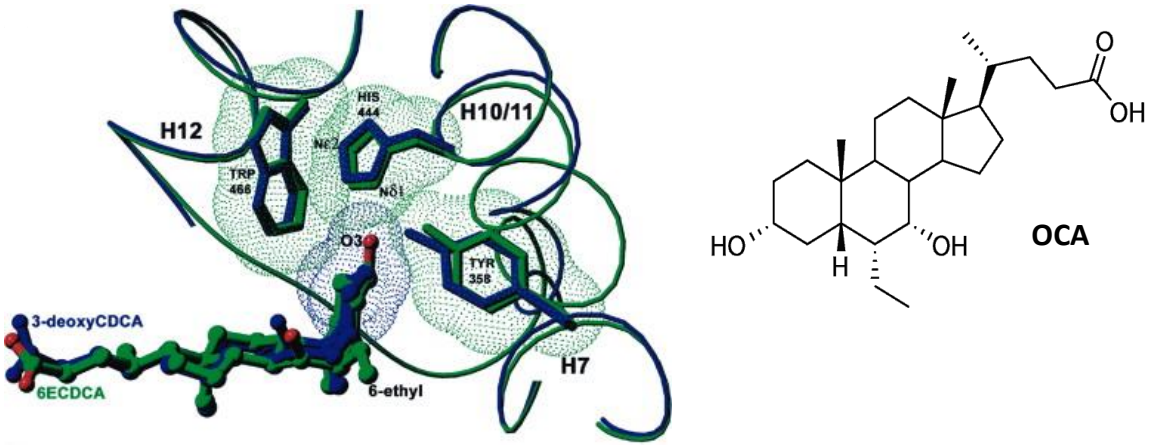


Figure 13. The activation mechanism of FXR. In the left side 6-ECDCA (OCA) and 3-deoxyCDCA interactions with the activation switch of FXR. Shown is the superposition of their two crystal structures in the vicinity of the ligand and residues His444 and Trp466 (Mi et al. 2003).

A range of preclinical studies have shown that obeticholic acid increases insulin sensitivity and regulates glucose homeostasis, modulates lipid metabolism, and exerts anti-inflammatory and anti-fibrotic effects in the liver, kidney and intestine (X. X. Wang et al. 2009). In fact, OCA treatment inverts insulin resistance and Non-Alcoholic Fatty Liver Disease (NAFLD) in Zucker *fa/fa* rats, protecting against body weight gain and fat deposition in liver and muscle, which are associated with decreased expression of genes involved in fatty acid synthesis, lipogenesis and gluconeogenesis. For this reason OCA reached the phase III clinical trials for treatment of NAFLD/NASH (Cipriani et al. 2010).

OCA also displays marked immunomodulatory and anti-inflammatory properties. This molecule selectively inhibits NF-κB-mediated hepatic inflammatory responses, while maintaining or improving the cell survival response (Wagner et al. 2008). Obeticholic acid has been shown to inhibit vascular smooth muscle cell inflammation by downregulating NF-κB-dependent inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) expression (Li et al. 2007).

Moreover, OCA has been observed to protect against liver fibrosis through inhibition of Hepatic Stellate Cells (HSCs) activation in the porcine serum and bile duct ligation models (Verbeke et al. 2014). Indeed, OCA administration has shown to treat cholestatic liver disease, decreases insulin resistance and hepatic steatosis (Zhang et al. 2006; Pellicciari et al.

2002; Mudaliar et al. 2013). At present, obeticholic acid is in phase I clinical trials for the treatment of alcoholic hepatitis, phase II clinical trials for Non-Alcoholic Steatohepatitis (NASH) and Type II Diabetes Mellitus (T2DM), and phase III clinical trials of Primary Biliary Cirrhosis (PBC) (Mudaliar et al. 2013; Ali et al. 2015; Neuschwander-Tetri et al. 2015).

4.2.4 FXR synthetic ligands

Bile acids do not display strong selectivity for Farnesoid X Receptor, but they activate also the Pregnane X Receptor (PXR), Vitamin D Receptor (VDR) and other nuclear receptors (Reschly et al. 2008). Thus, it was necessary to develop novel ligands, characterised by higher affinity and selectivity. The first lead compound synthetized was TTNPB, with preferential binding for RXR and low affinity for FXR (Parks et al. 1999; Zavacki et al. 1997). Starting from TTNPB, different *stilbene* derivatives were subsequently synthesized (Love et al. 2002; Maloney et al. 2000). Among these, GW9047 showed good selectivity, but low affinity for FXR. Modulations of the structure of GW9047 led to [3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole] GW4064, a selective and potent activator of FXR (EC_{50}=90 nM), that is still considered the reference compound. Although GW4064 is an FXR agonist, active both *in vitro* and *in vivo*, it displays a limited bioavailability, excluding its use in clinical phases.

In 2003, new modifications on TTNBP backbone were realized, in order to avoid retinoic acid receptor binding, yielding other FXR ligands, such as AGN29, AGN31 and AGN34 (Dussault et al. 2003). AGN29 and AGN31 revealed agonistic properties for both FXR and RXR with an EC_{50}=1 µM, while AGN34 acted as an FXR and RXR antagonist. The benzopyran structure of AGN compounds was used to generate FXR agonists and *fexaramine* resulted a selective and potent FXR agonists with an EC_{50}=38 nM (Downes et al. 2003). Interestingly, CDCA, GW4064 and *fexaramine* induced different FXR target genes, suggesting that these FXR ligands may also act as gene–selective FXR modulators. This means that they can modulate the expression of various FXR target genes in different manners, either as agonists or antagonists.

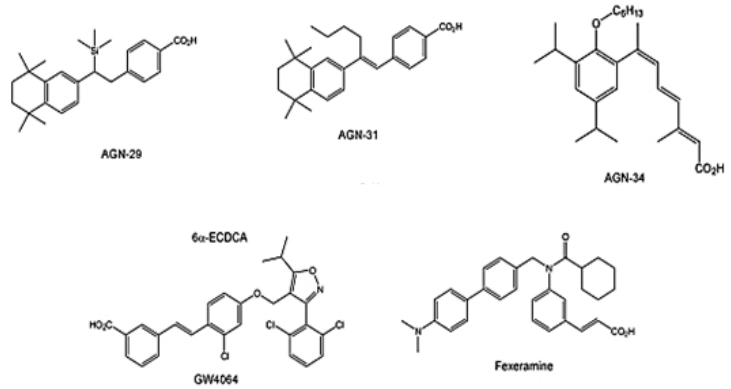


Figure 14. Several FXR agonists chemical structure. Chemical structure of the different FXR and RXR agonists.

Lately, WAY-362450 (FXR-450/XL335) was developed and it resulted a potent and selective FXR agonist with an oral bioavailability of 38% and a protracted half-life of 25 hours. WAY-362450 treatment determines a consistent lowering of serum triglyceride (TG) levels and serum cholesterol levels and inhibits aortic atherosclerotic formation in a dose-dependent manner. These data suggest that WAY-362450 could be useful in treating patients with atherosclerotic vascular diseases (Ali et al. 2015).

4.2.5 FXR antagonists

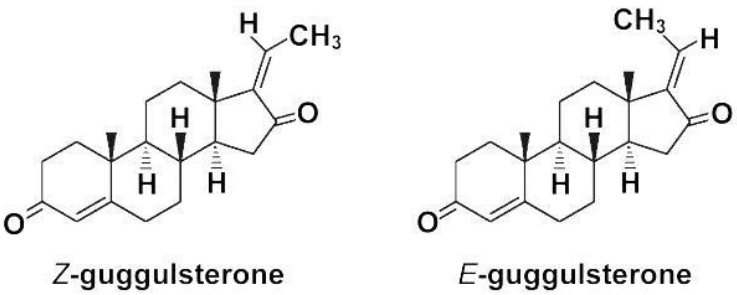
An FXR antagonist is a molecule that binds with high affinity to the LBD of the nuclear receptor, but does not activate it and, indeed, does not evoke the physiological effects of FXR activation. Moreover, an FXR antagonist is supposed to inhibit the activation of the receptor by agonistic ligands. For example, this could be possible by stabilizing complexes of FXR with co-repressors, such as the nuclear co-repressor, or by binding the LBD with high affinity, but without stabilizing AF2. Binding the Farnesoid X Receptor with high affinity, without stabilizing helix 12 in an active conformation, would impede co-activator recruitment and, therefore, not evoke FXR activation (Gronemeyer et al. 2004).

The first FXR antagonist described in the literature was guggulsterone, the active compound of guggulipid, an extract from *Commiphora mukul*, an Indian remedy used to reduce serum levels of cholesterol and triglycerides (Wu et al. 2002).

Guggulsterone appears in two isomers (E/Z) and its metabolic effects are mediated by several nuclear receptors, including the estrogen receptor, the glucocorticoid receptor, the mineralocorticoid receptor, the PXR and FXR. *In vitro* experiments with guggulsterone indicated that it acts as an FXR antagonist, inhibiting the activation of FXR by CDCA (Urizar et al. 2002). However, it was also demonstrated that guggulsterone enhances CDCA and

GW4064 to induce Bile Salt Export Pump (BSEP) expression, enzyme involved in bile secretion from liver to intestine (Cui et al. 2003) (Figure 15).

Figure 15. Guggulsterone chemical structure. Chemical structure of the two isoforms of guggulsterone, an active antagonist of many nuclear receptors.



Another natural highly selective FXR antagonist recently discovered is theonellasterol, a 4-methylenesteroid isolated from the *Theonella swinhoei* sponge (Figure 16). Although theonellasterol was known since long time (Renga et al. 2012), its effect on nuclear receptors has never been studied. Theonellasterol has shown to directly inhibit FXR *trans*-activation induced by CDCA, and reverses the effect of CDCA on the expression of canonical FXR target genes. In rodent models of cholestasis, theonellasterol is able to attenuate liver injury caused by bile duct ligation (Burris et al. 2005).

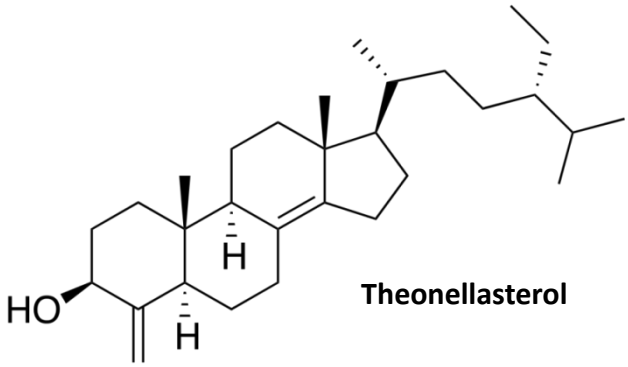


Figure 16. Chemical structure of theonellasterol. Chemical structure of theonellasterol, another natural highly selective FXR antagonist, isolated from the *Theonella swinhoei* sponge.

4.3 Role of FXR

Farnesoid X Receptor (FXR), once activated by its endogenous ligands, such as Bile Acids (BAs), or by synthetic ligands, like Obeticholic Acid (OCA), regulates a wide range of target genes, which modulate BA homeostasis, lipoprotein and glucose metabolism and inflammation processes (Kalaany & Mangelsdorf 2006).

4.3.1 FXR in bile acid homeostasis

Bile Acids (BAs) are amphipathic molecules synthetized from cholesterol by multiple enzymatic steps taking place in different cellular compartments of the liver: cytosol, mitochondria, endoplasmic reticulum and peroxisomes (Russell, 2003). The classical (or neutral) pathway begins from 7α-hydroxylase cytochrome P450 (CYP7A1), producing equal amounts of Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA), whereas the alternative (or acidic) pathway is initiated by sterol 27-hydroxylase (CYP27A1), generating predominantly CDCA (Chiang 2002). The primary bile acids CA and CDCA undergo conjugation with glycine or taurine, before secretion into the bile to form Glycocholic Acid (GCA), Taurocholic Acid (TCA), Glycochenodeoxycholic Acid (GCDCA), and Tauroglycochenodeoxycholic Acid (TCDCA). In the intestine, they can be dehydroxylated by anaerobic bacteria to produce Deoxycholic Acid (DCA) and Lithocholic Acid (LCA) (Noel et al. 2016) (Figure 17).

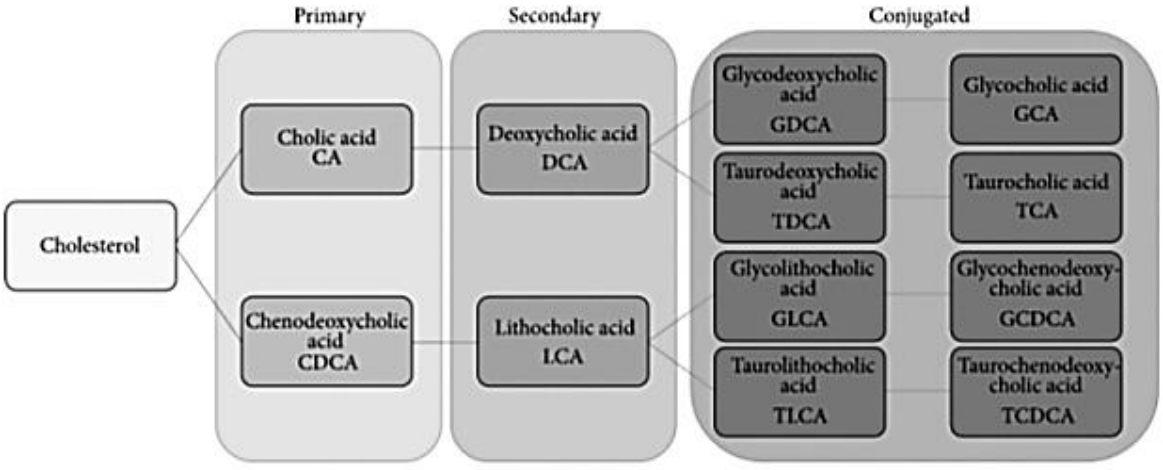


Figure 17. Major bile acids. The primary bile acids are synthesized from cholesterol in the liver. Secondary bile acids are formed by dehydroxylation of primary bile acids by intestinal bacteria. Primary and secondary bile acids can also undergo conjugation with taurine or glycine in the liver (Noel et al. 2016).

The BA biosynthetic pathway involves almost 15 genes, representing an energetically expensive process. To limit this drawback, BAs are re-cycled through the enterohepatic circulation. Although BAs present the advantage that they simplify the intestinal absorption of lipophilic nutrients, solubilizing them, the accumulation of high levels of bile acids can be detrimental. Remarkably, BA cytotoxicity grows linearly with the hydrophobic degree: UDCA, CA, CDCA, DCA and LCA, respectively. In order to make bile acids less hydrophobic, less cytotoxic and more readily secretable into bile, BAs undergo different modifications (Vessey et al. 1977).

Bile acids can exert a negative regulation on their own synthesis through an unclear FXR involvement, both in the liver and in the intestine. For example, in liver CDCA activates the transcriptional regulatory function of FXR, which suppresses CYP7A1 gene expression. Since FXR does not appear to bind directly to CYP7A1 promoter (A. Okamoto, unpublished results), it seems that FXR represses this gene together with additional DNA binding factors (Tu et al. 2000). Activation of FXR induces the expression of the Small Heterodimer Partner (SHP), which interacts with Liver Receptor Homolog-1 (LRH-1), repressing its activity. Moreover, SHP-LRH-1 interaction reduces CYP7A1 expression, diminishing in turn bile acid synthesis (Goodwin et al. 2000; Lee & Moore 2008; Lu et al. 2000). It is also known that, in murine distal ileum, FXR induces Fibroblast Grow Factor (FGF) 15 (for mouse) and 19 (for human) to bind to the hepatic Fibroblast Growth Factor Receptor 4 (FGFR4), repressing CYP7A1 *via* a c-Jun N-terminal kinase-dependent pathway (Inagaki et al. 2005). This latter mechanism suggests an important crosstalk between the intestine and the liver for bile acid synthesis regulation.

Another crucial cytochrome involved in the classical pathway of bile acid synthesis is CYP8B1, which controls the CA/CDCA ratio by regulating the synthesis of CA. As for CYP7A1, CYP8B1 is repressed by FXR *via* SHP-mediated repression of Hepatocyte Nuclear Factor-4α (HNF-4α) (Goodwin et al. 2000).

In order to be less cytotoxic, bile acids are firstly amidated by Bile Acid-CoA Synthetase (BACS), and then, conjugated with taurine and glycine through Bile Acid-CoA amino acid N-Acetyltransferase (BAAT) (Solaas et al. 2000). Unconjugated bile acids, as well conjugated ones, activate FXR, which positively regulates the expression of BACS and BAAT. Bile acids are then secreted in the gall bladder by Bile Salt Export Pump (BSEP) and Multidrug Related Protein 2 (MRP2). Both these transporters are activated by FXR at the transcriptional level. Besides, FXR is able to induce the expression of other ABC transporter implicated in the

biliary secretion of phosphatidylcholine (Modica et al. 2009). The modulation of these transporters has a pivotal role in avoiding bile acid accumulation in the liver, leading to hepatic injury.

While unconjugated bile acids are passively re-absorbed, conjugated bile acids are re-absorbed at the distal ileum level through the Apical Sodium-dependent Bile salt Transporter (ASBT) (Wong et al. 1994; Shneider 2001). Once transported into ileal enterocytes, bile acids are reversibly bound by the Intestinal Bile Acid-Binding Protein (IBABP), also called Fatty Acid-Binding Protein subclass 6 (FABP6) (Wong et al. 1994; Kramer et al. 1993), which shuttles bile acids from the apical to basolateral membrane (Coppola et al. 1998). Finally, Organic Solute Transporter α and β (OSTα and OSTβ) secrete bile acids in the portal blood (Dawson et al. 2005). This process is coordinated by FXR activation, that downregulates the expression of ASBT and, at the same time, induces IBABP, OSTα and OSTβ expression (Grober et al. 1999; Kok et al. 2003; Landrier et al. 2006; Rao et al. 2008). Further studies reveal that bile acids produce a negative feedback on ASBT expression by FXR-mediated induction of SHP, able to bind to and repress the transcriptional activity of LRH-1 for CYP7A1 (Chen et al. 2003), while IBABP and OSTs expression is directly induced by FXR at their promoters (Neimark et al. 2004).

Analogous to the intestine, the active transport system for bile acid absorption modulated by FXR is present also in the bile duct and in the kidney. In cholangiocytes, conjugated bile acids are absorbed at the canalicular membrane by ASBT and secreted by MRP3 and OSTs at the basolateral membrane (Ballatori et al. 2005). In kidney cells, conjugated bile acids are absorbed by ABST in the apical membrane of proximal renal tubular cells, and successively secreted by OSTα and OSTβ in the blood circulation (Christie et al. 1996; Craddock et al. 1998). Therefore, FXR controls the entire transport of bile acids from the intestinal lumen to the enterocytes, within the enterocytes and, finally, to the blood vessel for transportation to the liver, and, under physiological conditions, bile acids are not excreted into urine (Figure 18).

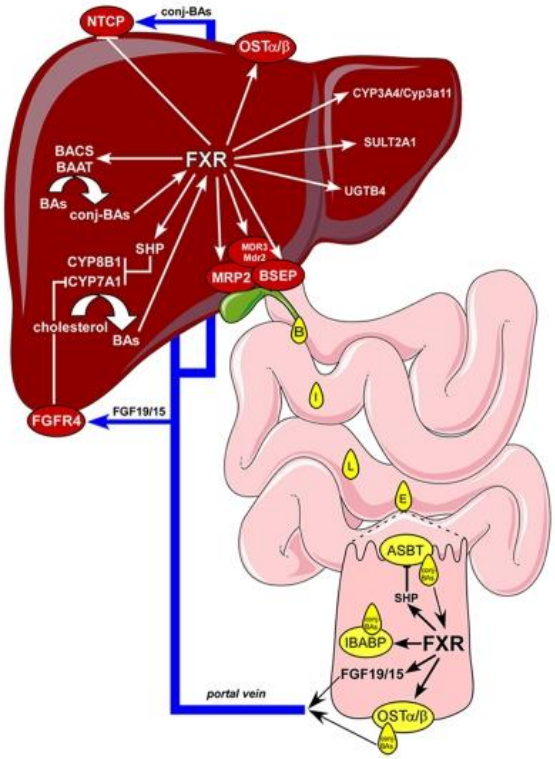


Figure 18. FXR regulates BA metabolism. Schematic illustration of BA biosynthetic pathways. BAs are recycled through the enterohepatic circulation. They can exert a negative regulation on their own synthesis through an unclear FXR involvement, both in the liver and in the intestine (Modica et al. 2010).

FXR plays a critical role also in the biotransformation process. Bile acids are modified in a two-steps process. In the first phase, oxidation reactions occur, generating more polar products. Then, during the second phase, these products are conjugated with endogenous molecules to further increase their water solubility. Once conjugated, bile acids are ready to be eliminated from the body. At the hepatic level, CYP3A4/CYP3A11, the most crucial enzyme involved in phase I reactions, is induced by FXR activation. Moreover, FXR positively regulates also phase II-enzymes involved in glucuronidation and sulfonation reactions, such as UGT2B4 and SULT2A1, respectively (Song et al. 2001).

4.3.2 FXR in lipid metabolism

FXR-deficient mice exhibit marked hypercholesterolemia, hypertriglyceridemia and increased intestinal cholesterol absorption (Lambert et al. 2003). In fact, when cholesterol is not converted into bile acids, it accumulates at hepatic and serum level. FXR regulates the

expression of Phospholipid transfer Protein (PLPT), essential for the transfer of phospholipids and cholesterol from Low-Density Lipoprotein (LDL) to High-Density Lipoprotein (HDL). Moreover, FXR leads to the decrease of plasma triglyceride levels by regulating the expression of several lipoproteins, such as ApoE, ApoC-I, ApoC-IV. Scavenger Receptor B1 (SRB1), involved in the hepatic uptake of HDL and in the clearance of serum HDL cholesterol esters, is another FXR-positively regulated protein (Mak et al. 2002; Sirvent et al. 2004). An alternative FXR-dependent mechanism to reduce triglyceride concentration is by regulating the expression of different lipid-modulating proteins. For example, ApoC-II stimulates Lipoprotein Lipase (LPL)-mediated triglyceride release from Very Low Density Lipoprotein (VLDL) and chylomicrons and promotes triglyceride hydrolysis into fatty acids. Conversely, the expression of ApoC-III, an inhibitor of LPL, is negatively regulated by FXR (Kast et al. 2001; Claudel et al. 2003). Moreover, FXR induces the expression of Peroxisome Proliferator Activated Receptor α (PPARα), which stimulates Pyruvate Dehydrogenase Kinase-4 (PDK-4) to promote fatty acid oxidation (Pineda Torra et al. 2003).

In addition, FXR controls triglyceride metabolism by affecting hepatic *de novo* lipogenesis. The active FXR, *via* SHP, is able to downregulate the expression of Sterol-Regulatory-Element-Binding-Protein-1c (SREBP-1c), a transcription factor that stimulates fatty acid synthesis and lipogenesis by inducing the expression of Fatty Acid Synthase (FAS), Acetyl-CoA Synthase (AceCS), Acetyl-CoA (ACC) and Glycerol-3-Phosphate (GPAT) (Watanabe et al. 2004; Horton et al. 2002).

4.3.4 FXR in glucose metabolism

Bile acids are able to bind to and activate FXR, which in turn exerts a feedback regulation. As well, glucose controls FXR gene expression in a dose- and time-dependent manner, and FXR itself acts on glucose metabolism (Duran-Sandoval et al. 2004). According to the unfed or fed state, FXR activation exerts opposite effects. In fasting conditions, FXR upregulates gluconeogenesis by stimulating Phosphoenolpyruvate Carboxykinase (PEPCK), while in fed state FXR activation by cholic acid suppresses, in a SHP-dependent manner, the expression of multiple genes in the gluconeogenic pathway, including PEPCK, PGC-1α, and Glucose-6-Phosphatase (G6P-ase) (Ma et al. 2006). Recent studies have revealed that mouse FGF15 and human FGF19, FXR-activated factors, inhibit hepatic gluconeogenesis through a mechanism involving the dephosphorylation and inactivation of the transcription factor Cyclic Adenosine Mono Phosphate (cAMP) Regulatory Element Binding Protein (CREB) (Potthoff et al. 2011).

This discrepancy could be explained in this way: in unfed condition, FXR activation could be weak because of bile acid storage in gallbladder, while after postprandial stimuli bile acids are circulating and can activate FXR. Farnesoid X Receptor has a role also in glycolysis and lipogenesis regulation, as a result of *trans*-repressing the expression of different glycolytic genes by interference with Carbohydrate Response Element Binding Protein (ChREBP) transcriptional activity (Duran-Sandoval et al. 2005; Caron et al. 2013).

Since the close association between glucose and insulin, it is not surprising that insulin and FXR expression are linked. In fact, insulin regulates FXR expression, and FXR makes cells insulin sensitive. Insulin sensitivity includes two mechanisms, one genomic and the other one non-genomic. Firstly, FXR activation induces the expression of glucose-dependent transcription factor Krueppel-Like Factor 11 (KLF11), which is essential in the modulation of insulin gene transcription. Then, the non-genomic approach relies on a Protein Kinase B (PKB)-mediated stimulation of translocation of the Glucose Transporter 2 (GLUT2) in pancreas β-cells, which can increase the glucose uptake by cells (Renga et al. 2010). The importance of Farnesoid X Receptor in carbohydrate metabolism suggests a potential application of FXR agonists to manage insulin resistance in diabetics patients.

4.3.5 FXR & liver regeneration

Liver regeneration is an adaptive response induced by specific stimuli and consists of sequential changes in gene expression and morphologic reconstruction. It comprises a variety of well-coordinated phases, with rapid stimulation of proliferative factors that activate the quiescent hepatocytes and prime their subsequent progression through the cell cycle, followed by re-establishment of normal liver size and renewed hepatocyte quiescence (Fausto 2000). This complex process is defined mainly by three major actors: cytokine, growth factor and metabolic signals (Fausto et al. 2006). Among metabolic signals necessary for liver regeneration there are bile acids. The effect of bile acids is dependent on Farnesoid X Receptor activation, able to transform the BA stress into the force of promoting liver regeneration. During the early stage of liver regeneration, CYP7A1 expression is suppressed both *via* SHP and Mitogen-Activated Protein Kinase (MAPK). Treating livers with the toxin Carbon Tetrachloride (CCl4), massive cell death occurs. FXR has shown to prevent hepatocytes from apoptosis (Meng et al. 2010), because it can upregulate ERK pathways both *in vitro* and *in vivo* (Wang, Yang, et al. 2008). During liver repair after injury, the role of FXR

in cell survival may be linked to the activation of Signal Transducer and Activator of Transcription 3 (STAT3), which is a key factor in cell survival (Meng et al. 2010).

The importance of FXR has been also demonstrated in FXR null-mice, which spontaneously develop liver tumours, after injury and when they age (Yang et al. 2007; Kim et al. 2007). In fact, liver is subjected to enter endless cycles of injury/repair, that will keep producing inflammatory cytokines and other growth factors, potential tumour promoters. Furthermore, bile acids, being toxic, cause DNA damage, inducing cell transformation when their levels are not controlled by FXR anymore. Therefore, FXR role in promotion of liver repair could be an intrinsic mechanism to protect liver from carcinogenesis (Chen et al. 2010).

4.3.6 FXR in vessel wall

FXR has been observed in vascular smooth muscle cells (Bishop-Bailey et al. 2004) and endothelial cells (He et al. 2006), but the evidence on the physiological and pathophysiological roles of FXR in the vasculature is still partial. Farnesoid X Receptor might be considered a direct target for the treatment of proliferative diseases because Vascular Smooth Muscle Cells (VSMCs) underwent apoptosis when treated with FXR ligands (Bishop-Bailey et al. 2004; Li et al. 2007). FXR agonists downregulate Interleukin-1β (IL-1β)-induced inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) expression in rat aortic smooth muscle cells, inhibiting vascular inflammation and suppressing smooth muscle cell migration (Li et al. 2007). When bile acids levels are elevated in circulation, they can also induce the expression of Vascular Cell Adhesion Molecule 1 (VCAM1) and Intercellular Adhesion Molecule 1 (ICAM1) by stimulation of NF-κB and p38 MAPK signalling pathways, through the increase of ROS in vascular endothelial cells (Qin et al. 2006).

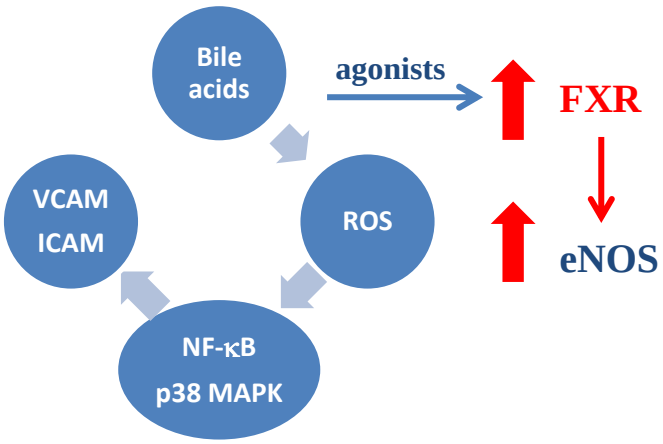


Figure 19. FXR in vessel wall. Schematic illustration of FXR activity in Vascular Smooth Muscle Cells (VSMC).
FXR, activated by bile acids, promotes the VCAM and ICAM expression *via* ROS-NF-κB-p38 MAPK.

The endothelium plays a critical role in regulation of vascular tone. Alterations in the gene expression profile and an affected release of endothelium-derived factors occur in metabolic diseases, characterized by an amplified production of pro-inflammatory cytokines, decreased secretion of adiponectin from adipose tissue and high circulating levels of free fatty acids and hyperglycemia (Rask-Madsen & King 2007; Biddinger et al. 2008). A defective endothelium displays uncoupling of endothelial Nitric Oxide Synthase (eNOS) followed by a decreased production of nitric oxide and increased expression of Endothelin 1 (ET1). FXR activation in the endothelial cells leads to downregulation of ET1 and to upregulation of eNOS mRNA and protein (Li et al. 2008). Furthermore, FXR might also affect the endothelium-derived nitric oxide activity by modulation of serum Asymmetric Dimethylarginine (ADMA). ADMA, an endogenous NOS inhibitor (Hu et al. 2006), at high concentration, reduces nitric oxide synthesis in patients with hypercholesterolemia, hypertriglyceridemia, hypertension, type II diabetes, chronic renal failure and chronic heart failure, while reduction of ADMA levels improves nitric oxide synthesis (Mallamaci et al. 2004; Zoccali & Kielstein 2006). *In vitro* studies have revealed that reduced ADMA levels could be obtained by positively regulating the expression/activity of hepatic Dimethylarginine Dimethylaminohydrolase 1 (DDAH-1), the major catabolic pathway of ADMA, by FXR activation. In fact, on intron I region of the DDAH-1 gene a FXR responsive element has been identified (Hu et al. 2006), indicating that DDAH-1 gene expression is FXR-mediated.

4.3.7 FXR in inflammation

NF-κB is present not only in vascular endothelial cells, but it is considered a hallmark of general inflammatory response and a critical mediator of tumour promotion. The inflammatory response occurs during viral and bacterial invasion, and also during the onset and the progression of several diseases, for example in cholestasis, atherosclerosis, in fibrotic tissues. Bile acids have been known to act directly on innate immunity cells. Growing data demonstrate that the Farnesoid X Receptor is involved in the pathological and inflammatory processes of various diseases (Wang & Wan 2008). CDCA, one of the endogenous ligand of FXR, attenuates IL-1β, IL-6 and TNF-α release from lipopolisaccharide primed monocytes (Calmus et al. 1992). Experiments from three double knockout mice evidenced that FXR ablation might result in a pro-inflammatory phenotype. *In vivo* studies have revealed that FXR activation decreased the severity of inflammation extent of the intestine (Gadaleta, van Erpecum, et al. 2011), and pro-inflammatory cytokines that stimulate NF-κB pathway

repressed FXR activation in the intestine and liver (Wang, Chen, et al. 2008; Gadaleta, Oldenburg, et al. 2011). At the same time, FXR SUMOylation might antagonize the NF-κB pathway (Hollman et al. 2012).

4.3.8 FXR & diseases

Because of the widespread localization of Farnesoid X Receptor and because of its involvement in the regulation of several pathways regarding mainly bile acid homeostasis, lipid and glucose metabolism, it is clear that FXR could be considered an interesting target in treating many diseases.

Cholestasis is a pathological condition characterized by an impairment or a reduction of bile flow. We can distinguish two types of cholestasis: intrahepatic cholestasis, due to defects in bile formation process, and extrahepatic one, caused by physical obstruction in bile ducts, in presence of tumours or stones. The subsequent accumulation of toxic bile acids leads to hepatic fibrosis and inflammation, which may progress to cirrhosis, cancer and liver failure. It is well known that FXR negatively regulates bile acids synthesis by inhibiting CYP7A1 and CYP8B1 expression, reduces hepatic bile acid uptake by downregulating the Na^+/Taurocholate Co-transporting Polypeptide (NTCP), increases the expression of bile serum export pump for the secretion of bile acids from liver to bile and induces OSTα and OSTβ expression, in order to reverse bile acids into the blood flow. Moreover, FXR is involved in phase I and II reactions to make bile acids more hydrophilic. For these reasons, FXR activation by agonists could reduce cholestasis, as described for rats administered with GW4064 and obeticholic acid (Modica et al. 2010). Other evidences suggest that FXR inhibition might be protective in cholestasis. In fact, in FXR knockout mice subjected to bile duct ligation, the hepatic basolateral transporter MRP4 increases, stimulating bile acid efflux into the systemic circulation before reaching kidney for elimination (Mennone et al. 2006).

Since the close relationship between liver and intestine for bile acids, reduced intestinal bile acid reabsorption and increased fecal bile acid excretion contribute to chronic diarrhea, occurring in Crohn's disease, short-bowel disease and post-gastrectomy syndrome. A genetic cause for defective bile acid reabsorption is attributable to mutation in sodium-dependent bile acid transport (ASBT) gene. The use of a sequestering bile acid drug shows protective effects on the bowel, but it exhibits several adverse effects. However, the FXR agonist administration leads to a decrease in bile acids, due to CYP1A7 inhibition (Modica et al. 2010).

Non-Alcoholic Fatty Liver Disease (NAFLD) cases are more and more common, mainly in Western countries. NAFLD correlates with adiposity, obesity and insulin resistance and type II diabetes mellitus (Farrell & Larter 2006). Peripheral insulin resistance impairs uptake of glucose from blood into skeletal muscle and adipose tissue, increasing serum non-esterified fatty acid concentration, since lipolysis is suppressed. It is known that lipid and carbohydrate metabolisms, as well insulin sensitivity, are tightly controlled by Farnesoid X Receptor. FXR activation reduces SREBP-1c expression, increasing insulin sensitivity and glycogen synthesis, suggesting that FXR agonist could have beneficial effects in sick patients (Fiorucci et al. 2007).

Bile acids are also involved in the induction of hepatocyte proliferation and liver regeneration (Barone et al. 1996), while a block of enterohepatic bile acid circulation results in the delay and inhibition of liver regeneration (Ueda et al. 2002). Hepatocytes proliferation occurs not only after partial hepatectomy, to regenerate the liver, but also in tumour propagation. In a Hepatitis B Virus (HBV) transgenic mouse model, animals were treated with a bile acid-rich diet, which promotes the onset of hepatic tumour (Barone et al. 2003). FXR-knockout mice, in which high chronic levels of bile acids are present, spontaneously develop Hepatocellular Carcinoma (HCC) (Kim et al. 2007). The activation of Farnesoid X Receptor could play a protective role, acting as a metabolic onco-suppressor, by keeping bile acid concentration under the physiological threshold, reducing in this way their toxic potential.

Indeed, Farnesoid X Receptor represents a feasible pharmacological target for homeostasis restoration in several disorders. FXR activation leads to a complex response that combine beneficial as well undesirable side effects. So, the identification of selective Farnesoid X Receptor modulators might be essential for the development of molecules targeting specific tissues and specific cluster of genes (Kuipers et al. 2004).

Aim of the Study

The hepatoprotective effects of Nitric Oxide (NO) have been well documented in several models of liver injury, including Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH), cholestatic hepatitis and Ischemia/Reperfusion (I/R) injury (Yagnik et al. 2002).

All three isoforms of NOS, endothelial (eNOS), inducible (iNOS) and neuronal (nNOS), can be inhibited by Asymmetric Dimethylarginine (ADMA), an amino acid naturally-produced during protein methylation process. ADMA can compete with arginine for NOS active site and, in this way, it reduces NO production (Vallance et al. 1992). Intracellular and extracellular ADMA affects the regulation of NO concentration. Since endothelial NO plays a crucial role in protection against the onset and progression of many organ disorders, the inhibition of endothelial Nitric Oxide Synthase (eNOS) could exacerbate many already compromised conditions.

ADMA is synthesized by Protein Arginine Methyltransferases (PRMTs) and it is metabolized to citrulline and dimethylamine, by Dimethylarginine Dimethylaminohydrolase (DDAH) enzyme. Finally, its import/export across cell membranes depends on Cationic Amino-acid Transporters (CATs) activity (Reade et al. 2002).

Elimination of ADMA is partially due to the kidneys, but approximately the 80% of circulating ADMA is excreted after DDAH-1 metabolism in the liver (Teerlink 2005). So, it is reasonable to affirm that the liver plays a critical role in ADMA handling and increased ADMA concentration is related to the degree of hepatic dysfunction in patients suffering from liver diseases, with varying aetiologies (Ferrigno, Laura G Di Pasqua, et al. 2015).

Cardiovascular diseases, whose ADMA is a sensible marker, are the major cause of death in patients suffering from NAFLD. Other conditions, occurring in NAFLD/NASH such as insulin resistance, hepatic dysfunction and chronic inflammation, are also strictly related to the increased incidence of cardiovascular events in NAFLD (Boga et al. 2015).

Moreover, several studies showed in a rat model of cholestasis, higher plasma ADMA, SDMA and L-Arginine levels in comparison with control rats (Sheen et al. 2010; Yang et al. 2012). During the development of cholestatic hepatitis, from 2 to 4 weeks in BDL-rats, in fact, progressive plasma ADMA accumulation occurs, whereas plasma L-Arginine levels diminish in a proportional way when compared with sham rats (Schwedhelm et al. 2008).

Since ADMA inhibits NOS, it could have a role in NAFLD/NASH, cholestasis and acute hepatic I/R injury.

In these three types of hepatic injury, a dysregulation of ADMA/DDAH/NOS pathway occurs, leading to various consequences that worsen liver damage.
In the last few years, several factors that could affect the ADMA/DDAH/NOS pathway have been discovered. Recently, it has been shown that a Farnesoid X Receptor (FXR) agonist, GW4064, increases hepatic DDAH-1 gene (but not the DDAH-2 gene) expression, with a concomitant decrease in plasma ADMA levels (Hu et al. 2006). Moreover, FXR agonists, such as physiologic Chenodeoxycholic Acid (CDCA) or semi-synthetic GW4064, lead to upregulation of eNOS mRNA and protein expression, resulting in NO production increase (Laffitte et al. 2000). Lastly, recently published data revealed also that the FXR agonists may positively affect CAT-1 expression both in liver and kidney (Li et al. 2009).

Recently, two other transporters have been identified to be important in ADMA pathway: OCT-2, similarly to CAT transporters, is involved in cellular uptake of ADMA; meanwhile MATE-1 is mainly involved in ADMA cellular efflux and excretion into urine (Strobel et al. 2013).
More, in MCD-rat model the high level of reactive oxygen species may affect the SH-groups present in the catalytic site of DDAH-1, causing enzyme inactivity. The same occurrence may take place also in BDL-rats, accompanied by reduced eNOS activity and increased iNOS one (Sarela et al. 1999; Wei et al. 2002).

Major understanding of ADMA/DDAH/NOS pathway and targeting the FXR to induce changes in ADMA levels may be considered as a potential point of interest to reduce the effect of several hepatic diseases.
Thus, in this study we propose several goals: first of all, how changes in ADMA/DDAH/NOS axis occur in three different models of liver diseases, considering ADMA production, elimination after DDAH-1 metabolism and excretion.
Secondly, we wanted to assess any changes in hepatic ADMA uptake, considering its import/export across membranes, mediated by CATs.
Finally, since we know that FXR plays an important part in regulating CATs, we will investigate how FXR agonist Obeticholic Acid (OCA) administration could induce changes in ADMA handling and its possible clinical implication in liver injury treatment.

Materials & Methods

Animal Models & Operation Procedures

Before running our experiments, animals had to be subjected to different treatments depending on the type of investigation to conduct. To this purpose, we used three different animal models to mimic three different human diseases, having as main theme the study of ADMA in all three models examined.

5.1 Methionine-Choline Deficient-rat model (MCD): animals

Male Wistar rats eight weeks old purchased from Charles River-Italy were subjected to a methionine-choline deficient diet, obtained from Piccioni Laboratory-Italy, for 2 (n=5), 4 (n=5) or 8 (n=5) weeks or to a control diet for 2, 4 and 8 weeks (n=3 each group). The animal model used was approved by the Italian Ministry of Health and the Pavia University Animal Care Commission (Document number 2-2012). Blood samples and hepatic biopsies from the left lobe were collected at the indicated times and snap frozen in liquid nitrogen.

5.1.1 Experimental groups

Animals were divided into two groups:

- **Group 1:** Control: rats fed with diet supplemented with choline and methionine.
- **Group 2:** MCD: rats fed with choline and methionine deficient diet.

The rats, both control and MCD-diet treated, were sacrificed at the following time intervals:

- **II week** MCD (n= 5) control (n= 3)
- **IV week** MCD (n= 5) control (n= 3)
- **VIII week** MCD (n= 5) control (n= 3)

5.2 Bile Duct Ligation-rat model (BDL): animals

Sixteen Male Wistar rats, provided by Charles River company (Italy) and weighing about 200-250 grams with free access to water and food, were used for these experiments. Their use was approved by Italian Ministry of Health and by the University Commission for Animal Care (Document number 2-2010) and the animals were cared for according to its guidelines.

5.2.1 Obstructive cholestasis (BDL) procedure

Rats were anesthetized by intraperitoneally administration of sodium pentobarbital (40 mg/Kg). Afterwards, the abdomen was opened by a median incision and the common bile duct was double-ligated and cut between the ligatures (BDL) (n=8). Sham-operated control animals (n=8) had similar manipulation but not bile duct ligation and were kept under anaesthesia for an equal length of time. Once the operation was completed, the abdomen was saturated and rats woke up from anaesthesia. After 72 hours, blood samples were collected and immediately centrifuged to separate serum. Hepatic biopsies from Left Lobe (LL), Median Lobe (ML) and Right Lobe (RL) lobe were collected and snap frozen in liquid nitrogen.

5.2.2 Experimental groups:

Animals were divided into two groups:

- **Group 1:** Bile Duct Ligation (BDL; n=8): rats in which obstructive cholestasis is induced for a period of 72 hours, by means of double-ligation of bile duct.
- **Group 2:** Sham-operated control (Sham; n=8): rats subjected to the same procedure of group 1 animals, but without ligation of bile duct.

5.3 Ischemia/Reperfusion-rat model (I/R): animals

Male Wistar rats (supplied by the Charles River company, Italy) were used in this study. The animals were allowed free access to water and food in all the experiments. The use and care of animals was approved by the Italian Ministry of Health and by the University Commission for Animal Care (Document number 2-2010).

5.3.1 Ischemia/Reperfusion (I/R) procedure

After 5 days of OCA or vehicle alone treatment, rats were anesthetized with sodium pentobarbital (40 mg/Kg) administered intraperitoneally. The abdomen was opened *via* a midline incision, and the bile duct was cannulated (PE-50). Ischemia to the left and median lobes was induced by clamping the left branch of both the portal vein and the hepatic artery, for 30 or 60 minutes with microvascular clips in the abdomen temporarily closed with a suture. Then, the clips were removed and it was allowed to reperfuse the liver for 60 minutes. By using partial, rather than total hepatic ischemia, portal vein congestion and subsequent bacterial translocation into the portal venous blood was avoided. Sham-operated control

animals underwent similar manipulation, with a midline incision but without the clamping of the vessels, they were kept under anaesthesia for an equal length of time.

A set of 60 minutes ischemia experiments followed by 60 minutes reperfusion was performed after 5 hours of BDL. Also in this case, sham-operated control rats underwent similar manipulation without vascular occlusion or BDL procedure.

At the end of the reperfusion period, all groups were subjected to abdominal re-opening, bile, blood and tissue samples were collected.

5.3.2 Experimental groups (a)

Four experimental groups were taken into account (Figure 20):

- **Group 1:** Ischemia/Reperfusion (I/R; n=8): rats in which partial ischemia is induced for a period of 30 minutes, by means of the closure with atraumatic clips of the left branch of the hepatic artery and the portal vein, followed by 1-hour reperfusion.
- **Group 2:** Ischemia/Reperfusion (I/R; n=8): rats in which partial ischemia is induced for a period of 60 minutes, by means of the closure with atraumatic clips of the left branch of the hepatic artery and the portal vein, followed by 1-hour reperfusion.
- **Group 3:** Bile Duct Ligation + Ischemia/Reperfusion (BDL+I/R, n=6): rats in which obstructive cholestasis is induced for a period of 5 hours, by means of double-ligation of bile duct. This procedure was followed by ischemia induced for a period of 60 minutes, by means of the closure with atraumatic clips of the left branch of the hepatic artery and the portal vein, followed by 1-hour reperfusion.
- **Group 4:** Operated control (Sham; n=8 for each group): rats subjected to the same procedures of group 1, 2 and 3 animals, but without clips application to avoid ischemia, or BDL procedures.

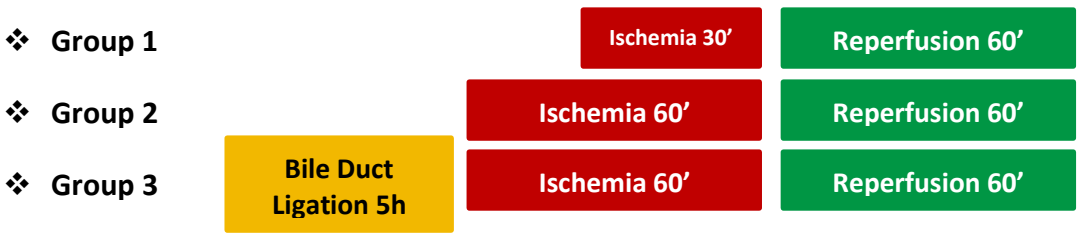


Figure 20. Schematic illustration of experimental groups.

5.3.3 Pharmacological treatment

The FXR agonist (Obeticholic Acid, OCA) was kindly provided by Intercept Pharmaceuticals, San Diego, California, USA. Animals were orally administered 10 mg/Kg/day of the OCA in vehicle (methylcellulose 1%) for 5 days or with vehicle alone.

5.3.4 Experimental groups (b)

Four experimental groups were taken into account (Table 1):

- **Group 1:** Ischemia/Reperfusion (I/R; n=8): rats in which partial ischemia is induced for a period of 60 minutes, by means of the closure with atraumatic clips of the left branch of the hepatic artery and the portal vein, followed by 1-hour reperfusion.
- **Group 2:** Ischemia/Reperfusion + Obeticholic Acid (I/R + OCA; n=8): 5 day-OCA-treated rats in which partial ischemia, followed by reperfusion, is induced.
- **Group 3:** Operated control (Sham; n=7): rats subjected to the same procedure of group 1 animals, but without clips application to avoid ischemia.
- **Group 4:** Operated control + Obeticholic Acid (Sham + OCA; n=7): 5 day-OCA-treated rats subjected to the same procedure of group 1 animals, but without clips application to avoid ischemia.

Experimental groups	Treatment	n° animals
Group 1	I/R	n=8
Group 2	I/R + OCA	n=8
Group 3	Sham	n=7
Group 4	Sham + OCA	n=7

Table 1. Schematic representation of experimental groups.

5.4 Samples collection

Blood samples were collected after all three experimental procedures. Blood was drawn from the vena cava and centrifuged at 3000 x g for 10 minutes at 4°C, to isolate serum.

Bile is a fluid secreted by hepatocytes and participates to the digestion process, helping to emulsify the lipids present in food. Thus, bile production requires intact hepatocytes. For this reason it is considered a strong and reliable indicator of overall liver quality and viability. Bile was collected in darkened vials during the reperfusion period of I/R procedure and in Sham-Operated rats of BDL procedure. Bile and serum samples were snap frozen in liquid nitrogen until they were analysed.

Afterwards, the liver was removed, washed in ice-cold PBS, dried on paper towels, then a sample of tissue from the left lobe, or all three lobe in the case of BDL, was collected. The biopsy has been preserved in liquid nitrogen.

Biochemical Assays

6.1 Transaminases evaluation

Aspartate Transaminase (AST) is an enzyme belonging to the class of transferases detectable in very high concentration in the bloodstream as a result of liver and muscle damage. Alanine Aminotransferase (ALT), also belonging to the class of transferases, differently from AST, is a generic cell necrosis index, present in elevated plasma concentration as result of muscle damage of different nature. Therefore it is assessed in combination with more specific indicators. For this reason, the AST/ALT ratio is commonly used as an index of liver damage and can be quantified in serum obtained from all our experimental model rats.

Alkaline Phosphatase (AP) is an hydrolase. There are three different isoforms: at hepatobiliary level, in the skeletal system and in the gut. High levels of AP are associated to hepatic injury.

Bilirubin is a yellow compound that occurs in the normal catabolic pathway that breaks down heme in vertebrates. Bilirubin is excreted in bile and urine, and elevated levels may indicate liver dysfunction.

Liver injury was assessed by serum levels of ALT, AST, AP, total and direct bilirubin. Liver injury markers and glucose, total cholesterol and triglycerides were all assessed by an automated Hitachi 747 analyser (Roche/Hitachi, Indianapolis, IN, USA), with the contribution of Doctor Vittoria Rizzo, Department of Molecular Medicine, IRCCS Policlinico San Matteo, Pavia.

6.2 ADMA in bile, serum and tissue evaluation

ADMA levels in bile, serum and tissue were evaluated by direct ELISA kit (Immundiagnostik, AG Germany), that is based on a method of competitive enzyme-linked immunosorbent. In this kind of ELISA assay were used microplates in which the wells were

pre-treated with an anti-ADMA antibody. Standards and samples were dispensed into appropriate wells with an antibody specific for the ADMA conjugated to peroxidase.

During incubation time, ADMA present in our sample competed with the abovementioned molecule for binding with polyclonal antibody. Therefore, ADMA from sample displaced the antibody binding. The concentration of the tracer bound to the antibody will be inversely proportional to the concentration of ADMA in the sample.

Later, the microplate was treated with Tetramethylbenzidine (TMB), a peroxidase substrate. Finally the enzymatic reaction was stopped by adding an acidic stop solution. The colour shift from blue to yellow indicated that the reaction has taken place and the absorbance was measured in a spectrophotometer at 450 nm.

6.3 DDAH activity assay

DDAH activity evaluation was performed using the method proposed by Tain and Baylis (Tain & Baylis 2007). Tissue samples were homogenized in cold phosphate buffer 100 mM, pH 6.5; urease (100 U/mL) was added and samples were incubated at 37°C for 15 minutes. ADMA 1 mM in phosphate buffer was added (final ADMA concentration: 0.8 mM) and samples were incubated at 37°C for 60 minutes; the reaction was stopped by mixing 1:1 with 4% sulphosalicylic acid and samples were centrifuged for 10 minutes at 3000 x g. Finally, the supernatants were assayed for citrulline as follows. Solution A (diacetyl monoxime 80 mM, thiosemicarbazide 2 mM) and solution B (H_2PO_4 3 M, H_2SO_4 6 M, $NH_4Fe(SO_4)_2$ 1.75 mM) were prepared, mixed 1:3, and added 1:1 to the samples. Samples were then incubated at 60°C for 110 minutes and read spectrophotometrically at 528 nm against citrulline standards (Ferrigno et al. 2014).

6.4 ATP assay

Adenosine triphosphate is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. ATP is also a marker of the energetic status of the cell, as it rapidly falls in detrimental conditions.

Tissue ATP is measured with the ATPlite monitoring system, based on firefly (*Photinus pyralis*) luciferase. The production of light is caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration and it was measured by using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

ATP + D-Luciferin + O_2 ⟶ Oxyluciferin + AMP + PPi + CO_2 + LIGHT

Briefly, frozen tissue was homogenized in ice-cold 100 mM phosphate buffer with 3 mM EDTA; the homogenate was immediately precipitated in TCA 30% and centrifuged at 3000 x g for 15 minutes at 4°C. The supernatant was diluted 50x in 100 mM phosphate buffer pH 7.75 and assayed. Tissue ATP was measured with the luciferin-luciferase method using the ATPlite luminescence assay kit (Perkin Elmer Inc., USA), according to manufacturer's instructions with minor changes.

Afterwards, it was performed the ADP evaluation treating the same samples with Phosphoenolpyruvate (PEP) and Pyruvate Kinase enzyme (PK). PK is able to catalyse the pyruvate production, starting from ADP and PEP.

ADP + PEP → ATP + pyruvate

By this step all the ADP present in the sample was converted to ATP and, in this way, it was possible to obtain a total ATP value, given adding ATP and ADP. The values of ATP and ADP were calculated by subtraction (Gorman et al. 2003).

6.5 Reactive Oxygen Species (ROS) assay

Reactive Oxygen Species (ROS) include both the so-called "free radicals" (superoxide radical, hydroxyl radical) or molecules containing oxygen, but not radical (hydrogen peroxide). The production takes place during normal metabolic processes involving oxygen, but an excessive release of ROS causes a lot of damages to proteins, lipids and DNA, molecules highly sensitive to oxidative stress.

The hepatic determination of ROS was obtained using the conversion of the 2',7'-Dichlorofluorescein Diacetate (H_2DCFDA) to a fluorescent molecule, the 2',7'-Dichlorofluorescein (DCF). The H_2DCFDA works as a probe: it enters the cell where its two acetyl groups are removed by intracellular esterases and, in the presence of ROS, it is oxidized to DCF.

Tissue samples were homogenized (50 mg/ml) in Locke's buffer (120 mM NaCl, 2.5 mM KCl, 5 mM $NaHCO_3$, 6 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4) and incubated for 20 minutes at room temperature with 10 mM H_2DCFDA (Molecular Probes Inc.). Production of the fluorescent derivative DCF as a function of time was measured using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

6.6 Lipid peroxidation (TBARS) assay

Lipid peroxidation is a process triggered by free radicals. Subsequently those lipids containing unsaturated fatty acids chains are directly oxidized by molecular oxygen. Then, the oxidative damage spreads with a chain reaction because the electron-deprived lipids try to refill the loss by oxidizing neighbouring molecules. This process grows and involves proteins and also DNA, resulting in the end in the production of carcinogenic and toxic secondary products, such as aldehydes and ketones. One of these is the Malondialdehyde (MDA) that was used as lipid peroxidation index.

The extent of lipid peroxidation was evaluated in terms of Thiobarbituric Acid Reactive Substances (TBARS) formation, according to Esterbauer and Cheeseman's method (Esterbauer & Cheeseman 1990) and then the samples absorbance was detected spectrophotometrically at the wave length of 535 nm. The calibration curve was built using MDA as standard.

6.7 Nitrate/Nitrite assay

Total NO production was evaluated by measurement of the tissue Nitrite/Nitrate (NOx) content. The levels of NOx in serum were determined by Cayman Nitrate/Nitrite Colorimetric Assay Kit. The samples were filtered through a 30-kDa molecular weight cut-off filter to eliminate any proteins and then they were mixed with an equal volume of Griess reagent, incubated for 10 minutes at room temperature in the dark, and measured at a wave length of 540 nm.

6.8 Glutathione assay

Glutathione is one of the most important antioxidant molecules produced by the human body. Glutathione has an antioxidant action both against free radicals and against molecules such as hydrogen peroxide, nitrites, nitrates, benzoates, and other. From a structural standpoint, Glutathione is a tripeptide composed by glycine, glutamate and cysteine. The latter binds to

the glycine through a canon peptide bond, whereas it establishes an atypical peptide bond with the glutamate. This kind of bond involves the side chain carboxylic group of glutamate and the amine group of cysteine. Glutathione is produced by the glutathione reductase enzyme, that restores reduced Glutathione (GSH) from oxidized Glutathione (GSSG) by means of NADPH as electron donor.

The hepatic concentration of total GSH was established following the instruction of the enzymatic Glutathione Assay Kit (purchased by Cayman Chemicals) and exploiting the activity of glutathione reductase. GSSG concentration was measured after derivatization of GSH with 2-vinylpyridine.

6.9 Lipid assay: Nile Red

Hepatic lipid assay was performed according to Lyn-Cook *et al.* method (Lyn-Cook et al. 2009). Frozen tissue samples (50–70 mg each) were homogenized in 200 µl of water, and 4 µl aliquots were utilized to determine protein concentration by means of Lowry assay. To extract lipids 1 ml chloroform–methanol solution (2:1) was added to the homogenized samples and then they were incubated for 1 hour at room temperature (RT) with intermittent agitation. After centrifuging of the samples at 3000 x g for 5 minutes RT to separate the lower lipid-containing layer, the latter was transferred to a clean tube and air-dried. Pellets were re-suspended in 100 µl of 100% ethanol, 5 µl aliquots of lipid extract were added to 190 µl of Phosphate-Buffered Saline (PBS) in a 96-well polystyrene black plate, and then, 5 µl of Nile Red solution (1 mg/ml in DMSO) were added to each well. Samples were light-protected with aluminum foil and incubated at room temperature for 10 minutes, under gentle agitation. Fluorescence intensity (Ex 485/Em 572) was measured using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader. Results are expressed as total lipid/liver weight (mg/g).

6.10 Lowry protein assay

The Lowry assay (Folin-Ciocalteau) is used to quantify the protein content in a specific sample. The Folin reactive (a mixture of tungstate, molybdate and sodium phosphate) and a copper sulphate solution are added to the sample, determining a colour change directly related to the protein amount. The absorbance of the sample is measured spectrophotometrically at 550 nm and the high sensitivity of this method allows to quantify up to 5 µg proteins in a 0.2 ml volume.

Two reagents, A and B (Table 2), were mixed in a ratio of 50 parts of A and 1 part of B, making a final solution C (A+B= C, Table 2). The samples were analysed in duplicate.

Copper ions (Cu^{2+}) in C solution react in a basic environment with $CO\text{-}NH_2$ protein groups, causing the violet colour of the mixture. This reaction is specific for polypeptides because almost two $CO\text{-}NH_2$ groups are required.

Subsequently, Folin reactive was added (D solution, Table 2). Tungstic acid and molybdic acid were reduced by the copper protein complex, to tungstate blue and molybdate blue. Folin reactive was added only at the end, because in absence of Cu^{2+} ions, it would bind exclusively to aromatic residues, leaving proteins undetected.

To quantify the sample protein content, it was necessary to build a calibration curve, by means of Bovine Serum Albumin (BSA) standard at the concentration of 2 mg/ml. Seven points of the curve were prepared, making serial dilution in bi-distilled water and adjusting the final volume to 200 µl. As the samples, also the curve was analysed in duplicate. To fix the zero point of the curve, a blank sample was prepared and treated like other samples. Both standards and samples were supplemented with 1 ml of C solution (Table 2), stirred and incubated for 10 minutes at room temperature. Then, 100 µl of Folin reactive (D solution) were added to all tubes. After 30 minutes of incubation, the absorbance was detected spectrophotometrically at 550 nm.

The absorbance value was used to determine the protein concentration according to Lambert-Beer law:

$$\mathbf{A = \varepsilon_\lambda \times C \times l}$$

(**A**= absorbance; $\boldsymbol{\varepsilon_\lambda}$= molar extinction coefficient; **C**= sample concentration; **l**= optical path in cm).

Solution A	NaK 268 mg/l, Na_2CO_3 23.4 g/l, NaOH 4 g/l
Solution B	$CuSO_4$ at 1.56 % in distilled water
Solution C	Solution A + Solution B with a ratio of 50:1
Solution D	Folin + distilled water with a ratio of 1:1

Table 2. Summary of Lowry solutions.

6.11 Real Time PCR

Farnesoid X Receptor (FXR), Cationic Amino-acid Transporter 1 (CAT-1), Cationic Amino-acid Transporter 2A (CAT-2A), Cationic Amino-acid Transporter 2B (CAT-2B), Organic Cation Transporter 1 (OCT-1), Multidrug And Toxin Extrusion protein 1 (MATE-1), Protein Arginine Methyltransferase 1 (PRMT-1) and Dimethylarginine Dimethylaminohydrolase 1 (DDAH-1) mRNAs were analysed using Real Time Polymerase Chain Reaction (RT-PCR): total RNA was isolated from frozen liver samples with Tri reagent (Sigma-Aldrich). The cDNA was generated using iScript Supermix (Bio-Rad). The RNA was assayed by measuring the absorbance at 260/280 nm. FXR, CAT-1, CAT-2A, CAT-2B, OCT-1, MATE-1, PRMT-1, DDAH-1, Ubiquitin C (UBC), Actin (ACT), Tubulin (TUB), Ribosomal Protein 9 (RS9) and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) gene amplification efficiencies were established by means of calibration curves (respectively 115.6%, 109.5%, 115.7%, 107.5%, 95%, 166.8%, 105%, 190.3%, 105.1%, 100%, 100.1%, 136.9%, 151.3%). The expression of the house keeping genes remained constant in the considered experimental groups. Primers had the following sequence: **FXR:** 5'-CGCCTCATCGGCGGGAAGAA-3' (forward) 3'-TCACGCAGTTGCCCCCCGTTC-5' (reverse); **CAT-1:** 5'-GGG TCC GGT TCG CAG TGT GG-3' (forward) 3'-GCA CCC GTC AAC CGC TGT CA-5' (reverse); **CAT-2A:** 5'-TAC GTT GTC GCC GCA GGC TC-3'(forward), 3'-TCG TGG CAG CAA CGG GTG AC-5' (reverse); **CAT-2B:** 5'-TAC GTT GTC GCC GCA GGC TC-3' (forward), 5'-GCT GCC ACT GCA CCC GAT GA-3' (reverse); **OCT-1:** 5'-TCT GTG TCC GGT GTG CTA AC-3' (forward), 3'-TGC AGC TCA TGC GGG ATA AA-5' (reverse); **MATE-1:** 5'-TCC CCA TTT ACG CTG TGT CC-3' (forward), 3'-ACC ACA GAC CAA TCA CTC CC-5' (reverse); **PRMT-1:** 5'-TGC TGC ACG CTC GTG ACA AGT-3' (forward), 3'-TCC ACC ACG TCC ACC AGG GG-5' (reverse); **DDAH-1:** 5'-CAA CGA GGT CCT GAG ATC TTG GC-3' (forward), 3'-GGA TCA GTA GAT GGT CCT TGA GC-5' (reverse); **UBC:** 5'-CAC CAA GAA GGT CAA ACA GGA A-3' (forward), 3'-AAG ACA CCT CCC CAT CAA ACC-5' (reverse); **ACT:** 5'-GTG ACG AGG CCC GCA AGA G-3' (forward), 3'-AGG GGC CGG ACT CAT CGT-5' (reverse); **TUB:** 5'-AGA AGC AAC ACC TCC TCC TCG-3' (forward), 3'-ATA CAC TCA CGC ATG GTT GCT G-5' (reverse); **RS9:** 5'-CCC TTC GAG AAA TCG CGT CT-3' (forward), 3'-GCA GAG CGT TGC CTT CAA AC-5' (reverse); **GAPDH:** 5'-AAC CTG CCA AGT ATG ATG AC-3' (forward), 3'-GGA GTT GCT GTT GAA GTC A-5' (reverse). Gene expression was analysed using Platinum Syber Green qPCR mix UDG. UBC, ACT, TUB, RS9 and GAPDH were used as reference genes. The amplification was performed through two-step cycling (95°–60°C) for 45 cycles, in an ABI prism 7000 sequence detection system (Applied Biosystems Deutschland Inc., Darmstadt,

Germany), following the instructions of the supplier. All samples were assayed in triplicate. The results were normalized to the endogenous controls, and fold change of the gene expression was calculated using threshold cycle (Ct) values.

6.12 SDS-PAGE

6.12.1 Reagents & Antibodies

The CelLytic Buffer and Protease Inhibitor Cocktail were purchased from Sigma-Aldrich (Milan, Italy), as well as the mouse antibody anti-Tubulin. The rabbit polyclonal antibodies anti-iNOS and anti-eNOS were provided respectively by Cayman Chemical and Santa Cruz Biotechnology, INC., anti-CAT-1 and anti-CAT-2 were purchased from Proteintech and Aviva Systems Biology, respectively, while anti-PRMT-1 was from Abcam. The goat polyclonal antibody anti-DDAH-1 was provided from Abcam.

Specific peroxidase-conjugated anti-IgG antibodies were from Santa Cruz Biotechnology.

The Enhanced Chemiluminescence substrate (ECL) was from Bio-Rad, as well as the Blotting-Grade Blocker (non-fat dry milk).

All reagents were of the highest grade of purity available and were purchased from Sigma-Aldrich (Italy).

6.12.2 Liver tissue extracts preparation

Liver tissue samples (about 50 mg) were homogenized in ice-cold CelLytic Buffer (500 µl) supplemented with Protease Inhibitor Cocktail (10 µl) and centrifuged at 15000 x g for 10 minutes. The collected supernatant was divided into new tubes and 4 µl of it were used to quantify the protein content by means of the Lowry method. After that, the supernatant was divided into aliquots containing the same amount of proteins and it was reduced with SDS 2XR, 2% β-mercaptoethanol.

6.12.3 Electrophoresis & Immunoblotting

Samples of liver extracts, containing the same amount of proteins, were separated in SDS-PAGE on 7.5% or 10% acrylamide gels, and transferred to PVDF membrane that was blocked for 2 hours with 5% Blotting-Grade Blocker in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) at 4°C.

After blocking, membranes were washed twice in TBS and incubated with appropriate primary antibodies overnight at 4°C, under gentle agitation. The dilution for primary antibodies used in the present study were: anti-eNOS, anti-iNOS, anti-CAT-1, anti-CAT-2, anti-PRMT-1 and anti-Tubulin (DM1A), 1:1000, while for anti-DDAH-1 1:3000.

After washing in PBS (Na_2HPO_4 8 mM, NaH_2PO_4-H_2O 2 mM, NaCl 140 mM, pH 7.4, 0.1% Tween 20), membranes were incubated with appropriate peroxidase-conjugated secondary antibodies diluted 1:2000 for all primary antibodies, except for anti-PRTM-1 that was 1:3000. The membranes were washed again in PBS and, then, reactive proteins were visualized with a chemiluminescence reaction with Bio-Rad Chemidoc XRS+.

Membranes were incubated for 2 hours at room temperature with 1% NaN_3 in PBS to inhibit the peroxidase signal and then they were reprobed with anti-Tubulin primary antibody as a control for equal loading.

Bands intensity quantification was performed by computer using the Bio-Rad Image Lab software.

6.12.4 Polyacrylamide Gel Electrophoresis (SDS PAGE)

Electrophoresis is a technique consisting in differentiated migration of charged molecules in electric field. Molecules are separated according to their charge, size and according to the applied electric field.

Sodium Dodecyl Sulphate (SDS) is an anionic detergent that stably binds to proteins leading to denaturation. In case of excess of SDS, the proteins acquire a constant amount of negative charge *per* unit mass: all the protein-SDS complexes will move toward the anode and, according to the gel sorting properties, their mobility (and therefore the distance travelled after a certain period) is inversely proportional to $\log_{10}$ of their molecular weight. Using standards with known molecular weight together with samples, it is possible to establish the molecular weight of the protein in the sample.

Protein sorting in SDS-PAGE was carried out thanks to the use of a chamber for electrophoresis MINI-PROTEAN III, Bio-Rad. The sorting gel dimensions were 88x55 mm and a thickness of 1.5 mm.

Proteins package themselves in the stacking gel, that had a polyacrylamide concentration of 3%, while they separated each other in the linear running gel, that had a polyacrylamide concentration of 7.5% or 10%.

6.12.5 Preparing acrylamide gels

Two glass plates, outer and inner, were joined together vertically, using a special support.

The prepared running gel solution (Table 3) was poured between the two glass plates by the use of a syringe. Once the gel polymerized, it formed a texture capable of separating proteins, according to their molecular weight.

Afterwards, we proceeded inserting the stacking gel (Table 3), a gel spacer at the 3% of polyacrylamide, which was poured onto the running gel by means of the syringe. It allows to a special teflon comb to form the sample loading wells.

Solutions	Running Gel 7.5%	Running Gel 10%	Stacking Gel 3%
Acrylamide 30%	2250 µl	3000 µl	250 µl
Tris HCl 1.5 M pH 8.8	2250 µl	2250 µl	-
Tris HCl 1.25 M pH 6.8	-	-	250 ml
SDS 10%	90 µl	90 µl	25 ml
Water	3870 µl	3120 µl	1700 µl
TEMED 1%	450 µl	450 µl	250 µl
APS 10%	90 µl	90 µl	25 µl
Total Volume	**9000 µl**	**9000 µl**	**2500 µl**

Table 3. Polyacrylamide gel solutions.

TEMED (N,N,N,N'-Tetra-Methyl-Ethylendiamine) and APS (Ammonium Persulphate), which are the two cross-linking and polymerizing agents in the reaction, were added just before pouring the solutions between the glass plates.

The samples run simultaneously with a standard mixture of known molecular weight protein. The electrophoresis was carried out for about 2 hours at a constant voltage of 100 Volts, at room temperature. The used buffer for the run was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

6.12.6 Western Blotting

This technique consists of moving proteins, previously separated through SDS-PAGE, from within the gel onto a membrane made of Polyvinylidene Difluoride (PVDF). A MINI TRANS-BLOT Bio-Rad chamber was utilized and the transfer took place through the application of an electric field with constant amperage of 200 mA, at 4°C for 2 hours. The procedure consisted in preparing a "sandwich" putting in contact the polyacrylamide gel and the PVDF membrane, the latter was previously activated by soaking it first in methanol for few seconds and then in water.

Both the gel and the PVDF were squeezed together by suitable supports. Therefore, to ensure a more closely contact, two layers of sponges and blotting paper were used, soaked in blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol). During the transfer, the gel was turned towards the negative pole, while the membrane was close to the positive one. This is necessary because, since proteins have a negative charge, will tend to migrate toward the positive pole and, therefore, towards the PVDF membrane.

6.12.7 Immunoblotting

The PVDF membrane was incubated for two hours at 4°C, under mild stirring, with 5% Blotting-Grade Blocker in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) to block any non-specific sites. Afterwards, the membrane was washed twice in TBS 1X 0.1% Tween 20 and incubated overnight, under mild stirring, with specific primary antibodies depending on the protein of interest: anti-iNOS, anti-eNOS, anti-CAT-1, anti-CAT-2, anti-PRMT-1 and anti-Tubulin, diluted 1:1000 and anti-DDAH-1 1:3000.

After that, the PVDF was washed every 5 minutes for 30 minutes, under stirring and at room temperature with washing buffer PBS 1X (Na_2HPO_4 8 mM, NaH_2PO_4-H_2O 2 mM, NaCl 140 mM, pH 7.4, 0.1% Tween 20), in order to remove the excess of antibody. Then the membrane was incubated for 1 hour at 4°C with appropriate peroxidase-conjugated secondary antibody diluted 1:2000 for all primary antibodies, except for anti-PRTM-1 that was 1:3000.

Subsequently, the membranes were washed again with PBS 1X. In the presence of the substrate, the peroxidase conjugated secondary antibody, developed a chemiluminescent reaction which identified the protein of interest, exposing the membrane to the analyser of digital images ChemiDoc XRS+, Bio-Rad.

For the chemiluminescence reaction the Bio-Rad kit, which included two reagents to be mixed in the ratio 1:1, was used. Reagents must be added on the membrane in order to allow

the development of the light reaction. At the end, the PVDF was exposed for different times (from a minimum of 30 seconds to a maximum of 10 minutes) to the analyser of digital images ChemiDoc XRS+, Bio-Rad.

The intensity quantification of the bands was performed to the computer with the software Image Lab, Bio-Rad.

It is possible to inhibit the secondary antibody conjugated with peroxidase, in order to cover again the same PVDF membrane with new antibody, without any interference in the new marking due to the previous one.

In practice, at the end of the immunoblotting development, the PVDF membrane was rehydrated using the washing buffer for few minutes. Subsequently, it was placed in a 1% solution of NaN_3 in PBS 1X and left under stirring at room temperature for about 2 hours. At this point, the excess NaN_3 was removed with several washes, and it was possible to re-probe the membrane with another antibody, without the need of performing again the non-specific sites blocking procedure.

6.13 Statistical analysis

In the case of two experimental groups, comparisons between groups were performed by unpaired t test, or by the Mann-Witney test for non-parametric data. In the case of three or more experimental groups, data were analysed by one-way ANOVA, followed by Tukey's multiple comparisons test or, when data distribution was not normal, according to the Shapiro test, Dunn's test was used. The value of $*p \leq 0.05$ was considered to indicate statistical significance. The accompanying table and graphs present the mean value ± standard error of the mean (SEM).

Results

Results Part I

7.1 Transaminases & biochemical parameters

7.1.1 Transaminases

Serum levels of AST, ALT and AP, have been used as indicators of liver injury due to the progression of steatosis. Blood samples were collected at the end of II, IV and VIII week of MCD-diet administration. In our established *in vivo* model of steatohepatitis we found a significant increase in serum AST, ALT and AP in MCD-rats, as compared with their respective control groups (Figure 21).

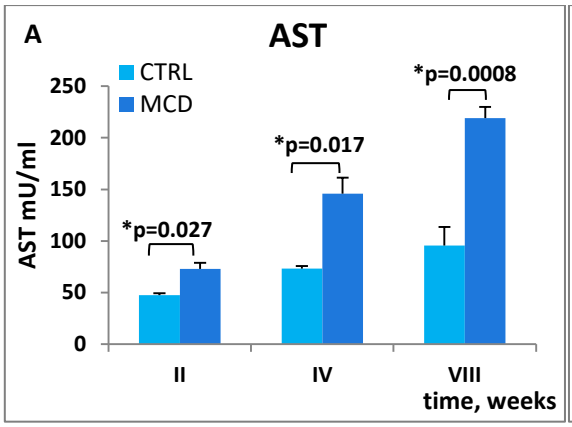


Figure 21A. Serum AST levels. A significant increase in serum AST levels was found in MCD treated rats at any considered time.

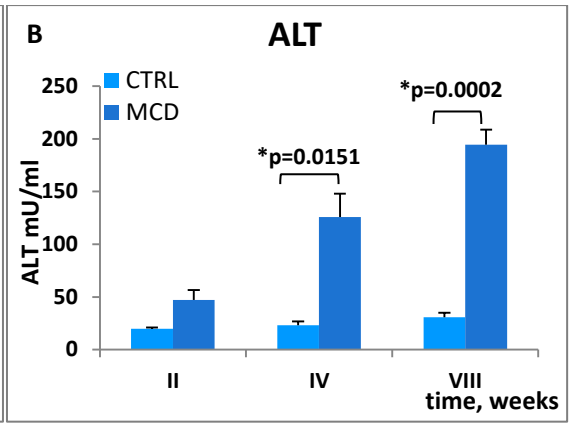


Figure 21B. Serum ALT levels. A significant increase in serum ALT levels was found in MCD treated rats at IV and VIII week of treatment.

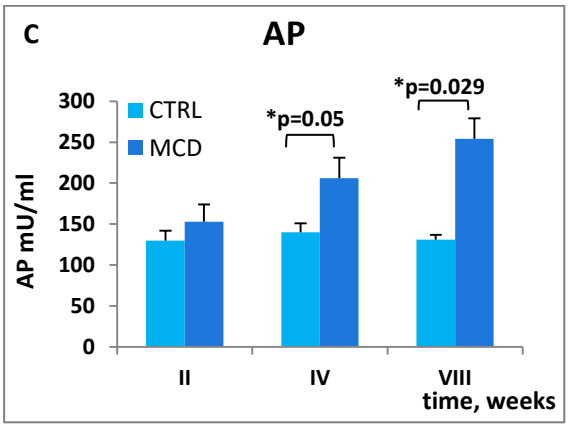


Figure 21C. Serum AP levels. A significant increase in serum AP levels was found in MCD treated rats at IV and VIII week of treatment.

Figure 21. Transaminases release.
Transaminases release in serum was evaluated. A marked and significant increase in AST **(Figure 21A)**, ALT (**Figure 21B**) and AP (**Figure 21C**) in MCD treated rats serum (II, IV, VIII week respectively) confirmed hepatocyte damage in well-established NASH-rat model.
The significant **p values* are indicated in each chart and, in any case, are **p<0.05*. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.1.2 Biochemical parameters

The same trend was found for the total bilirubin serum levels, meanwhile a time-dependent decrease in serum glucose concentration was observed in MCD-treated rats and it became significant from the II week. Similar data were also found for the serum lipid profile, such as cholesterol and triglycerides: the differences were markedly significant after the II week of treatment yet (Table 4).

Biochemical parameters	II week		IV week		VIII week	
	CTRL	MCD	CTRL	MCD	CTRL	MCD
Total bilirubin (mU/ml)	0.12±0.01	0.16±0.01	0.12±0.01	0.33±0.05*	0.15±0.04	0.79±0.1*
Glucose (mU/ml)	211±5	159±9*	173±13	135±7*	174±5	121±5*
Cholesterol (mU/ml)	53±1	34±3*	68±3	24±2*	75±2	23±2*
Triglycerides (mU/ml)	57±11	23±1*	101±8	18±2*	98±13	23±4*

Table 4. Biochemical parameters evaluation. Biochemical parameters like total bilirubin, glucose, cholesterol and triglycerides were evaluated. They increased in MCD-diet treated rats, compared with control groups. **p<0.05*, *versus* corresponding control. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.2 Total lipid content

Hepatic content of lipids was evaluated by means of Lyn-Cook *et al.* method (Lyn-Cook et al. 2009). The increase in hepatic total lipid amount is considered a severity marker for steatosis. In our model, the evaluation of hepatic lipid content just showed a significant increase already after 2 weeks of MCD-diet in comparison with control groups (Figure 22).

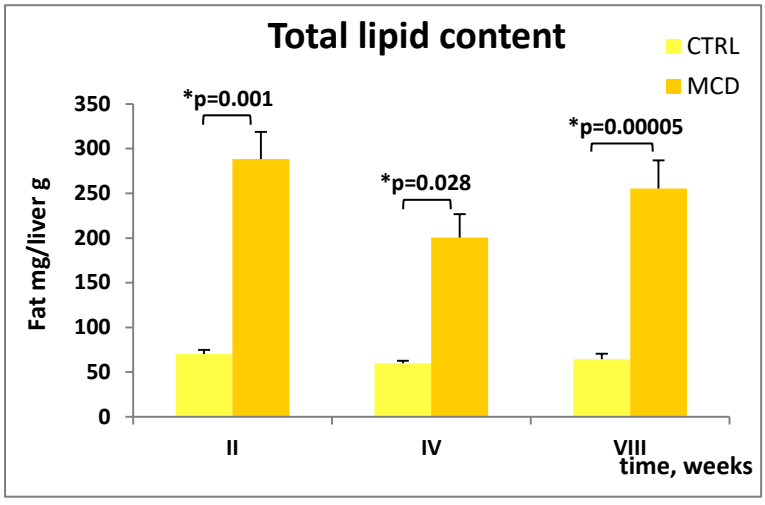


Figure 22. Evaluation of hepatic lipid content.
Liver lipid total content just showed a significant increase already after 2 weeks of MCD-diet in comparison with control groups. The significant **p values* are indicated in the chart and, in any case, are **p<0.05*. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.3 Serum & tissue ADMA levels

ADMA levels in serum and tissue were evaluated by means of the ELISA kit (Immundiagnostik, AG Germany). Serum samples and hepatic biopsies were collected at the end of the II, IV and VIII week of MCD-diet treatment. After 4 weeks of treatment, as shown in the panel, a significant decrease in serum ADMA levels occurred in MCD animals, reaching its lowest level at 8 weeks of treatment as compared with control animals (Figure 23).

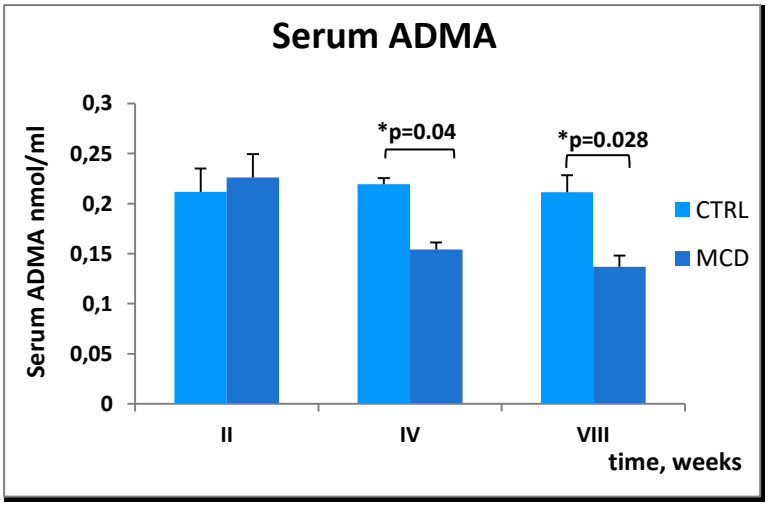
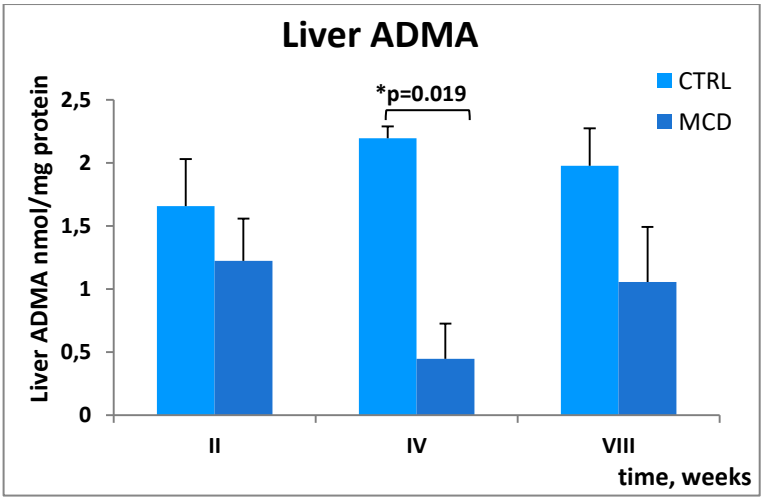


Figure 23. Serum ADMA content evaluation. A significant decrease in serum ADMA levels occurred in MCD-diet treated rats, when compared with controls. Data became significant after 4 weeks of treatment (**p=0.04*) and they are maintained after 8 weeks (**p=0.028*). The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

As concerning the ADMA hepatic content, it was observed a reduction in all treated MCD-rats when compared to their respective control groups. The decrease became significant after 4-week MCD- diet treatment (Figure 24).

Figure 24. Liver ADMA content evaluation. Hepatic ADMA content significantly decreased, respectively, after 4 (**p=0.019*) and 8 weeks of diet, compared with controls. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.



7.4 Oxidative stress and mitochondrial function: TBARS, Glutathione & ATP/ADP ratio

7.4.1 Lipid peroxidation, TBARS

Lipid peroxidation is a process triggered by free radicals and affecting membrane phospholipids. Then, the oxidative damage spreads with a chain reaction oxidizing neighbouring molecules and worsening the injury. The extent of lipid peroxidation was evaluated in terms of Thiobarbituric Acid Reactive Substances (TBARS) formation, using Malondialdehyde (MDA) as a standard. Higher lipid peroxides (TBARS) were detected in MCD rats: TBARS significantly increased after 4 weeks of diet as compared with control group and even more at 8 weeks. These data indicated the high level of oxidative stress (Figure 25).

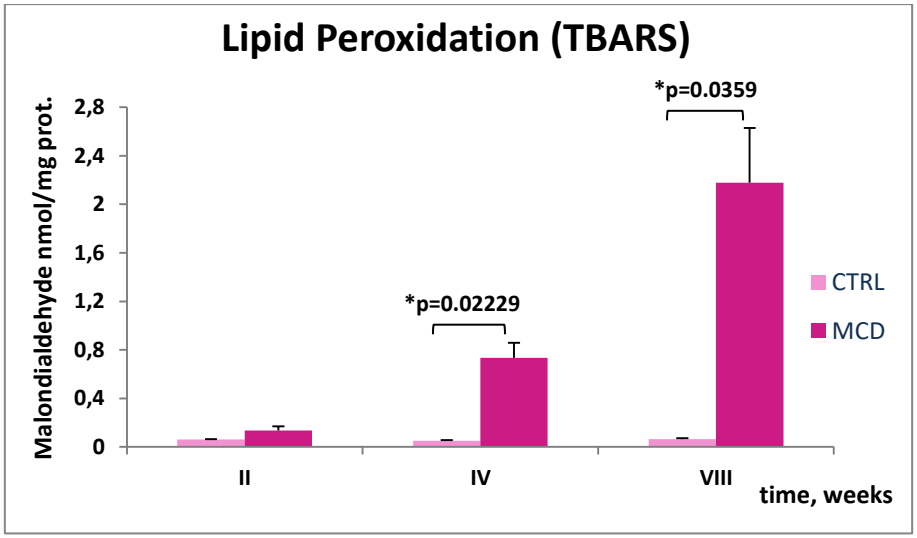


Figure 25. Lipid peroxidation (TBARS) evaluation. In MCD-diet fed rats higher levels of TBARS were found both after 4 (**p=0.02229*) and 8 (**p=0.0359*) weeks of treatment. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.4.2 Glutathione content

The hepatic concentration of total Glutathione was established following the instruction of the enzymatic Glutathione Assay Kit (Cayman Chemicals).

Glutathione is one of the most important antioxidant molecules produced by the human body, thus reduced Glutathione (GSH) content is considered a marker of oxidative stress-induced injury.

As showed in the panel below, in our model during the progression from steatosis to steatohepatitis (from II to VIII week) there was a time-dependent decrease in GSH stores of MCD-treated animals, compared with control ones. The decrease was significant already after 2 weeks of MCD-diet administration and it was maintained for all treatment time (Figure 26).

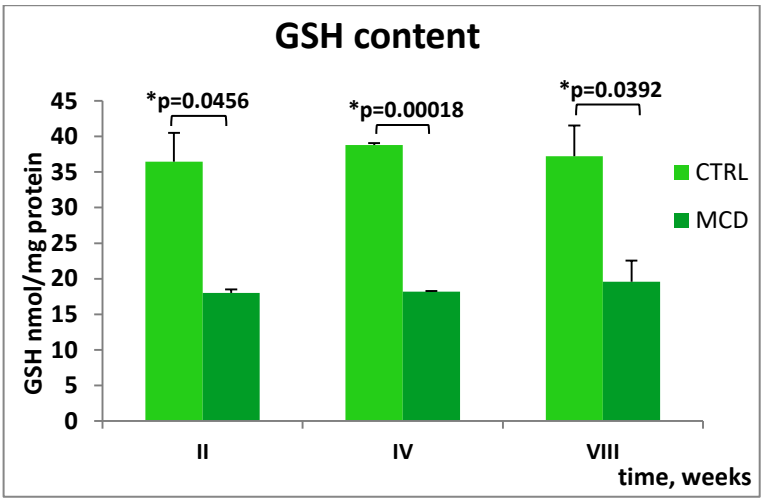


Figure 26. Hepatic GSH stores content evaluation. A time-dependent decrease in GSH stores of MCD-treated animals was found, as compared with controls. The decrease was significant already after 2 weeks of MCD-diet administration and it was maintained for all treatment time. The significant **p values* are shown in the chart. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.4.3 ATP/ADP ratio

ATP content is a marker of the energetic status of the cell, as it rapidly falls in detrimental conditions, leading to necrosis or apoptosis. ATP and ADP were established by means of ATPlite luminescence assay kit (Perkin Elmer Inc., USA).

Further confirmation that high oxidative stress occurs during progression from NAFLD to NASH in our MCD-rat model came from the ATP/ADP ratio. The mitochondrial function was critically affected by oxidative stress, in fact, the hepatic energy content showed a marked decrease already after 2 weeks of treatment, which remained stable up to 8 weeks, as it is showed in the panel of ATP/ADP ratio in Figure 27.

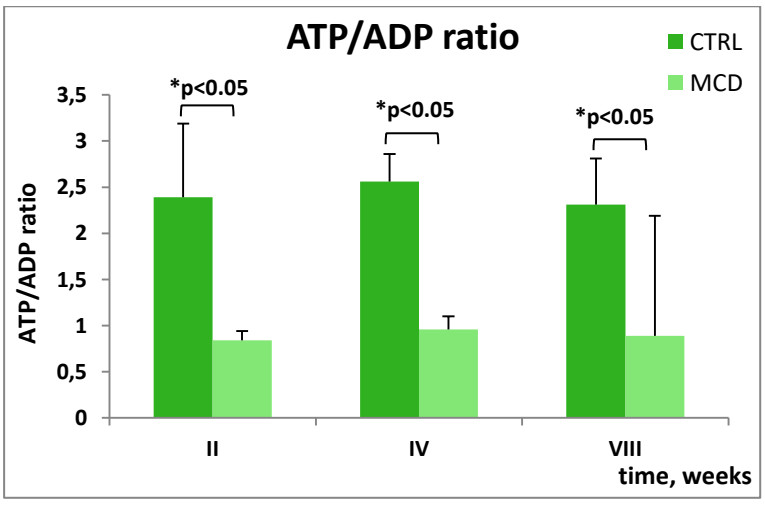


Figure 27. Hepatic energy content evaluation. Hepatic energy content, given by ATP/ADP ratio, showed a marked decrease already after 2 weeks of treatment, which remained stable up to 8 weeks. Significant **p values* are indicated in the panel. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.5 Serum NOx levels

Total NO production was evaluated by measurement of the serum nitrite/nitrate (NOx) content, determined by Cayman Nitrate/Nitrite Colorimetric Assay Kit.

A significant increase in serum NOx, another oxidative stress index, was found in MCD rats after 8 weeks of treatment as compared with the control group (Figure 28).

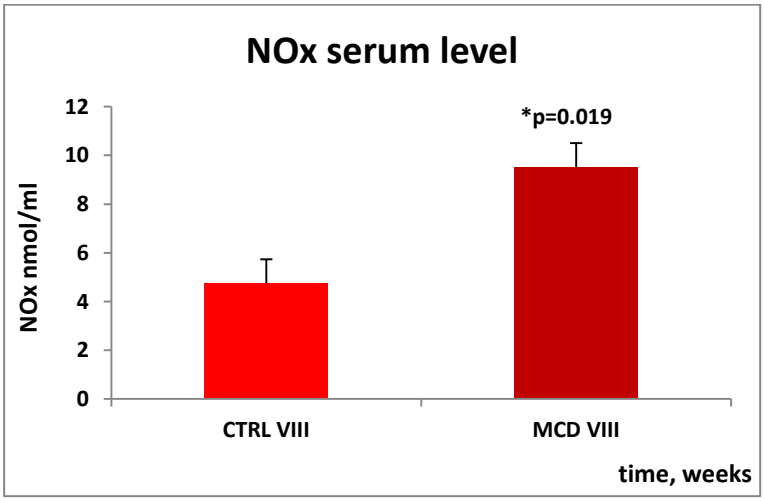


Figure 28. Serum NOx content evaluation. A significant increase in serum NOx occurred in MCD treated rats after 8 weeks, when compared to controls, (**p=0.019*). The results are reported as the mean ± S.E. of 5 animals for MCD group and n=3 for control group.

7.6 PRMT-1 mRNA & protein expression

To assess the enzyme involved in ADMA synthesis, PRMT-1 mRNA and protein were detected. The RT-PCR analysis showed a significant higher PRMT-1 mRNA expression in liver obtained after 4 weeks MCD-diet fed rats. The significance was maintained also after 8 weeks of treatment (Figure 29).

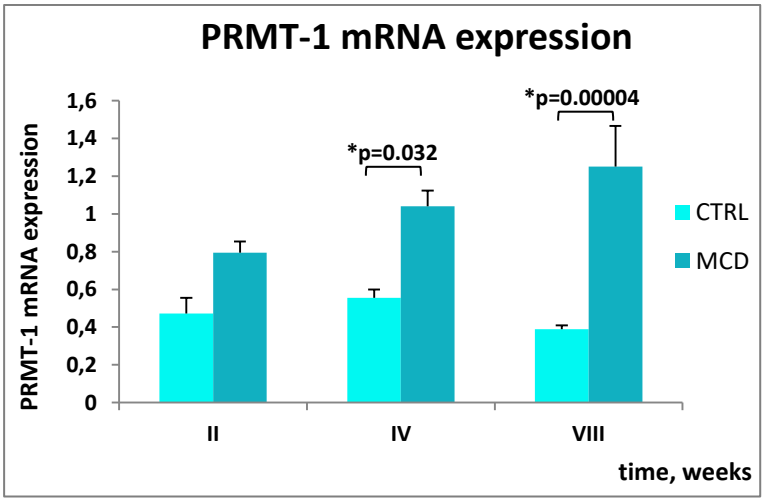


Figure 29. PRMT-1 mRNA expression evaluation. PRMT-1 mRNA expression increased in a significant manner in MCD treated rats after 4 weeks (**p=0.032*) of treatment. The significance was maintained up to 8 weeks (**p=0.00004*). The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

The same trend was observed after Western Blot analysis of PRMT-1 protein expression. After 8 weeks of MCD-diet treatment, livers showed significantly higher PRMT-1 protein expression compared with controls (Figure 30).

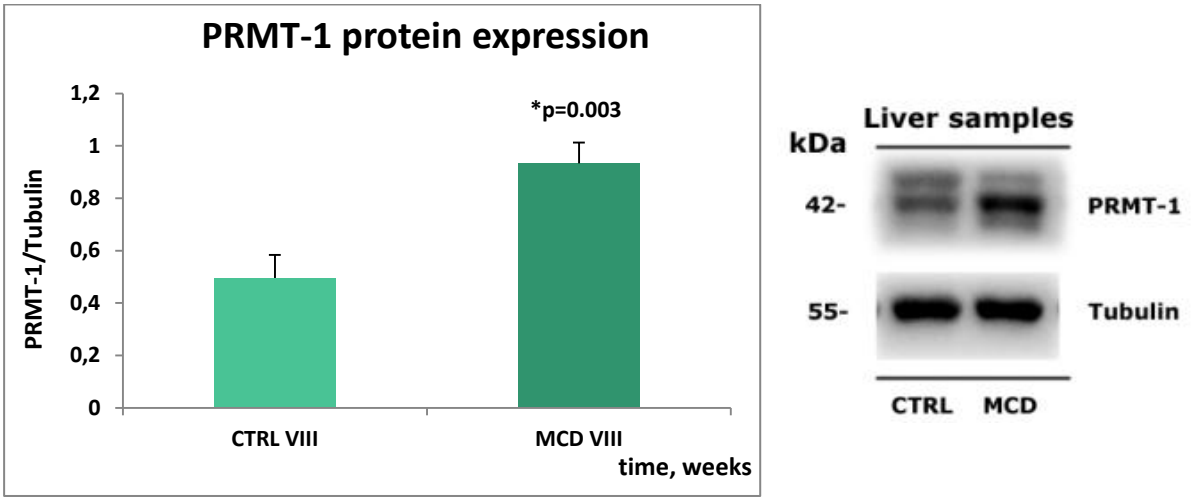


Figure 30. Western Blot analysis of PRMT-1 protein expression. The figure shows immune-reactive bands and their quantification. A significant increase (**p=0.003*) in MCD treated rats, after 8 weeks of diet was observed, as compared with control group. The results are reported as the mean ± S.E. of 5 animals for MCD group and n=3 for control group.

7.7 DDAH-1 mRNA, protein expression & activity

The main enzyme involved in ADMA metabolism to L-Citrulline and DMA is DDAH-1, which has high activity in the liver and kidney. In order to investigate its expression and activity in our experimental model of NASH, we performed RT-PCR, Western Blot and enzymatic assay analysis of DDAH-1.

As reported in the chart, an increase in DDAH-1 mRNA was observed after 8 weeks in MCD animals as compared with control group (Figure 31).

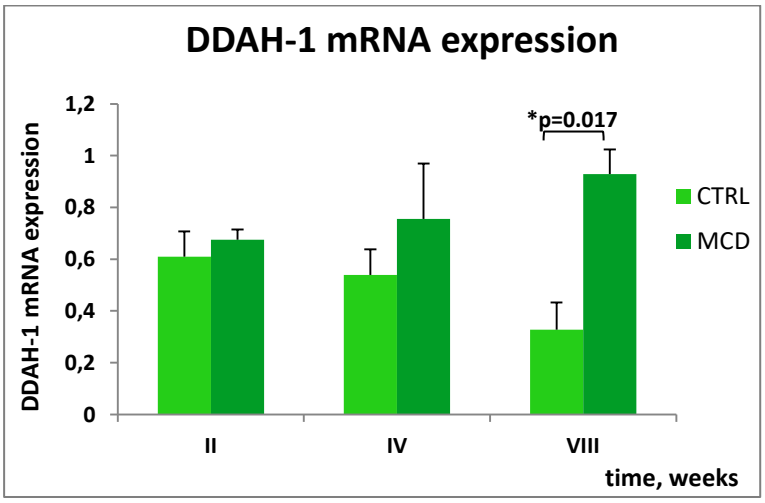


Figure 31. DDAH-1 mRNA expression evaluation. DDAH-1 mRNA expression increased in a significant manner in MCD treated rats after 8 weeks (**p=0.017*) of treatment. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

As concerning the protein expression of DDAH-1, the Western Blot analysis revealed no changes in 8-week fed MCD-rat, compared with control group (Figure 32).

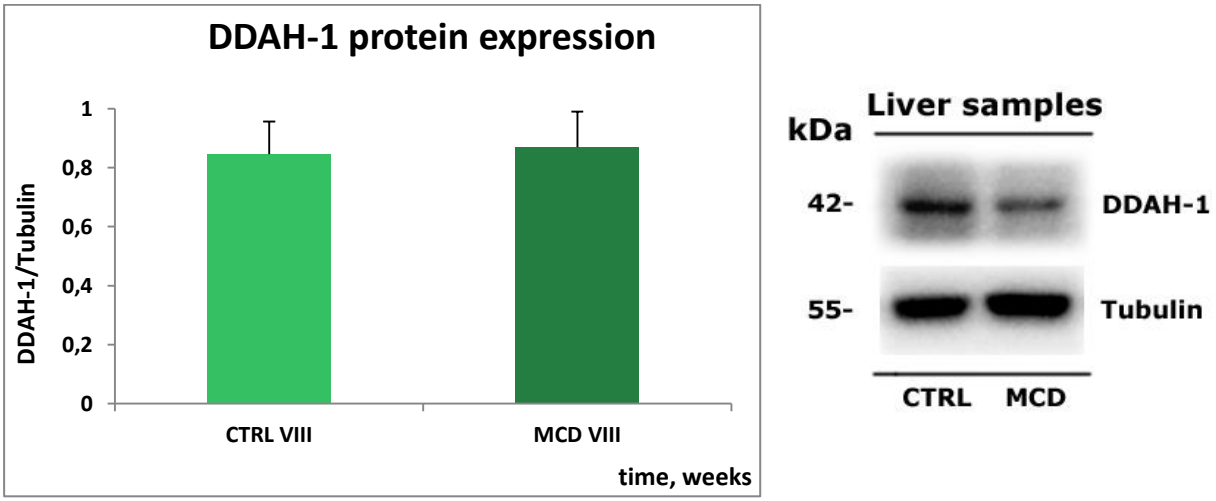


Figure 32. Western Blot analysis of DDAH-1 protein expression. The figure shows immune-reactive bands and their quantification. No changes were found between 8-week MCD diet fed rats and control group. The results are reported as the mean ± S.E. of 5 animals for MCD group and n=3 for control group.

Despite what was obtained for mRNA and protein expression, DDAH activity showed a significant decrease already at 4 weeks of treatment and it was maintained such 8 weeks, when compared with control groups (Figure 33).

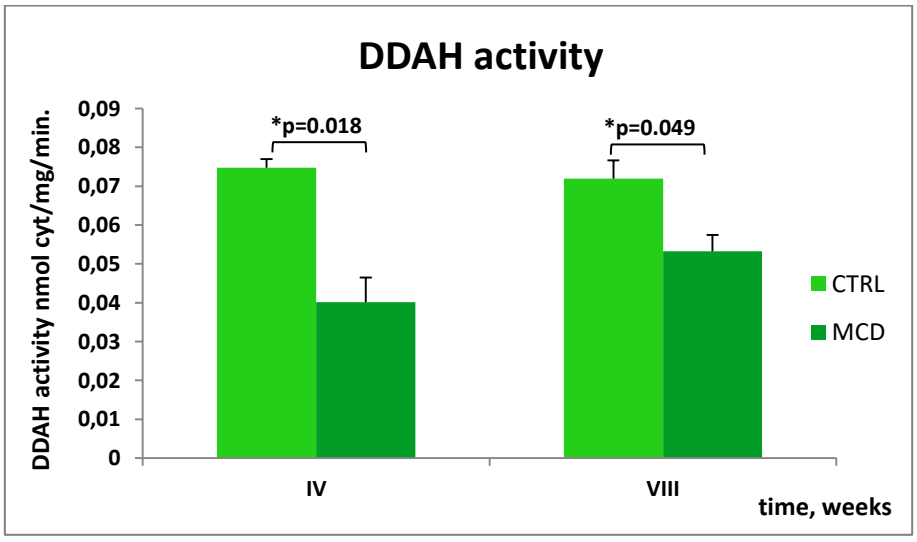


Figure 33. Evaluation of hepatic DDAH activity. DDAH activity showed a significant decrease already at 4 weeks (**p=0.018*) of treatment and it was maintained such 8 weeks (**p=0.049*), when compared with control groups. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

7.8 Cationic Amino-acid Transporters mRNA & protein expression

7.8.1 CAT-1

ADMA can be released in extracellular environment by CATs, involved also in the removal of circulating ADMA by the liver. To investigate how ADMA import/export traffic is regulated by CATs in our MCD-rat model, we analysed mRNA and protein expression of CAT-1. As we can see in the panel, a significant time-dependent increase in CAT-1 mRNA expression was found after 8 weeks in MCD animals, as compared with control group.

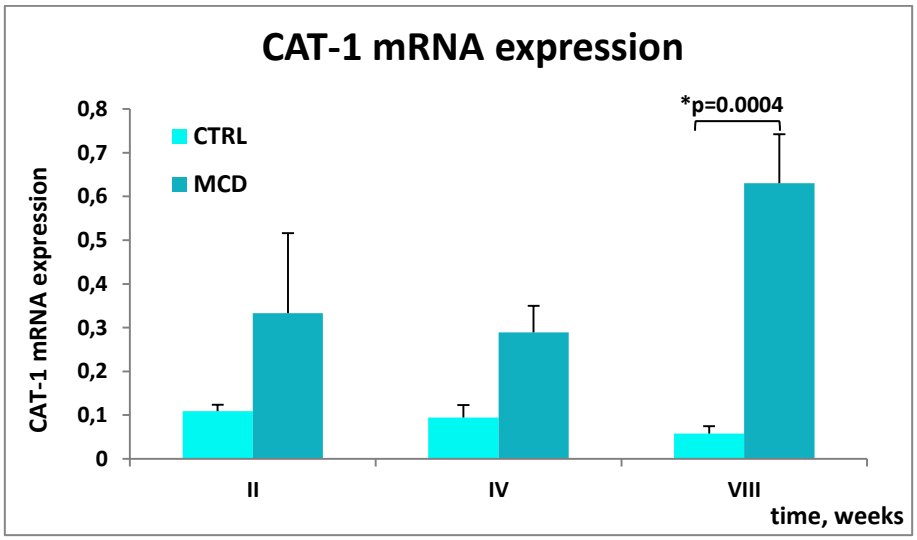


Figure 34. CAT-1 mRNA expression evaluation. A significant time-dependent increase in CAT-1 mRNA expression was found after 8 weeks (**p=0.0004*) in MCD animals, as compared with control group. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

The figure shows that no changes were detected in the CAT-1 protein expression in MCD animals after 8 weeks of treatment, compared with control group.

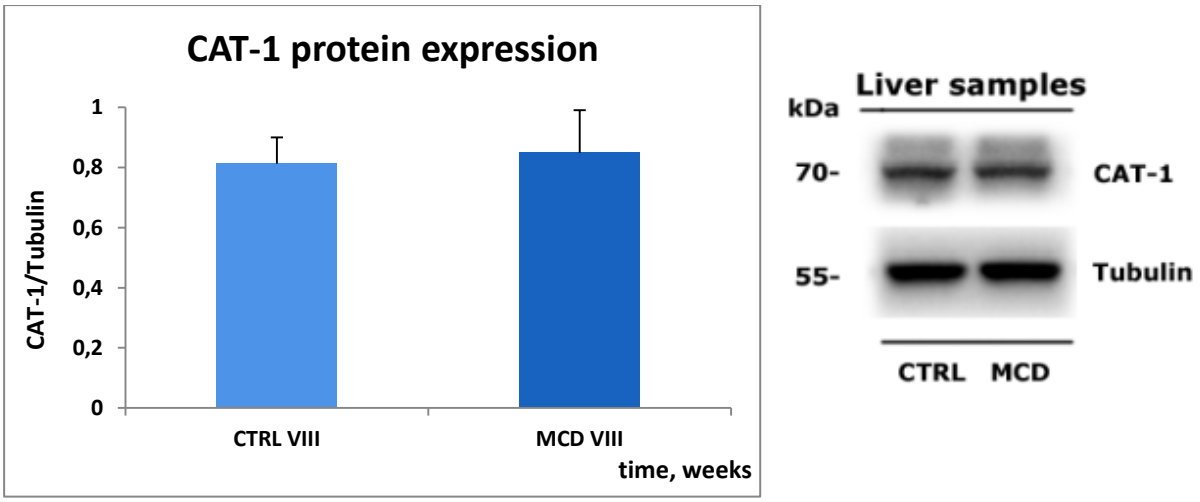


Figure 35. Western Blot analysis of CAT-1 protein expression. The figure shows immune-reactive bands and their quantification. No changes were found between 8-week MCD diet fed rats and control group. The results are reported as the mean ± S.E. of 5 animals for MCD group and n=3 for control group.

7.8.2 CAT-2A & CAT-2B

The mRNA expression of CAT-2A and CAT-2B isoforms decreased already in 2-week fed MCD-rats. The reduction became significant after 4 weeks of treatment, maintaining this significant trend up to 8 weeks (Figure 36 e 37).

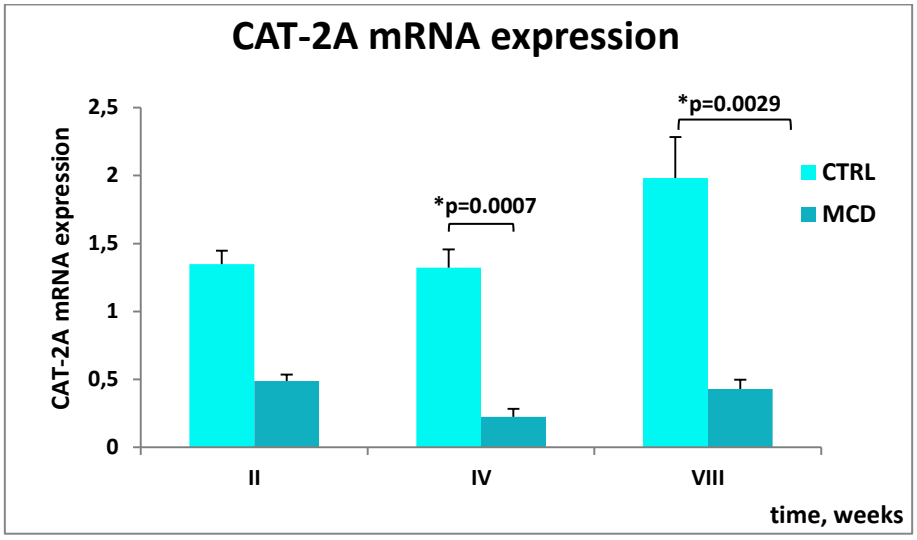


Figure 36. CAT-2A mRNA expression evaluation. A time-dependent decrease in CAT-2A mRNA expression was found already after 2 weeks in MCD-diet fed rats. This reduction became significant after 4 weeks (**p=0.0007*) and it was maintained also after 8 weeks (**p=0.0029*), as compared with control groups. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

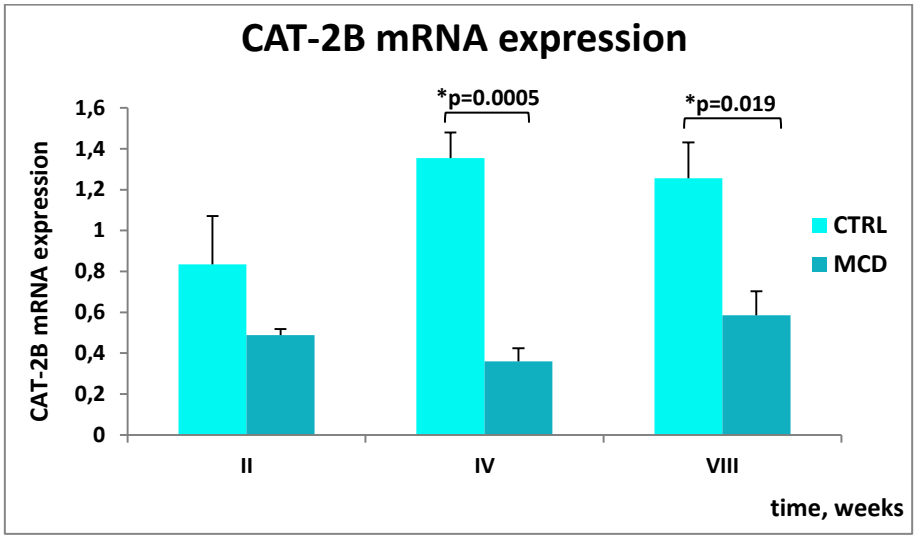


Figure 37. CAT-2B mRNA expression evaluation. A time-dependent decrease in CAT-2B mRNA expression was found already after 2 weeks in MCD-diet fed rats. This reduction became significant after 4 weeks (**p=0.0005*) and it was maintained also after 8 weeks (**p=0.019*), as compared with control groups. The results are reported as the mean ± S.E. of 5 animals for MCD groups and n=3 for control groups.

The protein content in CAT-2 (no antibodies for CAT-2A and CAT-2B are available) increased in a significant manner in MCD animals after 8 week-diet, when compared with control group (Figure 38).

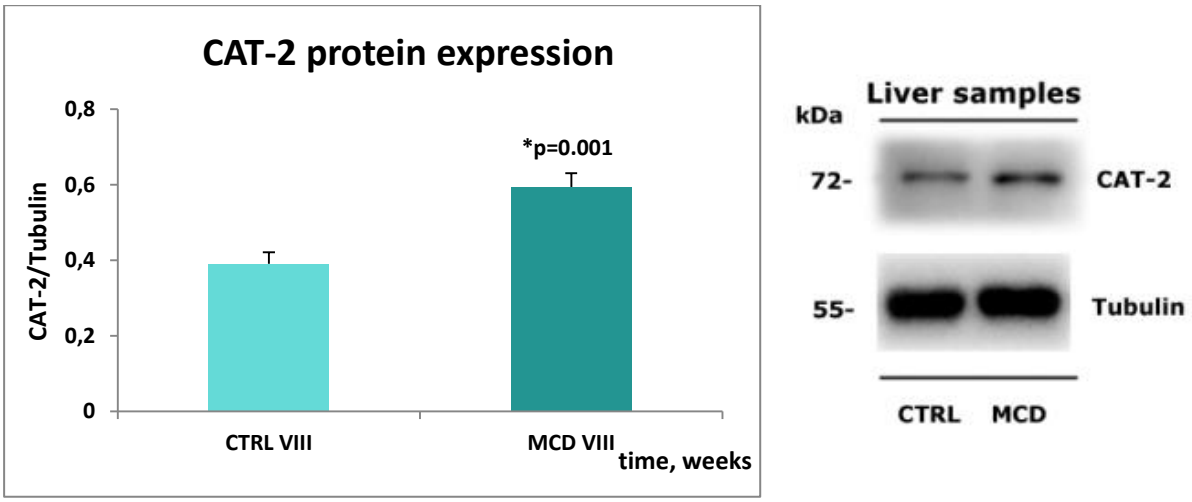


Figure 38. Western Blot analysis of CAT-2 protein expression. The figure shows immune-reactive bands and their quantification. No antibodies for CAT-2A and CAT-2B are available, so the figure represents the expression of both isoforms of CAT-2. A significant increase in MCD animals after 8 weeks (**p=0.001*) of diet was detected, when compared with control group. The results are reported as the mean ± S.E. of 5 animals for MCD group and n=3 for control group.

Results part II

8.1 Transaminases & biochemical parameters

To assess the extent of liver injury in an *in vivo* model of obstructive extrahepatic cholestasis, transaminases and total and direct bilirubin were evaluated. Serum AST, ALT and AP were significantly higher in animals subjected to bile duct ligation when compared with the sham-operated group (control). Also total and direct bilirubin concentration was significantly increased after BDL, as compared with sham-operated rats (Table 5).

Biochemical parameters	Sham	BDL
AST (mU/ml)	87.2 ± 8.3	647.7 ± 105*
ALT (mU/ml)	35.1 ± 2.5	308.3 ± 45*
Alkaline Phosphatase (mU/ml)	264.5 ± 15	333.8 ± 33*
Total bilirubin (mg/dl)	0.08 ± 0.02	8.8 ± 0.13*
Direct bilirubin (mg/dl)	0.05 ± 0.01	7.04 ± 0.1*

Table 5: Serum enzymes and total and direct bilirubin levels in 3-day BDL and sham-operated rats. Transaminases and bilirubin values were significantly higher in BDL animals compared with sham-operated group *(*p<0.05*). These are the mean results of eight different experiments ± S.E.

8.2 Serum & tissue ADMA levels

The hepatic ADMA content was comparable among the three lobes of sham-operated rats, while, after 3-day BDL, tissue ADMA levels were significantly higher in the right lobe and in the median one, when compared with the left lobe. Moreover, ADMA concentration in ML and RL in BDL animals significantly increased, compared with sham-operated ML and RL, respectively (Figure 39).

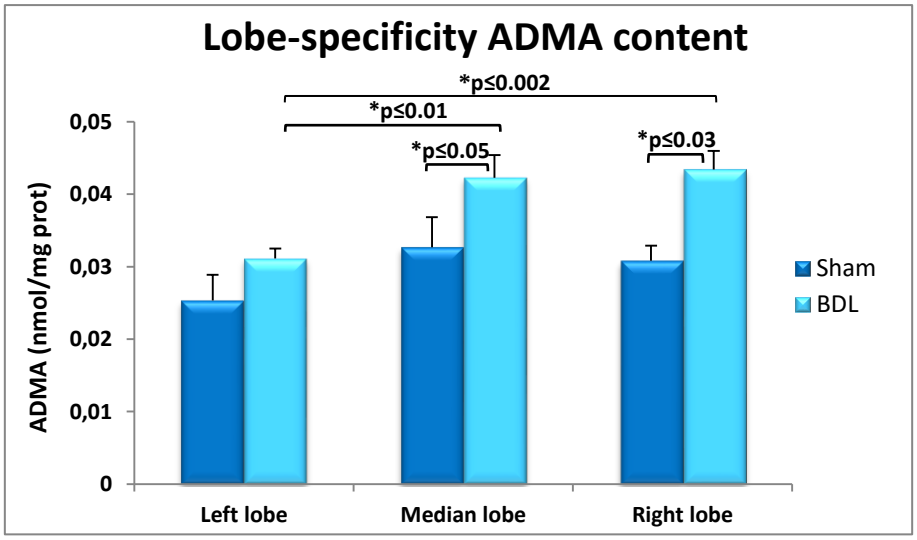


Figure 39. ADMA content in the three different lobes evaluation. ADMA concentration was significantly higher in the right and median lobes of BDL rats compared with both BDL-left lobe and the respective lobes in sham-operated animals. The significant **p values* are indicated in the graph. These are the mean results of eight different experiments ± S.E.

No changes in serum ADMA after BDL were detected as compared with sham-operated group (0.88 ± 0,06 *vs* 0.87 ± 0,02 nmol/ml, **p≤0.49*, respectively – data not shown).

8.3 Lipid peroxidation, TBARS

The extent of oxidative stress was assessed evaluating TBARS levels. Animals subjected to bile duct ligation showed significant higher TBARS concentration in all the three lobes in comparison with the three lobes of control groups. The sham-operated right lobe displayed a significant decrease in TBARS level compared with the respective LL (Figure 40).

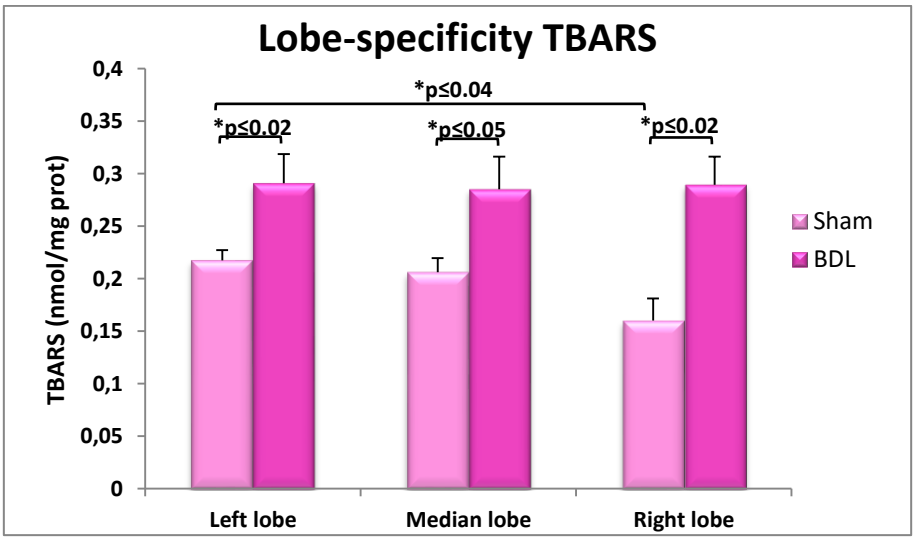


Figure 40. TBARS levels, as oxidative stress marker, evaluation. TBARS values were significantly higher in BDL lobes compared with control lobes. Sham-operated RL showed a significant reduction compared with the respective LL. The significant **p values* are indicated in the graph. These are the mean results of eight different experiments ± S.E.

8.4 PRMT-1 mRNA expression

In order to evaluate modulation in the ADMA synthesis, PRMT-1 expression was detected after 72-hour BDL: significantly higher expression was found in all hepatic lobes, when compared with the respective lobes from sham-operated groups. No differences in PRMT-1 mRNA expression among the lobes were found neither in sham-operated groups nor in BDL groups (Figure 41).

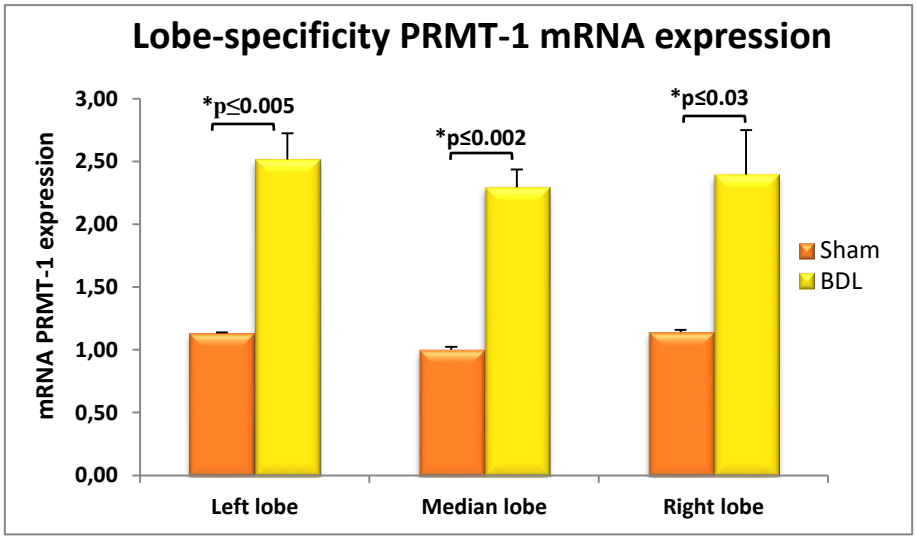


Figure 41. PRMT-1 mRNA expression evaluation. BDL lobes displayed significant higher PRMT-1 mRNA expression, when compared with control groups. No changes were found among the lobes neither in sham-operated animals or BDL rats. The significant **p values* are indicated in the graph. These are the mean results of eight different experiments ± S.E.

8.5 DDAH mRNA expression & activity

DDAH-1 mRNA expression in tissue significantly increased in all three lobes after 72-hour bile duct ligation, as compared with the respectively lobes obtained from sham-operated groups. Median and right lobes in BDL groups showed a significant increase also in comparison with BDL-left lobe. No differences, instead, were found among the three lobes in sham-operated animals (Figure 42).

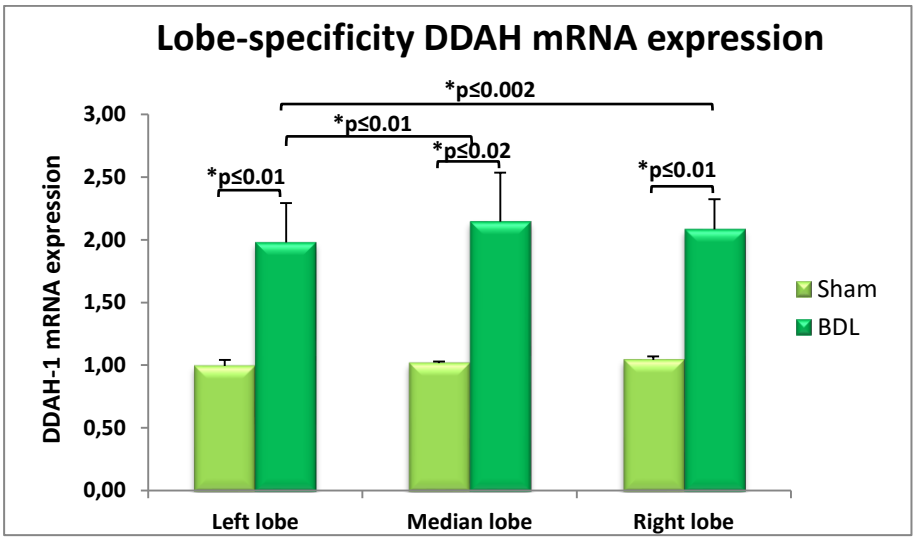


Figure 42. Tissue DDAH-1 mRNA expression evaluation. All the three lobes in BDL animals displayed a significant increase in DDAH-1 mRNA expression, compared with the control lobes, among which there are no differences. The DDAH-1 mRNA expression in the median and the right BDL-lobes was significantly higher than in the left lobe. The significant **p values* are indicated in the graph. These are the mean results of eight different experiments ± S.E.

DDAH activity was evaluated in RL, ML and LL, but no changes were found in both BDL and in sham-operated groups (Figure 43).

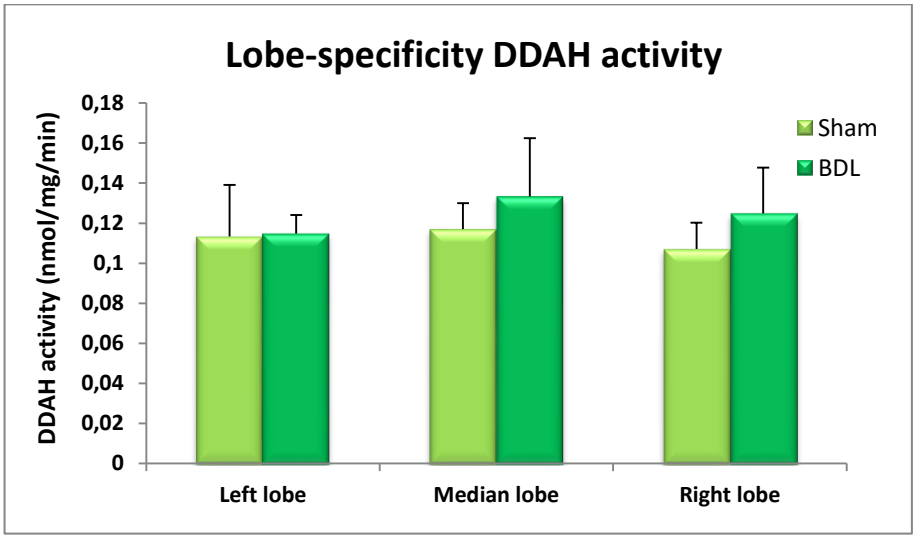


Figure 43. DDAH activity evaluation. No differences among the three lobes and in both experimental groups were found. These are the mean results of eight different experiments ± S.E.

8.6 Cationic Amino-acid Transporters-2, CAT-2 protein expression

ADMA interferes with NO synthesis by competing with arginine and SDMA for cellular transport across CATs. In sham-operated animals, higher levels of CAT-2 were found in RL and ML lobes as compared with LL. CAT-2 protein expression significantly decrease in RL and ML after BDL as compared with the respective sham-operated groups, although no differences among the three BDL lobes were found (Figure 44).

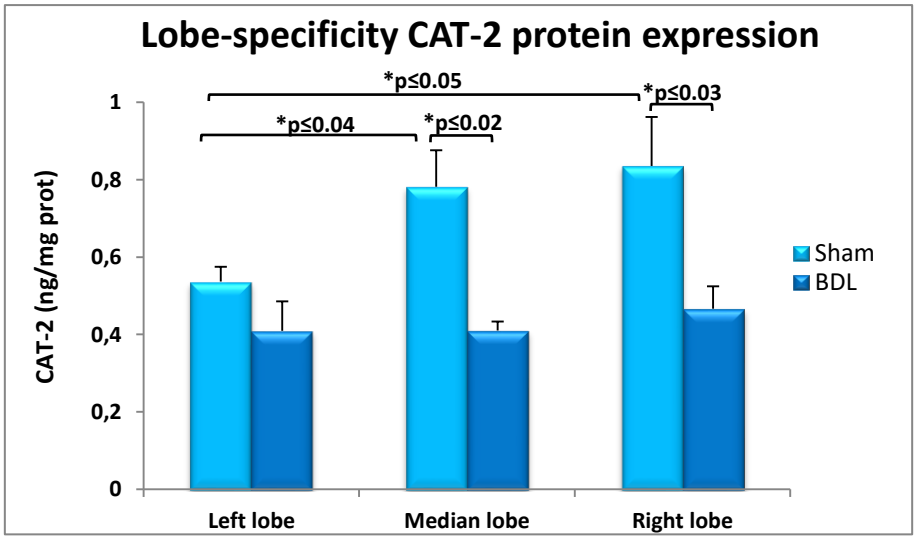
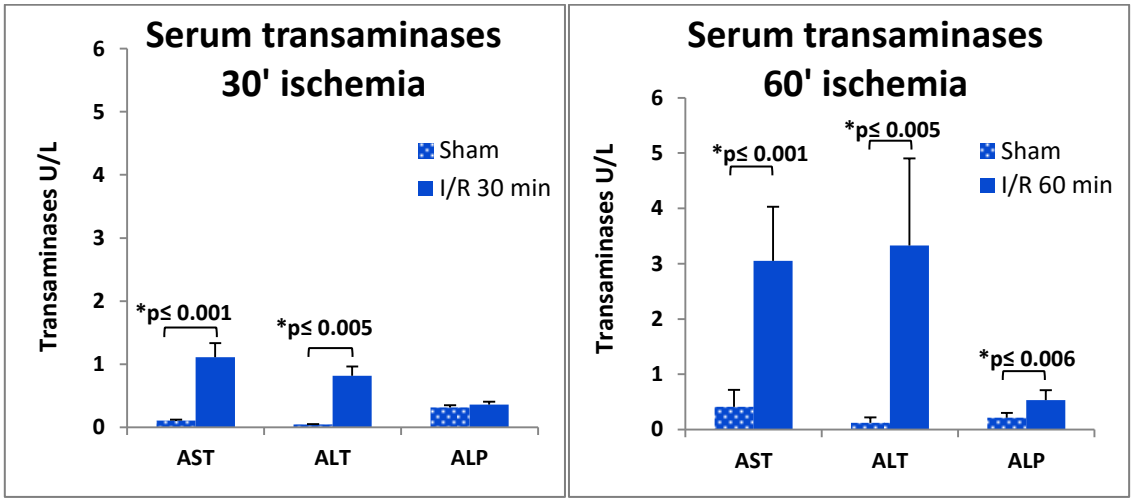


Figure 44. CAT-2 protein expression evaluation. The graph shows protein expression obtained by means of direct ELISA kit (Cayman Chemicals). In control groups, ML and RL showed a significant increase in CAT-2 protein expression, in comparison with LL. CAT-2 protein expression remained constant in the three lobes of BDL animals, while in ML and RL a significant decrease, compared with shamp-operated respective lobes, occurred. The significant **p values* are indicated in the graph. These are the mean results of eight different experiments ± S.E.

Results Part IIIa

9.1 Transaminases & biochemical parameters in serum

Serum levels of AST, ALT, AP and direct bilirubin have been used as indicators of liver damage. Samples were collected at the end of reperfusion period in our *in vivo* model of Ischemia/Reperfusion (I/R) injury. As expected, transaminase levels increased in rats subjected to ischemia and reperfusion as compared with sham-operated group. Liver injury was strictly related to the ischemia period: a time-dependent significant increase was detected, mostly after 60 minutes of ischemia. The same trend was verifiable in serum direct bilirubin, that was significantly higher in I/R group then in sham one (Figure 45).



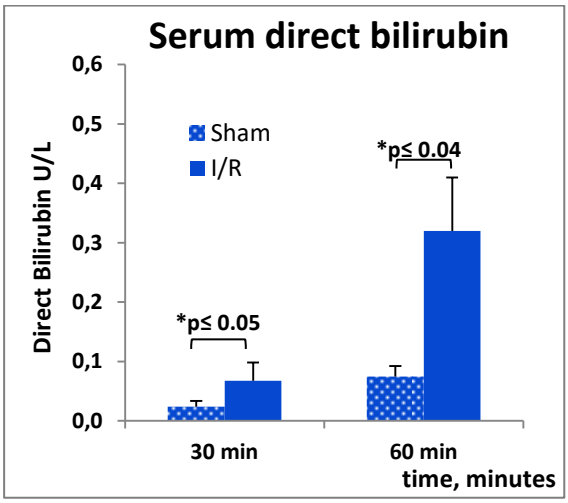
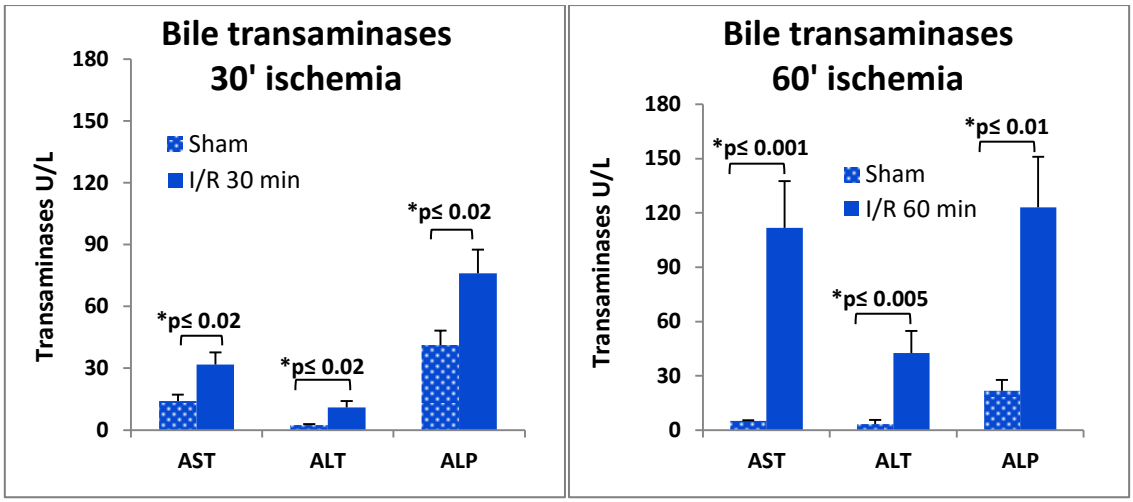


Figure 45. Serum enzymes and direct bilirubin in I/R and sham rats after 30 and 60 minutes of ischemia. Livers were subjected to 30 or 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. Analysis on blood samples showed that transaminases and direct bilirubin levels were significantly higher in I/R animals, than in sham ones, both after 30 and 60 minutes of ischemia. The significant **p values* are indicated in the graphs. The results are reported as the mean ± S.E. of 8 different experiments.

9.2 Transaminases & biochemical parameters in bile

Transaminases levels and direct bilirubin were also evaluated in bile. As it is possible to observe in the panels below, a time-dependent significant increase in AST, ALT and AP release occurred, especially in 60-minutes subjected ischemia rats. Similar results were found in bilirubin release after the same times of ischemia (Figure 46).



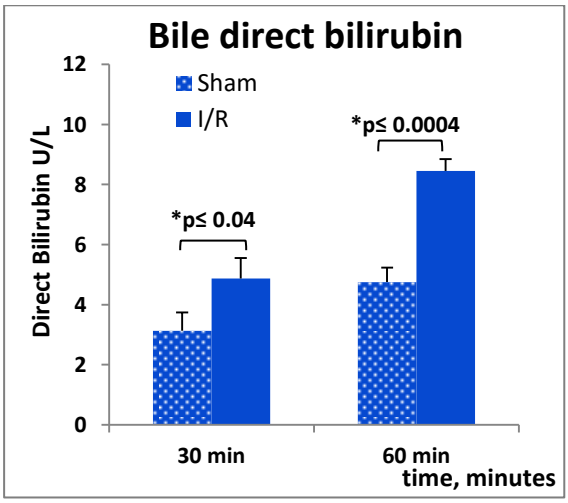


Figure 46. Biliary enzymes and direct bilirubin in I/R and sham rats after 30 and 60 minutes of ischemia. Livers were subjected to 30 or 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. Analysis on bile samples showed that transaminases and direct bilirubin levels were significantly higher in I/R animals, than in sham ones, both after 30 and 60 minutes of ischemia. The significant **p values* are indicated in the graphs. The results are reported as the mean ± S.E. of 8 different experiments.

9.3 Biliary, serum & tissue ADMA levels

ADMA levels in bile, serum and tissue were evaluated by means of the ELISA kit (Immundiagnostik, AG Germany). Surprisingly, ADMA was detected in bile for the first time. It showed a time-dependent and significant increase, both after 30 and 60 minutes of ischemia. To confirm our data, bile samples were also analysed by the HPLC method equipped with a fluorescence detector and no differences were detected, comparing HPLC results and ELISA kit results (Figure 47).

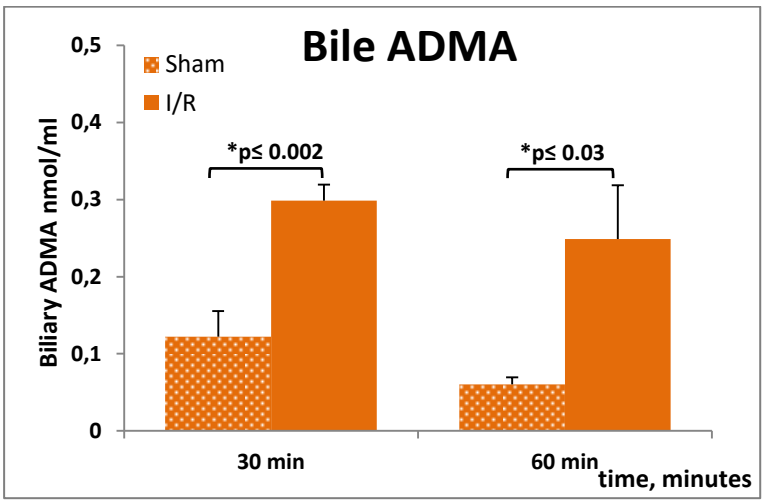


Figure 47. Biliary ADMA levels evaluation. Ischemia injury induced a significant increase in biliary ADMA levels both after 30 and 60 minutes, when I/R group is compared with sham-operated one. The significant **p values* are indicated in the graph. The results are reported as the mean ± S.E. of 8 different experiments.

Only after 60 minutes of ischemia injury, a significant increase in serum ADMA levels occurred. On the contrary tissue ADMA content, after the same time of I/R decreased significantly. Submitting animals to 5 hours of BDL, no changes in serum ADMA were found, while a significant increase in tessue ADMA levels was detected (Figure 48).

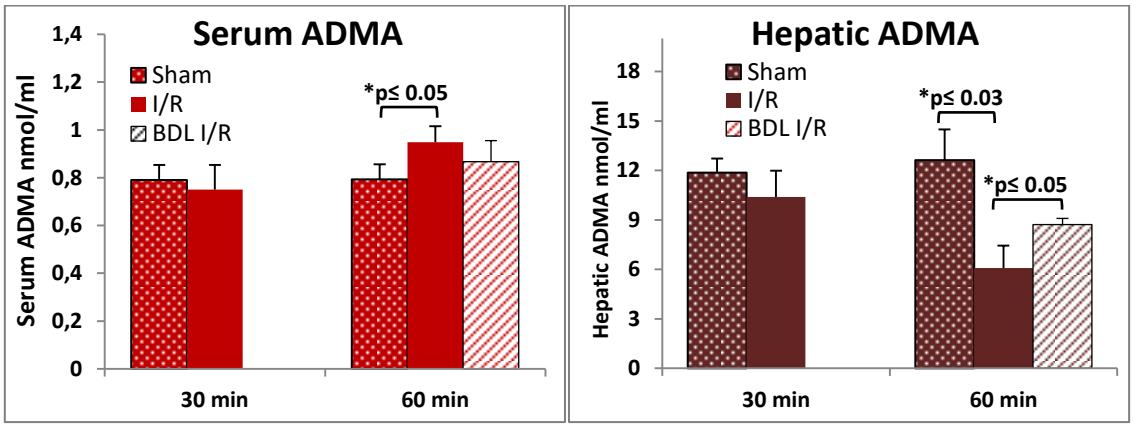


Figure 48. Serum and hepatic ADMA content evaluation. Livers were subjected to 30 or 60 minutes of ischemia, followed by 60 minutes of reperfusion. A set of 60 minutes ischemia experiments, followed by 60 minutes reperfusion was performed at the end of 5 hours of BDL. Sham-operated control rats had similar manipulation, without vascular occlusion. Analysis on serum and tissue samples showed that ADMA levels were significantly higher in I/R animals serum, while they decreased in tissue, after 60 minutes of ischemia. No changes and significant increase were found in serum and tissue ADMA of BDL animals, respectively. The significant **p values* are indicated in the graphs. The results are reported as the mean ± S.E. of 6-8 different experiments.

9.4 PRMT-1 mRNA expression

In order to evaluate any changes in ADMA producing enzyme, PRMT-1 mRNA expression was evaluated after 60 minutes of ischemia, followed by 60 minutes of reperfusion. The PRMT-1 mRNA expression decreased significantly in I/R group, when compared with sham-operated group (Figure 49).

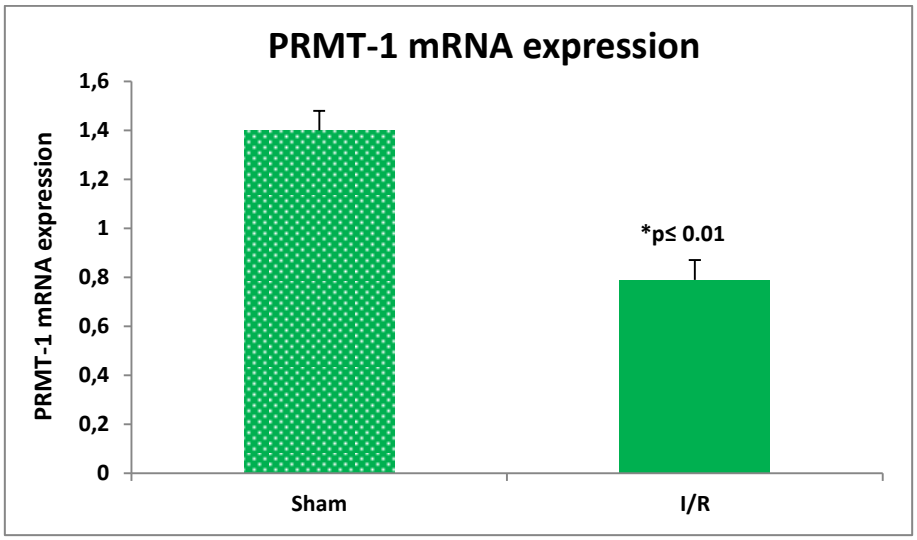


Figure 49. PRMT-1 mRNA expression evaluation. Livers were subjected to 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. A significant decrease (*$p \leq 0.01$) in PRMT-1 mRNA expression was found in I/R rats, compared with sham-operated rats. The results are reported as the mean ± S.E. of 8 different experiments.

9.5 DDAH mRNA, protein expression & activity

Evaluation of DDAH-1 mRNA expression was performed by RT-PCR analysis after 60 minutes of ischemia, followed by 60 minutes of reperfusion. A significant decrease in DDAH-1 mRNA expression was recorded in ischemic group, compared with sham-operated group (Figure 50).

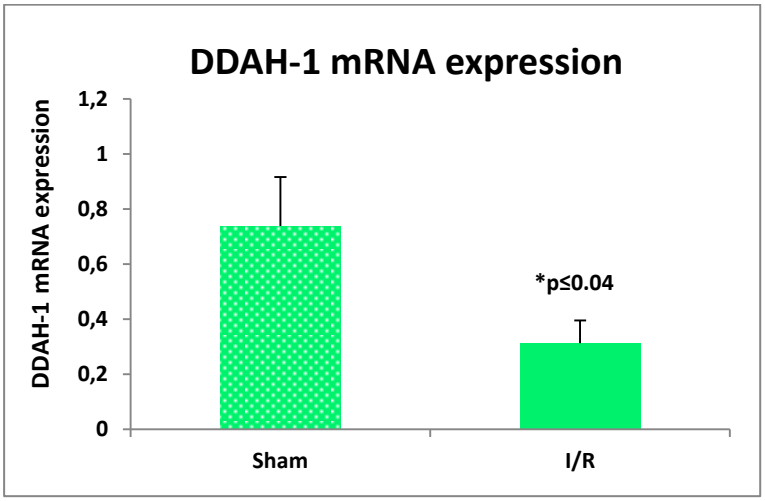
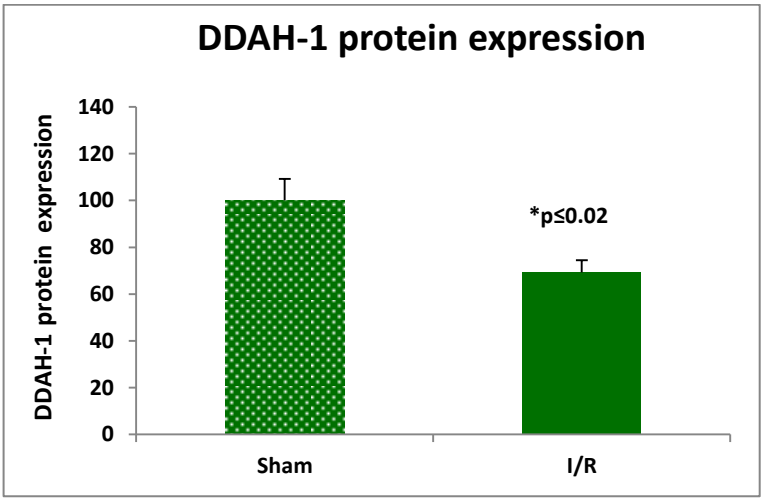


Figure 50. DDAH-1 mRNA expression evaluation. Livers were subjected to 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. A significant (*$p \leq 0.04$) decrease in mRNA expression of DDAH-1 was detectable comparing I/R group with sham rats. The results are reported as the mean ± S.E. of 8 different experiments.

A similar trend was found in DDAH-1 protein expression, performed at the end of reperfusion period in rat livers subjected to 60 minutes of ischemia, by means of direct ELISA kit. A significant decrease was recorded in I/R group, compared with sham one (Figure 51).

Figure 51. DDAH-1 protein expression evaluation. Livers were subjected 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. A significant (*$p \leq 0.02$) decrease in protein expression of DDAH-1 was recorded, comparing I/R group with sham rats. The results are reported as the mean ± S.E. of 8 different experiments.



DDAH-1 activity was also evaluated. At the end of reperfusion period, DDAH-1 activity was significantly decreased both after 30 and 60 minutes of ischemic damage, when compared with sham-operated control groups (Figure 52).

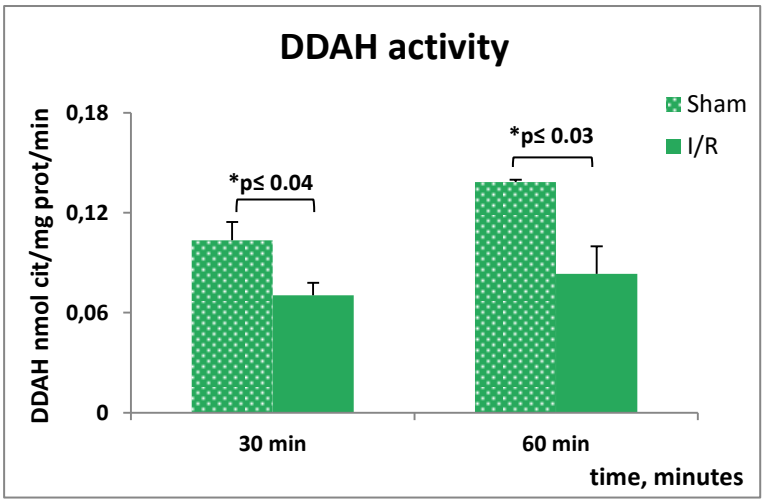
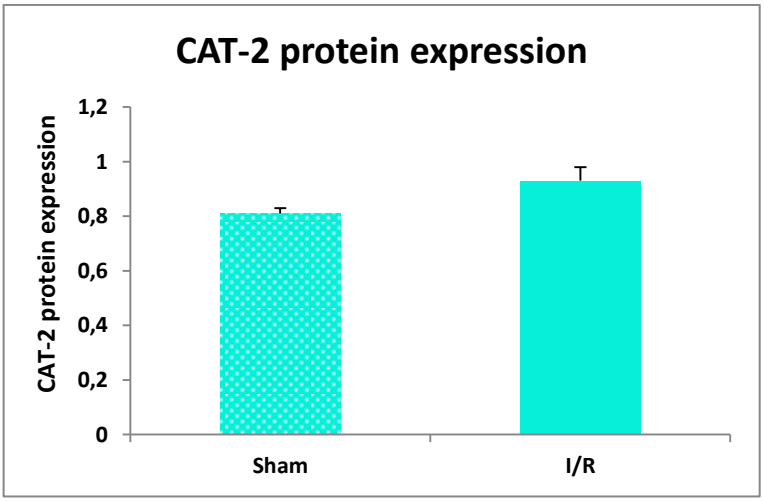


Figure 52. DDAH-1 activity evaluation. Livers were subjected to 30 or 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. A significant decrease in DDAH-1 activity was detected in I/R groups, compared with sham rats, both after 30 and 60 minutes of ischemia. The significant **p values* are indicated in the graph. The results are reported as the mean ± S.E. of 8 different experiments.

9.6 Cationic Amino-acid Transporters-2, CAT-2 protein expression

In order to evaluate any significant changes in ADMA transport after I/R injury, CAT-2 protein expression was performed by means of direct ELISA kit. No changes were recorded in CAT-2 protein expression after I/R injury, compared to sham-operated rats (Figure 53).

Figure 53. CAT-2 protein expression evaluation. Livers were subjected to 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. No changes were detected in I/R group, compared with sham rats. The results are reported as the mean ± S.E. of 8 different experiments.



9.7 Oxidative stress markers: TBARS, ROS, GSH/GSSG

Since it was noticed a marked decrease in the activity of DDAH-1, that it is highly sensitive to reactive species, we evaluated three different markers of oxidative stress. As reported in the table below, no significant changes were found in GSH/GSSG ratio, as well as in TBARS and ROS hepatic content (Table 6).

Oxidative stress markers	30' ischemia & 60' reperfusion		60' ischemia & 60' reperfusion	
	Sham	I/R	Sham	I/R
GSH/GSSG	9.1 ± 0.9	8.1 ± 0.5	9.3 ± 1.3	8.3 ± 0.7
TBARS (nmol/mg liver)	1.9 ± 0.3	1.8 ± 0.5	2.3 ± 0.4	2.4 ± 0.9
ROS (arbitrary unit)	1849 ± 39	1982 ± 71	2019 ± 34	2055 ± 56

Table 6. Oxidative stress markers evaluation. Livers were subjected to 30 or 60 minutes of ischemia, followed by 60 minutes of reperfusion. Sham-operated control rats had similar manipulation, without vascular occlusion. No significant changes were found in GSH/GSSG ratio, as well as in TBARS and ROS hepatic content. The results are reported as the mean ± S.E. of 8 different experiments.

Part IIIb: Pharmacological treatment in I/R *in vivo* model

10.1 Transaminases & biochemical parameters

Serum levels of AST, ALT, AP, total and direct bilirubin have been used as indicators of liver injury. Samples were collected at the end of reperfusion period in our *in vivo* model of Ischemia/Reperfusion (I/R) injury. Rats were orally administrated with 10 mg/Kg *per* day Obeticholic acid (OCA), or vehicle alone (methylcellulose 1%).

No changes in serum AST, ALT, AP, total and direct bilirubin levels were observed comparing OCA-treated sham rats with vehicle-treated sham group (Table 4). OCA administration reduced, although not significantly, the hepatic serum enzyme release in I/R group (Table 4). The same trend was found for the serum levels of total bilirubin, while a significant decrease was detected for the direct bilirubin in the I/R group treated with OCA as compared with vehicle-treated I/R animals (Table 7).

Biochemical Parameters	Sham	Sham + OCA	I/R	I/R + OCA
AST (mU/ml)	243 ± 57	141 ± 23	3572 ± 906	2457 ± 162
ALT (mU/ml)	66 ± 17	47 ± 7	3785 ± 802	2733 ± 169
AP (mU/ml)	410 ± 46	379 ± 34	582 ± 50	620 ± 62
Total Bilirubin (mU/ml)	0.14 ± 0.018	0.10 ± 0.004	0.25 ± 0.05	0.201 ± 0.067
Direct Bilirubin (mU/ml)	0.045 ± 0.014	0.033 ± 0.002	0.17 ± 0.03	0.082 ± 0.015*

Table 7. Serum enzymes and total and direct bilirubin levels in I/R and Sham OCA-treated rats *vs* their respective control groups. Serum parameters, like transaminases and bilirubin, were evaluated. Lower level of them was observed in OCA-treated rats, compared with vehicle *(*p<0.05 vs* I/R). The results are reported as the mean ± S.E. of 7-8 different experiments.

10.2 Farnesoid X Receptor, FXR mRNA expression

In order to obtain this part of our results, we performed the experiments in presence or absence of Obeticholic Acid (OCA), a Farnesoid X Receptor (FXR) agonist, and we assessed the FXR mRNA expression in our *in vivo* model of Ischemia/Reperfusion (I/R) injury. As it is possible to observe in the chart below, there was a significant decrease in FXR mRNA expression only in sham OCA-treated group, compared with sham one. No differences were detected between I/R groups, with or without OCA treatment (Figure 54).

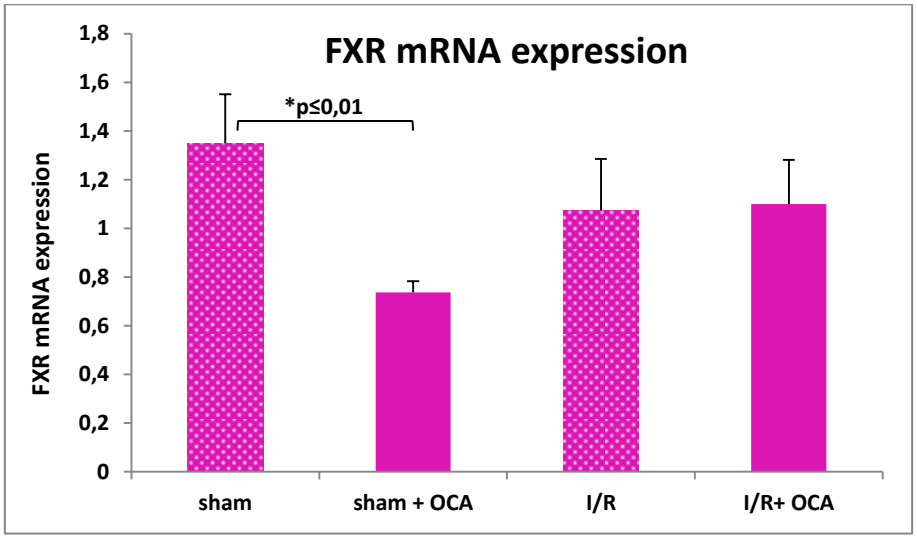


Figure 54. FXR mRNA expression evaluation. The pharmacologically administration of obeticholic acid induced a significant decrease (**p≤0.01*) only in sham OCA-treated rats, compared with sham ones. No changes in I/R groups, with or without OCA, were found. The results are reported as the mean ± S.E. of 7-8 different experiments.

10.3 Biliary, serum & tissue ADMA levels

ADMA levels in bile, serum and tissue were evaluated by means of the ELISA kit (Immundiagnostik, AG Germany).

After OCA or vehicle alone (methylcellulose 1%) administration, biliary ADMA levels, in both OCA-treated groups, increased significantly compared with their respectively untreated groups. An increase in biliary ADMA levels was observed also in I/R group, compared with the sham one, as reported in the panel in Figure 55.

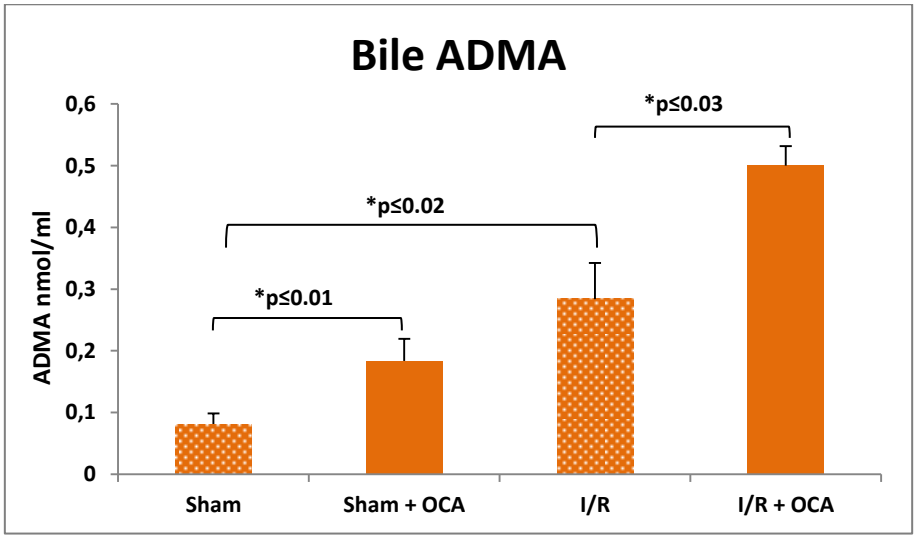


Figure 55. Biliary ADMA levels evaluation. OCA administration induced a significant increase in biliary ADMA levels, both in sham and I/R groups. An increase in biliary ADMA levels was also observed in I/R group, compared with the sham one. The significant **p values* are indicated in the graph. The results are reported as the mean ± S.E. of 7-8 different experiments.

Sham rats treated with OCA showed a marked increase in serum ADMA levels compared with vehicle-treated rats. As evidenced in Figure 56, no differences in serum ADMA were detected comparing the I/R groups, with or without OCA treatment. Moreover, a significant increase in serum ADMA levels was observed in I/R group, compared with the sham one (Figure 56).

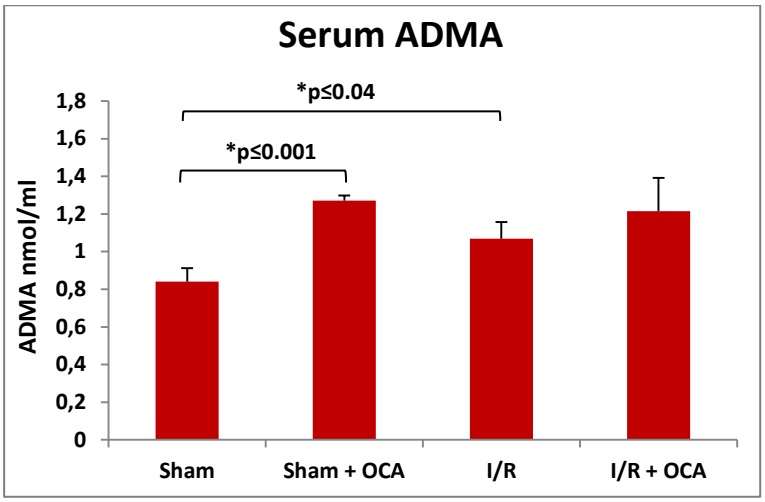
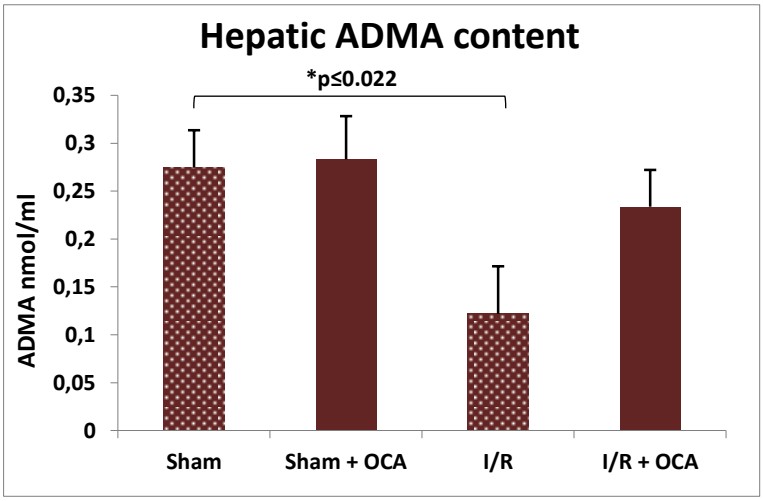


Figure 56. Serum ADMA levels evaluation. A marked increase in serum ADMA levels was found in sham rats treated with OCA, compared with vehicle-treated rats. No differences in serum ADMA were detected comparing the I/R groups, with or without OCA treatment. Moreover, a significant increase in serum ADMA levels was observed in I/R group, compared with the sham one. The significant **p values* are indicated in the graph. The results are reported as the mean ± S.E. of 7-8 different experiments.

No differences in hepatic ADMA content were found in vehicle and OCA-treated sham groups. An increase, although not significant, in hepatic ADMA, was detected in OCA-treated I/R group as compared with vehicle-treated I/R rats. We can observe a significant decrease of hepatic ADMA levels in I/R group, compared with the sham one (Figure 57).

Figure 57. Tissue ADMA levels evaluation. No changes in tissue ADMA content was found in vehicle and OCA-treated sham groups. A significant reduction in hepatic ADMA content was detected in I/R rats compared with control group (**p≤0.022*). An increase, although not significant, in hepatic ADMA was detected in OCA-treated I/R group, when compared with vehicle-treated I/R rats. The results are reported as the mean ± S.E. of 7-8 different experiments.



10.4 PRMT-1 mRNA expression

PRMT-1 is the enzyme able to synthetize ADMA, starting from arginine. The PRMT-1 mRNA expression was evaluated after 5 days of OCA treatment: significantly hepatic lower expression was found in OCA sham rats compared with vehicle-treated rats. A significant decrease was observed comparing PRMT-1 mRNA expression in sham *versus* I/R groups, both in presence or absence of OCA treatment (Figure 58).

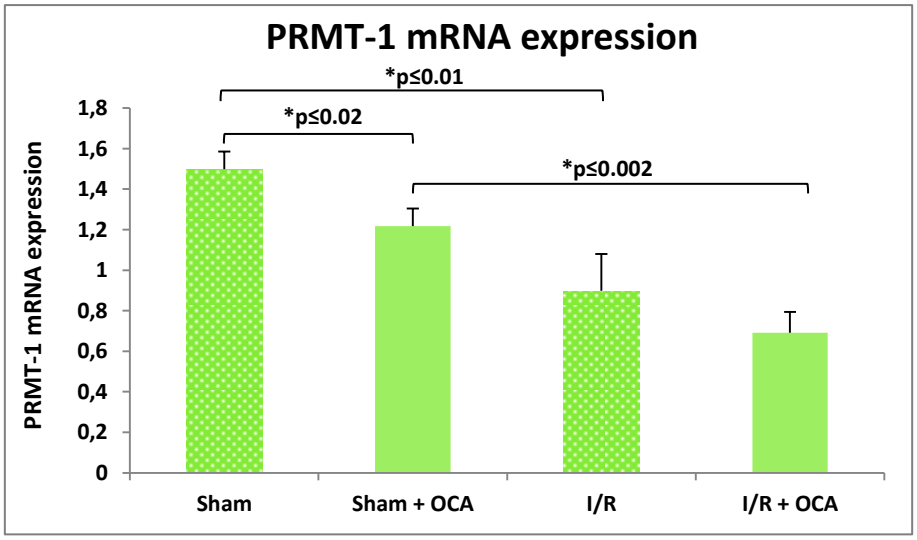


Figure 58. PRMT-1 mRNA expression evaluation. A significant decrease in PRMT-1 mRNA expression was found in sham rats treated with OCA, compared with vehicle-treated rats. A significant decrease was observed comparing PRMT-1 mRNA expression in sham *versus* I/R groups, both in presence or absence of OCA treatment. The significant **p values* are indicated in the chart. The results are reported as the mean ± S.E. of 7-8 different experiments.

10.5 DDAH mRNA expression & activity

The evaluation of mRNA expression and activity of DDAH showed that no changes were detectable after 5 days of OCA administration both in sham and I/R rats.

Comparing DDAH-1 mRNA expression, a significant decrease was found in sham *versus* I/R groups both, in presence or absence of OCA treatment (Figure 59).

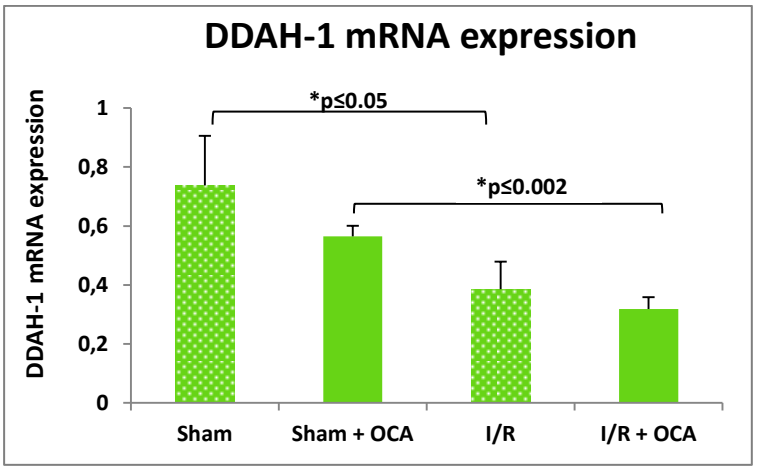
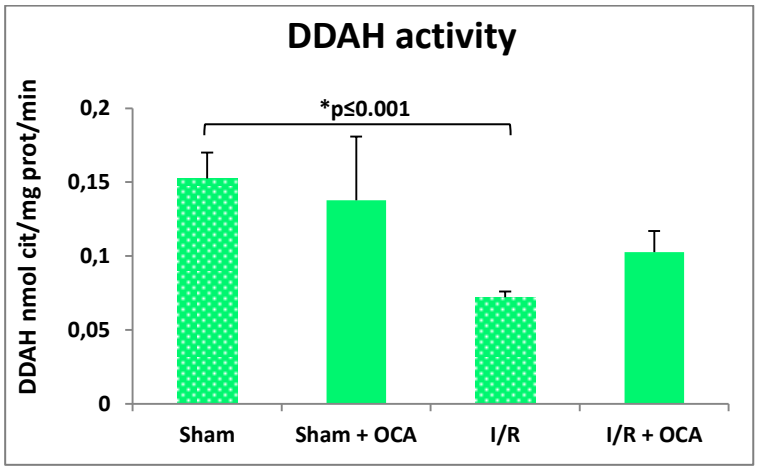


Figure 59. DDAH-1 mRNA expression evaluation. A decrease in mRNA expression in DDAH-1 was detectable comparing I/R groups with sham rats, both in presence or absence of OCA treatment. The significant **p values* are indicated in the chart. The results are reported as the mean ± S.E. of 7-8 different experiments.

No differences were found in sham groups with or without OCA treatment. A decrease in DDAH activity occurred in I/R groups in presence or absence of OCA administration, when compared with the sham groups. The DDAH activity decreased significantly in I/R group when compared with the sham one. Although not significant, an increase in DDAH activity was found in the I/R group treated with OCA compared with I/R rats (Figure 60).

Figure 60. DDAH-1 activity evaluation. No differences were found in sham control groups with or without OCA. A total decrease in DDAH activity was found in I/R groups. The DDAH activity decreased significantly in I/R group (**p≤0.001*), when compared with the sham one. The results are reported as the mean ± S.E. of 7-8 different experiments.



10.6 Cationic Amino-acid Transporters, CATs mRNA expression

10.6.1 CAT-1

ADMA can be released in extracellular environment by CATs, involved also in the removal of circulating ADMA by the liver. In our *in vivo* experimental model of I/R injury, a significant decrease in CAT-1 expression was evident in sham rats treated with OCA, when compared with vehicle-treated rats. No difference in CAT-1 was detected comparing the I/R groups with or without OCA treatment. In I/R group, CAT-1 mRNA expression was significantly lower than in sham group (Figure 61).

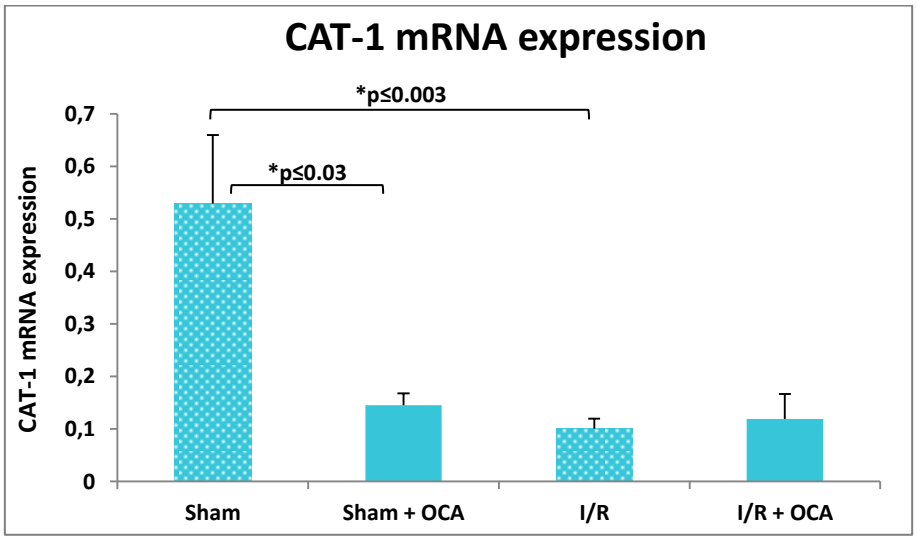


Figure 61. CAT-1 mRNA expression evaluation. A marked decrease in CAT-1 expression was found in sham rats treated with OCA compared with vehicle-treated rats (*$*p \leq 0.03$*). As evidenced here, OCA did not induce differences in CAT-1 expression in I/R groups with or without OCA treatment. In I/R group, CAT-1 mRNA expression is significantly lower (*$*p \leq 0.003$*) than sham group. The results are reported as the mean ± S.E. of 7-8 different experiments.

10.6.2 CAT-2A & CAT-2B

A significant reduction in CAT-2A mRNA expression was detected in sham group after 5 day-OCA treatment. A decrease in CAT-2A occurred in I/R groups both in presence or absence of OCA administration. Reduced CAT-2A expression was found in I/R OCA-treated group as compared with vehicle-treated rats (sham + OCA) (Figure 62).

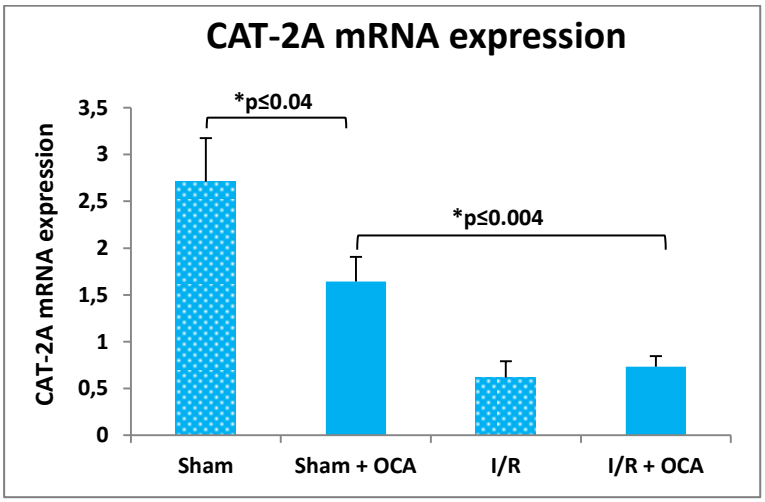
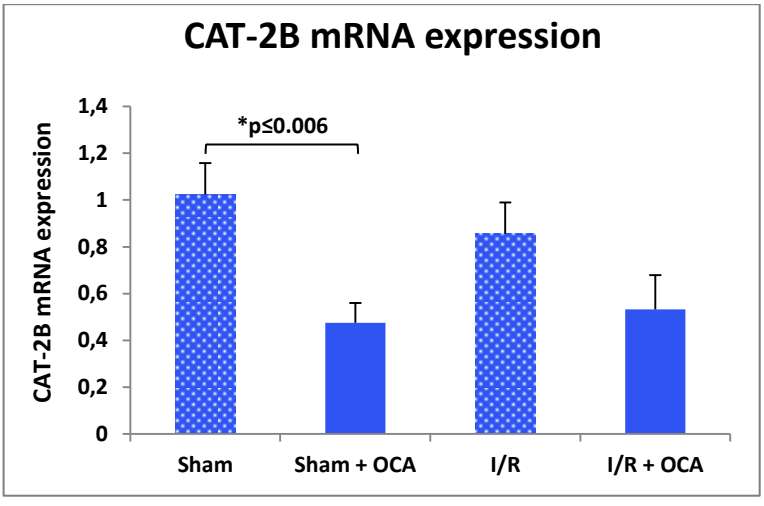


Figure 62. CAT-2A mRNA expression evaluation. A decrease in CAT-2A mRNA expression was found in OCA-treated sham rats compared with vehicle-treated rats. I/R groups, with or without OCA treatment, showed a marked decrease in CAT-2A mRNA expression when compared with sham groups. A decreased CAT-2A expression was found in I/R OCA-treated group as compared with vehicle-treated rats. The significant **p values* are indicated in the panel. The results are reported as the mean ± S.E. of 7-8 different experiments.

The same trend was detected for CAT-2B mRNA expression. CAT-2B decreased in OCA-treated sham rats, compared with vehicle. Lower CAT-2B mRNA expression, although not significant, was evaluated in I/R group treated with OCA when compared with vehicle-treated I/R rats (Figure 63).

Figure 63. CAT-2B mRNA expression evaluation. A decrease in CAT-2B mRNA expression was found in sham rats treated with OCA (**p≤0.006*) compared with vehicle-treated rats. A decrease, even if not significant, in CAT-2B mRNA expression was detected in I/R group treated with OCA, when compared with vehicle-treated I/R rats. The results are reported as the mean ± S.E. of 7-8 different experiments.



10.7 Organic Cation Transporter 1, OCT-1 mRNA expression

The most relevant carrier of organic cation transporter family is OCT-1, which can carry in or out hepatocytes some positively charged endogenous compounds, as well as ADMA and drugs (Strobel et al. 2013). The evaluation of OCT-1 mRNA expression showed that no changes occurred comparing the sham groups with or without OCA. A significant increase in OCT-1 mRNA expression was detected in I/R group compared with the sham one. Moreover, this significant increase in I/R group was completely restored to the levels observed in the sham group after OCA administration (Figure 64).

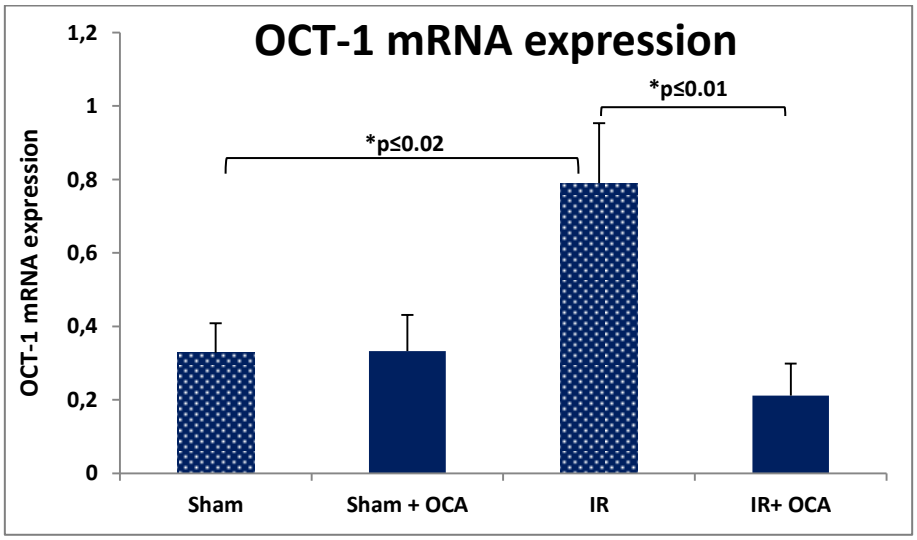


Figure 64. OCT-1 mRNA expression evaluation. No changes in OCT-1 mRNA expression occurred in sham rats treated with OCA compared with vehicle-treated rats. A significant increase in OCT-1 mRNA expression was detected in I/R group compared with the sham one. The significant increase in OCT-1 mRNA expression obtained in I/R rats was restored to the levels of sham groups after OCA administration. The significant **p values* are indicated in the chart. The results are reported as the mean ± S.E. of 7-8 different experiments.

10.8 Multidrug & Toxin Extrusion Transport 1, MATE-1 mRNA expression

MATE-1 is placed in the apical membrane of renal tubule cells and bile *canaliculi* (Otsuka et al. 2005). It performs its function especially as efflux protein and it shares several substrates with OCT-1, particularly those ones showing a similar structure to L-Arginine and ADMA.

A significant upregulation of MATE-1 mRNA expression was found both in sham and I/R groups treated with OCA, compared with the respectively vehicle-treated groups. No increase in MATE-1 mRNA expression occurred in I/R animals *versus* sham group (Figure 65).

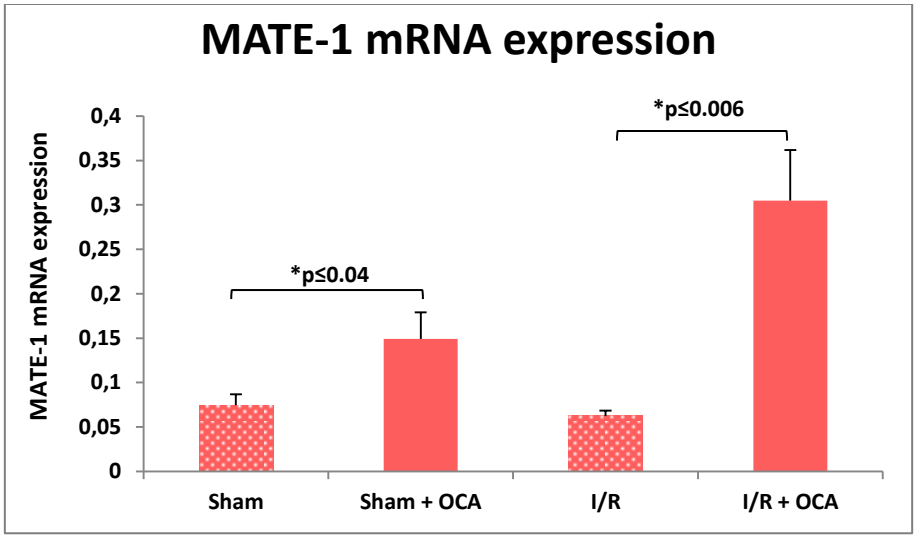


Figure 65. MATE-1 mRNA expression evaluation. Upregulation of MATE-1 mRNA expression was detectable both in sham and I/R groups treated with OCA, compared with the respectively vehicle-treated groups (*$p \leq 0.04$* and *$p \leq 0.006$* respectively). No increase in MATE-1 mRNA expression occurred in I/R animals *versus* sham group. The results are reported as the mean ± S.E. of 7-8 different experiments.

10.9 eNOS & iNOS protein expression

The eNOS and iNOS protein expression was quantified using Western Blot analysis. The treatment with OCA in I/R group induced a significant increase in eNOS protein expression compared with the I/R group. No significant changes occurred comparing sham *versus* sham treated with OCA (Figure 66).

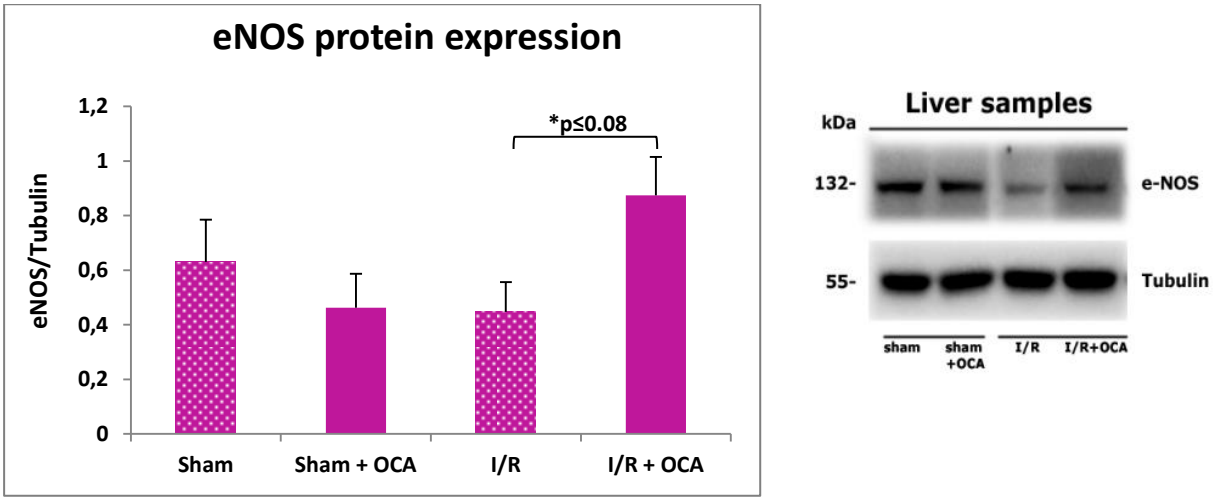


Figure 66. Western Blot analysis of eNOS protein expression. The figure shows immune-reactive bands and their quantification. No changes in eNOS expression were detected comparing sham rats with sham treated with OCA rats. A significant increase in eNOS expression (**p≤0.08*) was found in I/R rats treated with OCA, compared with vehicle-treated rats. The results are reported as the mean ± S.E. of 7-8 different experiments.

The I/R injury induced a significant increase in iNOS expression in I/R group compared with the sham one. The OCA treatment decreased the iNOS expression after I/R as compared with I/R vehicle-treated animals (Figure 67).

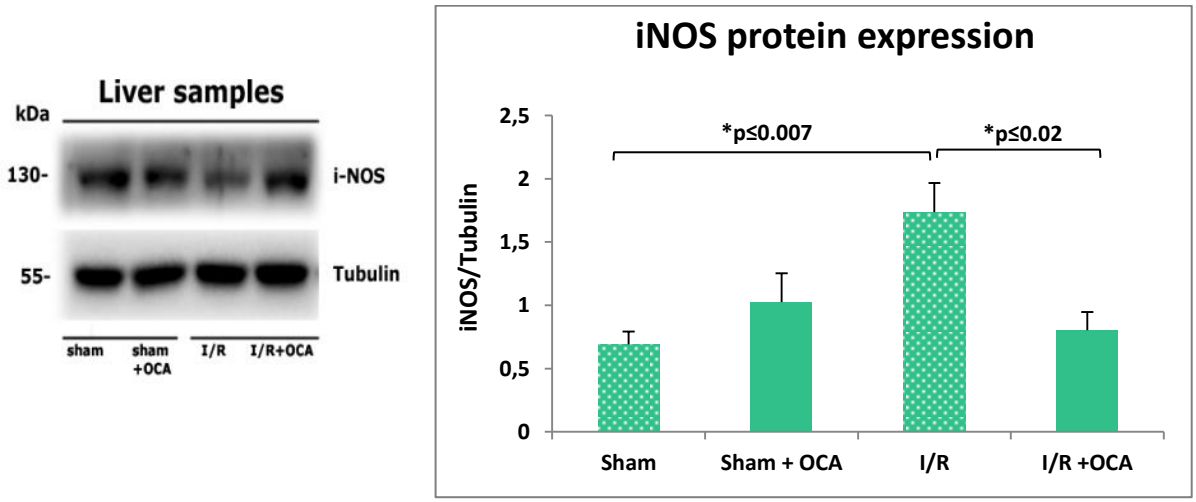


Figure 67. Western Blot analysis of eNOS protein expression. The figure shows immune-reactive bands and their quantification. I/R injury induced a significant increase in iNOS expression comparing I/R rats with sham group (**p≤0.007*). The OCA administration in I/R rats induced a significant decrease in iNOS expression (**p≤0.02*). The results are reported as the mean ± S.E. of 7-8 different experiments.

Discussion

All three isoforms of Nitric Oxide Synthase (NOS) can be inhibited by a naturally-produced amino acid, known as Asymmetric Dimethylarginine (ADMA), which competes with arginine for NOS active site, decreasing the synthesis of NO (Vallance et al. 1992). The ADMA-mediated regulation of NO production is determined by the intracellular and extracellular ADMA. ADMA is synthesized by Protein Arginine Methyltransferases (PRMTs) and it is metabolized to citrulline and dimethylamine, by Dimethylarginine Dimethylaminohydrolase (DDAH).

ADMA is partially excreted by the kidney, but the most important way of ADMA elimination is by metabolism, in particular by hepatic isoform of DDAH (Teerlink 2005). Accordingly, increased ADMA concentration is related to the degree of hepatic dysfunction in patients suffering from liver diseases, with varying aetiologies (Ferrigno, Laura G Di Pasqua, et al. 2015).

11.1 ADMA in NAFLD/NASH

We have documented, for the first time, that a diet deprived of methionine and choline induced a significant decrease in serum ADMA levels after 4 weeks and mainly after 8 weeks of treatment. This result was accompanied by an increase in serum NOx, detected in 8-week MCD fed rats. Noteworthy, literature reported that monkeys fed with an enriched methionine diet, displayed elevated ADMA plasma levels. Similar data, about high concentration of ADMA in serum, were also found in pigs given a diet supplemented with methionine (Kusku-Kiraz et al. 2015). Since, it was hypothesized that methyl groups of ADMA may be derived from the demethylation of methionine to homocyst(e)ine (Böger et al. 2000), this is in accordance to what we found in our study on which animals fed by a methionine deficient diet, where the absence of this amino acid probably caused a decrease in available methyl groups. In addition, the absence of choline, too, should be considered. Since choline is an essential vitamin in metabolic reactions, low dietary choline reduced methionine formation, leading to a marked increase in *S*-Adenosylmethionine (SAM) utilization in the liver and making it a significant factor in the low serum levels of asymmetric dimethylarginine (Kucera & Cervinkova 2014).

We reported also that MCD diet administration induces a manifest decrease in serum levels of glucose, cholesterol and triglycerides, accordingly with previous published data (Mas et al. 2013). Interestingly, a positive correlation between glucose and ADMA levels was found. It is important to note that the opposite can also occur, as in diabetes: diabetic rats displayed

increased serum levels of glucose, cholesterol and triglycerides accompanied by high ADMA levels (Leng et al. 2014).

Moreover, PRMT-1 mRNA and protein expression, one of the enzymes involved in ADMA synthesis, increased in MCD animals, as a possible hepatic response to the decrease in serum or tissue ADMA levels, shown in our nutritional model of NAFLD/NASH. Similarly, in another model of diet-induced NAFLD, the hepatic levels of the PRMT-1 protein expression raised significantly in 52-week High-Fat High-Cholesterol (HFHC) diet fed mice (Pacana et al. 2015). The hepatic ADMA depletion recorded in our study after only 4 weeks of MCD diet, also occurred in mice after a 52-week HFHC diet; differently from this work, in the HFCF model, plasma levels of ADMA were significantly higher than in controls, and this occurrence was associated with a concomitant partial reduction in hepatic methionine (30% less than in controls) (Pacana et al. 2015).

For what concerns DDAH-1, the enzyme that metabolizes ADMA, a significant increase has been observed in its mRNA expression after 8 weeks of diet, suggesting, at first sight, that ADMA decrease could be due to its greater degradation. Unfortunately, despite the trend displayed for mRNA, there were no changes in DDAH-1 protein expression and its activity was even significantly reduced in rats subjected to MCD diet, already after 4 weeks. It is well known that DDAH is an oxidant-sensitive enzyme, and in MCD rats we observed elevated levels of ROS and TBARS, concomitant to low GSH content, suggesting that the oxidative stress increase could have affected its catalytic site, probably inducing protein degradation (Aiken et al. 2011; Y.-L. Tain, Huang, et al. 2010). Subsequently, to investigate whether changes in ADMA levels were due to modifications in its transport, Cationic Amino-acid Transporters (CATs) were taken into account. ADMA can be released in the extracellular space and, at the same time, liver and kidneys can remove it from circulation by means of these transporters (Teerlink 2005). It has been reported, that blood ADMA content decreased after the administration of a Farnesoid X Receptor (FXR) agonist in mice, probably, because of the upregulation of CAT-1 (Magné et al. 2015). Similarly, in our study we observed an increase in CAT-1 mRNA expression, concomitantly with a decrease in serum ADMA, but no changes in CAT-1 protein were detected in MCD rats, when compared with controls. This occurrence is probably due to the extensive rate of oxidative stress that can affect protein stability, as previously suggested for DDAH-1. On the other hand, CAT-2A and CAT-2B mRNA, expressed particularly in the liver (Teerlink et al. 2009), decreased after 4 week-MCD diet, but an increase in CAT-2 protein content was detected, indicating that, in NASH, CAT-2

undergoes post-transcriptional modifications as predominant regulation mechanism. In fact, this kind of regulation has been elucidated previously for other hepatic transporters in a model of obstructive cholestasis, where a progressive significant increase in MRP4 protein occurred, without changes in MRP2 mRNA (Denk, Soroka, Takeyama, et al. 2004; J. Lee et al. 2001). Significantly, multiple changes in ADMA transporters have been detected in the MCD dietary model of NASH (Teerlink 2005). This is of particular interest because ADMA contributes to a wide range of pathologies, probably because both low and high concentrations of this dimethylarginine markedly modify the expression gene profile. In fact, relatively small changes in ADMA content are sufficient to affect gene expression in human coronary endothelial cells (Smith et al. 2003), so studying how changes in ADMA/DDAH/NOS pathway can play a role in the onset and progression of NAFLD/NASH could be worthy of further investigation.

11.2 ADMA in obstructive cholestasis

While serum ADMA levels decreased in a steatotic animal model, in a cholestatic model of 2-week bile duct ligation (BDL), an increase in serum ADMA content has been found (Chang et al. 2013; Yang et al. 2012). However, in our 72-hour BDL model no increase in serum ADMA concentration was observed, likely due to the short period of occlusion: before obtaining an increase in serum, a hepatic increase of ADMA had to occur in the days immediately after BDL, as described in this study. Moreover, this is the first evidence documenting that ADMA is differently distributed in the three lobes: in fact, it is significantly higher in the Right (RL) and Median Lobes (ML) in comparison with the Left Lobe (LL). It is known that in Wistar rats, the right and the caudate lobes perform a different functionally role respect to the median and the left lobes, especially in terms of protein synthesis (Garcia-Moreno et al. 1994). On the other hand, it is also recognized that the vascular tree is divided into 3-dimensional unit in each lobe (Teutsch et al. 1999). Besides, the left lobe has only one primary portal branch, whereas the median lobe appears to have two portal branches (DUCHEN 1961). Many published data support the present finding on the different hepatic lobe functionality in response to a pathological situation, such as obstructive cholestasis. To explain the raised hepatic ADMA content in RL and ML, we evaluated the lobe heterogeneity of the enzymes involved in the synthesis and degradation of ADMA, PRMT-1 and DDAH-1, respectively. A significant increase in PRMT-1 mRNA expression was observed in BDL group, compared with sham-operated animals, while no changes were detected in PRMT-1

mRNA expression among the three BDL lobes. As in our MCD model, also in this case, we detected an increase in DDAH-1 mRNA expression in BDL rats, compared with control ones, but DDAH activity showed no changes between the above mentioned groups. Probably, also in this case, as previously reported for NASH model, the failure in DDAH activity is attributable to the high oxidative stress, as confirmed by high levels of thiobarbituric acid reactive species found in BDL rats, in each lobe, when compared whit sham-control group. High level of damage was also confirmed by increased release of transaminases in the BDL rats serum, respect to sham ones. Since we could not attribute tissue ADMA heterogeneity to a different level of DDAH activity, different ADMA distribution was likely associated with a decreased mRNA expression of CAT-2 transporters. The downregulation of CAT-2 was particularly evident in the right and median lobes, when compared with their respective sham-operated group and to the left lobe. Previous works demonstrated that in rat livers subjected to obstructive cholestasis, a downregulation of cationic transporters took place, already after 3 days of BDL (Denk, Soroka, Mennone, et al. 2004). In addition, a decrease in CAT-2 transporters related to an increase in tissue ADMA was also elucidated in a 2-week BDL model (Chang et al. 2013). As further confirmation, Nies and colleagues recently reported that, in Caucasian suffering from cholestasis, the hepatic expression of the cationic drug uptake transporters was significantly affected (Nies et al. 2009). Thus, in the BDL model, we confirmed the reduction in cationic transporters expression and we also documented that, immediately after the bile duct ligation, this event occurred in a lobe-specific manner. The mechanism involved in this inter-lobe variation is largely undiscovered yet, although factors such as portal streamlining of blood to the liver (DUCHEN 1961) and differences in the metabolic capacity of each lobe have been proposed for elucidating the heterogeneous liver lobe response (Lawson & Pound 1974).

11.3 ADMA in Ischemia/Reperfusion injury

By means of an *in vivo* model of Ischemia/Reperfusion (I/R) injury we documented, for the first time, that ADMA was significantly excreted by bile and a time-dependent increase was observed after 30 and 60 minutes of ischemia. Moreover, ischemia/reperfusion injury induced changes in biliary, serum and hepatic levels of ADMA, affecting *per se* the enzymes involved in ADMA synthesis and metabolism. Thus, this finding supports the hypothesis that ADMA/DDAH/NOS pathway may play a central role in acute hepatic I/R injury (Ferrigno et al. 2014).

Our data about biliary excretion of ADMA are useful to explain previous Laleman's *et al.* published data: they observed a 50%-increase in circulating ADMA, accompanied by a decrease in ADMA removal rate, in a Bile Duct Ligation (BDL) rat-model. Contrarily, Thioacetamide-(TTA)-induced cirrhosis did not affect neither the circulating ADMA, nor the ADMA removal rate (Laleman et al. 2005).

In addition, our results demonstrated that in a *in vivo* I/R model, following a short BDL period, a significant increase in intracellular content of ADMA took place, respect to animals undergoing simple I/R without BDL (Ferrigno et al. 2014). Furthermore, patients suffering from alcoholic cirrhosis displayed high plasma ADMA levels, strictly related with high plasma bilirubin levels (Lluch et al. 2004). This reported result, accompanied by our results, suggested that a possible link between biliary excretion and ADMA plasma concentration exists.

With regards to the increased plasma ADMA content, many data demonstrated that in patients affected by several diseases, such as liver cirrhosis (Lluch et al. 2004), alcoholic hepatitis (Mookerjee, Malaki, et al. 2007) and acute liver failure, ADMA concentration is significantly increased in plasma (Mookerjee, Dalton, et al. 2007), as well as in the first postoperative day after cardiopulmonary bypass due to the extensive ischemia/reperfusion damage, suggesting the use of ADMA as a reliable and feasible marker of early I/R injury (Siroen et al. 2004). Confirming also the previous data reported by Trocha *et al.* (Trocha et al. 2010), we observed higher serum ADMA levels after 60 minutes of ischemia, when compared with control livers. At the same time, the hepatic ADMA content decreased significantly in the ischemic group, in comparison with the sham-operated one.

ADMA is already considered a marker for graft rejection, in fact, it is present at high concentration in serum of patients rejecting the liver graft (Brenner et al. 2012). However, the increase of biliary excretion of ADMA, occurring already after 30 minutes of ischemia and preceding even its increased appearance in serum, could be considered a very early and new marker of dysfunction of liver graft in post-transplantation period (Ferrigno et al. 2014).

At the best of our knowledge, ADMA, being a potent inhibitor of all isoforms of NOS, plays a crucial role during ischemia/reperfusion injury. So, any agent able to affect ADMA/DDAH/NOS pathway in order to reduce the ischemic damage, further worsened by ADMA-mediated inhibition of eNOS, could be a strategy to prevent and treat this kind of occurrence.

Thus, once obtained these innovative results, our next goal was to try to understand the role of Farnesoid X Receptor (FXR) in excretion of ADMA. Hence, we conducted further investigations using the Obeticholic Acid (OCA), an FXR agonist, in our model of ischemia/reperfusion injury. In fact, recent data demonstrated that, under hypoxic conditions, the farnesoid X receptor could regulate the expression of various genes, among which those ones strictly related to ADMA metabolism and transport, such as DDAH-1 and CAT-1. The sequencing of DDAH-1 gene revealed the presence of an FXR Response Element (FXRE) in its promoter, and a dose-dependent response to the FXR agonist GW4064, in terms of DDAH-1 gene expression, has been demonstrated (Fujino et al. 2009). On the other hand, the pharmacological activation of FXR seems to downregulate CAT-1 transporter gene expression (Hyrsova et al. 2016).
We performed our experiments using the same *in vivo* model of I/R injury in rats, following 5-day OCA (10 mg/Kg/day) treatment.
Afterwards, our results documented, for the first time, that the pharmacological administration of OCA, further increases, in a significant manner the biliary excretion of ADMA, during hepatic ischemia/reperfusion injury.

To clarify the molecular mechanism of this finding, we investigated the involvement of ADMA transporters, such as Multidrug and Toxin Extrusion Transport 1 (MATE-1) and Organic Cation Transporter 1 (OCT-1). It is known that MATE efflux transporters are proton-coupled antiporters, mostly expressed in the liver and kidney and positioned at apical membranes of renal tubular epithelia and bile *canaliculi* (Motohashi & Inui 2013). Since MATE-1 is the main responsible of elimination of organic cations across the apical membrane in the liver, its mRNA increase suggests its involvement in the further promotion of ADMA biliary clearance, detected in animals subjected to ischemia and reperfusion and treated with FXR-agonist. Moreover, we consider MATE-1 the best candidate for ADMA efflux into bile, because this event occurred concomitantly with a downregulation of all other transporters, such as CAT-1, CAT-2A, CAT-2B and OCT-1.
Obeticholic acid is responsible for the increased ADMA elimination by bile also in sham-operated group, representing an FXR-mediated effect, associated with a decreased expression of CAT transporters. Interestingly, the function of MATE-1 is pH environment-dependent (Strobel et al. 2013) and an extracellular acidification improves MATE-1 export activity. Therefore, as it is well known, since pH acidification typically occurs in ischemic tissue, in order to protect from anoxic damage, an increase in MATE-1 expression is an additional

confirmation of our thesis (Currin et al. 1991; Vairetti et al. 2006; Ferrigno, Laura Giuseppina Di Pasqua, et al. 2015). Our results in I/R model could be summarized by the above evidences: the increased ADMA excretion by bile, that occurred in the I/R group, was attributable to MATE-1 and may represent an adaptive mechanism to limit the dangerous ADMA accumulation in the liver. Since high levels of ADMA could be detrimental for the mitochondrial function (Chen et al. 2011), it is reasonable to suppose that the liver tried to protect mitochondria already damaged by ischemia. Previously, elevated endogenous ADMA was found to contribute to hepatic mitochondrial dysfunction in diabetic rats and, in 2016, it has been evidenced that ADMA could also be imported into mitochondria by Solute Carrier family 25 member 2 (SLC25A2) transporters (Porcelli et al. 2016).

In this study, the OCA treatment induced an impairment in the ADMA exchange by decreasing its transporters, CATs and OCT-1, and these events could clarify the additional increase in biliary ADMA observed in the I/R animals treated by FXR agonist. In addition, all CATs isoforms decreased in the sham group treated with OCA: in particular, we observed a downregulation of the CAT-2B isoform, a low capacity transporter, that has an high affinity for cationic amino acids and presents a marked affinity for ADMA (Closs, Gräf, et al. 1997). In contrast, CAT-2A, the alternative splice variant of CAT-2B, that displays low affinity but high transport capacity, was not reduced by OCA administration. In I/R, we found that the OCA administration had no effect on these two transporters, that were already decreased by ischemia. It has been reported that high dose administration of FXR agonist GW4064 induced an increase in CAT-1 expression and a decrease in plasma levels of ADMA (Li et al. 2009). Probably, we saw no changes in CAT-1 expression because we used a lower dose of obeticholic acid, compared with the dose of a similar FXR agonist used in Li *et al.* 2009.

Of note our results about OCT-1: its expression increased in our model of hepatic I/R. The involvement of OCT-1, belonging to the Solute Carrier family (SCL22A1), in the ADMA import/export, has been recently established (Strobel et al. 2013). In humans, OCT-1 is largely found in the hepatic tissue and it is localised within the hepatocytes and, at lesser extent, in the cholangiocytes (Motohashi & Inui 2013). Our results documented that OCA administration induced a significant decrease in OCT-1 mRNA expression in ischemic rats; conversely, no changes were detected in the sham group treated with or without OCA. The OCT-mediated transport is categorized as facilitate diffusion: OCTs translocate the substrate in both direction across the membrane and function as electrogenic uniporters of organic cations (Hyrsova et al. 2016). Bile acid-mediated FXR activation seems to be linked with cell-

specific membrane transporters expression (Mazuy et al. 2015). Of note, previously data demonstrated that the cholic acid treatment led to a marked decrease in OCT-1 expression, in wild-type mouse livers (Maeda et al. 2004). Since OCTs typically support basolateral organic cations entry, while MATEs support apical organic cations efflux (Pelis & Wright 2014), it was supposed that in the liver, OCT-1 works in concert with MATE-1 to mediate, respectively, the hepatic uptake and biliary excretion of cationic drugs and their metabolites (Nies et al. 2011).

Recently, it has been demonstrated that FXR activation by OCA can lead to DDAH-1 expression increase, in a dose dependent manner in HepG2 cells (Mookerjee et al. 2015). Moreover, administration of high-salt diet and obeticholic acid in rats, at 10 or 30 mg/Kg/day for 6 weeks, makes DDAH-1 protein expression higher than in control animals (Ghebremariam et al. 2013). In addition, in 2015, Mookerjee *et al.* described that, in a model of BDL-induced cirrhosis, the administration of OCA (5 mg/Kg for 5 days) led to an increase in DDAH-1 protein (Mookerjee et al. 2015). Using a model of cirrhosis induced by BDL, an increase in serum ADMA takes place (Laleman et al. 2005), while our results show that during I/R, the regular biliary excretion allows ADMA elimination. We reported that OCA did not affect DDAH-1 expression and activity and a possible explanation of our data could be provided by the comparison of the two experimental models, BDL and I/R, in which different mechanisms control ADMA elimination: only in the I/R model the ADMA can be excreted by bile and different ADMA clearance is associated with different effect on DDAH-1 expression. Not surprisingly, the inability to eliminate ADMA by bile produces the compensatory effect of increasing the expression of DDAH-1 in BDL rats, on the contrary it is diminished in I/R model.

It is known that ADMA inhibits all isoforms of nitric oxide synthase and competes with arginine for its transport. When ADMA levels increase, nitric oxide is not produced anymore, causing several complications to the systemic circulation. An important role in the vascular smooth muscle cells (Bishop-Bailey et al. 2004) and endothelial cells (He et al. 2006) eNOS regulation is played by FXR activation (Li et al. 2008). Recently, it has been demonstrated that, treating cirrhotic animals with OCA, an increase in eNOS activity appeared (Mookerjee et al. 2015). Moreover, FXR activation mediated by the physiological ligand Chenodeoxycholic Acid (CDCA) in hypertensive rats, upregulated the eNOS expression, reducing blood pressure (Li et al. 2015). Furthermore, endothelial cells treated with

obeticholic acid displayed upregulation of eNOS expression and the enhanced NOS activity results in vasodilation (Zhang et al. 2016).

Our results confirm again the OCA ability in increasing the eNOS protein and, interestingly, they demonstrate a decrease in the inducible isoform of NOS content, in particular during the hepatic I/R injury. Thus, both eNOS and iNOS are involved in the progression of hepatic I/R injury: while eNOS mediates protection, iNOS is involved in pro-inflammatory process. This event explained the decrease in the serum enzyme release in the I/R group treated with OCA. Lastly, recent studies reported that, in patients with Primary Biliary Cirrhosis (PBC), OCA produced a marked decrease in bilirubin, a well known marker of PBC outcomes (Hirschfield et al. 2015). Our results, using an I/R model, showed a similar decrease, although not significant, in total and direct bilirubin.

	MCD MODEL	BDL MODEL	I/R MODEL OCA
Serum ADMA	↓	=	↑
Hepatic ADMA	↓	↑	↓
Biliary ADMA	/	/	↑
PRMT-1 mRNA/protein	↑	↑	↓
DDAH-1 mRNA/protein	↑	↑	↓
DDAH-1 activity	↓	=	↓
CAT-1	↑	/	↓
CAT-2A	↓	/	↓
CAT-2B	↓	/	↓
CAT-2	/	↓	↓
OCT-1	/	/	↓
MATE-1	/	/	↑
iNOS		/	↓
eNOS		/	↑

Table 8. Schematic summery of the results obtained in three different experimental models of liver injury.

Conclusions

ADMA uptake and metabolism are performed by the liver, by means of Cationic Amino-acid Transporters (CATs) and Dimethylarginine Dimethylaminohydrolase (DDAH) activity. The discovery of CATs expression at hepatic level strengthened the theory that the liver plays a critical role in ADMA metabolism (Hattori et al. 1999). In fact, the liver can eliminate ADMA by means of DDAH-1 activity (Ogawa et al. 1989; Kimoto et al. 1993), after catching it from bloodstream, through CATs (Closs, Basha, et al. 1997). Therefore, it seems clear that, since the liver has a key role in ADMA elimination, any hepatic dysfunction may affect the correct metabolism of this dimethylarginine, leading to more complicated consequences.

Non-Alcoholic Fatty Liver Disease (NAFLD) is a widespread pathological condition, which is characterized by relevant intracellular lipid deposition in the hepatic parenchyma. About 10% of patients suffering from NAFLD progresses to Non-Alcoholic Steatohepatitis (NASH), a more serious pathological condition, that involves liver inflammation and apoptotic cell death and, ultimately, it can result in cirrhosis and/or liver failure (Reddy & Rao 2006).
Different therapeutic strategies in NAFLD/NASH have been unsuccessful, but in recent years, a category of drugs, able to reduce hepatic inflammation without associated side effect of weight gain, has become the "holy grail" of NASH therapeutics. The best candidates for NASH treatment are hydrophilic Bile Acids (BAs), which modulate, through the activation of Farnesoid X Receptor (FXR) (Makishima et al. 1999; Wang et al. 1999), both glucose and lipid homeostasis (Lefebvre et al. 2009) and show a significant anti-inflammatory activity (Hollman et al. 2012). Several agonists for the FXR have been produced, although the Obeticholic Acid (OCA) is the first in-class synthetic BA under analysis for NASH treatment (Reddy & Rao 2006).
In our MCD-rat model of NAFLD/NASH, we found that ADMA/DDAH/NOS pathway was affected too, in particular in terms of ADMA producing/metabolizing enzymes and its transporters. Since we know that FXR plays a pivotal role in regulating CAT-1, DDAH-1 and eNOS gene expression, we suggest that the treatment with OCA, that has reached the phase III clinical trial for NASH, could be a feasible strategy to regulate also ADMA handling.

Moreover, in our model of cholestasis, the BDL-rat model, we reported that tissue ADMA content increased significantly after bile duct blockade, and that it occurs in a lobe-specific manner. On the contrary we demonstrated that in our *in vivo* ischemia/reperfusion injury model, tissue ADMA decreased both in ischemic group and in ischemic group following a short BDL period. This finding is explained by biliary excretion of ADMA. In fact, after I/R

injury, we found increased ADMA concentration in bile. This is one of the main results of our work, because biliary ADMA excretion, that increased after I/R injury is a considerable step forward in ADMA/DDAH/NOS pathway knowledge and we suggest that it, coupled with a CATs downregulation, is part of a "selfish" liver protective strategy to reduce detrimental accumulation of ADMA in its own tissue.
Indeed, by the pharmacological administration of OCA in our I/R injury model, we demonstrated that biliary ADMA clearance further increased in I/R group, suggesting that regulation and modulation of its transporters, by the FXR agonist OCA, could have a crucial role in helping the "selfish" liver protection from I/R injury. The two recently identified transporters, in fact, elucidated how transport of ADMA is completed by its cellular uptake by OCT-1 and cellular excretion by MATE-1.

In conclusion, the present study addresses several "hot" topics in liver pathophysiology and it provides noteworthy and original insights into the effect of obeticholic acid in increasing the biliary excretion of ADMA, by upregulating MATE-1 and downregulating OCT-1.
Even though the real consequence of these findings have to be further elucidated, in our work, we also demonstrated that, thanks to an eNOS increase and an iNOS decrease both mediated by OCA treatment, a reduction in the hepatic vasoconstriction, representative event that occurs during I/R, was obtained, explaining the observed reduction in liver injury.

Our results support the idea that the advancement in the understanding of the molecular pathophysiology of liver injury might lead to the development of novel therapeutic alternatives, whose the FXR agonists represent a feasible and truthful example.

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