UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biochimiche, Nutrizionali e Metaboliche

Dipartimento di Scienze Farmacologiche e Biomolecolari

Dottorato di Ricerca in Biochimica XXVI ciclo

BIO/10

Cholesterol Homeostasis: involvement of histone deacetylases in the molecular regulatory pathway of Cholesterol 7α-hydroxylase

Erika FIORINO

Matr. R09261

Tutor: Prof. **Maurizio CRESTANI**

Coordinatore: Prof. **Francesco BONOMI**

Anno accademico 2013-2014

SUMMARY

INTRODUCTION

HYPERCHOLESTEROLEMIA and CVD RISK

Several epidemiological studies revealed lipid disorders, and in particular hypercholesterolemia, as one of the major risk factors for the onset and development of cardiovascular disease (CVD) (Prospective Studies et al., 2007, Kannel et al., 1961, Gordon et al., 1977, Yusuf et al., 2004). High cholesterol plasma levels lead, in fact, to Low Density Lipoproteins (LDL)-cholesterol accumulation in artery wall, predisposing to inflammation and consequently to the development of the atherosclerotic plaque underlying CVD. CVD is considered one of the leading causes of death in western countries (Go et al., 2014). Clinical risk factors, such as hypertension and high cholesterol, are the main targets of primary prevention in clinical practice. Hypolipidemic therapies, such as statins, lowering LDL-cholesterol reduced CVD related morbidity and mortality in primary and secondary prevention (Baigent et al., 2005) in association with a low cholesterol and saturated fat content diet. However, some patients, in particular patients with familial hypercholesterolemia, fail to reach the recommended LDL-cholesterol levels with statins (Rader et al., 2003) or develop statin intolerance leading to myopathy, or do not lower CVD risk as a consequence of other lipid disorders (Bhatt et al., 2006, Barter et al., 2007, Holmes et al., 2014). Treatment with drugs displaying different mechanisms of action, such as bile acid (BA) sequestrants and fibrates is available but, although the improvement in lipid profile, they do not seem to ameliorate CVD risk (Drexel, 2009). In this scenario the discovery of new therapeutic approaches and consequently of new pharmacological targets is desiderable to attain the reduction of CVD risk. In light of these evidences, it is therefore fundamental to deepen the molecular mechanisms underlying the maintenance of cholesterol and lipid homeostasis.

CHOLESTEROL

GENERAL CHARACTERISTICS

Cholesterol is a molecule consisting of 27 carbon atoms characterized by a tetracyclic steroid nucleus formed by four linked hydrocarbon rings, 1 made by 5 carbon atoms and the other 3 made by 6 carbon atoms, in the "chair" conformation. The cyclopentanoperhydrophenanthrenic nucleus is unsaturated at position 5-6 with an alkene group and it has a rigid planar structure with a flexible tail, consisting of a isooctyl side chain at carbon 17. Cholesterol is an amphipathic molecule due to its polar hydrophilic hydroxyl group at carbon 3 and to the non-polar lipophilic hydrocarbon body (Fig. 1). The peculiar structure of cholesterol confers the ability to intercalate between phospholipids in lipid bilayers of the cellular membrane, interacting both with the polar heads and with the apolar tails of phospholipids, thus modulating membrane fluidity and permeability.

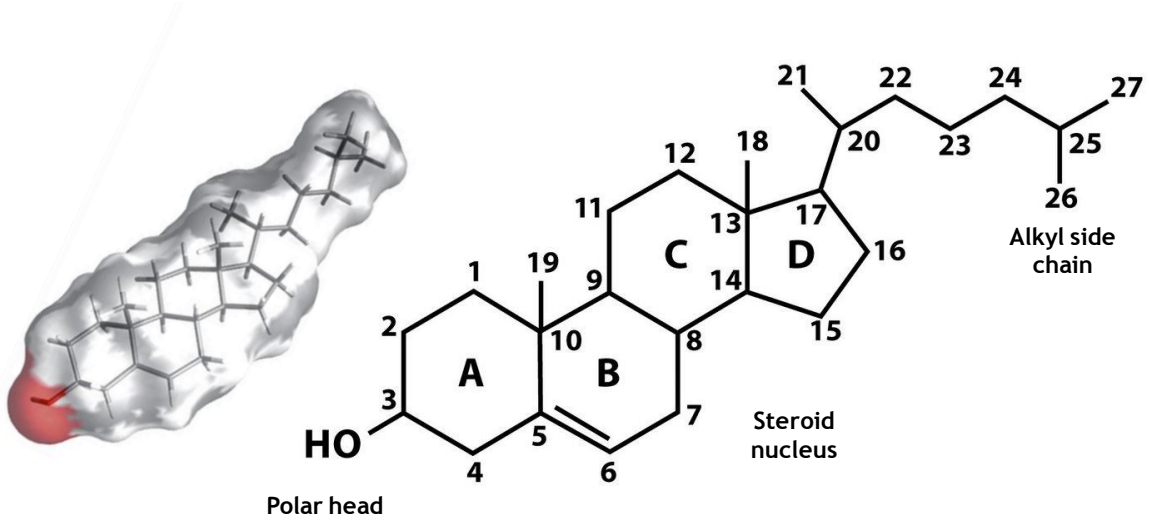


Fig.1: Cholesterol structure

Cholesterol is, in fact, an essential component of cellular membranes, nervous fibers and myelin sheaths. Moreover, it is the precursor of BA, steroidal hormones and vitamin D. Cholesterol is the most abundant sterol in animals tissues; in plants it is a minor component only of a complex mixture of structurally related 'phytosterols', such as stigmasterol. Prokaryotes lack cholesterol entirely, apart from a few species that acquire it from eukaryotic hosts. Supply of cholesterol is ensured by the endogenous biosynthetic pathway and it is synthesized mainly in the liver by the mavalonate pathway, whereas the remaining amount derives from the diet. Since it is not readily and completely biodegradable, cholesterol does not serve as a fuel for animal tissues. Animals, in fact, lack the enzymes necessary to catabolize steroid rings constituting cholesterol so that high levels of cholesterol in the blood are detrimental as they may lead to excessive accumulation in vessel walls, predisposing to the development of pathological conditions such as atherosclerosis and myocardial infarction. The major route of cholesterol elimination from the intact organism is its conversion to BA or its secretion into bile.

CHOLESTEROL HOMEOSTASIS

Biosynthesis and catabolism of cholesterol occur mainly in the liver. Cholesterol biosynthesis is a highly complex process of about thirty different enzymatic reactions and it is regulated by an elegant system of feedback inhibition, which senses cholesterol intracellular levels and subsequently modulates the expression of the key proteins involved in cholesterol homeostasis (Ye and DeBose-Boyd, 2011). Cytosolic acetyl-CoA in hepatocytes is the precursor of cholesterol synthesis passing through the formation of the important intermediate mevalonic acid (Russell and Setchell, 1992). Mevalonate is synthesized from ß-hydroxy-ß-methyglutaryl-CoA (HMG-CoA) through a reaction catalyzed by HMG-CoA reductase, which is the rate limiting enzyme of cholesterol biosynthesis localized in the endoplasmic reticulum. Recently, it has been demonstrated that squalene epoxidase (SQLE) may be a further control

point in cholesterol synthesis (Gill et al., 2011). HMGCoA reductase activity is regulated by several factors: post translational modifications, protein half-life modulation and regulation of gene transcription. Post translational modifications are mediated by glucagon and insulin that induce respectively phosphorylation/dephosphorylation events leading to the inhibition/activation of the enzyme (Beg and Brewer, 1982). Some metabolites of mevalonate pathway, such as a non-sterol isoprenoid signal derived from farnesyl diphosphate (FPP) and 24,25-oxidolanosterol, modulate HMGCoA reductase half-life (Gardner et al., 2001). Peculiar sequences, Sterol Regulatory Element (SRE) localized on HMGCoA reductase, regulate gene transcription. Sterol Regulatory Element Binding Protein (SREBP), sensitive to intracellular cholesterol levels, binds SRE and induces gene transcription (Hua et al., 1993). At low cholesterol levels, in fact, insulin sensitive gene 1 (INSIG-1) dissociates from SREBP/SREBP Cleavage Activating Protein (SCAP) complex and it is degraded; the complex can migrate into the Golgi apparatus where SREBP is activated by sequential cleavages. Active SREBP translocates into the nucleus binding SRE sequences thus activating cholesterol synthesis (Ye and DeBose-Boyd, 2011). On the other hand, when intracellular cholesterol levels increase SCAP is inhibited and consequently cholesterol synthesis is blocked (Goldstein and Brown, 1987).

Due to its insoluble nature cholesterol cannot dissolve in the blood, therefore it must be transported to different tissues by carriers called lipoproteins consisting of apolipoprotein core, phospholipids, cholesterol, triacylglycerols and steryl esters. Apolipoproteins associated with lipids form different classes of lipoproteins with hydrophobic lipids inside and hydrophilic side chain outside. Due to the different composition in lipids and proteins there are different density lipoproteins: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL). Cholesterol is esterified in the hepatocytes by acyl-CoA-cholesterol-acyltransferase (ACAT), transferring an acyl group from CoA to the hydroxyl group of cholesterol. This more hydrophobic form of cholesterol can be stored in lipid droplet in the liver or assembled in VLDLs.

Through the blood stream VLDLs delivered from the liver reach tissues. During this process they increase cholesterol content and are converted to LDLs. LDLs are internalized into the cell by the LDL-receptor (LDLR) delivering cholesterol inside the cell. In contrast, HDLs are the mediators for reverse cholesterol transport, collecting cholesterol from peripheral tissues and bringing it back to the liver. Nascent lipid poor HDL particles, also known as prebeta-HDL, collect free cholesterol and phospholipids from peripheral tissues; lecithin-cholesterol acyl transferase (LCAT) esterifies cholesterol forming HDL2 particles. Finally they reach the liver via Scavenger Receptor B1 (SR-B1) (Kingwell et al., 2014).

Elimination of cholesterol from the body is achieved only promoting its conversion to BA. In fact, approximately one third of daily cholesterol produced, both endogenous and exogenous, is eliminated as fecal bile acids and BA biosynthesis represents quantitatively the most important pathway to maintain cholesterol homeostasis in mammals. In addition, cholesterol is eliminated as such through the bile.

BILE ACIDS

BA are steroid acids secreted by parenchimal cells in the biliary duct and stored in the gallbladder, from which they are released in the duodenum after hormonal stimuli. In the intestine BA act as powerful detergents or emulsifying agents to aid the digestion and absorption of dietary cholesterol, lipids, fat soluble vitamins (A, D, E, K) and other essential nutrients and to prevent the precipitation of cholesterol in bile. BA return to the liver via the enterohepatic circulation through the portal vein. Approximately 95% of the non-conjugated bile acids are reabsorbed passively throughout the small and large intestines (Hofmann and Hagey, 2008). This continuous flux of BA between the liver and the intestine requires several BA transporters (Alrefai and Gill, 2007). For instance, bile salt export pump (BSEP) mediates the release of BA into the bile across the

canalicular membrane, while apical sodium-dependent bile acid transporter (ASBT) and organic solute transporter (OSTα-OSTß) reabsorb BA from the ileum, and finally sodium-taurocholate cotransporter polypeptide (NCTP) extracts BA from the portal blood (Dawson et al., 2009). A small amount of BA is lost in the feces but it is reintegrated by *de novo synthesis*, therefore in the steady state the fraction of bile acid pool that is excreted from the body each day is replaced by an equal amount of newly synthesized bile acid. Cholesterol reabsorbed from intestine in the bile is instead only 50%. In light of these processes the pharmacological interruption of the enterohepatic circulation of BA with BA sequestrants has been used as an effective strategy for lowering plasma LDL-cholesterol in the treatment of atherosclerosis.

Bile salts are hydrophilic on one side and hydrophobic on the other side consequently, they aggregate around lipid droplets to form micelles, with the hydrophobic sides towards the fat and hydrophilic sides facing outwards. The hydrophilic sides are negatively charged, and this charge prevents fat droplets coated with bile from re-aggregating into larger fat particles. BA release is regulated by hormones that are in turn induced by food: when the chyme enters the duodenum, low pH value induces secretin that stimulates pancreatic release of HCO_3^- in the small intestine to reach pH 7; secretin promotes bile secretion from hepatocytes. Moreover, chyme in the duodenum causes cholecystokinin release that stimulates gallbladder emptying.

Cholesterol conversion to BA requires at least 17 enzymes and occurs in multiple intracellular compartments including cytosol, endoplasmic reticulum, mitochondria, and peroxisomes. Two different BA biosynthetic pathways have been identified: the “classic” or “neutral” pathway , exclusively hepatic, and the “alternative” or “acidic” pathway whose reaction may also take place in extrahepatic cells (Martin et al., 1993). The most important BA present in the bile are cholic acid and chenodeoxycholic acid. These primary BA can be converted by microbiota in deoxycholic and lithocholic acid respectively. About 98% of BA are conjugated with glycine or taurine in the liver generating bile salts

(Johnson et al., 1991), the most abundant constituent of bile together with cholesterol and lecithin.

BILE ACID BIOSYNTHESIS: THE "CLASSIC" or "NEUTRAL" PATHWAY

The "classic" or "neutral" pathway occurs only in the liver by more than ten different enzymes located in different subcellular compartments of the hepatocyte. It is also known as "neutral" since the intermediates mainly generated in this pathway are neutral sterols. The "classic" pathway is initiated by CYP7A1, the major check-point and the rate limiting enzyme of BA biosynthesis (Myant and Mitropoulos, 1977), which catalyzes cholesterol hydroxylation at the 7α position. CYP7A1 belongs to cytochrome P450 superfamily and it is located in the endoplasmic reticulum. NADPH, O_2 and the electron transport chain of the cytochrome P450 take part in the esoergonic and irreversible reaction. 3ß-hydroxy-C27-steroid dehydrogenase/isomerase (3ß-HDS) converts 7α-hydroxycholesterol in 7α-hydroxi-colest-4-ene-3-one, precursor both of chenodeoxycholic and of cholic acid. 12α-hydroxylase (CYP8B1), an enzyme localized in the endoplasmic reticulum, converts 7α-hydroxi-colest-4-ene-3-one in 7α,12α-dihydroxy-colest-4-ene-3-one (Chiang, 1998). Δ4-3-ossosteroid-5ß-reductase and 3α-hydroxysteroid dehydrogenase (HDS) converts these intermediates in 5ß-cholestan-3α,7α-diol to synthesize chenodeoxycholic acid (CDCA), and in 5ß-cholestan-3α,7α,12α-triol to synthesize cholic acid (CA). Their side chain is finally oxidized to a carboxylic group by sterol 27-hydroxylase (CYP27A1), a mitochondrial enzyme that leads to the formation of CDCA and CA respectively (Björkhem et al., 1992). After their synthesis primary BA are then conjugated with taurine or glycine before being secreted into bile. (Fig. 2)

BILE ACID BIOSYNTHESIS: THE "ALTERNATIVE" or "ACIDIC" PATHWAY

CYP27A1 catalyzes the first reaction of this biosynthetic pathway. This enzyme is the only member of the CYP27 cytochrome P450 superfamily and it is

localized in the inner mitochondrial membrane of endothelial cells, fibroblasts and macrophages (Martin et al., 1993). CYP27A1 plays an important role in the extrahepatic cholesterol disposal (Lund et al., 1996): it promotes extrahepatic cholesterol conversion to 27-hydroxycholesterol and in 3ß-hydroxy-5-cholestenoic acid that released in the blood stream are picked up by hepatocytes and used for BA biosynthesis. After the hydroxylation at 27 position, another hydroxylation occurs at 7α position catalized by oxysterol 7α-hydroxylase (CYP7B1) (Schwarz et al., 1997). The following reactions take place in the liver and they are catalyzed by the same enzyme of the "classic" pathway. The most abundant product of the "alternative" pathway is CDCA. (Fig.2)

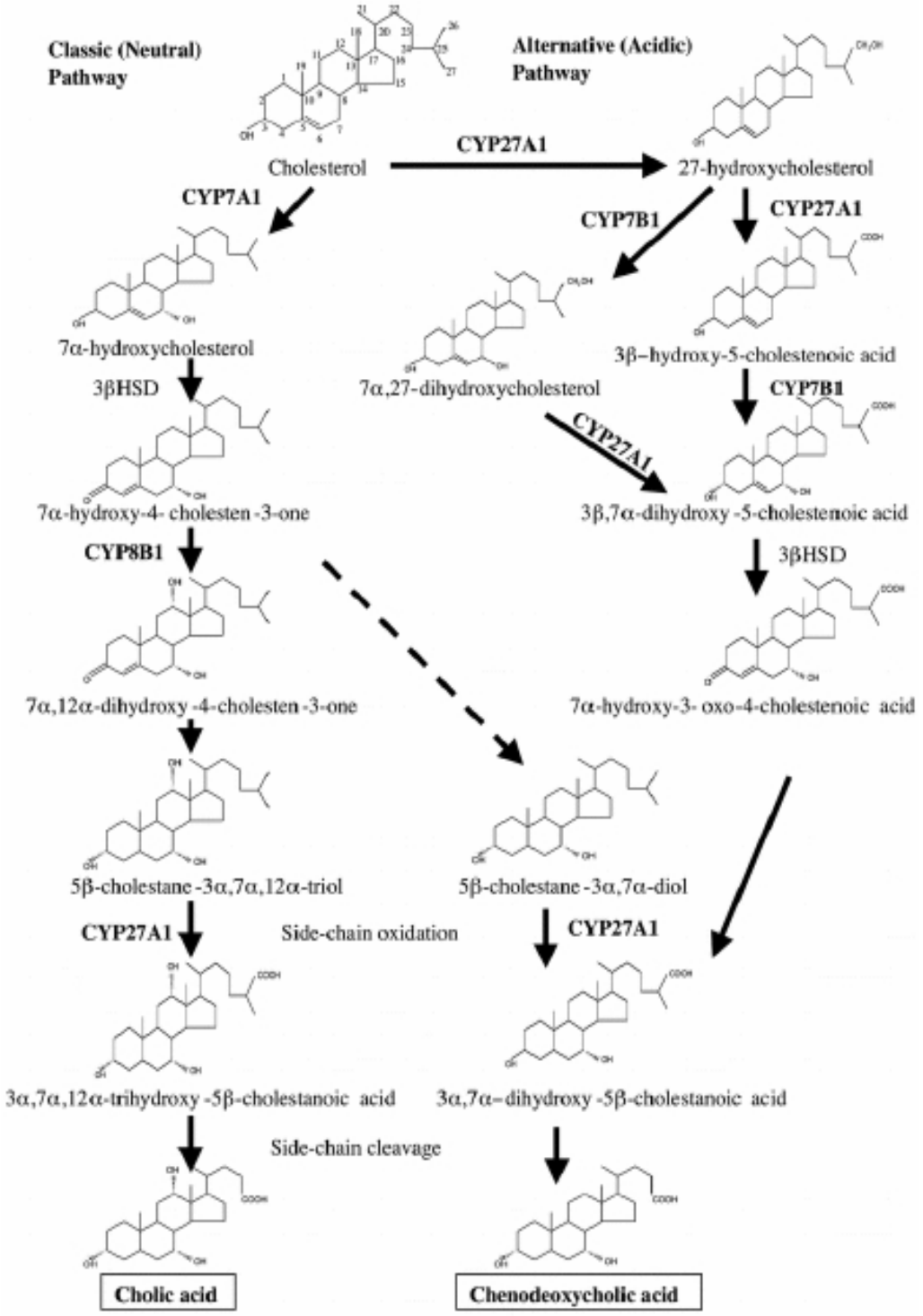


Fig.2: Classic and anternative biosynthetic pathway of BA biosynthesis

CHOLESTEROL 7α-HYDROXYLASE (CYP7A1)

Cholesterol 7α-Hydroxylase (CYP7A1) is a 57 kDa enzyme consisting of 503 or 504 aminoacidic residues respectively in rats and in humans (Ogishima et al., 1987, Chiang et al., 1990). CYP7A1 is anchored in the membrane of endoplasmic reticulum by an N-terminal hydrophobic region, and possesses a binding region for heme and a sterol binding site; moreover the aromatic aminoacid rich region is important for electron transfer; these regions are conserved in humans, rats and hamsters (Crestani et al., 1993).

CYP7A1 gene was cloned in rats (Jelinek and Russell, 1990), humans (Wang and Chiang, 1994), hamsters (Crestani et al., 1993) and mice (Tzung et al., 1994). In hamsters, rats and humans gene length is about 11Kb, made of 6 exons and 5 introns, and their sequence is high conserved among species. In humans only a single copy of the enzyme is present on the chromosome 8 in centromeric region q11-q12 (Cohen et al., 1992). *CYP7A1* gene, differently from other CYP450 possessing 7-13 exons, contains only 6 exons, suggesting that the evolutionary origin of this gene is rather old.

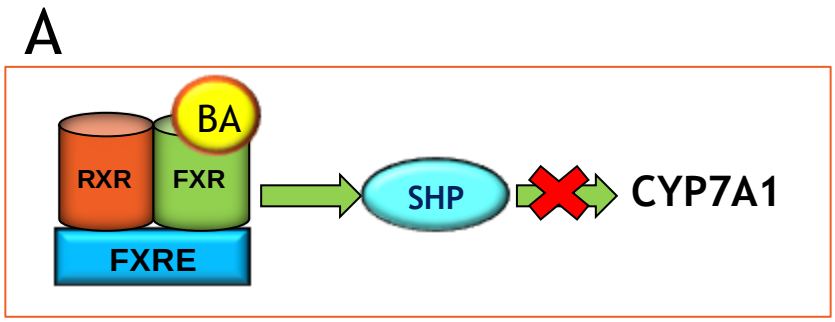
CYP7A1$^{-/-}$ mice show hyperkeratosis, hypercholesterolemia, hypertriglyceridemia, behavioural and vision defects due to the inability to assume liposoluble vitamins (Pullinger et al., 2002, Ishibashi et al., 1996). On the contrary, it has been demonstrated that overexpression of *CYP7A1* gene in hamsters by adenoviruses, decreases LDL cholesterol in animals fed both chow and high fat diet. By using adenovirus-mediated overexpression in hypercholesterolemic mice lacking the LDL receptor it was shown that *Cyp7a1* dramatically reduced serum cholesterol levels and improved the ratio of high-density lipoproteins/LDL (Spady et al., 1998). An interesting paper by Li and coworkers showed that mice overexpressing *Cyp7a1* in the liver were resistant to HFD–induced obesity, fatty liver, and insulin resistance. These mice displayed

increased hepatic cholesterol catabolism and an increased bile acid pool, increased secretion of hepatic VLDL but maintained plasma triglyceride. Moreover, the authors observed induction of fatty acid oxidation genes in the brown adipose tissue in association with increased body energy expenditure in Cyp7a1-tg mice (Li et al., 2010b), linked to an increased hydrophobic BA pool (Li et al., 2011b). Improvement of energy expenditure in these mice is consistent with a paper recently published demonstrating that BA act also on TGR5, another BA receptor localized in brown adipose tissue, whose stimulation leads to deiodinase 2 (D2) activation causing increase of 3,5,3′-tri-iodothyronine (T3) and consequently uncoupled protein 1 (UCP1) induction, a well known activator of non-shivering thermogenesis (Watanabe et al., 2006).

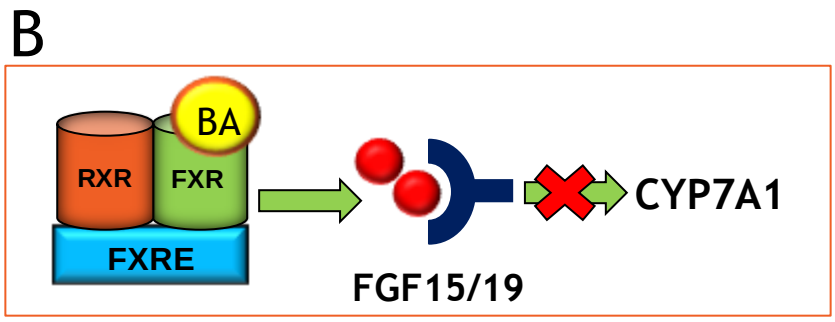
CHOLESTEROL 7α-HYDROXYLASE REGULATION: the role of BA

CYP7A1 gene is regulated mainly at the transcriptional level in response to BA, cholesterol and hormones and is fundamental for the maintenance of cholesterol homeostasis (Ramirez et al., 1994). The main regulatory mechanism of *CYP7A1* transcription is the feedback regulation operated by BA returning to the liver via the enterohepatic circulation. The sequence responsible of this response was first discovered by Stroup and co-workers (Stroup et al., 1997) and it is localized at nucleotides−148/−129 relative to the transcription start site of the rat gene. This sequence is highly conserved in all CYP7A1 from different species and was named BARE (bile acid-responsive element). The BARE is the binding site of some nuclear receptors such as the orphan nuclear receptor hepatocyte nuclear factor 4 (HNF4), and the mouse homologue liver receptor homologue-1 (LRH-1) (Crestani et al., 1998). A further characterization of this region showed that the rat *CYP7A1* proximal promoter contains 2 BAREs, with high sequence similarity. BARE-I corresponds to the LXRE (Lehmann et al. 1997), and BARE-II has been shown to be the binding site for LRH-1 and HNF4, which are required for hepatic basal expression of CYP7A1 (Nitta et al., 1999). First evidences of *CYP7A1* feedback regulation mechanism operated by BA appear in

the literature in 2000, with the discovery of the mechanism of action of bile acid receptor farnesoid X receptor (FXR, NR1H4). In the liver (Fig. 3A) BA interact with their nuclear receptor FXR, inducing the expression of the orphan nuclear receptor small heterodimer partner (SHP, NR0B2), an atypical orphan receptor lacking the DNA binding domain. SHP heterodimerizes with LRH-1 and represses the transcription activity of the receptor and consequently the expression of *CYP7A1* (Goodwin et al., 2000) (Lu et al., 2000c).



Goodwin et al., *Mol. Cell*, **2000,** 6: 517 - 525



Inagaki et al., *Cell metabolism*, **2005,** 4: 217-25

Fig.3: BA-induced activation of FXR in the liver (A) and in the intestine (B)

An apparently contradictory study by Kerr and coworkers showed that in mice lacking *Shp* gene, BA negative feedback on their own synthesis and particularly on *CYP7A1* gene was impaired but not completely eliminated. This evidence revealed the existence of other compensatory repression pathways of bile acid signaling (Kerr et al., 2002). Further investigations, in fact, lead to the discovery of additional mechanisms adopted by BA to regulate *CYP7A1* transcription. Subsequently, it was demonstrated that in the intestine (Fig. 3B),

FXR activation by BA induces the release of fibroblast growth factor 15/19 (FGF15 in mouse or FGF19 in human), which activates hepatic FGF receptor 4 (FGFR4) signaling to inhibit *CYP7A1* transcription and BA biosynthesis (Inagaki et al., 2005). Moreover, De Fabiani and coworkers in 2001 demonstrated that BA decrease *CYP7A1* transcription in a FXR/SHP-independent manner, via the mitogen-activated protein kinase pathway, which depresses the transactivation potential of HNF-4. Moreover, they showed that the pro-inflammatory cytokine TNFα inhibits *CYP7A1* through the same mechanism (De Fabiani et al., 2001). Further investigations, allowed the same authors to better define the molecular events underlying *CYP7A1* repression; they found that BA induce the dissociation of a co-activator complex from HNF-4 (De Fabiani et al., 2003), interfering with the recruitment of PPARγ co-activator-1α (PGC-1α) and cAMP-response element binding protein-binding protein (CBP; p300), two transcriptional coactivators, to *CYP7A1* promoter. Since the selective FXR ligand, GW4064 does not affect the transactivation potential of HNF-4, the authors demonstrated that this dissociation is not mediated by FXR/SHP.

These evidences stimulated several studies to investigate chromatin remodeling induced by BA (Fang et al., 2007, Kemper et al., 2004, Ponugoti et al., 2010). An interesting study conducted in our laboratory analyzed chromatin remodeling elicited by BA and showed the dissociation of PCG-1α and CBP from *CYP7A1* promoter and recruitment of Histone Deacetylases (HDACs) and corepressors after BA treatment in the human liver cell line HepG2. This effect occurs earlier than the FXR/SHP-mediated repression, demonstrating also that the feedback repression of *CYP7A1* transcription by BA is a multiple process involving several mechanisms (Mitro et al. 2007). In particular, it was shown that BA induce the translocation of HDAC7 from cytoplasm to nucleus and the sequential recruitment of HDAC 7, 3, and 1, and of the corepressors Silencing Mediator of Retinoid and Thyroid receptors-α (SMRT-α) and N-CoR1 forms a repressive complex on *CYP7A1* promoter that leads to the repression of *CYP7A1* gene transcription (Fig. 4).

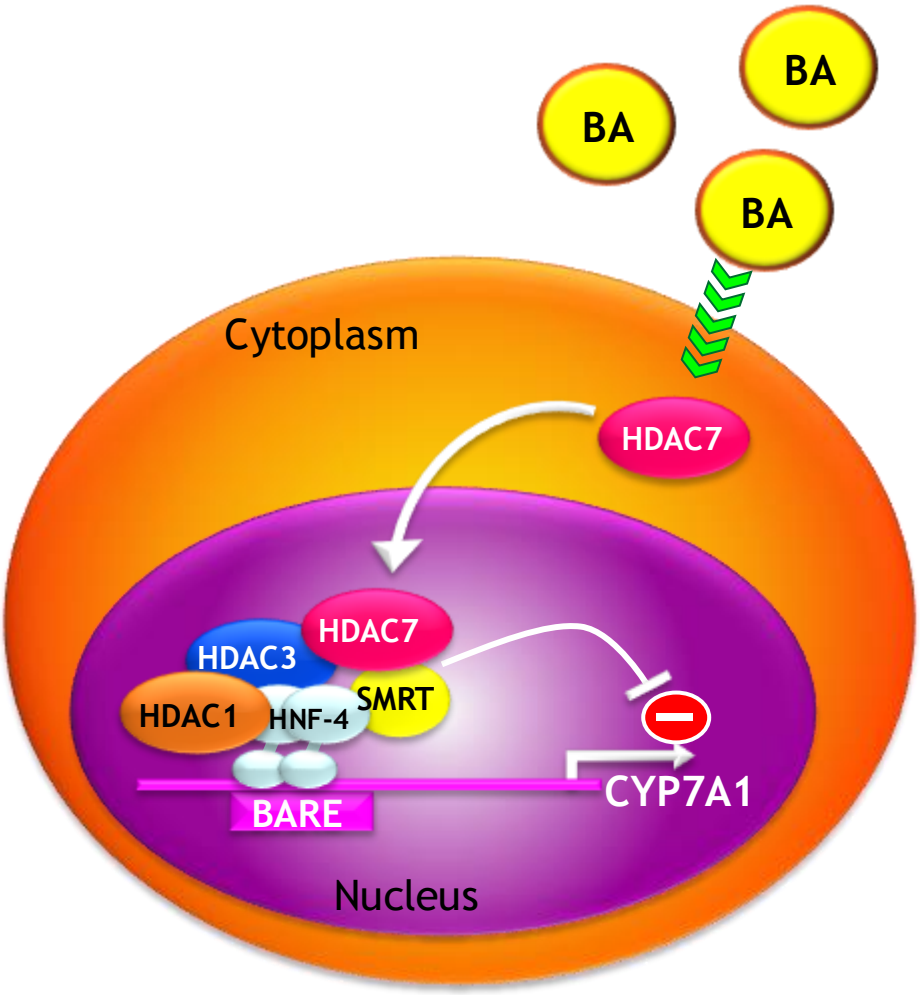


Fig. 4: BA-induced chromatin remodeling of *CYP7A1* promoter

Adapted by Mitro, *Frontiers in Bioscience* 13, 6276-6288, May 1, 2008

CHOLESTEROL 7a-HYDROXYLASE REGULATION: other regulatory factors

As mentioned above, BA are important actors in the digestive processes and in the absorption of dietary lipids. Therefore, it is reasonable to assume that their synthesis can be regulated by the fasted-to-fed cycle. In mice CYP7A1 expression increases in the fasting state and returns to normal upon feeding (De Fabiani et al., 2003), suggesting the modulation by the two principal hormones regulating fasting-feeding cycle, insulin and glucagon. In particular, insulin

inhibits *CYP7A1 i*n different species (De Fabiani et al., 2000, Crestani et al., 1998). Moreover, it has been demonstrated that physiological concentrations of insulin rapidly increase mRNA levels in primary human hepatocytes but, on the contrary, an extended treatment inhibits *CYP7A1* expression. This occurs because the insulin-regulated transcription factors, forkhead box O1 (FoxO1) and SREBP-1c, inhibit HNF-4 and PGC-1α transactivation of the *CYP7A1*, while the rapid insulin-induced activation of the p38 kinase pathway increases HNF-4α and could lead to *CYP7A1* induction in the shorter time (Xu et al., 2007). On the other side, during fasting glucagon activates cAMP signalling inhibiting *CYP7A1* transcription in primary rat and human hepatocytes (Song and Chiang, 2006).

Furthermore, it has been demonstrated that *CYP7A1* expression is also regulated by thyroid hormones (Ness et al., 1990), extrogens (Kushwaha and Born, 1991) and inibite by the proinflammatory cytochines TNFα e IL-1ß (De Fabiani et al., 2001). The activity of CYP7A1 withstands diurnal variation in rat livers and in rodents it is increased during the night whereas decreased during the day (Gielen et al., 1969). In humans, CYP7A1 undergoes daytime changes as well but it is independent of food intake and opposite from the circadian rhythm of cholesterol synthesis (Gälman et al., 2005).

EPIGENETICS

Inheritable influence on gene activity that modifies chromatin structure without changes in DNA sequence is known as epigenetics. Nucleosomes are the building blocks of chromatin and histone octamers wrapped twice by DNA constitute their core structure in eukaryotic cells. Chromatin is a dynamic structure that adopts a locally accessible and transcriptionally active form, also known as euchromatin during the activation of gene transcription. Alternatively, when chromatin is in a highly condensed and transcriptionally inhibited state, is referred to as heterochromatin (Richards and Elgin, 2002) (Fig. 5).

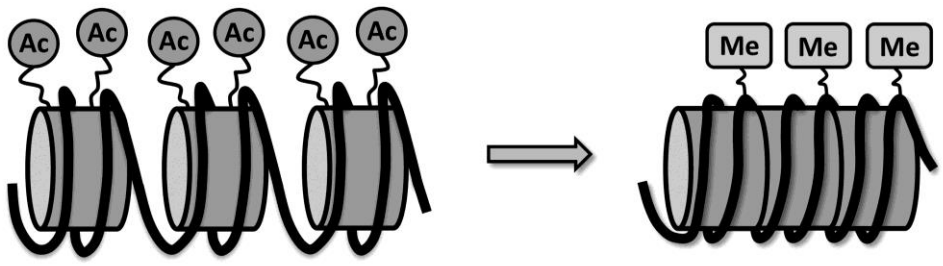


Fig. 5: Conformational changes of chromatin induced by histone modifications

On the left, euchromatin represents acetylated and transcriptionally active state. On the right, DNA methylation and histone deacetylation induce chromatin packaging (heterochromatin) making it less accessible to transcriptional factors. (Ferrari et al., 2012)

The main epigenetic modifications include DNA methylation and histone modifications (Vaillant and Paszkowski, 2007). Posttranslational modifications, including acetylation, ADPribosylation, methylation, phosphorylation, and ubiquitination occurring at amino acids in the N-terminal of histone tails strongly regulate chromatin packaging and consequently gene expression. One of the most studied post-translational modifications is acetylation of lysine residues of

histone tails resulting from the balance between HAT (histone acetyltransferase) and HDAC activity (Grunstein, 1997b); acetylation carried out by HAT increases the distance between DNA and histones and the accessibility of transcription factors to gene promoter regions, while deacetylation operated by HDACs induces chromatin packaging thus reducing the accessibility of transcription factors to local chromatin regions (López-Rodas et al., 1993a).

HISTONE DEACETYLASES

Eukaryotic HDACs belong to an ancient family of proteins comprising two subfamilies with different HDAC activity: the classical HDAC family and the sirtuins family (de Ruijter et al., 2003). Mammalian HDACs are listed into four classes based on their homology with yeast HDACs (Haberland et al., 2009) (Fig. 6). The classical HDAC family consists of eleven Zn_2^+-dependent enzymes harboring a Zn_2^+ ion in their catalytic pocket (Ferrari et al., 2012). They are divided in three classes:

Class I (HDAC1, 2, 3, and 8) closely related to the yeast transcriptional regulator Rpd3, are ubiquitously expressed and are mainly localized in the nuclear compartment where they exert the most relevant histone deacetylase activity.

Class II (HDAC4, 5, 6, 7, 9, and 10) similar to the yeast Hda1 localize in the cytoplasm, shuttling into the nucleus in response to specific cellular signals. Class IIa HDACs are characterized by post-translational regulation such as phosphorylation that determines their cytosolic localization, whereas dephosphorylation of Class IIa HDACs enables their translocation to the nucleus; Class IIa HDACs carry a large N-terminal with conserved binding sites for the transcription factor myocyte enhancer factor 2 (MEF2) and the chaperone protein 14-3-3. First of all, upon phosphorylation by different kinases, the calcium/calmodulin-dependent protein kinase and protein kinase D, class IIa

HDACs bind 14-3-3 and translocate from the cytoplasm to the nucleus (Passier et al., 2000, Vega et al., 2004, Lu et al., 2000a). When interacting with class IIa HDACs, MEF2 acts as transcriptional repressor; in contrast, its dissociation from HDACs promotes the recruitment of the histone acetyltransferase p300 and the conversion of MEF in a transcriptional activator (Lu et al., 2000b, Youn et al., 2000, Wang et al., 1999, Miska et al., 1999, Sparrow et al., 1999). Class IIa HDACs feature only minimal histone deacetylase activity due to a swap of a key tyrosine residue in the catalytic domain with a histidine (Lahm et al., 2007); nonetheless, they act as scaffold molecules to recruit class I HDACs. Class IIb HDACs are localized in the cytoplasm and act mainly on non-histone substrates such as cytoskeletal and transmembrane proteins (Haberland et al., 2009).

Class IV (HDAC11) role is still not clearly understood (Gregoretti et al., 2004).

Class	Protein domain	Members
Class I	Deacetylase catalytic domain Phosphorylation sites (serine residues) at C terminus	HDAC1 HDAC2 HDAC3 HDAC8
Class IIa	Deacetylase catalytic domain Phosphorylation sites (serine residues) at N terminus Myocyte enhancer factor binding sites Binding sites for 14-3-3 chaperone protein	HDAC4 HDAC5 HDAC7 HDAC9
Class IIb	Deacetylase catalytic domain Zinc finger domain or leucine rich region	HDAC6 HDAC10
Class III (Sirtuins)	Deacetylase catalytic domain, requiring NAD^+ as cofactor	SIRT1 SIRT2 SIRT3 SIRT4 SIRT5 SIRT6 SIRT7
Class IV	Deacetylase catalytic domain	HDAC11

Fig. 6: Superfamily of mammalian histone deacetylases

(Ferrari et al., 2012)

Class III HDACs comprises the sirtuins family; they are sensitive to changes in the intracellular NAD^+/NADH ratio and they rely upon NAD^+ hydrolysis for their deacetylase activity. In mammals, sirtuins regulate several functions, ranging from the control of cellular stress to energy metabolism (Finkel et al., 2009) (Fiorino et al., 2014). Sir2 homolog family in mammals comprises seven proteins with different localizations and functions and are classified in four classes based on phylogenetic analysis: SIRT1, 2, and 3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and 7 to class IV (Frye, 2000).

HISTONE DEACETYLASES INHIBITORS

From the structural point of view, synthetic HDAC inhibitors can be classified in four groups: short-chain fatty acids, hydroxamic acids, benzamide derivatives, and cyclic peptides. We will briefly highlight the main features of each class addressing interested readers to specific reviews on the topic (Mai and Altucci, 2009).

Short-chain fatty acids such as sodium butyrate, sodium phenylacetate, phenylbutyrate and valproate, are active at millimolar concentrations and can be defined pan-inhibitors although they seem to be less active against class IIb HDACs. The mechanism by which this class of compounds inhibits HDACs has not been fully elucidated.

The first compound bearing a hydroxamic acid moiety to be described as HDAC inhibitor was trichostatin A (TSA), which is produced by Streptomyces hygroscopicus and displays antifungal activities. However, the prototype compound of this class is suberoylanilide hydroxamic acid (SAHA) that was approved by the US FDA in 2006 for cutaneous manifestations of cutaneous T-cell lymphoma (Fig. 7). Hydroxamic acids are usually active in the nanomolar range and inhibit HDACs by complexing the zinc cation present in the catalytic pocket

of the enzyme with the hydroxamate group. Many evidences suggest HDAC inhibitors as an interesting approach in cancer therapy. DNA damage response is in fact modulated by the acetylation status of histone and non-histone proteins and HDACs protect cancer cells from genotoxic insults. Therefore, HDAC inhibitors can silence DNA repair pathways, inactivate non-histone proteins that are required for DNA stability and induce reactive oxygen species and DNA doublestrand breaks (Rajendran et al., 2011).

The cyclic peptide romidepsin (formerly known as FK-228) is produced by Chromobacterium violaceum and shows higher inhibitory activity toward class I HDACs, with IC50 in the nanomolar range. In the cellular reducing environment, this compound is converted into its reduced form that can interact with the enzyme active site. This molecule was very recently approved by US FDA for patients with cutaneous T-cell lymphoma.

Finally, MS-275 is the lead compound of the benzamide derivatives series. This compound is highly active against class I HDACs and almost completely inactive toward class II enzymes (Fig. 7).

In addition, several natural compounds have been added to the list of HDAC inhibitors in the last years. The beneficial effects of dietary isothiocyanates and allyl sulfides on human health, and in particular their cancer chemopreventive effects, have been known for a long time (Zhang and Talalay, 1994) however, only recently their positive actions have been linked with epigenetic mechanism.

A major understanding of HDAC active site structure and of the molecular features required to inhibit HDAC catalytic activity, in particular the presence of a spacer “arm” that allows the entry into the catalytic pocket and of a functional group interacting with the zinc cation, led to the reconsideration of the biological activity of several natural compounds.

Sulforaphane (SFN) is one of the most characterized isothiocyanates found in vegetables. It derives from the glucosinolate glucoraphanin present in

cruciferous vegetables, such as broccoli and broccoli sprouts. Like other isothiocyanates, it is metabolized via the mercapturic acid pathway to active metabolites, among which SFN-cysteine displays a good fit for HDAC active site according to computer modeling predictions (Nian et al., 2009). Indeed, the effects of SFN in vitro and in vivo systems are associated with increased global histone acetylation (Myzak et al., 2004, Myzak et al., 2006a, Myzak et al., 2006b).

Allyl compounds are garlic components comprising diallyl disulfide and S-allyl mercaptocysteine, which are both converted into the active metabolite allyl mercaptane. Docking simulation revealed a good fit between allyl mercaptane and HDAC active site, consistent with accumulation of acetylated histones and growth arrest in cancer cells treated with the active metabolite at micromolar concentrations (Nian et al., 2008).

Finally, sodium butyrate that we mentioned before as a synthetic HDAC inhibitor, should also be included in the list of naturally occurring inhibitors since it is generated during the fermentation of dietary fibers in the large intestine (Cummings and Englyst, 1987).

HDACs inhibitors might represent a novel and promising therapy in patients with heart failure (McKinsey et al., 2000) since HDACs play an important role in control of several cardiac events such as hypertrophy (Antos et al., 2003), autophagy (Cao et al., 2011), contractility (Gupta et al., 2008). Moreover, other recent evidences underline the importance of tubulin acetylation mediated by HDAC6 in the development of Huntington (Dompierre et al., 2007) and Parkinson diseases (Outeiro et al., 2007). Based on these results, the HDAC class II selective inhibitors have been theorized as new avenues for therapeutic intervention in some neurodegenerative disorders (Li et al., 2011a). Given the role of different HDACs in metabolic regulation, it is possible that in the future some specific inhibitors may find some clinical applications in metabolic disorders.

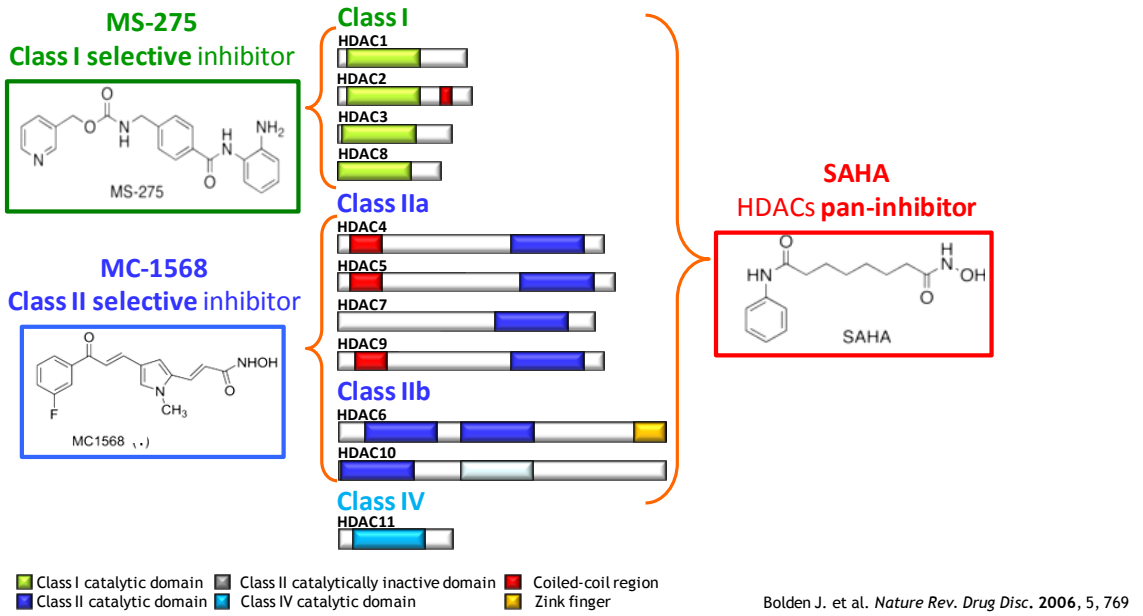


Fig. 7: Histone Deacetylases inhibitors

SAHA: Pan inhibitor of HDACs inhibits both class I and II HDACs; MS275: class I selective HDAC inhibitor and MC1568 class II selective HDAC inhibitor.

AIM OF THE STUDY

Hypercholesterolemia is one of the main risk factors for the onset and development of cardiovascular disease (CVD) and atherosclerosis. Nowadays several therapies are being used to reduce the rate of morbidity and mortality associated with hypercholesterolemia, targeting both the endogenous cholesterol biosynthetic pathway and the uptake of exogenous cholesterol form the diet but some patients respond poorly to them or show some adverse effects. Therefore, it is necessary to better understand genetic and molecular mechanisms involved in the pathophysiological regulation of cholesterol homeostasis to devise new and innovative therapeutic approaches aimed at lowering plasma cholesterol levels and the associated CVD risk.

Hepatic bile acid biosynthesis is quantitatively the chief metabolic pathway to maintain cholesterol balance in mammals. It has been demonstrated that increased bile acid synthesis strengthens cholesterol removal from liver and decreases circulating low density lipoprotein (LDL) levels. Cholesterol 7α-hydroxylase (CYP7A1) is the major check-point in the "classic" pathway of BA synthesis catalyzing the hydroxylation of cholesterol at the 7α position and it acts as the main regulator of bile acid biosynthesis, therefore it has deep impact on cholesterol homeostasis. The activity of this enzyme is mainly regulated at the transcriptional level by different bile acid-induced pathways which feedback inhibit *CYP7A1* gene transcription. A peculiar role in this scenario is played by HDACs. In recent years, in fact, it has become clear that epigenetic modifications leading to chromatin remodeling affect metabolic homeostasis. In particular, HDACs have been involved in the regulation of BA biosynthesis genes. Mitro and coworkers in 2007 described an FXR-independent *CYP7A1* inhibition mediated by the translocation of HDAC7 from cytoplasm to nucleus and the sequential recruitment of HDACs and corepressors elicited by BA on *CYP7A1* promoter, which is chronologically distinct from the FXR/SHP dependent regulatory pathway occurring at a later stage (Mitro et al., 2007). These observations highlight the crucial role of HDACs in the regulation of *CYP7A1* expression suggesting HDAC7 as a possible main actor in this regulatory pathway.

Moreover, they observed that non-selective HDAC inhibitors, such as valproic acid (VPA) and trichostatin A (TSA), prevent the repressive effect of BA on *CYP7A1 in vitro* (Fig. 8a). In addition, *in vivo* these inhibitors increase *Cyp7a1* levels and fecal bile acid excretion as an index of increased BA biosynthesis (Fig. 8b) leading to LDL-cholesterol reduction in a genetic model of hypercholesterolemia, such as LDL receptor knock out mice (Fig. 8C).

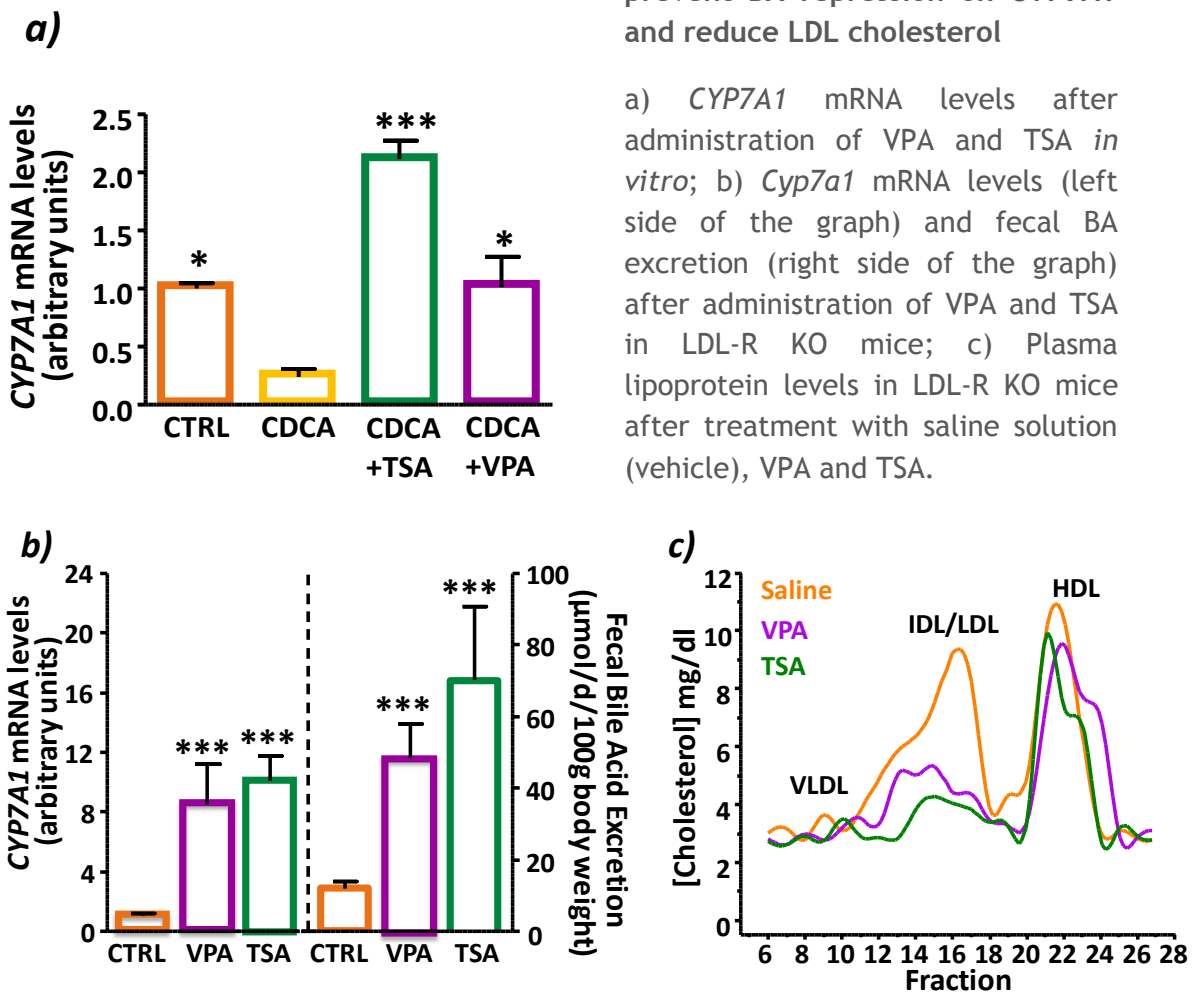


Fig. 8: Non-selective HDAC-i totally prevent BA repression on *CYP7A1* and reduce LDL cholesterol

a) *CYP7A1* mRNA levels after administration of VPA and TSA *in vitro*; b) *Cyp7a1* mRNA levels (left side of the graph) and fecal BA excretion (right side of the graph) after administration of VPA and TSA in LDL-R KO mice; c) Plasma lipoprotein levels in LDL-R KO mice after treatment with saline solution (vehicle), VPA and TSA.

Based on these evidences the *central hypothesis* is that HDACs are key regulators of *CYP7A1* expression and consequently of cholesterol homeostasis, thence, the aim of my doctorate project has been to define the role of specific HDACs and corepressors in the regulatory mechanism of *CYP7A1* gene

transcription and on cholesterol catabolism to BA and to confirm the central role of HDAC7 in this pathway *in vivo*.

To address this goal I used different strategies:

Class selective HDAC inhibitors *in vitro* and *in vivo:*

I tested class selective HDAC-inhibitors *in vitro* and *in vivo*, in a human hepatoma reporter cell line harboring a human *CYP7A1* minigene/luciferase reporter gene construct (HepG2 2.2.1 luc) and on C57BL/6J mice, respectively, with the aim to understand which class of HDACs played the predominant role in the regulatory pathway of *CYP7A1*.

RNAi approach:

To unravel the role of specific HDACs and corepressors and their single contribution in the modulation of *CYP7A1* expression, I set up adenovectors expressing shRNA that silence HDACs *in vitro* and *in vivo*.

Generation of a conditional KO mouse lacking liver HDAC7 (H7LivKO):

It has been shown that HDAC7 can translocate from cytoplasm to nucleus in response to BA and that the inhibition of this translocation with the phosphatase inhibitor Caliculin A totally prevents BA repressive effect on *CYP7A1* (Mitro et al., 2007), suggesting a key role of HDAC7 in the transcriptional regulation of this gene. Therefore, to better define the role of HDAC7 in *CYP7A1* regulation and on cholesterol and lipid homeostasis *in vivo*, I generated a conditional KO mouse lacking hepatic Hdac7 (H7LivKO) and I analyzed the metabolic features of H7LivKO mice on *Western Diet*, a diet enriched in cholesterol, in order to emphasize the effect of HDAC7 ablation on cholesterol and lipid metabolism.

MATERIALS and METHODS

CELL CULTURES

HepG2 2.2.1 luc

HepG2 2.2.1 luc cell line is produced by ATCC (American Type Culture Collection, Manassas, VA) and it is derived from the hepatocellular carcinoma cell line, HepG2. The parental cells were stably transfected at passage 48 with a human CYP7A1 minigene/luciferase construct. Luciferase reporter gene is upstream of h*CYP7A1* promoter, inserted within exon 2 (Fig. 9).

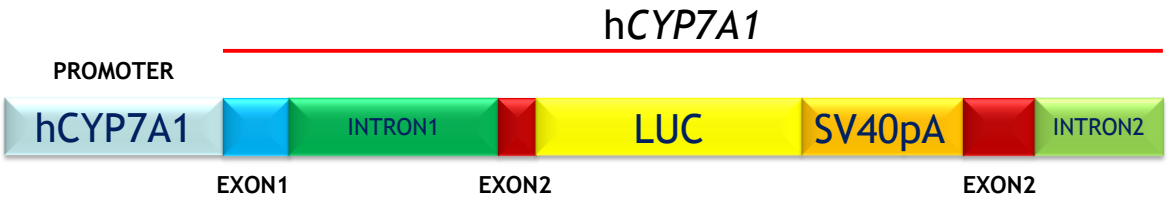


Fig. 9: Schematic representation of h*CYP7A1* minigene/luciferase construct

Manteinance medium:

Dulbecco's Modified Eagle's Medium and Ham's F12 (1:1) (Gibco, Life Tecnologies Paesley, Scotland) with fetal bovine serum to a final concentration of 10%, L-Glutamine (Gibco, Life Tecnologies Paesley, Scotland), Pen/Strep (Gibco, Life Tecnologies Paesley, Scotland) and supplemented with 0,4 mg/ml G418.

Temperature: 37.0°C

Subculturing protocol:

- Remove and discard culture medium.

- Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.53 mM EDTA solution (Gibco, Life Tecnologies Paesley, Scotland) to remove all traces of serum which contains trypsin inhibitor.
- Add 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5-7 minutes).
  Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
- Add 7.0 ml of complete growth medium and aspirate cells by gently pipetting.
- Add appropriate aliquots of the cell suspension to new culture vessels. Inoculate at a cell concentration between 1.5 X 10^5 and 1.0 X 10^6 viable cells/75 cm^2.

Incubate cultures at 37°C.

Medium Renewal: Every 2/3 days.

HDAC inhibitors treatment:

Day 1:

50000 cell/well were seeded in 96 well plates and incubate 24h at 37°C in DMEM F12 with 10% fetal bovine serum, 1% L-Glutamine, 1% Pen/Strep and 0,4 mg/ml G418.

Day 2:

O/N treatment with HDAC inhibitors (SAHA 2,5µM, MS275 2,5µM, MC1568 2,5µM) or 0,1%DMSO in the control wells, with or without CDCA 25µM in DMEM F12 with 1% L-Glutamine, 1% Pen/Strep and 0,4 mg/ml G418medium without serum.

MS275 and MC1568 are provided by Prof. Antonello Mai, Università di Roma "La Sapienza". SAHA by Cayman Michigan, USA.

Day 3:

96 well plates cells were lysed using briteliteTM plus (Perkin Elmer, Monza, Italy) and RLU were detected with EnVision (Perkin Elmer Waltham, MA). Data were normalized on the total protein amount by BCA assay (Pierce).

Transfection protocol:

Day 1:

Preparation of transfection mix, final volume 1200µl consisting of:

600µl of a solution containing siRNA 2 µM in DMEM F12 medium without Pen/Strep and serum; kindly mix and incubate 5 minutes RT.

600µl of a solution containing 38µl DharmaFECT 4 siRNA Transfection Reagent (GE Healthcare Lafayette, CO) in DMEM F12 medium without Pen/Strep and serum; kindly mix and incubate 5 minutes RT.

Mix together the solutions and incubate 20 minutes RT.

Add the transfection mix to DMEM F12 medium with 10% serum, 1% L-glutamin and without Pen/Strep and 0,4 mg/ml G418 containing cells previously counted, till reaching a final volume of 12000 µl at 500000 cells/ml.

Incubate the solution containing cells, medium and transfection mix for 30 minutes and then seed 500000 cells/ml in 96 and 12 well plates. Incubate cells at 37°C for 24h.

Day 2:

Add DMEM F12 medium with 1% L-glutamin, without serum, Pen/Strep and G418 with or without 25 µM CDCA and incubate O/N at 37°C.

Day 3:

RNA extraction of 12 well plates and Real Time PCR quantification of the target gene silenced by siRNA.

96 well plates cells were lysed using briteliteTM plus (Perkin Elmer, Monza, Italy) and RLU were detected with EnVision (Perkin Elmer Waltham, MA). Data were normalized on the total protein amount by BCA assay.

Hepa 1-6

Hepa 1-6 is produced by ATCC (American Type Culture Collection, Manassas, VA) and it is a derivative of the BW7756 mouse hepatoma that arose in a C57/L mouse.

Manteinance medium:

Dulbecco's Modified Eagle's Medium Advanced with fetal bovine serum to a final concentration of 10%, 2% L-Glutamine, 1% Pen/Strep.

Temperature: 37.0°C

Subculturing protocol:

- Remove and discard culture medium.
- Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
- Add 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5-7 minutes).
  Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

- Add 7.0 ml of complete growth medium and aspirate cells by gently pipetting.
- Add appropriate aliquots of the cell suspension to new culture vessels. Inoculate at a cell concentration between 1.5 X 10^5 and 1.0 X 10^6 viable cells/75 cm^2.

Incubate cultures at 37°C.

Medium Renewal: Every 2/3 days.

Transfection protocol:

Day 1:

320000 cells per well are seeded in DMEM Advanced medium with 10% of serum, 2% L-glutamin and 1% Pen/Strep.

Day 2:

Preparation of transfection mix, final volume 200µl consisting of:
100µl of a solution containing siRNA 2 µM in DMEM Advanced medium without Pen/Strep and serum; kindly mix and incubate 5 minutes RT.
100µl of a solution containing 4.8µl DharmaFECT 4 siRNA Transfection Reagent (GE Healthcare Lafayette, CO) in DMEM Advanced medium without Pen/Strep and serum; kindly mix and incubate 5 minutes RT.
Mix together the solutions and incubate 20 minutes RT. Add the mix to each well together with 1800 µl DMEM Advanced medium with 10% serum, 2% L-glutamin and without Pen/Strep. Incubate cells at 37°C for 6h. Change medium with DMEM Advanced medium with 10% serum, 2% L-glutamin and 1% Pen/Strep. Incubate at 37°C per 48h.

Day 4:

RNA extraction and Real Time PCR quantification of the target gene silenced by siRNA.

PRIMARY HEPATOCYTES

Isolation from mouse livers

Mouse hepatocytes were isolated using a two-step collagenase perfusion method. Animals were first anhestesized with ketamin/xylazin (attachment IV of Directive 2010/6 3/UE), then liver has been *in situ* perfused by mean of caval vein incannulation (using a caliber 26 G needle) with Liver Perfusion Medium (Gibco, Life Tecnologies Paesley, Scotland) supplemented with 100UI of penicillin G/mL and 100µg of streptomycin/mL at 37 °C. Perfusion was carried on for 10 minutes using a peristaltic pump (Gilson, Middleton, USA) setted at 15 ml/min of flow rate. Liver was then perfused for ten 10 minutes with Liver Digestion Medium (Gibco, Life Tecnologies Paesley, Scotland) at 37° C.

Digested liver has been removed from mouse body, gallbladder has been eliminated and, under sterility conditions, was moved to a 10 cm petri dish with 10 ml of Leibovitz L-15 medium (Sigma Aldrich St. Louis, MO, USA) added with 5% of fetal bovine serum (FBS).

Liver has been disgregated with 2 microscopy glasses to allow the extraction of hepatocytes from parenchimal tissue, then to harvest completely the hepatocytes, 2 washes with 10 ml of Leibovitz's L-15 were carried on. The obtained suspension has been filtered through a plastic mesh with 100 µm pores and collected in a 50ml falcon tube.

The test tube was then centrifuged at 1500 rpm for 2 minutes. Hepatocytes pellet was washed twice with 10 ml of Hepatocyte Wash Medium (Gibco, Life Tecnologies Paesley, Scotland) added with 100 UI of penicillin G/mL and 100 µg of streptomycin/mL followed by a centrifugation at 1500 rpm for two minutes . The supernatant was eliminated after every rinse. Hepatocytes pellet was suspended with 10 mL Percoll 42% (Sigma Aldrich St. Louis, MO, USA)

(diluited in sterile phosphate buffer, PBS) and centrifuged at 1500 rpm for 2 minutes: the cells that were still alive after the process were set in the bottom part of the tube. After removing the supernatant, another rinse was carried on with 10 mL of Hepatocyte Wash Medium added with 100 UI of penicillin G/mL and 100 µg of streptomycin/mL. Vital hepatocytes were re-suspended in 10 mL of Hepatocyte Attachment Medium (HAM), Williams medium E (WME) (Gibco, Life Tecnologies Paesley, Scotland) added with 5% of FBS, 100 UI of penicillin G/mL and 100 µg of streptomycin/mL, centrifuged at 1500rpm for 2 minutes and stored at 4 °C. To obtain the desired cell concentration, isolation process has been performed on 4 different animals. Hepatocytes viability was evaluated by trypan blue dye (viability should be grater then 90%) preparing a mix containing 85 µl of HAM, 5 µl of trypan blue and 10 µl of isolated hepatocytes. After isolation, hepatocytes from different livers were put in the same 50 ml tube, suspended in 10 mL of Hepatocyte Attachment Medium (Gibco, Life Tecnologies Paesley, Scotland), counted in Burker's chamber and then plated in 12 wells plates (360.000 cell/mL), coated with type I collagen (BD Biosciences). Cells were incubated at 37°C with 5% of CO_2. After 2 hours, medium has been changed with ***FBS-containing*** WME (WME added with 5% FBS, 100 UI of penicillin G/mL, 100 µg of streptomycin/mL, 2mM L-glutamine, 0,1 µM desametasone, 10µg insuline /mL, 5,5 µg transferrin/mL and 6.7 ng of selenium/mL) and hepatocytes were incubated for 4h at 37°C (Fig. 10).

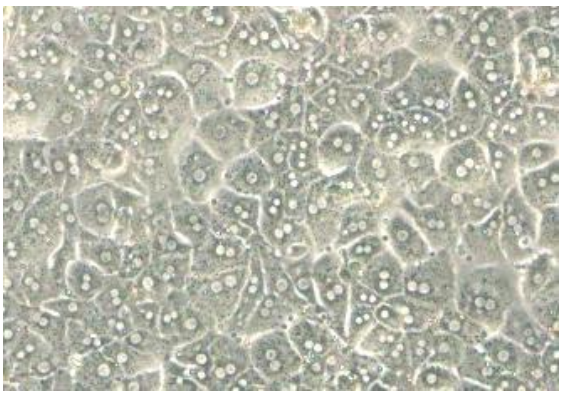


Fig. 10: Optical Microscopy of primary hepatocytes obtained with the protocol described above.

Primary hepatocytes trasduction with Adenoviruses

Hepatocytes were stored for about 4 hours in FBS WME, then the adenovirus treatments were performed each containing a different shRNA sequence (short hairpin RNA) specific for silencing Hdac1, 3, 4, 5, 7 and corepressor Smrt. Treatment was carried on four preparations following this experimental conditions:

AdLacZ: transduction control reference.

AdHdac1: contains Hdac1 specific shRNA.

AdHdac3: contains Hdac3 specific shRNA.

AdHdac4: contains Hdac4 specific shRNA.

AdHdac5: contains Hdac5 specific shRNA.

AdHdac7: contains Hdac7 specific shRNA.

AdSmrt: contains Smrt specific shRNA.

AdHdac4/5/7 co-treatment: contains Hdac4, 5 and 7 specific shRNA.

For every transduction reaction well, 100 MOI (Molteplicity Of Infection) of virus were added. MOI determine the viral particle number available for each cell transduction. Solutions containing the viral vectors were stored at -80°C and everyone has been characterized by means of a specific viral title expressed as PFU (plaque forming units). The following formula was used to calculate the exact amount of adenovirus solution that has to be added in each reaction well to obtain the correct MOI:

MOI x n°cells/mL = total PFU needed

(total PFU needed) / (PFU/mL) = mL adenovirus solution / well

24h after trasduction the medium was removed and changed with FBS-WME without adenoviruses for other 24h. At the end of this period the medium was again substituted with FBS-free WME.

72h after cell seeding, these were lysed with TRIzol® (Life Technologies) for mRNA extraction.

ADENOVIRUSES

ADENOVIRUSES PRODUCTION and PRIMARY HEPATOCYTES TREATMENT

Adenoviruses (Ad) were produced following BLOCK-iT™ Adenoviral RNAi Expression System (Life Technologies):

LR recombination was performed between pENTR/U6 entry construct and pAd/BLOCK-It DEST to generate the pAd/BLOCK-It expression construct (Fig. 11).

The purified plasmid was digested with Pac I to expose ITRs.

293A producer cell line was transfected with adenoviral expression clone till they showed 80/90% of CPE (cytophatic effect), occurring typically 14 days post-infection. Cell-containing medium was collected, transferred in centrifuge tubes thus lysed by means of 3 thaw/dethaw cycles, in which cells were freezed at -80°C for 30 min and then heated at 37°C for 20 minutes.

Cell lysates were then centrifuged at 4000 rpm for 12 min at 4°C, the supernatant, the crude viral lysate, was collected and the titer of the adenoviral stock was determined. Crude viral lysate of each AdHDACs produced was used to transduce primary hepatocytes cultures (Fig. 12).

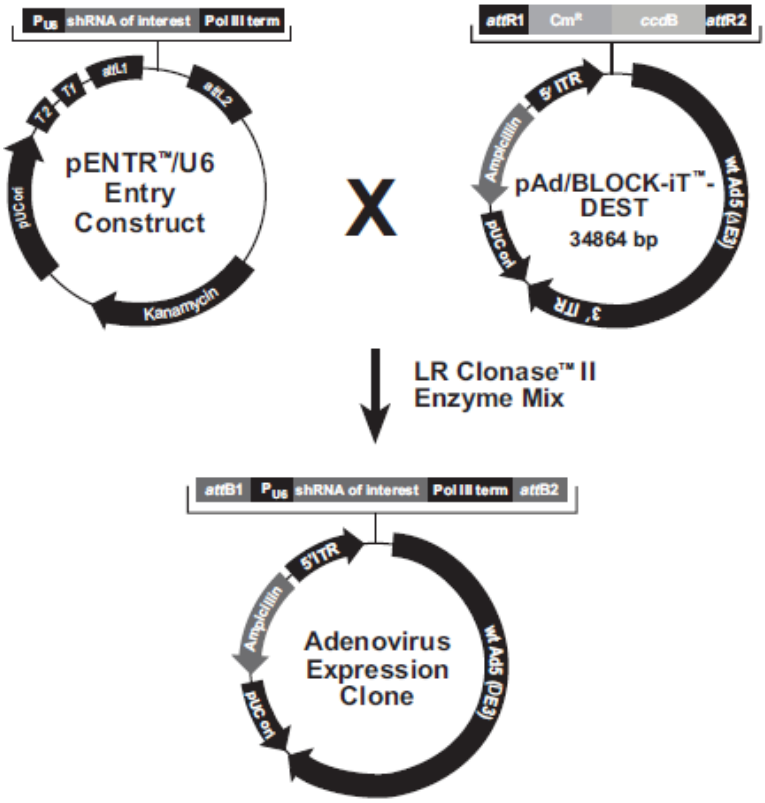


Fig. 11: BLOCK-iT™ Adenoviral RNAi Expression System

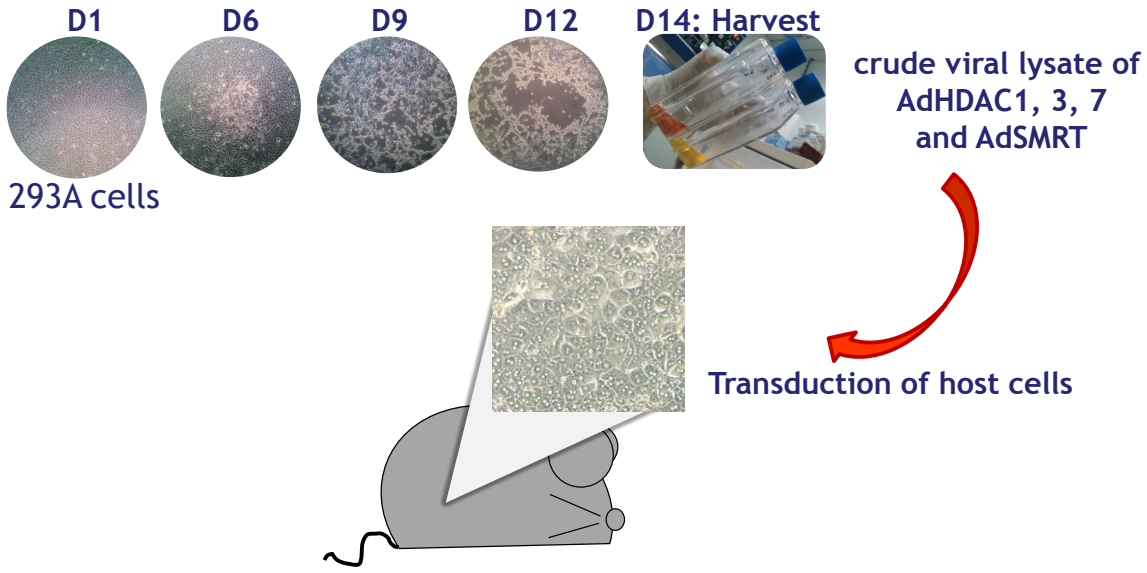


Fig. 12: Crude viral lysate production and transduction of host cells.

ADENOVIRUSES AMPLIFICATION and PURIFICATION

Adenovirus Titer Amplification

293A cells were plated in 15 T175 flasks and cultured till 80% of confluence. Infection solution was prepared adding 3 ml of crude viral lysate at 10^9 title to 72 ml of medium; 5 ml of this solution was diluted with 15 ml of medium and added to the cell culture of each flask. When about the 50-60% of cells were detached from the flask bottom, observed visually, the flask was manually shacked till whole the cells were floating. Cell-containing medium was collected and transferred in centrifuge tubes. After a centrifugation at 1000 rpm for 5 min at 4°C the supernatant was removed and the pellet was resuspended in 8ml of sterile PBS.

Cells then were lysed by means of 4 thaw/dethaw cycles, in which cells were freezed at -80°C for 30 min and then heated at 37°C for 20 minutes. The lysed cells were then centrifuged at 4000 rpm for 12 min at 4°C and the supernatant collected for the purification process.

Adenovirus Purification

After amplification Adenoviruses were purified on an ultracentrifuge (Beckmann). The first step provides for a 24000 rpm centrifugation in a SW28 rotor for 4 hours at 4°C on cesium chloride gradient. The full capsides were separated from other remnants and can be collected and prepared for the second centrifugation at 24000 rpm in a SW41 rotor O/N at 4°C on cesium chloride. 1-2 ml of the central band containing Adenoviruses were collected and purified with dialysis process.

Adenovirus Dialysis

Recovered Adenoviruses solutions were inserted in a dialysis membrane (Thermo-Pierce) and placed in a 500 ml beaker containing dialysis buffer consisting of:

BSA
NaCl
Tris-HCl 1M
Bidistilled water
Glycerol

The membrane was left for 24h in the dialysis solution that has been changed every 4 hours. Finally Adenoviruses titer was determined and Ad were stored at -80°C.

GENERATION OF LIVER CONDITIONAL KO MICE H7LivKO

Cre-Lox TECHNOLOGY

Conditional knock-out mice lacking Hdac7 only in the liver were obtained by mean of the Cre-Lox technology, which allows to delete a gene between two loxP sites by using a Cre enzyme that is able to recognize and catalyze the recombination between two specific LoxP sequences. LoxP sites are localized in the introns of the gene to be deleted, so that Cre causes the ablation of DNA sequence between the LoxP sites. Since a total body Hdac7 knock-out mouse is not viable cre is associated at the albumin promoter, so that this enzyme is produced only in the liver leading to the hepatic selective deletion of Hdac7 (Fig. 13).

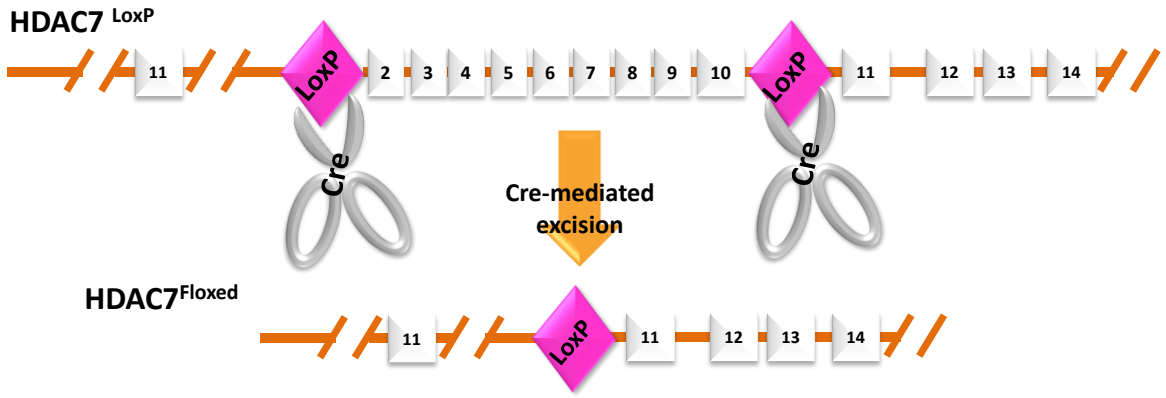
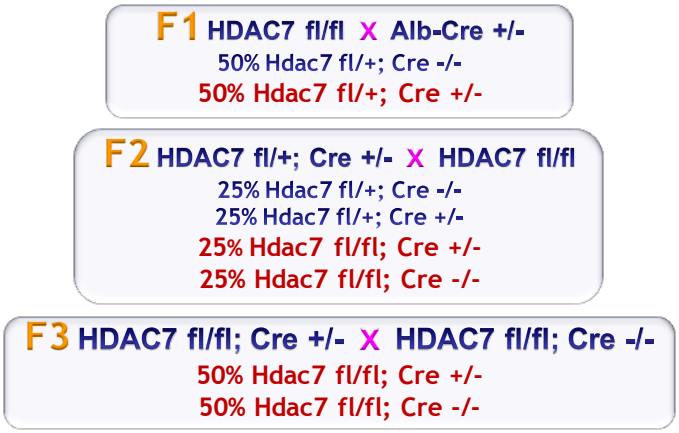


Fig. 13: Cre-Lox technology in the generation of H7LivKO mice

LoxP sites are inserted between exons 1 and 11 of Hdac7 gene. When albumin promoter is active Cre is expressed and HDAC7 gene deleted.

To generate H7LivKO mice we used Alb-Cre mice (Cre+/-) and Hdac7 LoxP/+ (Hdac7fl/+) heterozygous with a C57BL/6J genetic background obtained after crossing mice for 8 generations. Hdac7fl/+ heterozygous were crossed each other to generate Hdac7 LoxP/LoxP homozygous mice (Hdac7fl/fl). Hdac7fl/fl and Cre+/- were crossed as illustrated below to obtain H7LivKO mice (Hdac7fl/fl; Cre+/-).



GENOTYPING

To genotype these mice DNA was extacted from mice tails by NucleoSpin® Tissue (Macherey-Nagel) and quantified with Nanodrop (Thermo Scientific). Two different reaction mix were prepared to verify the presence of Cre and of LoxP on agarose gel (Fig. 14).

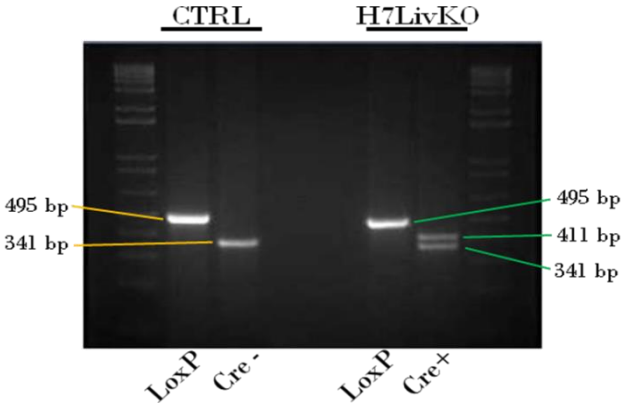


Fig. 14: Agarose gel of LoxP and Cre sequences in H7LivKO and Ctrl mice

495= LoxP; 341= MEH (Microsomal Epoxid Hidrolase, positive control); 411= Cre

PERCENTAGE OF EXCISION by REAL-TIME qPCR

In order to verify the deletion of Hdac7 gene, Cre excision efficiency was evaluated for each mouse. DNA was extacted from mice livers and tails by NucleoSpin® Tissue (Macherey-Nagel) and quantified with Nanodrop (Thermo Scientific). Liver gene expression was compared to the gene expression in the tail, where the gene is not deleted, by Real Time PCR with iScriptTM One Step RT-PCR for Syber (Bio-Rad, Milano, Italia) and 36B4 as housekeeping gene.

Gene	Sequenza
36B4	Fwd 5'-3' AGATGCAGCAGATCCGCAT Rev 5'-3' GTTCTTGCCCATCAGCACC
$Hdac7^{fl/fl}$	Geno-SA 5'-3' GTTGCAGGGTCAGCAGCGCAGGCTCTG Geno-SA 3'-5' CCAGTGGACGAGCATTCTGGAGAAAGGC

The oligonucleotides used for real-time PCR were synthesized by Eurofin MWG Operon (Ebersberg, Germany). Protocol: 4 min at 95°C; 30 sec at 95°C denaturation step; 30 sec at 60°C annealing step; 40 sec at 72°C extension step.

ANIMAL STUDIES

HDAC INHIBITORS TREATMENT

C57BL/6J mice (Charles River Laboratories) were intraperitoneally injected every day for 7 days with DMSO as vehicle, SAHA 25 mpk (mg per kilo), MS275 10 mpk and MC 1568 6,5 mpk. Livers were collected for total RNA extraction and analysis of *Cyp7a1* levels. All the compounds were dissolved in DMSO and stored at +4°C. Prior to use, the solutions were warmed in a 42°C water bath with agitation. Animal studies were conducted strictly following regulations of European Community (Directive 2010/63/EU) and local regulations for animal care (Decreto Legislativo 4 marzo 2014, n. 26).

Adenovirus infection of C57BL/6J

C57BL/6J mice (Charles River Laboratories) were divided in 4 groups (AdLacz, AdHdac1, AdHdac7, AdSmrt) each including 7 animals; they were first anhestesized with ketamin/xylazin. Animal studies were conducted strictly following regulations of European Community (attachment IV of Directive 2010/63/EU) and local regulations for animal care (Decreto Legislativo 4 marzo 2014, n. 26).

Virus titer (pfu/ml)/pfu to inject in vivo = ml of Adenovirus per mouse

ml of Adenovirus per mouse x n° of animals = total amount of Adenoviruses

Add physiological solution to Adenoviruses till a final volume calculated as 100µl x mouse.

100 µl containing 6 * 109 pfu of Adenovirus were intrajugular injected to each mouse. Mice were sacrificed 5 days after infection.

For blood analysis, blood was collected at sacrifice from the heart. Body weight was measured before and post infection. Cholesterol levels were determined using Plasma Cholesterol kit (Sentinel). Livers were collected for total RNA extraction and analysis of *HDACs* knock down and *Cyp7a1* levels.

H7LivKO

Six week old Hdac7 fl/fl;Cre-/- (CTRL) and six week old Hdac7 fl/fl;Cre+/- (H7LivKO) mice were fed for 16 weeks a Western type diet (Harlan), containing 21% milk derived fats, 34% sucrose and 0,2% cholesterol to increase cholesterol levels. Animal studies were conducted strictly following regulations of European Community (Directive 2010/63/EU) and local regulations for animal care (Decreto Legislativo 4 marzo 2014, n. 26). Animals had free access to food and water and the light cycle was from 7:00 A.M. to 7:00 P.M. After 16 weeks mice were anesthetize by mean of xylazin and ketamin and sacrificied with carbon dioxide (allegato IV della direttiva 2010/63/UE). Total RNA was extracted from livers and analyzed via Real-Time qPCR. Plasma, livers and tails were collected at sacrifice from individual animals. Feces were collected for 72h 1 week before sacrifice. Food consumption and body weight were evaluated. Cholesterol levels were determined by the Plasma Cholesterol Kit (Sentinel). Cholesterol distribution in plasma lipoprotein fractions was determined by FPLC using a Superose 6 column (Amersham, Milano, Italia). One-ml fractions were collected and assayed for cholesterol using an enzymatic kit (Sentinel) and triglycerides (Sentinel). An agarose electroforetic analysis of lipoproteins was performed on plasma samples. Liver slices were stained with E&E staining.

LIVER BILE ACID, CHOLESTEROL and TRIGLYCERIDES CONTENT

200mg of livers were homogenized in 1mL PBS in Tissue Lyser II (Qiagen®) and $2x10^5$ disintegrations per minute of [^{3}H]triolein were used to normalize for recovery. Total lipids were extracted by adding 10 mL chloroform/methanol (2:1). The organic phase was recovered and dried, and radioactivity was counted via liquid scintillation Tri-Carb 2100 TR (Perkin Elmer®). For the determination of BA, triglycerides and cholesterol content in liver extracts, samples were diluted 20-fold with 65 mM phosphate buffer (pH 7.0). BA, triglycerides and cholesterol concentrations were measured with enzymatic kits (Sentinel).

RNA EXTRACTION and REAL-TIME qPCR

Total RNA from murine primary hepatocytes, Hepa 1-6 cells and HepG2 2.2.1 luc and from mouse livers was double extracted with TRIzol Reagent® (Invitrogen), purified with commercial kit (Macherey-Nagel, Milano, Italia), and quantified with Nanodrop (Thermo Scientific, Wilmington, DE). Specific mRNA was amplified and quantitated by real time PCR, using iScriptTM One Step RT-PCR for Probes (Bio-Rad, Milano, Italia), following the manufacturer's instructions. Primer sequences are available on request. Data were normalized to 36B4 mRNA and quantitated setting up a standard curve.

Experiments were performed in triplicate and repeated at least twice with different cell preparations. Primers for real-time PCRs were designed by IDT

software available on line optimized to work in a one-step protocol (10 min at 50°C for reverse transcription, 40 cycles of amplifications each consisting of a denaturation step at 95° C for 10 s and an annealing/extension step at 60° C for 30 s). The oligonucleotides used for real-time PCR were synthesized by Eurofin MWG Operon (Ebersberg, Germany).

IMMUNOHISTOCHEMISTRY

Liver was fixed with Carnoy solution/Clorophorm, dehydrated, and embedded in paraffin, and 8 μm thick sections were subjected to hematoxylin-and-eosin staining. For immunohistochemistry, liver slices containing 8 μm sections were deparaffinized with xylene and rehydrated through graded ethanol. Antigen retrieval was performed with HCl 1N for 10 min on ice, 10 min at RT and with HCl 2N for 20 min at 37°C. Blocking step was performed incubating the slices in 5% BSA in TBS 1X for 2 hours RT. Polyclonal rabbit anti HDAC7 antibody (Abcam) was applied at 1:20 concentration in BSA 3% TBS O/N at 4°C. Slices were subsequently incubated with Alexa Fluor 488 goat anti-rabbit IgG (diluted 1:250 in TBS 3% BSA) (Invitrogen) was added as a secondary antibody for 1 hour at RT. To stain the nuclei slices were incubated with Hoechst 1:1000 in TBS 1X for 30 min RT. Slices were analyzed with a confocal microscope (Nikon Eclipse TE2000-S, Radiance 2100, Bio-Rad Laboratories). Image acquisition was performed with Laser Sharp software (Bio-Rad Laboratories).

LC-MS/MS BASED ASSAY for THE QUANTITATION of ENDOGENOUS BILE ACIDS

BA were quantified by means of a commercial kit sold by Biocrates Life Sciences (Innsbruck, Austria) that uses liquid chromatography coupled with mass spectrometry as detector. BA were quantified starting from 3 different matrix: Feces, Liver, Plasma. All these tissues were collected and treated differently due to their heterogeneity.

Feces sample preparation

Feces were collected from cages, every stool sample came from a single animal. Feces collection lasted 72h, the sample was homogenized and lyophilized. About 50mg of this sample were used for BA extraction with about 1ml of methanol, homogenized in Tissue Lyser II (Qiagen®) and centrifuged. 500µl of the supernatant were dried under nitrogen and the remnants dissolved in 40µl of methanol. A 10µl aliquot was transfered into the kit provided plate.

Liver sample preparation

About 200 mg of liver were extracted for the quantification of BA with 1ml of methanol, homogenized using a Tissue Lyser II (Qiagen®) and centrifuged. 500µl of the supernatant were dried under nitrogen and the remnants dissolved in 40µl of methanol. A 10µl aliquot was transfered into the kit provided plate.

Plasma sample preparation

Plasma samples were collected after whole blood centrifugation. Being already in solution plasma didn't need any further treatment and was directly transferred into the kit provided plate.

Kit parameters

LC-MS/MS platform: ABSciex API 4000 (ABSciex)

Ionization mode: Negative

Chromatographic column: column for Absolute IDQ Bile Acid Kit (BIOCRATES) equipped with Security Guard ULTRA Cartridge C18/XB-C18 (Phenomenex)

Sample preparation: Direct extraction on Biocrates 96 well Kit filter plate, ca. 1.5 hours

Sample Volume: 10 μl

Run time: 11 min/ sample

IS: 10 isotope labeled internal standard, provided with the kit

Mobile Phases:

A. MilliQ water (10mM NH4Ac, 0,015% formic acid)

B. CAN/MeOH/Water 65/30/5 (10mM NH4Ac, 0,015% formic acid)

STATISTICAL ANALYSIS

Statistical analyses were performed by Student's t test for the comparison of two different experimental groups or one-way ANOVA with the indicated post-

test for multiple testing comparisons. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as Mean +/- Standard Deviation; $p < 0,05$ was considered statistically significant.

RESULTS

MODULATION of CLASS I HDAC ACTIVITY with SELECTIVE HDAC INHIBITORS TOTALLY PREVENTS BA REPRESSION of CYP7A1 TRANSCRIPTION in vitro and INCREASES the EXPRESSION of Cyp7a1 in vivo

As previously published by our laboratory, HDACs are key regulators of *CYP7A1* gene transcription that in response to BA are recruited on the promoter of this gene leading to the inhibition of its transcription (Mitro et al., 2007). Since, it has been shown that only class I and class II HDACs are involved in the regulation of *CYP7A1*, to go further in the understanding of this regulatory mechanism, my first objective was to investigate whether either class I or class II of HDACs played a predominant role in the regulation of *CYP7A1* expression.

Therefore, to study the involvement of the specific classes of HDACs in the transcriptional regulation of *CYP7A1* gene promoter in the native chromatin context, I used a stable human hepatoma reporter cell line, HepG2 2.2.1 luc, containing the human *CYP7A1* promoter upstream of luciferase gene. The promoter activity of the luciferase reporter gene construct was studied in response both to chenodeoxycolic acid (CDCA) and to three different HDAC-inhibitors. In particular I used the pan-inhibitor SAHA, that inhibits both class I and class II, the class I selective inhibitor MS275 and the class II selective inhibitor MC1568 (Fig. 15).

These data showed that the decreased expression of h*CYP7A1* that occured after BA treatment was totally prevented in presence of the pan-inhibitor SAHA. Same results were obtained after treatment with the class I selective inhibitor MS275, that moreover greatly increased h*CYP7A1* basal level. On the other hand the treatment with the class II selective inhibitor MC1568 only elicited a partial recovery of *CYP7A1* promoter driven transcription. These data

highlight the peculiar role of class I HDACs in the regulation of h*CYP7A1* gene transcription in response to BA.

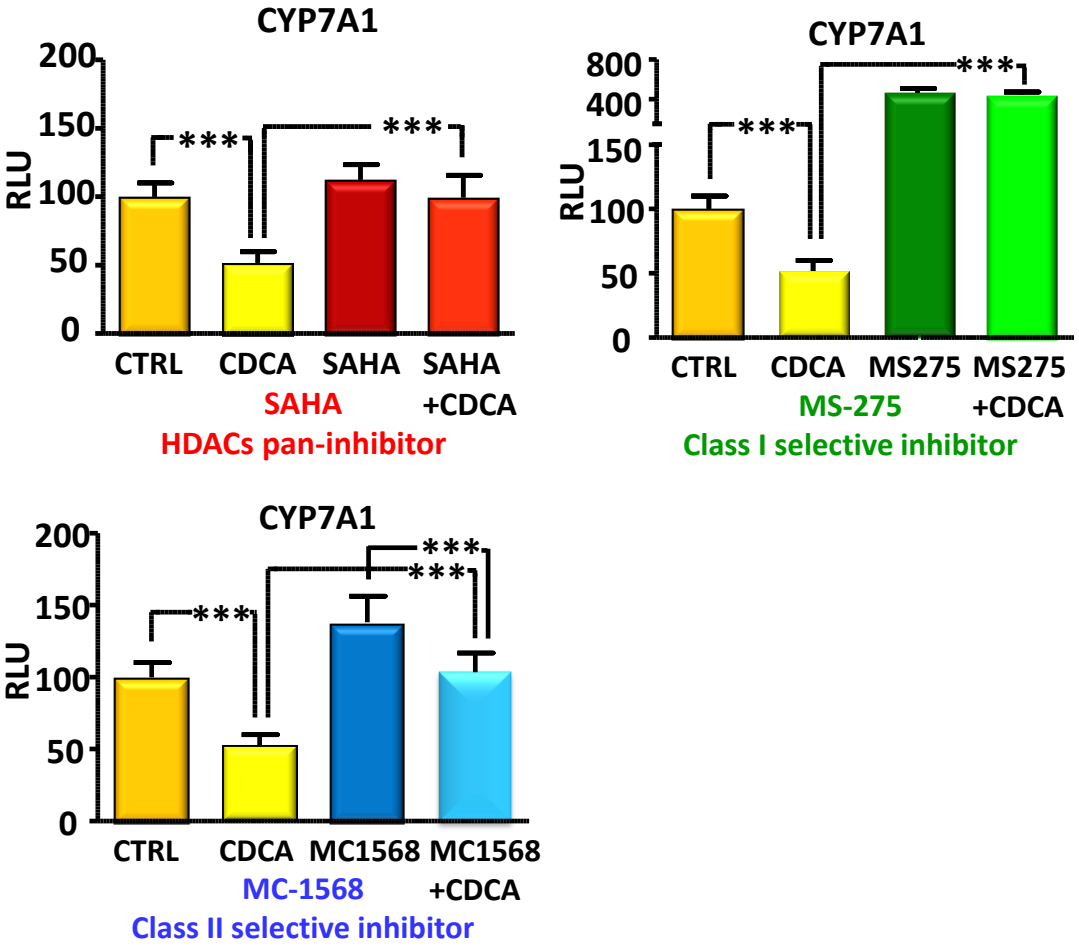


Fig. 15: Class I selective HDAC-i MS275 totally prevents the repressive effect of BA on h*CYP7A1* expression in HepG2 2.2.1 luc cell line

Evaluation of h*CYP7A1* transcriptional activity by luciferase activity assay in HepG2 2.2.1 luc cell line treated O/N with DMSO 0,1% as control, CDCA 25 μM and the three HDAC inhibitors, SAHA 2.5 μM, MS275 5μM or MC1568 5μM, alone and in combination with CDCA 25 μM. Data are expressed as percentage of luciferase activity compared to control (CTRL) and they are normalized on the total protein amount quantified by BCA assay. Representative data shown are the averages of six replicate assays in three experiments. Error bars indicate standard deviation from the mean. ANOVA statistical analysis was performed; *** indicates statistical significance with $p<0.001$.

To investigate the contribution of class I and II HDACs *in vivo*, I administered the same HDAC inhibitors by IP injection on C57BL/6J mice. The treatment with the class I selective HDAC-inhibitor MS275 showed a statistically significant increase of liver *Cyp7a1* gene expression also *in vivo*, confirming the *in vitro* data (Fig. 16), nonetheless I observed a mild induction of *Cyp7a1* gene expression, albeit not statistically significant, even after SAHA and MC1568 administration.

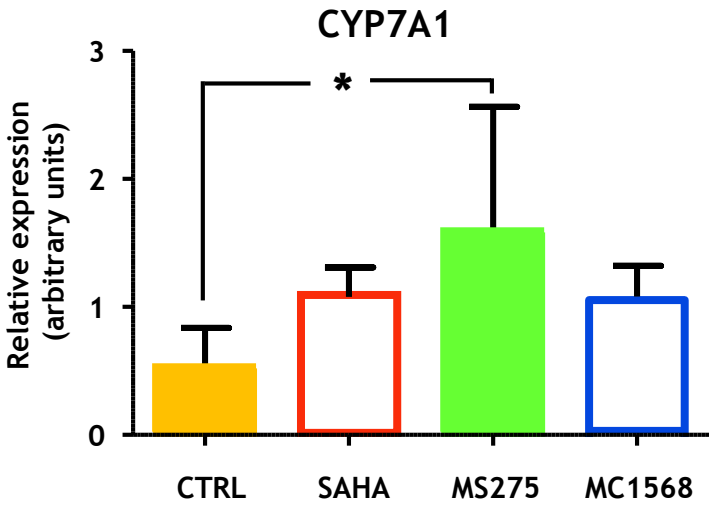


Fig. 16: Class I selective HDAC-i MS275 increases mCyp7a1 expression in vivo

mC*yp7a1* mRNA expression in C57BL/6J mice treated every day for 7 days with DMSO as vehicle, SAHA 25 mpk, MS275 10 mpk and MC1568 6,5 mpk. Liver samples were analyzed by real-time Q-PCR to determine mRNA levels. Data are expressed as mean ± SD. ANOVA statistical analysis was performed; * indicates statistical significance with $p<0.05$.

SILENCING of HDAC1, HDAC3 and HDAC7 INCREASES hCYP7A1 TRANSCRIPTION in HepG2 2.2.1 Luc

To corroborate the results obtained with HDAC inhibitors and to pinpoint the role of specific HDACs involved in the regulatory mechanism of *CYP7A1* gene transcription, I used an RNAi approach. I knocked-down HDACs using siRNA oligonucleotides silencing HDAC1, 3 belonging to Class I, and HDAC 7 belonging to Class II in HepG2 2.2.1 luc cell line.

After confirming their efficiency to silence their target gene expression (Fig. 17a), I measured the promoter activity of the luciferase reporter gene construct both in presence and in absence of BA.

As shown in Fig. 17b, I observed that HDAC1 silencing highly increased h*CYP7A1* transcription and it totally prevented BA repressive effect on the expression of this gene, highlighting an important role of HDAC1 in the regulation of h*CYP7A1* gene and pointing out the results previously obtained with class selective HDAC inhibitors. HDAC3 and HDAC7 silencing slightly increate h*CYP7A1* expression as an evidence of their involvement in the regulatory pathway of h*CYP7A1* transcription. Moreover, HDAC3 knock-down showed only a not statistically significant trend of prevention of BA effect while HDAC7 knock-down elicited a partial but significant recovery of h*CYP7A1* after BA treatment suggesting its peculiar role in this pathway.

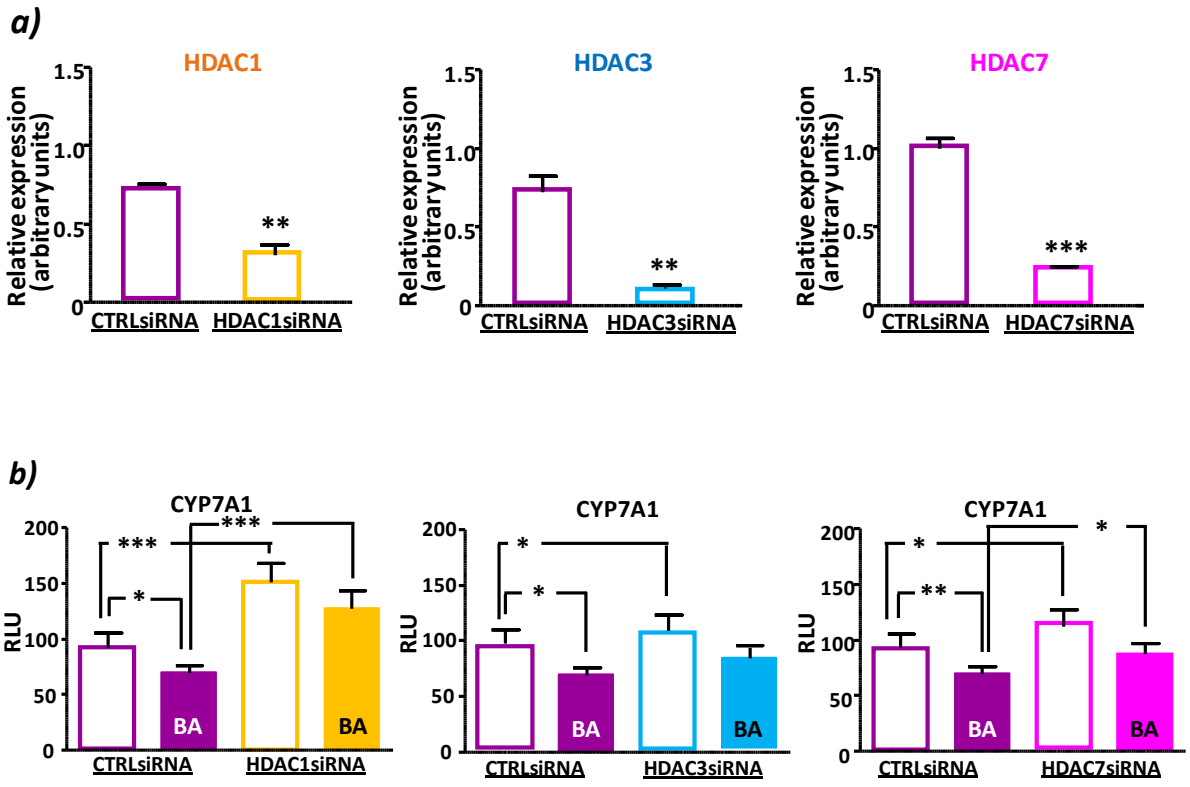


Fig. 17:

a) HDACs silencing by siRNA oligonucleotides in HepG2 2.2.1 luc cell line

Q-PCR determination of mRNA expression of HDAC1, HDAC3 and HDAC7 in HepG2 2.2.1 luc cell line after treatment with the respective siRNA sequences compared to control scramble (CTRLsiRNA); HDAC3 and HDAC7 silencing is higher than 85% and HDAC1 silencing is 60%. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; ** indicates statistical significance with $p<0.01$; *** indicates statistical significance with $p<0.001$.

b) h*CYP7A1* promoter activity in HepG2 2.2.1 luc cell line treated with siRNA oligonucleotides vs HDAC 1, 3 and 7

Evaluation of h*CYP7A1* transcriptional activity by luciferase activity assay in HepG2 2.2.1 luc cell line treated with HDAC1, HDAC3 and HDAC7 siRNA sequences 100nM alone or together with CDCA 25 μM, compared to control scramble (CTRLsiRNA). Data are expressed as mean ± SD. ANOVA statistical analysis was performed; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$; *** indicates statistical significance with $p<0.001$.

Hdac1, 7 and Smrt SILENCING SIGNIFICANTLY INCREASES Cyp7a1 EXPRESSION in MURINE PRIMARY HEPATOCYTES

To unravel the role of specific HDACs and corepressors *in vivo* and to study their contribution in *Cyp7a1* regulation, I planned to perform silencing experiments *in vivo* by using Adenoviral vectors expressing shRNA.

To this end I first identified murine siRNA oligonucleotides silencing *Hdac 1, 3, 7* and the corepressor *Smrt* by testing them in a murine model of hepatoma, Hepa 1-6 cell line. Three different siRNA sequences for each gene were analyzed and I selected the ones able to produce the best silencing (Fig. 18), which were used as templates to design shRNA against *Hdacs* and the corepressor *Smrt* to be used *in vivo* in adenoviral vectors.

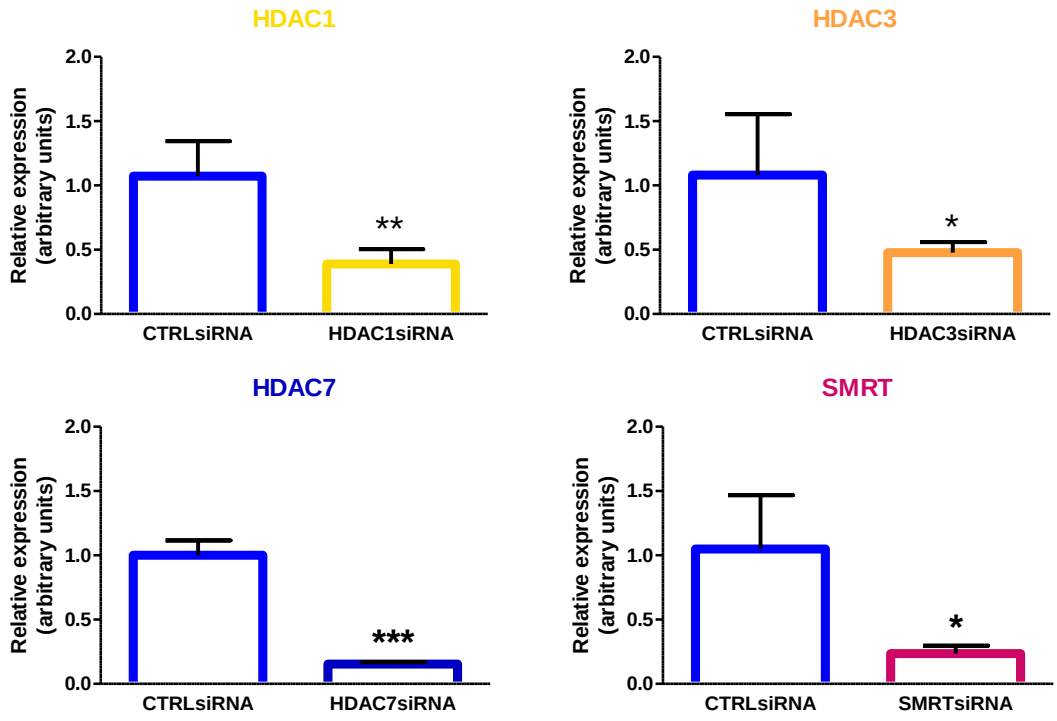


Fig. 18: HDACs gene silencing in murine Hepa 1-6 cell line

Relative expression of genes coding HDACs measured by Real-Time PCR in Hepa 1-6 cells total RNA. Cells were transfected with transfection reagent (CTRLsiRNA) or siRNA 30nM/100Nm. RNA was extracted 48h after transfection. I obtained 80-90% silencing of *Hdac7* gene and a 50-70% silencing of *Hdac1, Hdac3* and *Smrt/N-CoR* genes. Data are expressed as mean ± SD and are normalized with 36b4 as reference gene. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$; *** indicates statistical significance with $p<0.001$.

Based on these siRNA oligonuceotides I designed shRNA sequences silencing murine *Hdac1, Hdac3, Hdac7* and *Smrt* and I cloned them in adenovectors producing the respective Adenoviruses (Ad). Furthermore, since in literature it has been reported that cells can compensate for the lack of Hdac 7 increasing the amount of Hdac 4 and 5 (other two members of class II Hdacs) (Mihaylova et al., 2011), I also produced Ad against *Hdac 4* and *5*. To evaluate their silencing efficiency I treated murine hepatocytes cultures with the Ad, each containing a shRNA specific for the respective target gene silencing. I used 100 MOI (Molteplicity of Infection) of virus, that means the number of viral particles available for one cell trunsduction. Ad allows shRna to enter hepatocytes cytoplasm and to recognize a complementary RNA leading to its degradation. After transduction with adenoviral sh RNA, I analyzed *Hdac1, 3, 4, 5, 7* and *Smrt* gene expression by real time qPCR observing that the transduction significantly silenced *Hdac1, 4, 5, 7* e *Smrt* genes while Ad containing shRNA targeting Hdac3 was not able to silence its target (Fig. 19).

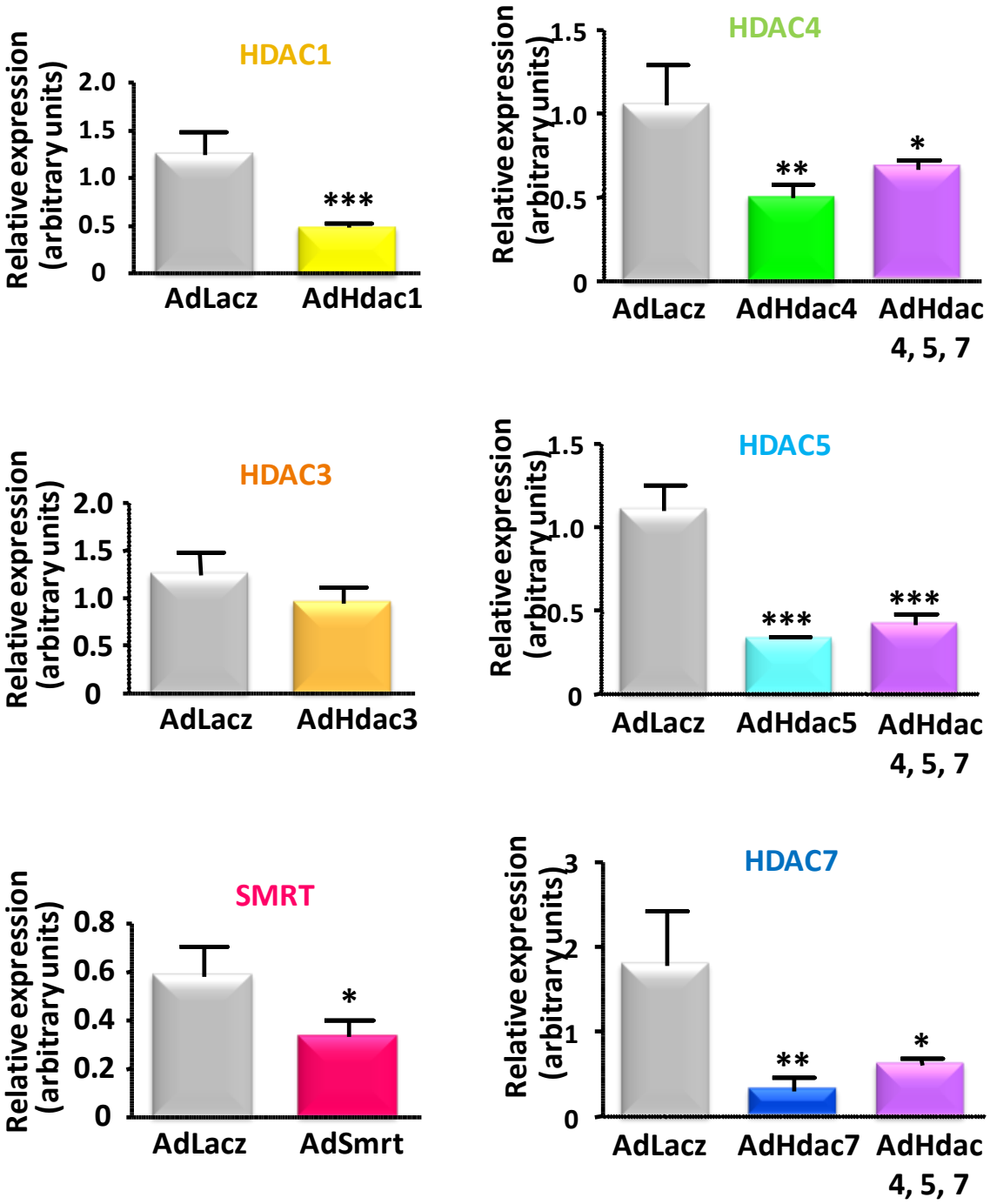


Fig 19: *Hdac1*, *Hdac4*, *Hdac5*, *Hdac7 and Smrt* silencing on murine primary hepatocytes

Relative expression of genes coding HDACs measured by Real-Time PCR on murine primary hepatocytes total RNA. Cells were transducted with the respective Ad (100 MOI) and compared to control AdLacz. RNA was extracted 72h after

transduction. AdHdac1, AdHdac4, AdHdac5, AdHdac7 e AdSmrt reduce the expression of their relative target genes respectively by 62%, 53%, 70%, 83% and 43%. Co-trasduction of AdHdac 4, 5 e 7 is able to silence *Hdac4* by 37%, *Hdac5* by 62% and *Hdac7* by 66%. Data are expressed as mean ± SD and are normalized on 36b4 as reference gene. Student's t test and ANOVA statistical analysis were performed; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$; *** indicates statistical significance with $p<0.001$.

Having confirmed that these Ad expressing shRNAs were able to repress their target gene expression, I investigated the contribution of the silencing of every specific *Hdac* or *Smrt* in the modulation of *Cyp7a1* gene expression in primary murine hepatocytes by real time qPCR in these cells after Ad treatment. *Hdac1, 7* and *Smrt* silencing significantly increased *Cyp7a1* expression. Furthermore, AdHdac4 and AdHdac5 raised *Cyp7a1* transcription but the co-silencing of AdHdac4 and AdHdac5 with AdHdac7 had neither additive nor synergistic effect, suggesting that *Hdac7* silencing is sufficient to induce *Cyp7a1* expression (Fig. 20). Since AdHdac3 was not able to reduce *Hdac3* expression, the contribution of *Hdac3* deletion in *Cyp7a1* regulation could not be investigated.

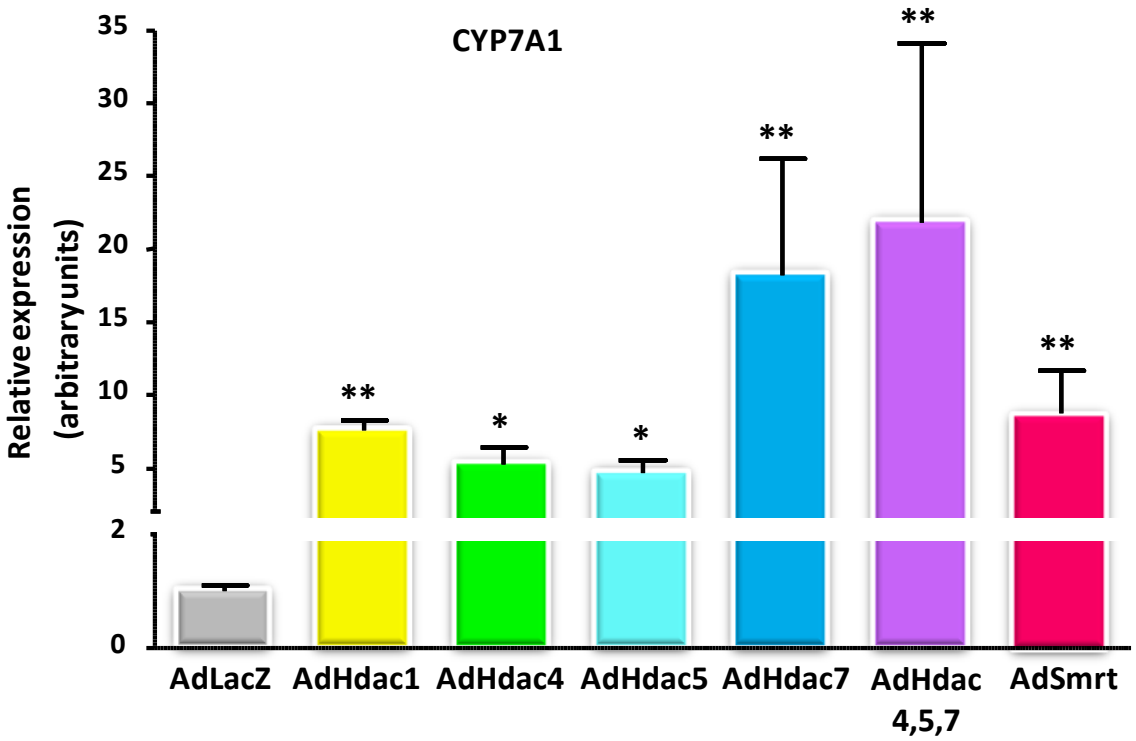


Fig. 20: Cyp7a1 gene expression levels in murine primary hepatocytes treated with AdHDACs and AdSmrt:

Q-PCR determination of *Cyp7a1* mRNA expression on murine primary hepatocytes. Cells were transducted with the respective Ad (100 MOI) and compared to control AdLacz. RNA was extracted 72h after transduction. *Cyp7a1* mRNA levels are 7 fold increased by AdHdac1 compared to the empty vector AdLacZ, 8 fold by AdSmrt and 21 fold by AdHdac7 alone or in co-treatment with AdHdac 4, and 5. Data are expressed as mean ± SD and are normalized on 36b4 as reference gene. ANOVA statistical analysis was performed with Dunnett's Multiple Comparison post test vs AdLacZ; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$.

Hdac7 and Smrt SILENCING SIGNIFICANTLY INCREASES Cyp7a1 EXPRESSION in vivo

To investigate the acute effect of silencing *Hdacs* and *Smrt*, I amplified and purified the Ad used in the experiment described above to proceed with the *in vivo* treatment. C57BL/6J mice were divided in 4 groups, one for each treatment, and injected in the jugular vein with $6*10^9$ pfu of AdLacz, AdHdac1, AdHdac7 and AdSmrt.

Body weight of mice at the beginning (B.I.) and at the end (A.I.) of the Ad infection (at sacrifice) did not yield any difference neither among the groups of treatment nor between B.I. and A.I. (Fig. 21), meaning that Ad did not affect health of infected mice.

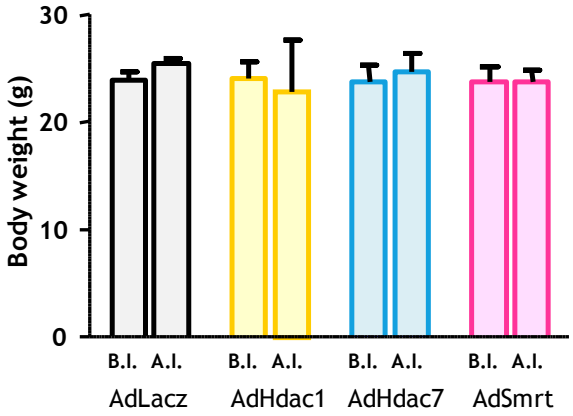


Fig. 21: Mice body weight

Mice body weight before (B.I.) and after (A.I.) infection with Ad in C57BL/6J mice. Data are expressed as mean ± SD. TWO WAY ANOVA statistical analysis was performed and no statistically significant difference was observed.

Real Time PCR analysis of total RNA from liver extracts of mice infected with Ad revealed that AdHdac1 (Fig. 22A) was ineffective in silencing its target gene, whose expression did not significantly differ from control AdLacz. AdHdac7 and AdSmrt infections, instead, efficiently reduced *Hdac7* and *Smrt* expression

respectively (Fig. 22B, C). Therefore, I measured the contribution of these knock-down on *Cyp7a1* expression and I found that AdHdac1, consistent with its inability in silencing its target gene, did not affect the expression of the gene, while AdHdac7 and AdSmrt significantly increased *Cyp7a1* mRNA levels (Fig. 23), confirming their important role in the regulation of BA metabolism also *in vivo*.

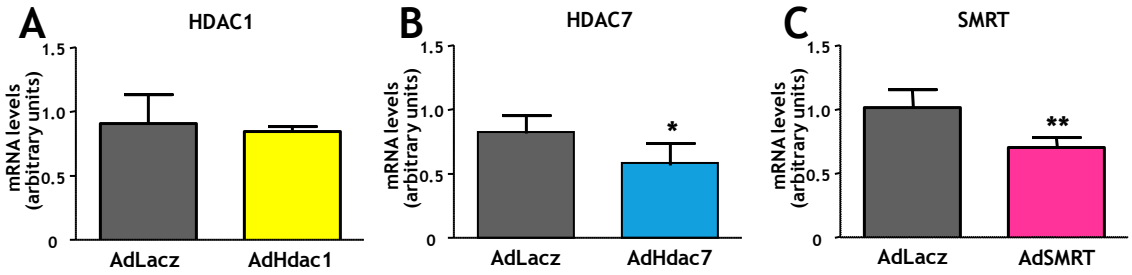


Fig. 22: *Hdac1*, *Hdac7 and Smrt* silencing in C57BL/6J mice

Relative expression of genes coding Hdac1, Hdac7 and Smrt measured by Real-Time PCR. Mice livers collected 5 days after intra-jugular injection with the respective Ad ($6*10^9$ pfu) were compared to to control AdLacz. Data are expressed as mean ± SD and are normalized on 36b4 as reference gene. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$.

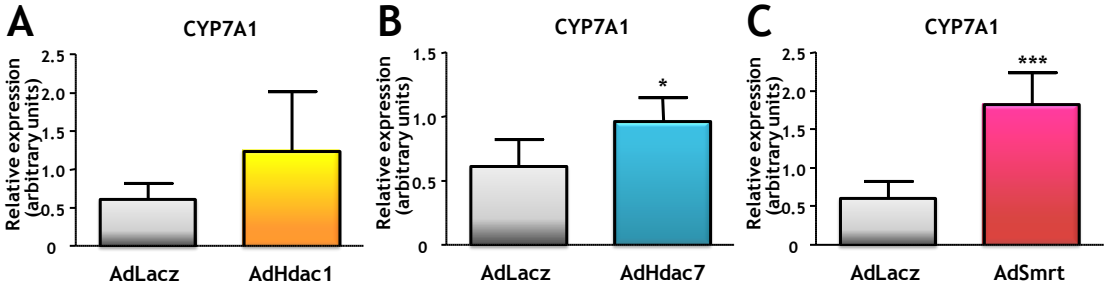


Fig. 23: *Cyp7a1* gene expression levels in C57BL/6J mice treated with AdHDACs and AdSmrt

Q-PCR determination of *Cyp7a1* mRNA expression on liver extracts of mice infected with AdHDACs and AdSmrt. Mice livers collected 5 days after intra-jugular injection with the respective Ad ($6*10^9$ pfu) were compared to control AdLacz. Data are expressed as mean ± SD and are normalized on 36b4 as reference gene. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$; *** indicates statistical significance with $p<0.001$.

Next, I measured total plasma cholesterol in these mice and, as expected, I did not observe differences among the groups, most likely because a 5 days treatment was not sufficient to elicit significant changes in cholesterol metabolism (Fig. 24).

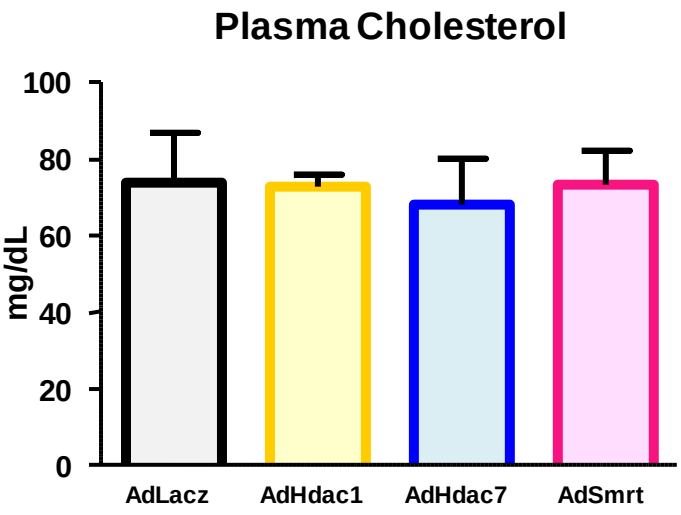


Fig. 24: Plasma Cholesterol levels after Ad treatments

Plasma Cholesterol levels after AdHdacs and AdSmrt treatment at sacrifice reveale any significant change among the groups. Data are expressed as mean ± SD. ANOVA statistical analysis was performed.

HDAC7 DELETION INDUCE PHENOTYPICAL IMPROVEMENT ON CHOLESTEROL and LIPOPROTEIN PROFILE of H7LivKO mice

Previous investigations performed in our laboratory demonstrated that HDAC7 shuttles from the cytoplasm to the nucleus in hepatic cells treated with BA, and subsequently is recruited to *CYP7A1* promoter as part of the repressive complex. This suggests a central role of HDAC7 in the inhibitory feedback of *CYP7A1* mediated by BA and consequently on cholesterol homeostasis (Mitro et al., 2007). Therefore, since it was shown that the total body knock-out is not viable, to investigate the role of HDAC7 *in vivo* I generated HDAC7 liver-specific knock-out mice (H7LivKO) with the Cre/Lox technology. Mice were challenged

with *Western Diet*, a diet enriched in cholesterol with the aim to emphasize the possible effect of *Hdac7* deletion on cholesterol and BA metabolism.

Actual deletion of *Hdac7* was assessed by real time qPCR of liver genomic DNA vs non hepatic genomic DNA. H7LivKO mice with excision percentage lower than 60% were excluded from the study (Fig. 25). Moreover, Hdac7 deletion was confirmed by immunofluorescence analysis observed with confocal microscopy (Fig. 26).

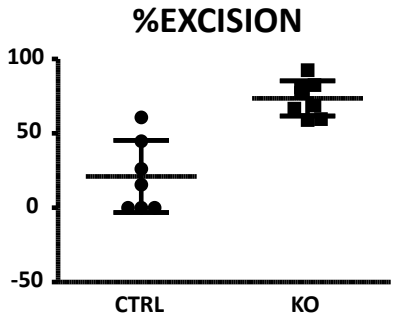


Fig. 25: *Hdac7* percentage of excision

Excision efficiency by Cre recombinase on LoxP sites insered into *Hdac7* gene in the liver was evaluated comparing liver expression to tail expression, in which the gene is not deleted.

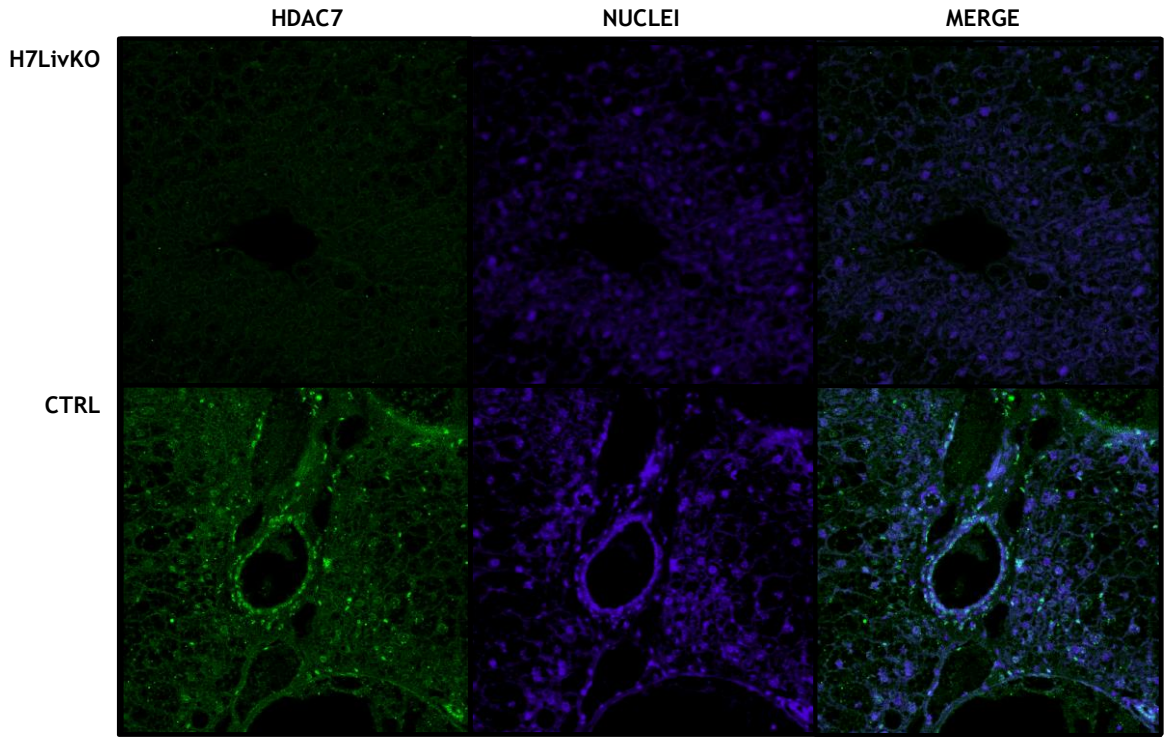


Fig. 26: Hdac7 deletion in H7LivKO

Immunofluorescence analysis of Hdac7 on liver slices of H7LivKO and wild type mice on western diet.

REDUCED BODY WEIGHT in H7LivKO on Western Diet

Body weight analysis showed 12% statistically significant reduction in H7LivKO mice compared to wild type mice (Fig. 27A), despite the equal food consumption (Fig. 27B), suggesting a possible role of HDAC7 on lipid metabolism.

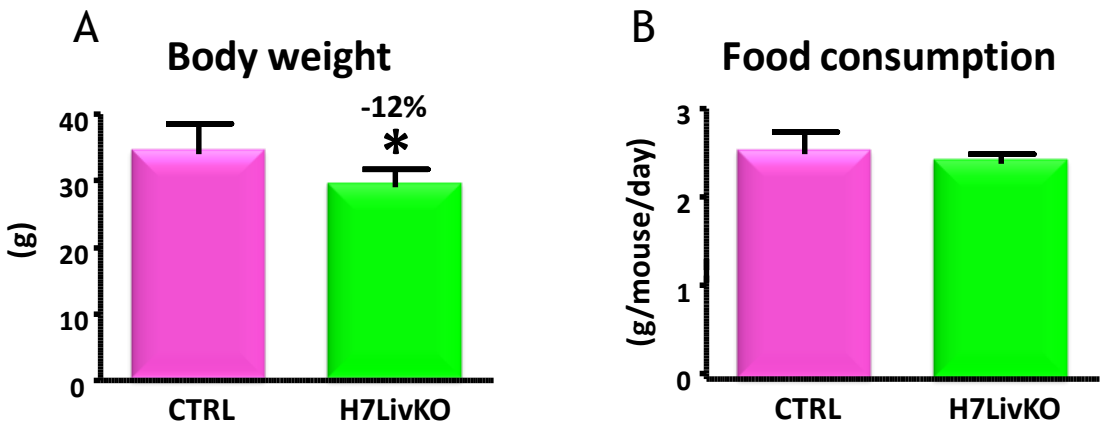


Fig. 27: Body weight reduction in H7LivKO mice fed WD:

Body weight (A) and food consumption (B) in H7LivKO mice compared to wild type mice after 16 weeks on *Western diet*. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$.

REDUCED LDL-CHOLESTEROL in H7LivKO on Western Diet

Mice on *Western diet* (WD) showed total plasma cholesterol levels significantly higher than mice on *Chow diet* (CD); moreover, I observed a statistically significant decrease of total plasma cholesterol, equal to 11%, in H7LivKO mice compared to wild type (wt) mice (Fig 28A).

In light of this evidence, I decided to better characterize this difference by performing Fast Protein Liquid Chromatography (FPLC) to analyze cholesterol levels in the different lipoprotein fractions. The FPLC analysis revealead a reduction of LDL-Cholesterol in H7LivKO mice compared to the respective controls (Fig. 28B).

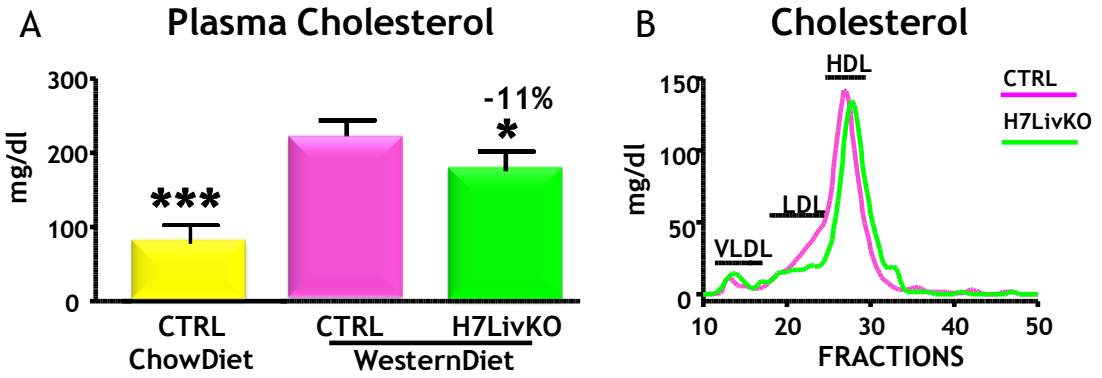


Fig. 28: Plasma cholesterol analysis on H7LivKO mice fed WD

A) Total plasma cholesterol levels of control mice on WD are significantly higher than mice on CD. 11% decrease of total plasma cholesterol is observed in mice lacking *HDAC7* in the liver compared to control on WD. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$.

B) Fast Protein Liquid Chromatography revealed a reduced amount in LDL-cholesterol in H7LivKO mice.

REDUCED LIVER LIPID ACCUMULATION and LIVER CHOLESTEROL in H7LivKO on Western Diet

We next analyzed the hepatic lipid content in wt and H7LivKI mice. Histological analysis by hematoxylin and eosin staining on liver slices highlighted lower lipid accumulation in H7LivKO compared to wt mice (Fig. 29).

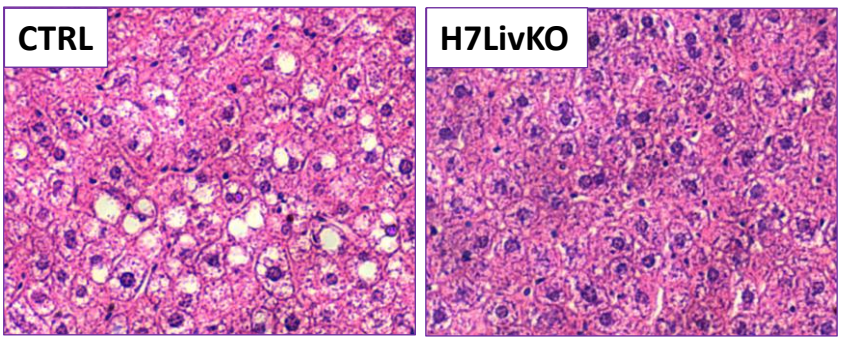


Fig. 29: Liver lipid accumulation

8 µm liver sections with hematoxylin and eosin staining of H7LivKO (right) and wyld type mice (left) on WD.

I extracted and quantified triglyceride and cholesterol levels, typical components of lipid droplets observed in hepatic steatosis. Surprisingly, liver triglycerides were reduced in H7LivKO though the difference did not reach statistical significance (Fig. 30A). On the other hand, I observed a statistically significant reduction of hepatic cholesterol levels in H7LivKO mice compared to controls (Fig. 30B).

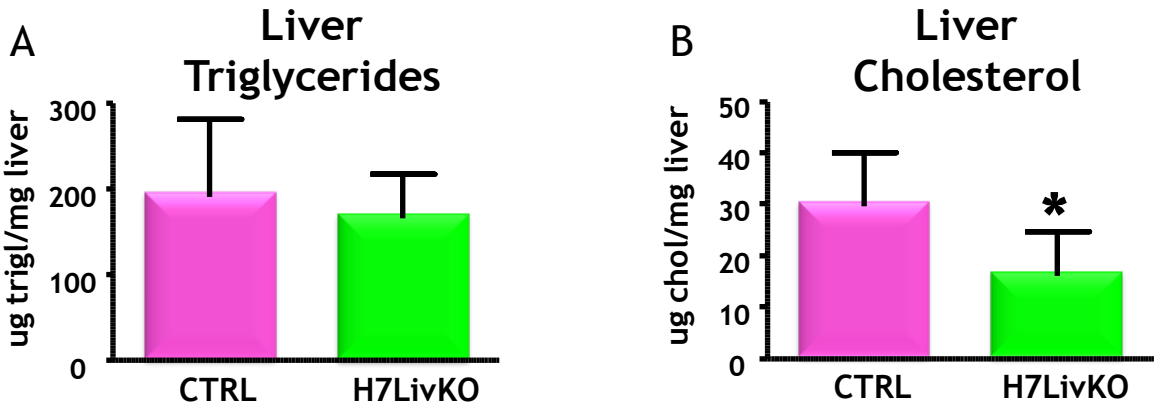


Fig. 30: Hepatic lipid analysis

A) Liver Triglycerides quantification after lipid extraction by methanol:chloroform using ^{3}H-triolein as internal standard. Data are expressed as mean ± SD.

B) Liver Cholesterol quantification after lipid extraction by methanol:chloroform using ^{3}H-triolein as internal standard. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$.

INCREASED Liver BILE ACIDS and Cyp7A1 in H7LivKO on Western Diet

Consistent with hepatic cholesterol decrease in H7LivKO mice, I also observed increased liver BA in knock out vs wt mice. Consistent with these results, *Cyp7a1* expression was higher in H7LivKO vs wt mice (Fig. 31A e B), suggesting that upregulation of the rate-limiting enzyme in BA synthesis resulted in increased BA production.

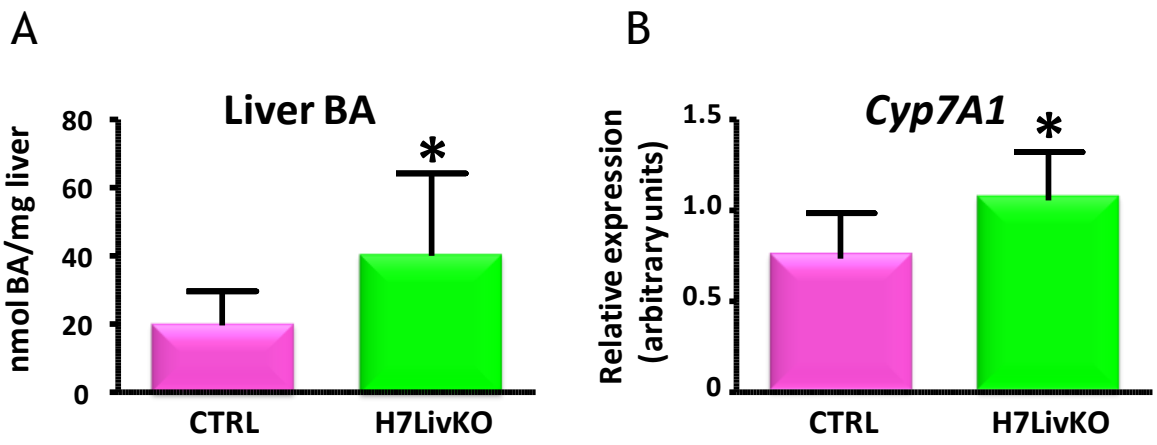


Fig. 31: Increased Liver BILE ACIDS and *Cyp7A1* in H7LivKO on Western Diet

A) Liver BA quantification after lipid extraction by methanol:chloroform using ^{3}H-triolein as internal standard. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$.

B) Q-PCR determination of *Cyp7a1* mRNA expression of liver extracts. Data are expressed as mean ± SD and are normalized on 36b4 as housekeeping gene. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$.

INCREASED LIVER, PLASMA and FECAL BA in H7LivKO on Western Diet

To better define the impact of Hdac7 ablation on BA metabolism, I performed a more detailed analysis of BA by LC-MS/MS for the quantitation of endogenous BA in plasma, liver and feces. Unconjugated BA were significantly increased in plasma of H7LivKO mice compared to wt mice (Fig. 32 A). The analysis of hepatic BA showed increased muricholic acids (MCA), α-MCA and ß-MCA and ursodeoxycholic acid (UDCA), three hydrophilic BA that are antagonists of the BA nuclear receptor FXR (Fig. 32 B). With the exception of cholic acid (CA), lithocholic acid (LCA) and UDCA, the content of the most Almost all the most abundant fecal BA, an index of increased BA biosynthesis, was higher in H7LivKO mice (Fig. 32 C).

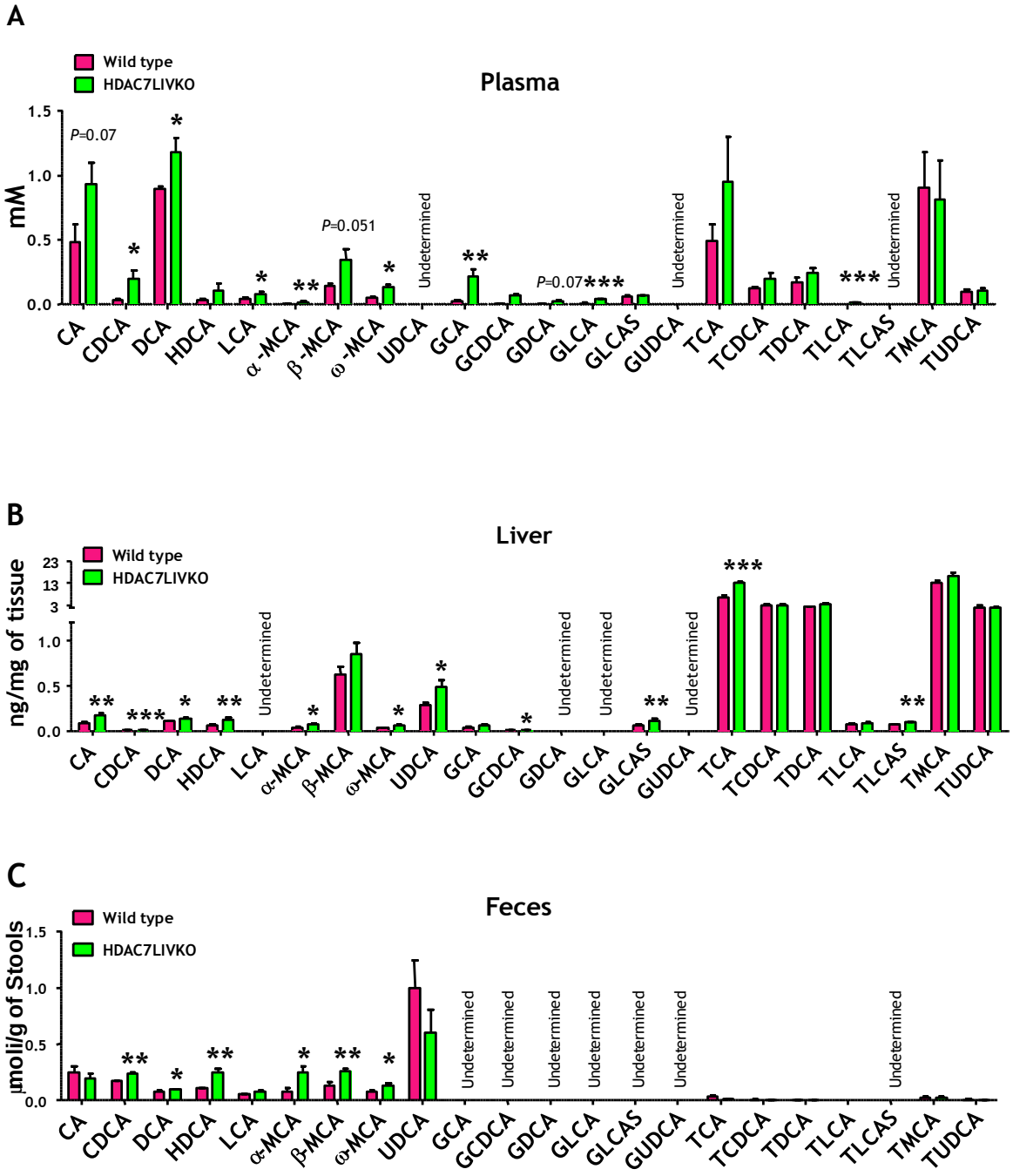


Fig. 32: LC-MS/MS based assay on plasma, liver and feces

BA quantitation of plasma and liver and feces extracts. Data are expressed as mean ± SD. Student's t test statistical analysis was performed; * indicates statistical significance with $p<0.05$; ** indicates statistical significance with $p<0.01$.

H7LivKO SHOW DIFFERENT HDL-CHOLESTEROL PROFILE, RICHER in SMALLER HDL

In depth analysis of lipoprotein subfractions brought up to our attention a different population of HDLs. Since FPLC is an exclusion chromatography technique, the size of molecules eluting in the late fractions, such as the ones displayed in the blue circle of Fig. 33, should be smaller. Therefore, I hypothesized that it might be a smaller population of HDL that is present only in H7LivKO mice.

To better investigate this aspect, agarose electroforesis analysis was performed. As shown in Fig. 34, plasma samples from control mice were more electropositive and enriched in alpha HDL, containing more cholesterol. On the other hand, plasma samples from H7LivKO mice migrated farther than controls, suggesting that they contained smaller HDLs, probably resembling lipid poor pre-beta HDLs.

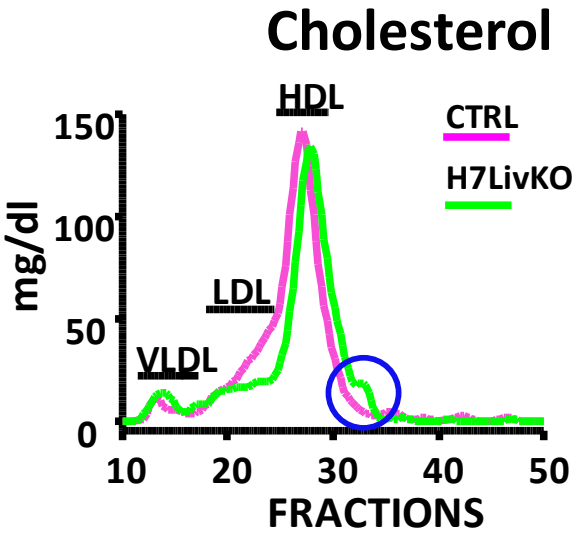


Fig. 33: Fast Protein Liquid Chromatography revealed a smaller population of HDL-cholesterol in H7LivKO mice

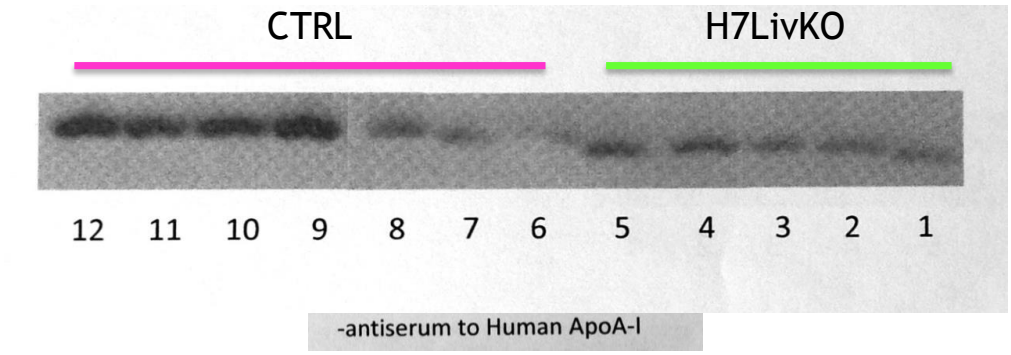


Fig. 34: Agarose electroforesis analysis of plasma lipoproteins

Ab anti ApoA-I was used for the detection of HDLs.

DISCUSSION

This study elucidates some peculiar aspects of the epigenetic transcriptional regulation of *CYP7A1*, the rate-limiting enzyme for cholesterol catabolism, deepening the important involvement of HDACs and corepressors in this pathway. Several epidemiological studies have well established the correlation between hypercholesterolemia and atherosclerosis and cardiovascular disease risk. Great attention is turned to the discovery of new pharmacological approaches aimed at reducing hypercholesterolemia to overcome side effects of the drugs nowadays available and the poor response that some patients develop towards these drugs. In this perspective, a deeper understanding of the genetic and molecular mechanisms involved in the pathophysiological regulation of cholesterol homeostasis is fundamental.

Quantitatively the most important route of cholesterol disposal in mammals is BA biosynthesis (Chiang, 2002). This biosynthetic pathway represents the final step of reverse cholesterol transport; in fact, excess of cholesterol transported to the liver is catabolized to BA, which play an essential role in maintaining cholesterol homeostasis. CYP7A1 is an enzyme expressed only in the liver that represents the major check-point of BA biosynthesis and its impact on cholesterol homeostasis has been fully demonstrated. The homozygous deletion mutation in *CYP7A1* locus in human patients, leading to the loss of the active site and enzyme function, causes hypercholesterolemia with high levels of LDL cholesterol (Pullinger et al., 2002). Moreover, Cyp7a1-tg mice show increased hepatic cholesterol catabolism and increased BA pool size, improving high-fat diet (HFD)–induced obesity, fatty liver and insulin resistance (Li et al., 2010a). In light of this critical role in the maintenance of cholesterol homeostasis, in the last decades many studies have been conducted to elucidate its regulatory mechanisms. Expression of *CYP7A1* gene is feedback inhibited by BA that act on their nuclear receptor FXR that in the liver induces the expression of SHP (Goodwin et al., 2000, Lu et al., 2000c) and in the intestine the release of FGF 15/19 (Inagaki et al., 2005), both leading to *CYP7A1* inhibition.

As previously published by our laboratory, BA also repress *CYP7A1* gene transcription via an FXR-independent pathway, chronologically preceding the

FXR-dependent mechanism. BA induce the dissociation of the coactivators PGC-1α and CBP from HNF-4α, a master regulator of *CYP7A1* (De Fabiani et al., 2003) and simultaneously induce the recruitment of transcriptional corepressors such as NcoR and SMRT, which create a repressive complex on *CYP7A1* promoter together with HDAC1, 3 and 7 (Mitro et al., 2007). These data highlighted epigenetic regulation of cholesterol homeostasis. Epigenetic modifications of chromatin induced by HDACs activity modulate gene transcription, impairing transcriptional activators accessibility to DNA (López-Rodas et al., 1993b, Grunstein, 1997a) and are associated to several pathological conditions, such as cancer (Miremadi et al., 2007), insulin resistance and obesity (Xiang et al., 2004, Gray and Ekström, 2001). In recent years a growing number of studies pointed out the contribution of HDACs in the modulation of lipid (Galmozzi et al., 2013, Knutson et al., 2008, Sun et al., 2011) and BA metabolism and on the regulation of *CYP7A1* (Kemper et al., 2004, Ponugoti et al., 2007). In addition, previous studies from our laboratory showed that HDAC inhibitors (HDAC-i) stimulate *CYP7A1* expression *in vitro* and *in vivo* by preventing the negative feedback exerted by BA, consequently decreasing serum cholesterol in mice (Mitro et al., 2007). Therefore, although the involvement of these enzymes in the regulation of these processes has been demonstrated, several questions remain unsolved. It is still unclear which class of HDACs is mainly involved, what is the contribution of specific HDACs and corepressors in the regulation of *CYP7A1* gene expression and ultimately their impact on BA and cholesterol metabolism.

To elucidate this issues, first of all, in this study I examined the effects of class-selective HDAC inhibitors on the promoter activity of h*CYP7A1 in vitro*. The data obtained suggest a major involvement of class I HDACs in the negative regulation of h*CYP7A1,* since class I selective HDAC inhibitor MS275 was able to totally prevent the BA-induced repressive effect on h*CYP7A1* promoter, while the class II HDAC inhibitor MC1568 yielded only a partial, although significant, recovery of gene transcription. The apparently lower contribution of class II HDACs might be ascribed, on one hand, to the minimal histone deacetylase activity of class IIa HDACs, the subclass involved in this regulation, due to a swap

of a key tyrosine residue in the catalytic domain with a histidine (Lahm et al. 2004); in fact, they mainly act as scaffold molecules to recruit class I HDACs (Nebbioso et al., 2009), thence the inhibition of their deacetylase activity does not impair their actual function. On the other hand, MC1568 has been well described as inhibitor of HDAC4 and HDAC5 (Nebbioso et al., 2009, Scognamiglio et al., 2008), but the role on HDAC7, the most involved in *CYP7A1* regulation among class IIa HDACs, has not been clarified. Therefore, this might explain the only partial recovery of gene expression that we observe after class II selective MC1568 treatment.

Moreover, the RNAi approach in the human hepatoma reporter cell line, HepG2 2.2.1 luc, allowed to investigate the contribution of HDAC1, HDAC3 and HDAC7, identified by Mitro et al. as main constituents of the repressive complex formed on h*CYP7A1* promoter upon BA treatment (Mitro et al., 2007). Although the silencing of these HDACs was able to significantly increase the basal level of h*CYP7A1*, pointing out their relevance in the regulatory mechanism of this gene, only HDAC1 and HDAC7 knock-down prevented the BA-induced inhibition of h*CYP7A1* transcription, highlighting their peculiar role in this pathway. The stronger effect of HDAC1 deletion might be partially due to the loss of interaction of HDAC1 with SHP that weaken SHP repression on *CYP7A1* gene transcription as well; in fact, it has been demonstrated that induction of SHP plays its repressive effect by recruiting Swi/Snf-Brm chromatin remodeling complex, which also contains an mSin3A/HDAC-1, to h*CYP7A1* promoter (Kemper et al., 2004).

In light of the possible differences between human and mouse regulation of cholesterol catabolism, in this study I also pinpointed the role of specific HDACs and corepressors firstly *in vitro* on murine primary hepatocytes and then *in vivo* on C57BL/6J mice. In the *in vitro* study I knocked-down *Hdac1, Hdac3, Hdac7* and *Smrt*. I also knocked down *Hdac4* and *Hdac5*, since it was previously described their possible compensatory role in the absence of *Hdac7* (Mihaylova et al., 2011). Our results underscore the major contribution of Hdac1, Hdac7 and Smrt in the repression of *Cyp7a1* mRNA levels. These evidences suggest HDAC7,

HDAC1 and SMRT as essential actors in the formation of the repressive complex acting on *Cyp7a1* promoter, indicating that their recruitment is critical to the feedback inhibition of *Cyp7a1* gene expression induced by BA.

The effects observed *in vitro* on murine primary hepatocytes were also confirmed *in vivo* in C57BL/6J mice, where AdHdac7 and AdSmrt significantly increase *Cyp7a1* mRNA levels, thus confirming their important role in the regulation of BA metabolism *in vivo* as well. However, AdHdac1 was ineffective in silencing its target gene *in vivo*, therefore further studies will be necessary to assess its contribution in the regulation of *Cyp7a1 in vivo*. In spite of the elevated expression of *Cyp7a1* gene upon administration of AdHdac7 and AdSmrt, no changes of plasma cholesterol levels were observed. This apparent discrepancy is actually ascribable to the adenoviral infection protocol. To avoid the onset of inflammatory response in the liver a single dose of Ad was injected to each mouse and after 5 days mice were sacrificed. Hence, only the *acute* effect of *Hdac7* and *Smrt* deletion on *Cyp7a1* expression could be observed whereas it was not possible to detect changes of plasma cholesterol levels, as they would have need longer time and multiple injections.

Both the *in vitro* and the *in vivo* evidences obtained after silencing, highlighted the role of the corepressor Smrt in the regulation of *Cyp7a1* gene expression. It has been well established, in fact, that NCoR and Smrt recruit HDACs and they repress gene transcription by interacting with class I and class II HDACs (Kao et al., 2000, Huang et al., 2000). It has been demonstrated that Smrt contains a histone interaction domain (HID) and a deacetylase activation domain (DAD) that binds and activates HDAC3, promoting the recruitment of repressive complex on target gene (Yu et al., 2003). Furthermore, it has been demonstrated that corepressors SMRT/N-CoR and mSin3A associate with Hdac7 in yeast and in mammalian cells, suggesting association of multiple repression complexes (Kao et al., 2000). In line with these evidences, this study suggests a peculiar role of the corepressor Smrt that, together with Hdac7 translocating in the nucleus after BA administration, might function as a *platform* in the formation of the repressive complex on *Cyp7a1* promoter.

These evidences together with previous results published by our laboratory showing that HDAC7 shuttles from cytoplasm to nucleus in response to BA and represses *CYP7A1* transcription (Mitro et al., 2007), suggest a key role of HDAC7 in cholesterol and BA metabolism. Therefore, to investigate more extensively the contribution of Hdac7 in the regulation of *Cyp7a1* and its implication in cholesterol homeostasis *in vivo*, I used liver specific *Hdac7* conditional knock-out mice (H7LivKO). A previous study by Chang and coworkers demonstrated that Hdac7 is expressed in the vascular endothelium during early embryogenesis and played a crucial role in angiogenesis since it represses matrix metalloproteinase (MMP) 10 expression that degrades the extracellular matrix and therefore it maintains vascular integrity. In *HDAC7* knock-out mice, the lack of this inhibition causes loss of endothelial cells adhesion, vascular dilation and rupture resulting in embryonic lethality by E11 (Chang et al., 2006). Therefore, to overcome the embryonic lethality, I generated a liver specific knock-out mouse, lacking *Hdac7* only in the liver. H7LivKO mice were fed *Western diet*, a diet enriched in cholesterol, to increase the low basal level of LDL cholesterol of wt mice and to emphasize the potential effect of *Hdac7* loss on BA and cholesterol metabolism. The potential role of the hepatic *Hdac7* in the regulation of *Cyp7a1* gene and the impact of its ablation on BA and cholesterol homeostasis *in vivo* has not been investigated previously. This study demonstrated that *Hdac7* is deeply involved in the molecular regulatory pathway of *Cyp7a1* gene expression thus affecting cholesterol metabolism and lipoprotein profile. *Hdac7* ablation significantly increased *Cyp7a1* transcription leading to higher content of BA in the liver and in plasma. Of note, fecal excretion of major BA, an index of increased BA biosynthesis, was higher in H7LivKO mice on *Western diet* and is likely to explain the reduction of liver and plasma cholesterol compared to control mice on *Western diet*. The importance of bile acid homeostasis in the resistance of High Fat Diet (HFD)-induced obesity and of hyperlipidemia in mice overexpressing *Cyp7a1* has been previously well described (Li et al., 2010b). In accordance to the evidences showed by Li and coworkers, our study reveals that the induced *Cyp7a1* expression by the ablation of *Hdac7* and the consequent increased BA

biosynthesis result in improvement of cholesterol and lipid homeostasis and in lipoprotein profile. Despite similar food consumption, H7LivKO mice showed 12% reduction of body weight compared to control. This might be a consequence of the higher circulating BA, which activate TGR5, the membrane BA receptor localized in brown adipose tissue, and increase energy expenditure via deiodinase 2 (D2) activation that causes increase of 3,5,3′-tri-iodothyronine (T3) and consequently uncoupled protein 1 (UCP1) induction, a well known activator of non-shivering thermogenesis (Watanabe et al., 2006); consistent with this evidence the increased pool of BA observed in Cyp7a1-tg mice induce fatty acid oxidation genes in brown adipose tissue (Li et al., 2010b). Furthermore, H7LivKO mice were protected against fatty liver induced by *Western diet*. The morphological evidence was supported by the significant decrease of hepatic cholesterol of *Western diet*-H7LivKO mice, a main component of lipid droplets in hepatic steatosis, even though the total hepatic triglycerides content displayed only a mild but not significant reduction. It is possible that reduced hepatic triglycerides content could be emphasized with a targeted analysis of single fatty acids.

A further evidence of phenotipical improvement of liver in H7LivKO mice pointed out by this study was the increased levels of hydrophilic BA in the liver, such as α-MCA, ß-MCA, ω-MCA and UDCA. Several publications underscore the association between hydrophobic BA and liver damage; a more hydrophobic BA pool was observed in TGR5 KO mice leading to liver injury (Péan et al., 2013); in addition, mice fed with a lithocholic acid–enriched diet exhibit a hydrophobic bile composition and bile duct obstruction leading to destructive cholangitis with bile infarcts (Fickert et al., 2006).

Another important evidence highlighted by this study was the reduction of LDL-cholesterol in H7LivKO mice fed *Western diet*. Interestingly, an important change also occured in HDL cholesterol. FPLC and agarose electroforesis analysis suggest the presence of smaller and less rich in cholesterol HDL poarticles that might resemble pre-beta HDL in H7LivKO mice. Since HDLs are major cholesterol

carriers in mice, this evidence probably has a major impact on cholesterol profile.

In fact, nascent or pre-beta HDL are a subset of HDL particles poor in lipids that are critical in collecting cholesterol from peripheral tissues as the first step in reverse cholesterol transport (Barter and Rye, 1996). They function as cholesterol acceptors from either cells or apoB-containing lipoproteins, by the lecithin:cholesterol acyltransferase (LCAT)-mediated esterification of cholesterol becoming mature, lipid-rich, and spherical HDL alpha (von Eckardstein et al., 2001). The higher presence of pre-beta HDL and the lower alpha HDL content in H7LivKO mice fed *Western diet*, thus might suggest a lower cholesterol deposition in peripheral tissues due to higher capacity to remove cholesterol from peripheral cells.

CONCLUSIONS

This study elucidated the importance of specific HDACs and corepressors in the regulatory machinery underlying *CYP7A1* expression, highlighting HDAC1, HDAC7 and SMRT as important players in this pathway. Moreover, it suggests HDAC7 as a crucial actor of this pathway, affecting cholesterol and lipid profile *in vivo*. The results obtained with the investigations performed during my doctorate training represent a step forward contributing to shed light on the mechanisms of *CYP7A1* regulation and their impact in lipid homeostasis. It remains to be elucidated the mechanism underlying the phenotypical improvement caused by the ablation of Hdac7. ChIP-seq experiments will help us to verify if the absence of HDAC7 impairs the recruitment of HDACs and SMRT in the transcriptional repressive complex on *Cyp7a1* promoter and it might suggest the involvement of other transcriptional coactivators or corepressors, in turn regulated by Hdac7, which can contribute to the phenotypical evidences observed in H7LivKO mice. Moreover, it will allow to investigate the possible cross talk between HDAC7 and FGF15/19 pathway in light of previous evidences that showed that HDAC inhibitors prevent the repressive effect of FGF15/19 on *Cyp7a1* promoter. Moreover, microarray analysis of H7LivKO mice compared to wt will reveal the effects of liver-specific HDAC7 ablation on global hepatic gene expression and it will elucidate possible changes in the expression of genes involved in lipid and lipoprotein metabolism that might be affected by the absence of HDAC7. In addition, H7LivKO serum will be analyzed by bidimensional electrophoresis and by quantitative FPLC to deepen lipoprotein changes.

BIBLIOGRAPHY

<1996-JLR HEPG2 2.2.1 LUC CONSTRUCT.pdf>.

ALREFAI, W. A. & GILL, R. K. 2007. Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharm Res,* 24**,** 1803-23.

ANTOS, C. L., MCKINSEY, T. A., DREITZ, M., HOLLINGSWORTH, L. M., ZHANG, C.-L., SCHREIBER, K., RINDT, H., GORCZYNSKI, R. J. & OLSON, E. N. 2003. Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors. *J Biol Chem,* 278**,** 28930-7.

BAIGENT, C., KEECH, A., KEARNEY, P. M., BLACKWELL, L., BUCK, G., POLLICINO, C., KIRBY, A., SOURJINA, T., PETO, R., COLLINS, R., SIMES, R. & CHOLESTEROL TREATMENT TRIALISTS, C. 2005. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet,* 366**,** 1267-78.

BARTER, P., GOTTO, A. M., LAROSA, J. C., MARONI, J., SZAREK, M., GRUNDY, S. M., KASTELEIN, J. J. P., BITTNER, V. & FRUCHART, J.-C. 2007. HDL Cholesterol, Very Low Levels of LDL Cholesterol, and Cardiovascular Events. *New England Journal of Medicine,* 357**,** 1301-1310.

BARTER, P. J. & RYE, K. A. 1996. Molecular mechanisms of reverse cholesterol transport. *Curr Opin Lipidol,* 7**,** 82-7.

BEG, Z. H. & BREWER, H. B. 1982. Modulation of rat liver 3-hydroxy-3-methylglutaryl-CoA reductase activity by reversible phosphorylation. *Federation proceedings,* 41**,** 2634-2638.

BHATT, D. L., STEG, P., OHMAN, E. & ET AL. 2006. INternational prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA,* 295**,** 180-189.

BJÖRKHEM, I., NYBERG, B. & EINARSSON, K. 1992. 7α-Hydroxylation of 27-hydroxycholesterol in human liver microsomes. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1128**,** 73-76.

CAO, D. J., WANG, Z. V., BATTIPROLU, P. K., JIANG, N., MORALES, C. R., KONG, Y., ROTHERMEL, B. A., GILLETTE, T. G. & HILL, J. A. 2011. Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. *Proc Natl Acad Sci U S A,* 108**,** 4123-8.

CHANG, S., YOUNG, B. D., LI, S., QI, X., RICHARDSON, J. A. & OLSON, E. N. 2006. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell,* 126**,** 321-34.

CHIANG, J. Y. 1998. Regulation of bile acid synthesis. *Front Biosci,* 3**,** d176-93.

CHIANG, J. Y., MILLER, W. F. & LIN, G. M. 1990. Regulation of cholesterol 7 alpha-hydroxylase in the liver. Purification of cholesterol 7 alpha-hydroxylase and the immunochemical evidence for the induction of cholesterol 7 alpha-hydroxylase by cholestyramine and circadian rhythm. *J Biol Chem,* 265**,** 3889-97.

CHIANG, J. Y. L. 2002. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev,* 23**,** 443-63.

COHEN, J. C., CALI, J. J., JELINEK, D. F., MEHRABIAN, M., SPARKES, R. S., LUSIS, A. J., RUSSELL, D. W. & HOBBS, H. H. 1992. Cloning of the human cholesterol 7 alpha-hydroxylase gene (CYP7) and localization to chromosome 8q11-q12. *Genomics,* 14**,** 153-61.

CRESTANI, M., GALLI, G. & CHIANG, J. Y. 1993. Genomic cloning, sequencing, and analysis of the hamster cholesterol 7 alpha-hydroxylase gene (CYP7). *Arch Biochem Biophys,* 306**,** 451-60.

CRESTANI, M., SADEGHPOUR, A., STROUP, D., GALLI, G. & CHIANG, J. Y. 1998. Transcriptional activation of the cholesterol 7alpha-hydroxylase gene (CYP7A) by nuclear hormone receptors. *J Lipid Res,* 39**,** 2192-200.

CUMMINGS, J. H. & ENGLYST, H. N. 1987. Fermentation in the human large intestine and the available substrates. *Am J Clin Nutr,* 45**,** 1243-55.

DAWSON, P. A., LAN, T. & RAO, A. 2009. Bile acid transporters. *J Lipid Res,* 50**,** 2340-57.

DE FABIANI, E., CRESTANI, M., MARRAPODI, M., PINELLI, A., GOLFIERI, V. & GALLI, G. 2000. Identification and characterization of cis-acting elements conferring insulin responsiveness on hamster cholesterol 7alpha-hydroxylase gene promoter. *Biochem J,* 347 Pt 1**,** 147-54.

DE FABIANI, E., MITRO, N., ANZULOVICH, A. C., PINELLI, A., GALLI, G. & CRESTANI, M. 2001. The negative effects of bile acids and tumor necrosis factor-alpha on the transcription of cholesterol 7alpha-hydroxylase gene (CYP7A1) converge to hepatic nuclear factor-4: a novel mechanism of feedback regulation of bile acid synthesis mediated by nuclear receptors. *J Biol Chem,* 276**,** 30708-16.

DE FABIANI, E., MITRO, N., GILARDI, F., CARUSO, D., GALLI, G. & CRESTANI, M. 2003. Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. *J Biol Chem,* 278**,** 39124-32.

DE RUIJTER, A. J. M., VAN GENNIP, A. H., CARON, H. N., KEMP, S. & VAN KUILENBURG, A. B. P. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.,* 370**,** 737-749.

DOMPIERRE, J. P., GODIN, J. D., CHARRIN, B. C., CORDELIÈRES, F. P., KING, S. J., HUMBERT, S. & SAUDOU, F. 2007. Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation. *J Neurosci,* 27**,** 3571-83.

DREXEL, H. 2009. Statins, fibrates, nicotinic acid, cholesterol absorption inhibitors, anion-exchange resins, omega-3 fatty acids: which drugs for which patients? *Fundamental & Clinical Pharmacology,* 23**,** 687-692.

FANG, S., MIAO, J., XIANG, L., PONUGOTI, B., TREUTER, E. & KEMPER, J. K. 2007. Coordinated recruitment of histone methyltransferase G9a and other chromatin-modifying enzymes in SHP-mediated regulation of hepatic bile acid metabolism. *Mol Cell Biol,* 27**,** 1407-24.

FERRARI, A., FIORINO, E., GIUDICI, M., GILARDI, F., GALMOZZI, A., MITRO, N., CERMENATI, G., GODIO, C., CARUSO, D., DE FABIANI, E. & CRESTANI, M. 2012. Linking epigenetics to lipid metabolism: focus on histone deacetylases. *Mol Membr Biol,* 29**,** 257-66.

FICKERT, P., FUCHSBICHLER, A., MARSCHALL, H.-U., WAGNER, M., ZOLLNER, G., KRAUSE, R., ZATLOUKAL, K., JAESCHKE, H., DENK, H. & TRAUNER, M. 2006. Lithocholic acid feeding

induces segmental bile duct obstruction and destructive cholangitis in mice. *Am J Pathol,* 168, 410-22.

FINKEL, T., DENG, C.-X. & MOSTOSLAVSKY, R. 2009. Recent progress in the biology and physiology of sirtuins. *Nature,* 460, 587-91.

FIORINO, E., GIUDICI, M., FERRARI, A., MITRO, N., CARUSO, D., DE FABIANI, E. & CRESTANI, M. 2014. The sirtuin class of histone deacetylases: regulation and roles in lipid metabolism. *IUBMB Life,* 66, 89-99.

FRYE, R. A. 2000. Phylogenetic Classification of Prokaryotic and Eukaryotic Sir2-like Proteins. *Biochemical and Biophysical Research Communications,* 273, 793-798.

GÄLMAN, C., ANGELIN, B. & RUDLING, M. 2005. Bile acid synthesis in humans has a rapid diurnal variation that is asynchronous with cholesterol synthesis. *Gastroenterology,* 129, 1445-53.

GALMOZZI, A., MITRO, N., FERRARI, A., GERS, E., GILARDI, F., GODIO, C., CERMENATI, G., GUALERZI, A., DONETTI, E., ROTILI, D., VALENTE, S., GUERRINI, U., CARUSO, D., MAI, A., SAEZ, E., DE FABIANI, E. & CRESTANI, M. 2013. Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue. *Diabetes,* 62, 732-42.

GARDNER, R. G., SHAN, H., MATSUDA, S. P. T. & HAMPTON, R. Y. 2001. An Oxysterol-derived Positive Signal for 3-Hydroxy- 3-methylglutaryl-CoA Reductase Degradation in Yeast. *Journal of Biological Chemistry,* 276, 8681-8694.

GIELEN, J., VAN CANTFORT, J., ROBAYE, B. & RENSON, J. 1969. [Circadian rhythm of 7-alpha-cholesterol-hydroxylase in rats]. *C R Acad Sci Hebd Seances Acad Sci D,* 269, 731-2.

GILL, S., STEVENSON, J., KRISTIANA, I. & BROWN, A. J. 2011. Cholesterol-dependent degradation of squalene monooxygenase, a control point in cholesterol synthesis beyond HMG-CoA reductase. *Cell Metab,* 13, 260-73.

GO, A. S., MOZAFFARIAN, D., ROGER, V. L., BENJAMIN, E. J., BERRY, J. D., BLAHA, M. J., DAI, S., FORD, E. S., FOX, C. S., FRANCO, S., FULLERTON, H. J., GILLESPIE, C., HAILPERN, S. M., HEIT, J. A., HOWARD, V. J., HUFFMAN, M. D., JUDD, S. E., KISSELA, B. M., KITTNER, S. J., LACKLAND, D. T., LICHTMAN, J. H., LISABETH, L. D., MACKEY, R. H., MAGID, D. J., MARCUS, G. M., MARELLI, A., MATCHAR, D. B., MCGUIRE, D. K., MOHLER, E. R., MOY, C. S., MUSSOLINO, M. E., NEUMAR, R. W., NICHOL, G., PANDEY, D. K., PAYNTER, N. P., REEVES, M. J., SORLIE, P. D., STEIN, J., TOWFIGHI, A., TURAN, T. N., VIRANI, S. S., WONG, N. D., WOO, D., TURNER, M. B., COMMITTEE, A. H. A. S. & SUBCOMMITTEE, S. S. 2014. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation,* 129, e28-e292.

GOLDSTEIN, J. L. & BROWN, M. S. 1987. Regulation of low-density lipoprotein receptors: implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis. *Circulation,* 76, 504-7.

GOODWIN, B., JONES, S. A., PRICE, R. R., WATSON, M. A., MCKEE, D. D., MOORE, L. B., GALARDI, C., WILSON, J. G., LEWIS, M. C., ROTH, M. E., MALONEY, P. R., WILLSON, T. M. & KLIEWER, S. A. 2000. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell,* 6, 517-26.

GORDON, T., CASTELLI, W. P., HJORTLAND, M. C., KANNEL, W. B. & DAWBER, T. R. 1977. High density lipoprotein as a protective factor against coronary heart disease: The Framingham study. *The American Journal of Medicine,* 62**,** 707-714.

GRAY, S. G. & EKSTRÖM, T. J. 2001. The human histone deacetylase family. *Exp Cell Res,* 262**,** 75-83.

GREGORETTI, I., LEE, Y.-M. & GOODSON, H. V. 2004. Molecular Evolution of the Histone Deacetylase Family: Functional Implications of Phylogenetic Analysis. *Journal of Molecular Biology,* 338**,** 17-31.

GRUNSTEIN, M. 1997a. Histone acetylation in chromatin structure and transcription. *Nature,* 389**,** 349-352.

GRUNSTEIN, M. 1997b. Histone acetylation in chromatin structure and transcription. *Nature,* 389**,** 349-52.

GUPTA, M. P., SAMANT, S. A., SMITH, S. H. & SHROFF, S. G. 2008. HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. *J Biol Chem,* 283**,** 10135-46.

HABERLAND, M., MONTGOMERY, R. L. & OLSON, E. N. 2009. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet,* 10**,** 32-42.

HOFMANN, A. F. & HAGEY, L. R. 2008. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci,* 65**,** 2461-83.

HOLMES, M. V., ASSELBERGS, F. W., PALMER, T. M., DRENOS, F., LANKTREE, M. B., NELSON, C. P., DALE, C. E., PADMANABHAN, S., FINAN, C., SWERDLOW, D. I., TRAGANTE, V., VAN IPEREN, E. P. A., SIVAPALARATNAM, S., SHAH, S., ELBERS, C. C., SHAH, T., ENGMANN, J., GIAMBARTOLOMEI, C., WHITE, J., ZABANEH, D., SOFAT, R., MCLACHLAN, S., CONSORTIUM, O. B. O. T. U., DOEVENDANS, P. A., BALMFORTH, A. J., HALL, A. S., NORTH, K. E., ALMOGUERA, B., HOOGEVEEN, R. C., CUSHMAN, M., FORNAGE, M., PATEL, S. R., REDLINE, S., SISCOVICK, D. S., TSAI, M. Y., KARCZEWSKI, K. J., HOFKER, M. H., VERSCHUREN, W. M., BOTS, M. L., VAN DER SCHOUW, Y. T., MELANDER, O., DOMINICZAK, A. F., MORRIS, R., BEN-SHLOMO, Y., PRICE, J., KUMARI, M., BAUMERT, J., PETERS, A., THORAND, B., KOENIG, W., GAUNT, T. R., HUMPHRIES, S. E., CLARKE, R., WATKINS, H., FARRALL, M., WILSON, J. G., RICH, S. S., DE BAKKER, P. I. W., LANGE, L. A., DAVEY SMITH, G., REINER, A. P., TALMUD, P. J., KIVIMÄKI, M., LAWLOR, D. A., DUDBRIDGE, F., SAMANI, N. J., KEATING, B. J., HINGORANI, A. D. & CASAS, J. P. 2014. Mendelian randomization of blood lipids for coronary heart disease. *Eur Heart J*.

HUA, X., YOKOYAMA, C., WU, J., BRIGGS, M. R., BROWN, M. S., GOLDSTEIN, J. L. & WANG, X. 1993. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proceedings of the National Academy of Sciences,* 90**,** 11603-11607.

HUANG, E. Y., ZHANG, J., MISKA, E. A., GUENTHER, M. G., KOUZARIDES, T. & LAZAR, M. A. 2000. Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev,* 14**,** 45-54.

INAGAKI, T., CHOI, M., MOSCHETTA, A., PENG, L., CUMMINS, C. L., MCDONALD, J. G., LUO, G., JONES, S. A., GOODWIN, B., RICHARDSON, J. A., GERARD, R. D., REPA, J. J., MANGELSDORF, D. J. & KLIEWER, S. A. 2005. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab,* 2**,** 217-25.

ISHIBASHI, S., SCHWARZ, M., FRYKMAN, P. K., HERZ, J. & RUSSELL, D. W. 1996. Disruption of cholesterol 7alpha-hydroxylase gene in mice. I. Postnatal lethality reversed by bile acid and vitamin supplementation. *J Biol Chem,* 271**,** 18017-23.

JELINEK, D. F. & RUSSELL, D. W. 1990. Structure of the rat gene encoding cholesterol 7 alpha-hydroxylase. *Biochemistry,* 29**,** 7781-5.

JOHNSON, M. R., BARNES, S., KWAKYE, J. B. & DIASIO, R. B. 1991. Purification and characterization of bile acid-CoA:amino acid N-acyltransferase from human liver. *J Biol Chem,* 266**,** 10227-33.

KANNEL, W. B., DAWBER, T. R., KAGAN, A., REVOTSKIE, N. & STOKES 3RD, J. 1961. Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. *Annals of internal medicine,* 55**,** 33-50.

KAO, H. Y., DOWNES, M., ORDENTLICH, P. & EVANS, R. M. 2000. Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. *Genes Dev,* 14**,** 55-66.

KEMPER, J. K., KIM, H., MIAO, J., BHALLA, S. & BAE, Y. 2004. Role of an mSin3A-Swi/Snf chromatin remodeling complex in the feedback repression of bile acid biosynthesis by SHP. *Mol Cell Biol,* 24**,** 7707-19.

KERR, T. A., SAEKI, S., SCHNEIDER, M., SCHAEFER, K., BERDY, S., REDDER, T., SHAN, B., RUSSELL, D. W. & SCHWARZ, M. 2002. Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. *Dev Cell,* 2**,** 713-20.

KINGWELL, B. A., CHAPMAN, M. J., KONTUSH, A. & MILLER, N. E. 2014. HDL-targeted therapies: progress, failures and future. *Nat Rev Drug Discov,* 13**,** 445-464.

KNUTSON, S. K., CHYLA, B. J., AMANN, J. M., BHASKARA, S., HUPPERT, S. S. & HIEBERT, S. W. 2008. Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. *EMBO J,* 27**,** 1017-28.

KUSHWAHA, R. S. & BORN, K. M. 1991. Effect of estrogen and progesterone on the hepatic cholesterol 7-alpha-hydroxylase activity in ovariectomized baboons. *Biochim Biophys Acta,* 1084**,** 300-2.

LAHM, A., PAOLINI, C., PALLAORO, M., NARDI, M. C., JONES, P., NEDDERMANN, P., SAMBUCINI, S., BOTTOMLEY, M. J., LO SURDO, P., CARFÍ, A., KOCH, U., DE FRANCESCO, R., STEINKÜHLER, C. & GALLINARI, P. 2007. Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. *Proc Natl Acad Sci U S A,* 104**,** 17335-40.

LI, G., JIANG, H., CHANG, M., XIE, H. & HU, L. 2011a. HDAC6 α-tubulin deacetylase: A potential therapeutic target in neurodegenerative diseases. *Journal of the Neurological Sciences,* 304**,** 1-8.

LI, T., MATOZEL, M., BOEHME, S., KONG, B., NILSSON, L.-M., GUO, G., ELLIS, E. & CHIANG, J. Y. L. 2011b. Overexpression of cholesterol 7α-hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis. *Hepatology,* 53**,** 996-1006.

LI, T., OWSLEY, E., MATOZEL, M., HSU, P., NOVAK, C. M. & CHIANG, J. Y. L. 2010a. Transgenic expression of cholesterol 7alpha-hydroxylase in the liver prevents high-fat diet-induced obesity and insulin resistance in mice. *Hepatology,* 52**,** 678-90.

LI, T., OWSLEY, E., MATOZEL, M., HSU, P., NOVAK, C. M. & CHIANG, J. Y. L. 2010b. Transgenic Expression of Cholesterol 7α-Hydroxylase in the Liver Prevents High-Fat Diet–Induced Obesity and Insulin Resistance in Mice. *Hepatology (Baltimore, Md.),* 52**,** 678-690.

LÓPEZ-RODAS, G., BROSCH, G., GEORGIEVA, E. I., SENDRA, R., FRANCO, L. & LOIDL, P. 1993a. Histone deacetylase. A key enzyme for the binding of regulatory proteins to chromatin. *FEBS Lett,* 317**,** 175-80.

LÓPEZ-RODAS, G., BROSCH, G., GEORGIEVA, E. I., SENDRA, R., FRANCO, L. & LOIDL, P. 1993b. Histone deacetylase: A key enzyme for the binding of regulatory proteins to chromatin. *FEBS Letters,* 317**,** 175-180.

LU, J., MCKINSEY, T. A., NICOL, R. L. & OLSON, E. N. 2000a. Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. *Proc Natl Acad Sci U S A,* 97**,** 4070-5.

LU, J., MCKINSEY, T. A., ZHANG, C. L. & OLSON, E. N. 2000b. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell,* 6**,** 233-44.

LU, T. T., MAKISHIMA, M., REPA, J. J., SCHOONJANS, K., KERR, T. A., AUWERX, J. & MANGELSDORF, D. J. 2000c. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell,* 6**,** 507-15.

LUND, E., ANDERSSON, O., ZHANG, J., BABIKER, A., AHLBORG, G., DICZFALUSY, U., EINARSSON, K., SJÖVALL, J. & BJÖRKHEM, I. 1996. Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. *Arterioscler Thromb Vasc Biol,* 16**,** 208-12.

MAI, A. & ALTUCCI, L. 2009. Epi-drugs to fight cancer: from chemistry to cancer treatment, the road ahead. *Int J Biochem Cell Biol,* 41**,** 199-213.

MARTIN, K. O., BUDAI, K. & JAVITT, N. B. 1993. Cholesterol and 27-hydroxycholesterol 7 alpha-hydroxylation: evidence for two different enzymes. *Journal of Lipid Research,* 34**,** 581-8.

MCKINSEY, T. A., ZHANG, C. L., LU, J. & OLSON, E. N. 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature,* 408**,** 106-11.

MIHAYLOVA, M. M., VASQUEZ, D. S., RAVNSKJAER, K., DENECHAUD, P.-D., YU, R. T., ALVAREZ, J. G., DOWNES, M., EVANS, R. M., MONTMINY, M. & SHAW, R. J. 2011. Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. *Cell,* 145**,** 607-21.

MIREMADI, A., OESTERGAARD, M. Z., PHAROAH, P. D. P. & CALDAS, C. 2007. Cancer genetics of epigenetic genes. *Hum Mol Genet,* 16 Spec No 1**,** R28-49.

MISKA, E. A., KARLSSON, C., LANGLEY, E., NIELSEN, S. J., PINES, J. & KOUZARIDES, T. 1999. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. *EMBO J,* 18**,** 5099-107.

MITRO, N., GODIO, C., DE FABIANI, E., SCOTTI, E., GALMOZZI, A., GILARDI, F., CARUSO, D., VIGIL CHACON, A. B. & CRESTANI, M. 2007. Insights in the regulation of cholesterol 7alpha-hydroxylase gene reveal a target for modulating bile acid synthesis. *Hepatology,* 46**,** 885-97.

MYANT, N. B. & MITROPOULOS, K. A. 1977. Cholesterol 7 alpha-hydroxylase. *J Lipid Res,* 18**,** 135-53.

MYZAK, M. C., DASHWOOD, W. M., ORNER, G. A., HO, E. & DASHWOOD, R. H. 2006a. Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice. *FASEB J,* 20**,** 506-8.

MYZAK, M. C., HARDIN, K., WANG, R., DASHWOOD, R. H. & HO, E. 2006b. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. *Carcinogenesis,* 27**,** 811-9.

MYZAK, M. C., KARPLUS, P. A., CHUNG, F. L. & DASHWOOD, R. H. 2004. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer Res,* 64**,** 5767-74.

NEBBIOSO, A., MANZO, F., MICELI, M., CONTE, M., MANENTE, L., BALDI, A., DE LUCA, A., ROTILI, D., VALENTE, S., MAI, A., USIELLO, A., GRONEMEYER, H. & ALTUCCI, L. 2009. Selective class II HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC–MEF2 complexes. *EMBO Reports,* 10**,** 776-782.

NESS, G. C., PENDLETON, L. C., LI, Y. C. & CHIANG, J. Y. 1990. Effect of thyroid hormone on hepatic cholesterol 7 alpha hydroxylase, LDL receptor, HMG-CoA reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysectomized rats. *Biochem Biophys Res Commun,* 172**,** 1150-6.

NIAN, H., DELAGE, B., HO, E. & DASHWOOD, R. H. 2009. Modulation of histone deacetylase activity by dietary isothiocyanates and allyl sulfides: studies with sulforaphane and garlic organosulfur compounds. *Environ Mol Mutagen,* 50**,** 213-21.

NIAN, H., DELAGE, B., PINTO, J. T. & DASHWOOD, R. H. 2008. Allyl mercaptan, a garlic-derived organosulfur compound, inhibits histone deacetylase and enhances Sp3 binding on the P21WAF1 promoter. *Carcinogenesis,* 29**,** 1816-24.

NITTA, M., KU, S., BROWN, C., OKAMOTO, A. Y. & SHAN, B. 1999. CPF: An orphan nuclear receptor that regulates liver-specific expression of the human cholesterol 7α-hydroxylase gene. *Proceedings of the National Academy of Sciences,* 96**,** 6660-6665.

OGISHIMA, T., DEGUCHI, S. & OKUDA, K. 1987. Purification and characterization of cholesterol 7 alpha-hydroxylase from rat liver microsomes. *J Biol Chem,* 262**,** 7646-50.

OUTEIRO, T. F., KONTOPOULOS, E., ALTMANN, S. M., KUFAREVA, I., STRATHEARN, K. E., AMORE, A. M., VOLK, C. B., MAXWELL, M. M., ROCHET, J.-C., MCLEAN, P. J., YOUNG, A. B., ABAGYAN, R., FEANY, M. B., HYMAN, B. T. & KAZANTSEV, A. G. 2007. Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science,* 317**,** 516-9.

PASSIER, R., ZENG, H., FREY, N., NAYA, F. J., NICOL, R. L., MCKINSEY, T. A., OVERBEEK, P., RICHARDSON, J. A., GRANT, S. R. & OLSON, E. N. 2000. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J Clin Invest,* 105**,** 1395-406.

PÉAN, N., DOIGNON, I., GARCIN, I., BESNARD, A., JULIEN, B., LIU, B., BRANCHEREAU, S., SPRAUL, A., GUETTIER, C., HUMBERT, L., SCHOONJANS, K., RAINTEAU, D. & TORDJMANN, T. 2013. The receptor TGR5 protects the liver from bile acid overload during liver regeneration in mice. *Hepatology,* 58**,** 1451-60.

PONUGOTI, B., FANG, S. & KEMPER, J. K. 2007. Functional interaction of hepatic nuclear factor-4 and peroxisome proliferator-activated receptor-gamma coactivator 1alpha in CYP7A1 regulation is inhibited by a key lipogenic activator, sterol regulatory element-binding protein-1c. *Mol Endocrinol,* 21**,** 2698-712.

PONUGOTI, B., KIM, D. H., XIAO, Z., SMITH, Z., MIAO, J., ZANG, M., WU, S. Y., CHIANG, C. M., VEENSTRA, T. D. & KEMPER, J. K. 2010. SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. *J Biol Chem,* 285**,** 33959-70.

PROSPECTIVE STUDIES, C., LEWINGTON, S., WHITLOCK, G., CLARKE, R., SHERLIKER, P., EMBERSON, J., HALSEY, J., QIZILBASH, N., PETO, R. & COLLINS, R. 2007. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet,* 370**,** 1829-39.

PULLINGER, C. R., ENG, C., SALEN, G., SHEFER, S., BATTA, A. K., ERICKSON, S. K., VERHAGEN, A., RIVERA, C. R., MULVIHILL, S. J., MALLOY, M. J. & KANE, J. P. 2002. Human cholesterol 7alpha-hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype. *J Clin Invest,* 110**,** 109-17.

RADER, D. J., COHEN, J. & HOBBS, H. H. 2003. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *The Journal of Clinical Investigation,* 111**,** 1795-1803.

RAJENDRAN, P., HO, E., WILLIAMS, D. E. & DASHWOOD, R. H. 2011. Dietary phytochemicals, HDAC inhibition, and DNA damage/repair defects in cancer cells. *Clin Epigenetics,* 3**,** 4.

RAMIREZ, M. I., KARAOGLU, D., HARO, D., BARILLAS, C., BASHIRZADEH, R. & GIL, G. 1994. Cholesterol and bile acids regulate cholesterol 7 alpha-hydroxylase expression at the transcriptional level in culture and in transgenic mice. *Molecular and Cellular Biology,* 14**,** 2809-2821.

RICHARDS, E. J. & ELGIN, S. C. R. 2002. Epigenetic Codes for Heterochromatin Formation and Silencing: Rounding up the Usual Suspects. *Cell,* 108**,** 489-500.

RUSSELL, D. W. & SETCHELL, K. D. 1992. Bile acid biosynthesis. *Biochemistry,* 31**,** 4737-49.

SCHWARZ, M., LUND, E. G., LATHE, R., BJÖRKHEM, I. & RUSSELL, D. W. 1997. Identification and characterization of a mouse oxysterol 7alpha-hydroxylase cDNA. *J Biol Chem,* 272**,** 23995-4001.

SCOGNAMIGLIO, A., NEBBIOSO, A., MANZO, F., VALENTE, S., MAI, A. & ALTUCCI, L. 2008. HDAC-class II specific inhibition involves HDAC proteasome-dependent degradation mediated by RANBP2. *Biochim Biophys Acta,* 1783**,** 2030-8.

SONG, K.-H. & CHIANG, J. Y. L. 2006. Glucagon and cAMP inhibit cholesterol 7alpha-hydroxylase (CYP7A1) gene expression in human hepatocytes: discordant regulation of bile acid synthesis and gluconeogenesis. *Hepatology,* 43**,** 117-25.

SPADY, D. K., CUTHBERT, J. A., WILLARD, M. N. & MEIDELL, R. S. 1998. Overexpression of cholesterol 7alpha-hydroxylase (CYP7A) in mice lacking the low density lipoprotein (LDL) receptor gene. LDL transport and plasma LDL concentrations are reduced. *J Biol Chem,* 273**,** 126-32.

SPARROW, D. B., MISKA, E. A., LANGLEY, E., REYNAUD-DEONAUTH, S., KOTECHA, S., TOWERS, N., SPOHR, G., KOUZARIDES, T. & MOHUN, T. J. 1999. MEF-2 function is modified by a novel co-repressor, MITR. *EMBO J,* 18**,** 5085-98.

STROUP, D., CRESTANI, M. & CHIANG, J. Y. 1997. Identification of a bile acid response element in the cholesterol 7 alpha-hydroxylase gene CYP7A. *Am J Physiol,* 273**,** G508-17.

SUN, Z., SINGH, N., MULLICAN, S. E., EVERETT, L. J., LI, L., YUAN, L., LIU, X., EPSTEIN, J. A. & LAZAR, M. A. 2011. Diet-induced lethality due to deletion of the Hdac3 gene in heart and skeletal muscle. *J Biol Chem,* 286**,** 33301-9.

TZUNG, K. W., ISHIMURA-OKA, K., KIHARA, S., OKA, K. & CHAN, L. 1994. Structure of the mouse cholesterol 7 alpha-hydroxylase gene. *Genomics,* 21**,** 244-7.

VAILLANT, I. & PASZKOWSKI, J. 2007. Role of histone and DNA methylation in gene regulation. *Curr Opin Plant Biol,* 10**,** 528-33.

VEGA, R. B., HARRISON, B. C., MEADOWS, E., ROBERTS, C. R., PAPST, P. J., OLSON, E. N. & MCKINSEY, T. A. 2004. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol Cell Biol,* 24**,** 8374-85.

VON ECKARDSTEIN, A., NOFER, J.-R. & ASSMANN, G. 2001. High Density Lipoproteins and Arteriosclerosis: Role of Cholesterol Efflux and Reverse Cholesterol Transport. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 21**,** 13-27.

WANG, A. H., BERTOS, N. R., VEZMAR, M., PELLETIER, N., CROSATO, M., HENG, H. H., TH'NG, J., HAN, J. & YANG, X. J. 1999. HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol Cell Biol,* 19**,** 7816-27.

WANG, D. P. & CHIANG, J. Y. 1994. Structure and nucleotide sequences of the human cholesterol 7 alpha-hydroxylase gene (CYP7). *Genomics,* 20**,** 320-3.

WATANABE, M., HOUTEN, S. M., MATAKI, C., CHRISTOFFOLETE, M. A., KIM, B. W., SATO, H., MESSADDEQ, N., HARNEY, J. W., EZAKI, O., KODAMA, T., SCHOONJANS, K., BIANCO, A. C. & AUWERX, J. 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature,* 439**,** 484-9.

XIANG, K., WANG, Y., ZHENG, T., JIA, W., LI, J., CHEN, L., SHEN, K., WU, S., LIN, X., ZHANG, G., WANG, C., WANG, S., LU, H., FANG, Q., SHI, Y., ZHANG, R., XU, J. & WENG, Q. 2004. Genome-Wide Search for Type 2 Diabetes/Impaired Glucose Homeostasis Susceptibility Genes in the Chinese: Significant Linkage to Chromosome 6q21-q23 and Chromosome 1q21-q24. *Diabetes,* 53**,** 228-234.

XU, Z., TAVARES-SANCHEZ, O. L., LI, Q., FERNANDO, J., RODRIGUEZ, C. M., STUDER, E. J., PANDAK, W. M., HYLEMON, P. B. & GIL, G. 2007. Activation of Bile Acid Biosynthesis by the p38 Mitogen-activated Protein Kinase (MAPK): HEPATOCYTE NUCLEAR FACTOR-4α PHOSPHORYLATION BY THE p38 MAPK IS REQUIRED FOR CHOLESTEROL 7α-HYDROXYLASE EXPRESSION(). *The Journal of Biological Chemistry,* 282**,** 24607-24614.

YE, J. & DEBOSE-BOYD, R. A. 2011. Regulation of cholesterol and fatty acid synthesis. *Cold Spring Harb Perspect Biol,* 3.

YOUN, H. D., GROZINGER, C. M. & LIU, J. O. 2000. Calcium regulates transcriptional repression of myocyte enhancer factor 2 by histone deacetylase 4. *J Biol Chem,* 275**,** 22563-7.

YU, J., LI, Y., ISHIZUKA, T., GUENTHER, M. G. & LAZAR, M. A. 2003. A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. *EMBO J,* 22**,** 3403-10.

YUSUF, S., HAWKEN, S., OUNPUU, S., DANS, T., AVEZUM, A., LANAS, F., MCQUEEN, M., BUDAJ, A., PAIS, P., VARIGOS, J., LISHENG, L. & INVESTIGATORS, I. S. 2004. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet,* 364**,** 937-52.

ZHANG, Y. & TALALAY, P. 1994. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res,* 54**,** 1976s-1981s.