Journal of Lipids

Interactions between Bile Acids and Nuclear Receptors and Their Effects on Lipid Metabolism and Liver Diseases

Guest Editors: David Q.-H. Wang, Brent A. Neuschwander-Tetri, Piero Portincasa, and William M. Pandak

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Editorial

Interactions between Bile Acids and Nuclear Receptors and Their Effects on Lipid Metabolism and Liver Diseases

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In this special issue of *Journal of Lipids*, we acknowledge the contributions by several experts offering timely perspectives on the complex interactions between bile acids and nuclear receptors (NRs) on lipid metabolism and liver diseases at different levels and contexts in the body.

NRs are found within the interior of cells and are defined as ligand-activated transcriptional regulators of several key aspects of body physiology and pathophysiology. NRs regulate gene transcription through interaction with cellular coactivators and corepressors. In the liver, NRs play a key role in a large variety of metabolic processes such as cholesterol, bile acid, fatty acid, and glucose homeostasis, as well as drug disposition. Also, additional critical processes involving the pathophysiology of liver diseases—inflammation and fibrosis, regeneration, cell differentiation, and tumor formation—are modulated by NRs. Of note, NRs are or might soon become drug targets. Despite the huge accumulation of knowledge in the field, the true comprehension of interactions between bile acids and NRs on lipid metabolism and hepatobiliary diseases has remained elusive. Thus continuous efforts are being made to understand the molecular functions of NRs, the significance of bile acid-controlled signaling pathways, and interactions of NRs on a number of metabolic and hepatic diseases.

The paper of T. Li and Y. L. Chiang is focused on the role of bile acid signaling in the regulation of glucose and lipid metabolism. Besides their detergent properties and key physiological functions, bile acids are also acting as potent metabolic regulators of glucose and lipid homeostasis. The identification of bile acid-activated nuclear receptor farnesoid X receptor (FXR) and cell surface G-protein-coupled receptor TGR5 has significantly advanced our understanding on how bile acid signaling regulates cellular metabolism in health and disease. Thus, novel therapeutic strategies can be envisioned which target bile acid metabolism for the treatment of metabolic disorders such as obesity, insulin resistance, and the metabolic syndrome.

NRs comprise one of the most abundant classes of transcriptional regulators of metabolic diseases and have emerged as promising pharmaceutical targets. The paper by G. Garruti et al. deals with the myriad roles of small heterodimer partner (SHP), a unique orphan nuclear receptor lacking a DNA-binding domain, but containing a putative ligand-binding domain. About half of mammalian NRs and several transcriptional coregulators can interact with SHP. SHP is a transcriptional regulator affecting multiple key biological functions and metabolic processes including cholesterol, bile acid, and fatty acid metabolism, as well as reproductive biology and glucose-energy homeostasis. In humans, studies are emerging on the association of SHP genetic variation with birth weight, high body mass index, obesity, insulin resistance, and diabetes. Future research must be

focused on synthetic ligands acting on SHP as a potential therapeutic target in a series of metabolic abnormalities.

One important issue in lipidology is the understanding of the molecular mechanisms whereby cholesterol and fatty acids are absorbed from the intestine and are transported to the liver. The cholesterol absorption inhibitor ezetimibe can significantly reduce plasma total and LDL cholesterol concentrations by inhibiting the Niemann-Pick C1-like 1 protein (NPC1L1), an intestinal sterol influx transporter that can actively facilitate the uptake of cholesterol for intestinal absorption. The paper by O. de Bari et al. emphasizes the novel concept that, ezetimibe treatment also induces a complete resistance to two frequent metabolic abnormalities, namely, cholesterol gallstones and nonalcoholic fatty liver disease (NAFLD). Furthermore, it prevented hypercholesterolemia in mice on a Western diet. This model has high translational value and points to a key role for chylomicrons, the natural lipid carriers used by enterocytes to transport cholesterol and fatty acids into the body. The hypothesis that ezetimibe could prevent two prevalent hepatobiliary diseases (i.e., cholesterol cholelithiasis and liver steatosis) possibly through the regulation of chylomicron-derived cholesterol and fatty acid metabolism in the liver is discussed here.

Because several proteins are implicated in determining biliary lipid secretion in the liver and are regulated by several transcription factors, including nuclear receptors liver X receptor (LXR) and FXR, the paper by M. C. Vázquez et al. is focused on molecular mechanisms underlying the link between nuclear receptor function and the formation of cholesterol gallstones. A potent role for estrogen receptors in the pathogenesis of cholesterol gallstone disease, involving both genomic and nongenomic activation of signaling pathways, is discussed. Evidence in this respect is heavily supported by human and murine genetic, physiological, pathophysiological, and pharmacological studies. Indeed, expanding the knowledge about the role of NRs in gallstone formation will certainly lead to the discovery of novel and more effective therapeutic strategies in a typical example of a metabolic "mass disease," that is, cholesterol cholelithiasis.

In the wide field of *lipopathy,* NAFLD is currently evolving as the most common liver disease worldwide, with potential costly and severe *sequelae,* including liver cirrhosis and hepatocellular carcinoma. In his paper, M. Fuchs underscored the concept that NAFLD not only represents an insulin resistance state characterized by a cluster of dysmetabolic cardiovascular risk factors, but also represents an independent risk factor for cardiovascular diseases. Of note, the bile acid-activated nuclear receptor FXR has been shown to play a role not only in bile acid but also in lipid (cholesterol and triglyceride) metabolism and glucose homeostasis. Specific targeting of FXR may be an elegant and very effective way to readjust dysregulated nuclear receptor-mediated metabolic pathways. Activation of FXR may result in not only beneficial actions but also potential undesirable side effects. One example is the (still unpredictable) balance between pro- and anti-atherogenic effects of FXR activation.

J. A. López-Velázquez et al. described the important role of several NRs in the liver as regulators of several critical metabolic steps involved in the pathogenesis of NAFLD. Such crucial steps include fat storage, export, uptake, oxidation, and lipolysis. A whole family of NRs is targeted by many ligands controlling lipid metabolism including fatty acids, oxysterols, and lipophilic molecules. Understanding the molecular mechanisms underlying the involvement of NRs in the pathogenesis of NAFLD may, therefore, offer targets for the development of new treatments of one of the most frequent chronic liver diseases worldwide.

In their paper, R. Müllenbach et al. provided an update on genetic variants of NRs involved in regulating important aspects of liver metabolism. One such aspect is the application of NRs in genetic diagnosis of monogenic (Mendelian) liver diseases and their uses in clinical diagnosis. Moreover, a role of NR polymorphisms in common diseases can be anticipated, linking regulatory networks to complex and variable phenotypes. Technical advances contribute to the restless expansion of knowledge and include transgenic animal models, expression quantitative trait loci (eQTL) mapping, and genomewide association studies (GWASs). Thus, it is highly likely that personal genome information might eventually be able to predict a variety of risks associated with an individual's lifestyle such as high fat diet and alcohol as well as susceptibility to infectious liver diseases such as hepatitis B or C.

Menopause is a consequence of the normal aging process in women and it is thought that menopause is associated with a higher risk for cardiovascular diseases. Indeed, the post-menopause lipid profile is often altered, which represents a risk factor for cardiovascular diseases. The paper by P. J. Oliveira et al. reports on the mechanisms linking alterations of mitochondrial bioenergetics in the heart, as a consequence from normal aging and/or from the menopausal process, to decreased fatty acid oxidation and accumulation of fatty acid intermediates in the cardiomyocyte cytosol. Such lipotoxic consequences might represent the important link to increased cardiovascular risk in the menopausal women.

In conclusion, the field of lipidology has become even more complex and exciting when considering that the discovery of NRs and their pleiotropic functions have opened the way to multidimensional, multidisciplinary and translational studies. Since NRs are involved in virtually all physiological functions, understanding how NRs work is therefore essential to explain the complex pathophysiological mechanisms underlying liver and extrahepatic diseases. A new era in which NRs will represent valid therapeutic targets for several disorders is hopefully approaching.

Lastly, we hope that this contribution will also help both young and experienced investigators in their daily difficult task to expand their research in the field of experimental and clinical lipidology in health and disease.

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Review Article

A Pleiotropic Role for the Orphan Nuclear Receptor Small Heterodimer Partner in Lipid Homeostasis and Metabolic Pathways

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Nuclear receptors (NRs) comprise one of the most abundant classes of transcriptional regulators of metabolic diseases and have emerged as promising pharmaceutical targets. Small heterodimer partner (SHP; NR0B2) is a unique orphan NR lacking a DNA-binding domain but contains a putative ligand-binding domain. SHP is a transcriptional regulator affecting multiple key biological functions and metabolic processes including cholesterol, bile acid, and fatty acid metabolism, as well as reproductive biology and glucose-energy homeostasis. About half of all mammalian NRs and several transcriptional coregulators can interact with SHP. The SHP-mediated repression of target transcription factors includes at least three mechanisms including direct interference with the C-terminal activation function 2 (AF2) coactivator domains of NRs, recruitment of corepressors, or direct interaction with the surface of NR/transcription factors. Future research must focus on synthetic ligands acting on SHP as a potential therapeutic target in a series of metabolic abnormalities. Current understanding about the pleiotropic role of SHP is examined in this paper, and principal metabolic aspects connected with SHP function will be also discussed.

1. Introduction

Nuclear receptors (NRs) constitute a unique family of ligand-modulated transcription factors. NRs mediate cellular response to small lipophilic endogenous and exogenous ligands [1, 2] and are responsible for sensing a number of hormones, including steroid and thyroid hormones, and act as positive and negative regulators of the expression of specific genes [3–5]. Therefore, NRs play a central role in many aspects of mammalian development, as well as lipid homeostasis, physiology, and metabolism. NRs make up one of the most abundant classes of transcriptional regulators in the body and have emerged as promising pharmaceutical targets.

Classically, NRs consist of several functional domains, that is, a variable N-terminal ligand-independent transactivation domain (which often exhibits a constitutive transcription activation function (AF-1)), a highly conserved DNA-binding domain (DBD) that contains two zinc fingers, a hinge domain (a variable linker region), and a multifunctional C-terminal domain. Furthermore, the C-terminal domain includes the ligand binding (LBD), the dimerization interface, and the ligand-dependent transactivation domain AF-2 [1, 6].

Small heterodimer partner (*SHP*; NR0B2 for nuclear receptor subfamily 0, group B, member 2; MIM number 604630, 601665) is a member of the mammalian NR

superfamily, due to the presence of a putative ligand-binding domain (LBD) [7]. SHP functions as a corepressor through heterodimeric interaction with a wide array of nuclear receptors and repressing their transcriptional activity. SHP achieves its goal via several members of the NR superfamily that are able to regulate SHP expression. However, SHP is also a unique and atypical NR because it lacks the classical DNA-binding domain (DBD), generally present in other NRs [8]. The *NR0B* family of NRs consists of 2 orphan receptors: SHP and DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1). DAX1 is a gene whose mutation causes the X-linked adrenal hypoplasia congenita [9] and is the only family member that lacks a conventional DBD. DAX-1 (NR0B1) is therefore seen as the closest relative of SHP in the NR superfamily [10–12]. Both SHP and DAX-1 appear to be specific to vertebrates. In this respect, no homologous genes have been found in *Drosophila melanogaster* or *Caenorhabditis elegans* [12]. Whereas SHP is different from other conventional NRs both structurally and functionally, it acts as a ligand-regulated receptor in metabolic pathways [13]. SHP belongs to the orphan subfamily since there is no known ligand for this receptor, except for some retinoid-related molecules [14]. SHP inhibits transcriptional activation by working on several other nuclear receptors, that is, directly modulating the activities of conventional nuclear receptors by acting as an inducible and tissue-specific corepressor [12, 15]. The discovery of SHP dates back to 1996 [10]; since then, this orphan NR has been identified as a key transcriptional regulator of signaling pathways [8, 16] involving fundamental biological functions and metabolic processes. Such processes include cholesterol, bile acid and fatty acid metabolism, glucose and energy homeostasis, and reproductive biology [17]. Experiments performed by fluorescence *in situ* hybridization (FISH) analysis of the human metaphase chromosome have shown that SHP is found at a single locus on chromosome 1 at position 1p36.1 and consists of two exons and a single intron spanning approximately 1.8 kb with 257 amino acids in humans [18]. In mice and rats, SHP resides on chromosomes 4 and 5, respectively, both consisting of 260 amino acids. SHP expression is predominantly observed in the liver [10, 18], but it is also detected at lower levels in other tissues, including the pancreas, spleen, small intestine, colon, gallbladder, kidney, adrenal gland, ovary, lung, brainstem, cerebellum, heart, and thymus (Table 1) [19–21].

The genomic structure and human SHP domain structure are depicted in Figure 1 [15]. SHP is indeed able to repress the transcriptional activities of its target NRs and transcriptional regulators through two functional Leu-Xaa-Xaa-Leu-Leu- (LXXLL-) like motifs [22–24]. Such motifs appear to be essential for the interaction with the (activation function 2) AF-2 domains of several sets of NRs [22, 23]. The human SHP is enriched by another 12 amino acids [25–36], and this region between helix 6 and 7 is also involved in the repression of the transactivation of NRs [37].

About half of all mammalian NRs and several transcriptional coregulators can interact with SHP [12]. Since SHP lacks DNA-binding domain, it exerts the inhibitory effects through protein-protein interaction [10]. SHP expression seems to follow a circadian rhythm in the liver, involving the CLOCK-BMAL1 pathway and suggesting that some of the regulatory functions of SHP and deriving functions must be temporal [19, 20, 38].

Table 1: Small heterodimer partner (SHP) expression [10, 18–21].

LIVER (greater)*
Spleen*
Pancreas*
Central nervous system (brainstem and cerebellum)
Adrenal gland*
Intestine (duodenum*, jejunum*, ileum*, and colon)
Gallbladder, stomach*, kidney*, ovary, lung, prostate, testis, uterus, heart*, thymus, and epididymis

All organs in the mouse. Astericks indicate SHP expression in humans [18, 133].

Gene expression of SHP is regulated by several factors including NRs, transcription factors, and a number of additional conditions and substances, as extensively reported in Table 2. Also, the central role of SHP is clear since this NR is able to act as a coregulator for wide range of targets, namely, NRs/transcription factors/transcriptional coregulators and few different molecules, as depicted in Table 3. In general, SHP acts as a repressor of the transcriptional activity of the specific interacting partner (via LBD of the partner and NR boxes of SHP) [12, 39–43]. However, it is also demonstrated that SHP is able to upregulate gene transcription, as in the case of PPARα and PPARγ [44–46] and NF-κB [44].

Both N-terminal NR interaction domain and C-terminal domain of SHP are important for repression [47, 48]. Overall, the SHP-mediated repression of target transcription factors occurs by at least three distinct transcriptional repression mechanisms (Figure 2).

A first mechanism involves direct interference with the AF-2 coactivator domain of NRs (competition for coactivator binding, leading to the repression of NR-mediated transcriptional activity). This is the case for the inhibition of estrogen receptor α (ERα) and estrogen receptor β (ERβ) [49].

A second mechanism for the SHP-mediated repression involves the recruitment of corepressors including direct interactions among mammalian homolog of the *Saccharomyces cerevisiae* transcriptional corepressor Sin3p (mSin3A), human Brahma (Brm), SWItch/Sucrose NonFermentable (SWI/SNF) complexes leading to the repression of cholesterol 7α-hydroxylase (CYP7A1) [50]).

A third mechanism of inhibition of SHP involves the direct interaction with the surface of NR or transcription factor, resulting in the blockade of DNA binding and the consequent inhibition of its transcriptional activity. This is the case for RAR-RXR heterodimers [10], PXR-RXR binding to DNA by SHP [1], interaction with hepatocyte nuclear factor (HNF4), or Jun family of the activator protein 1 (AP-1) transcription factor complex (JunD) [51, 52].

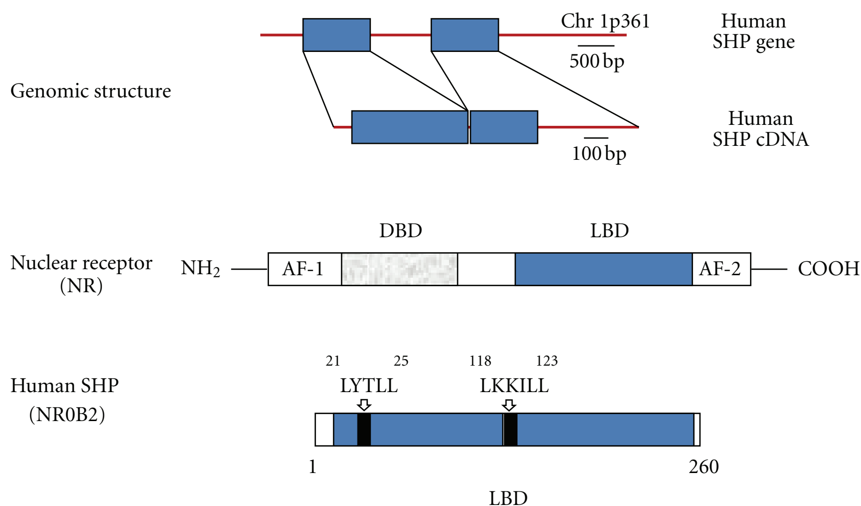

Figure 1: Top: the genomic structure of human SHP. Rectangles represent the two exons with a single intron spanning approximately 1.8 kilobases and located on a single locus on chromosome 1p36.1 [18]. The region 5′ includes ≈600 nucleotides from the transcription start site and is characterized by promoter activity. Bottom: typical nuclear receptor is compared with the domain structure of human SHP. The canonical structure of NR includes the N-terminal activation function 1 (AF1) domain, DNA-binding domain (DBD), ligand-binding domain (LBD), and C-terminal activation function 2 (AF2) domain. SHP lacks the DBD. Two functional LXXLL-related motifs (also named as NR boxes) are typical of the human SHP structural domains. Such motifs are located in the putative N-terminal helix 1 of the LBD and in the C-terminal region of the helix 5. While active NRs exhibit glutamic acid in AF-2, the SHP AF-2 domain is replaced with aspartic acid. Adapted from Chanda et al. [15] and Shulman and Mangelsdorf [130].

All three mechanisms might occur sequentially or alternatively according to type of cells and promoters [12].

Clearly, information on factors that increase or decrease SHP expression and that are regulated by SHP is essential for understanding the regulatory effects of this orphan NR. Few years of research have not been enough to identify a true ligand. Interestingly, it is suggested that targeting posttranslational modifications of SHP may be an effective therapeutic strategy. Selected groups of genes could be controlled to cure a vast range of metabolic and SHP-related diseases [53]. Overall, the huge amount of information on SHP function is currently available, making this NR essential in a number of functions involving cholesterol and bile acid metabolism, lipogenesis, glucose metabolism, steroid hormone biosynthesis, xenobiotic homeostasis/metabolism, and cell cycle.

In particular, the ability of SHP in interacting with different metabolic signaling pathways including bile acids and lipid homeostasis, fat mass, adipocytes, and obesity will be reviewed here.

2. Bile Acids and Lipid Homeostasis

The wide ability of SHP to target multiple genes in diverse signaling pathways points to the key role of SHP in various biological processes, including the metabolism of bile salts, glucose, and fatty acids. Both unique structure and functional properties account for the complexity of SHP signaling. Studies suggest that loss of SHP might positively affect cholesterol and bile acid homeostasis in pathophysiologically relevant conditions [54]. Bile acids (BAs) are amphipatic cholesterol metabolites which are synthesized in the liver, secreted into bile, stored in the gallbladder, and secreted postprandially into the duodenum. BAs are synthesized from cholesterol, and this pathway provides the elimination of excess cholesterol in the body [55]. Moreover, BAs should be seen as physiological detergents which, in the small intestine, are essential for the absorption, transport, and distribution of lipophilic molecules, including dietary lipids, steroids, and lipid-soluble vitamins. In the intestine, BAs undergo extensive metabolism by the intestinal microflora. A high efficient system is the enterohepatic circulation of BAs [55, 56], where more than 90–95% of BAs are returned to the liver from the terminal ileum via the portal vein. Thus, the concentration of BAs in serum, liver, and intestine is tightly regulated to prevent damage to enterohepatic tissues due to their strong detergent moiety [57–59]. The major rate-limiting step in biosynthetic pathway of BAs in humans is initiated by cholesterol 7α-hydroxylase (CYP7A1), the microsomal P450 liver enzyme, to produce two primary BAs, cholic acid, and chenodeoxycholic acid, essential in the overall balance of cholesterol homeostasis. Sterol 12α hydroxylase (CYP8B1) catalyzes the synthesis of cholic acid, a step which determines the cholic acid to CDCA ratio in the bile [60]. Secondary bile acids (deoxycholic acid and lithocholic acid) and tertiary bile acids (ursodeoxycholic acid) in humans are produced following intestinal dehydroxylation of primary bile acids by intestinal bacteria [58, 61].

Regulation of BA biosynthesis is highly coordinated and is mediated by key NRs including the orphan receptor, liver receptor homologue-1 (LRH1; NR5A2), the hepatocyte nuclear factor 4α (HNF4α), SHP, and the bile acid receptor farnesoid X receptor (FXR; NR1H4). Thus, the activation of FXR initiates a feedback regulatory loop via induction of SHP, which suppresses LRH-1- and HNF4α-dependent

Table 2: Regulators of the Shp gene promoter [12, 39–43].

(1) *Nuclear receptors*	
Protein	Model(s)/putative function
ERα	Uterus, pituitary, kidney, and adrenal gland, HepG2 cell lines/biological effects of estrogens, LDL/HDL metabolism [134].
ERRα, β, γ	SHP promoter is activated by the ERRγ, while SHP inhibits ERRγ transactivation (autoregulatory loop). SHP and ERRγ coexpressed in several tissues (e.g., pancreas, kidney, and heart). Role in some forms of moderate obesity? SHP also physically interacts with ERR α and β isoforms (yeast two-hybrid and biochemical assays) [133].
FXR	Downregulation of CYP7A1-mediated bile acid biosynthesis by the FXR/SHP/LRH-1 cascade in the liver [64].
LXRα	Direct regulation of SHP and repression of CYP7A1-mediated bile acid biosynthesis (in humans not in rodents). Effect on cholesterol homeostasis [135].
LRH-1	Liver/formation of heterodimeric SHP/LRH-1 complex > inactivation of LRH-1 > SHP repression (autoregulatory negative feedback) [64, 65, 136]. Also involved in the CLOCK-BMAL1 circadian activation of SHP [38].
PPARγ	Liver/PPARγ decreases gluconeogenic gene expression by the PPARγ/RXRα heterodimer binding to the PPRE in the human SHP promoter. A mechanism explaining the SHP-mediated acute antigluconeogenic effects of PPARγ [137].
SF-1	At least five binding sites for SF-1 detected in the promoter region of SHP. Rat testis and adrenal glands, human fetal adrenal gland [136].
(2) *Transcription factors*	
Protein	Model(s)/putative function
CLOCK-BMAL1	Liver/SHP displays a circadian expression pattern involving CLOCK-BMAL1 (core circadian clock component). Regulation of SHP promoter together with LRH-1 and SHP. Relevance for circadian liver function? [38].
E2A proteins (E47, E12, E2/5)	HepG2, HeLa, and CV-1 cells/bHLH transcription factors, the E2A proteins activate human (not mouse) hSHP promoter. E47 and SF-1 stimulate cooperatively SHP promoter. The Id protein inhibits E47 binding to hSHP promoter. A role for tissue-specific gene regulation, B-cell differentiation, tumor suppression? [138].
HNF-1α	Liver/modulation of bile acid and liver cholesterol synthesis via the FXR/SHP/LRH-1 complex and effect on CYP7A1 [69].
HNF4α	Pancreatic β-cells/decreased expression of SHP may be indirectly mediated by a downregulation of HNF4α. SHP can repress its own transcriptional activation by inhibiting HNF4 α function (feedback autoregulatory loop) and, indirectly (via HNF4 α), HNF1α function. Relevance for pancreatic islet differentiation, insulin secretion, synthesis [116].
JNK/c-Jun/AP-1	Primary rat hepatocytes/bile acid downregulation of CYP7A1-dependent bile acid biosynthesis via the JNK/cJun/AP1 pathway. SHP promoter is a direct target of activated c-Jun binding to AP-1 element [139]. Also, in HL-60 leukemia cells, c-Jun increases the transcriptional activation of the SHP promoter to activate the expression of Shp genes associated with the cascade regulation of monocytic differentiation [140].
SMILE	HEK-293T, HepG2, MCF-7, T47D, MDA-MB-435, HeLa, PC-3, C2C12, NIH 3T3, K28, Y-1, and TM4 cell lines/SMILE isoforms (SMILE-L and SMILE-S) regulate the SHP-driven inhibition of ERs transactivation in a cell-type-specific manner [25, 26, 39].
SREBP-1	Liver/effect on human (not mouse) SHP promoter. Cholesterol and bile acid homeostasis, fatty acid synthesis [27].
USF-1	HepG2, H4IIE, and AML12 cells/HGF activates AMPK signaling pathway in hepatocytes, E-box-binding transcription factor USF-1, and binding to the Shp gene promoter. SHP induction of gene expression leads to inhibition of hepatic gluconeogenesis due to SHP-repressed transcription factor HNF4α [28].
(3) *Transcriptional coregulators*	
Protein	Model(s)/putative function
RNF31	NCI-H295R (H295R) adrenocortical carcinoma cell line, COS-7 and HeLa cells/RNF31 interacts with SHP, stabilizes DAX-1, and is required for DAX-1-mediated repression of transcription. Relevant as coregulator of steroidogenic pathways [43].
SRC-1	Murine macrophage cell line RAW 264.7, HeLa, and CV-1 cells/SHP interacts negatively with SRC-1 (a transcription coactivator of nuclear receptors and other transcription factors including NF-κB). See also oxLDL in this table [44].

Table 2: Continued.

(4) *Other SHP inducers*	
Factor	Model(s)/putative function
Bile acids (final intermediates)	Experiments in HepG2 cells/treatment with chenodeoxycholic acid and late intermediates in the classic pathway of bile acid synthesis: 26-OH-THC (5β-cholestane-3α,7α,12α,26-tetrol), THCA (3α,7α,12α-trihydroxy-5 β-cholestanoic acid), 26-OHDHC (5β-cholestane-3α,7α,26-triol), DHCA (3α,7α-dihydroxy-5β-cholestanoic acid) resulted in 2.4-6.5-fold increase in SHP mRNA expression [132]. Confirmed by Ourlin et al. with the two FXR ligands chenodeoxycholic acid and cholic acid [1].
Guggulsterone (plant sterol)	Active extract from Commiphora Mukul. FXR antagonist. In Fisher rats, guggulsterone increased transcription of bile salt export pump (BSEP) mRNA and SHP expression [29].
GW4064 (ligand)	Synthetic FXR-selective agonist [29]. In primary cultured human hepatocytes, GW4064 treatment was associated with a marked induction of *SHP* ($\approx$70-fold) and complete suppression of *CYP7A*1 [64, 65]. In HepG2 cells, GW4064 (1uM) induced a 3.9-fold increase in SHP mRNA expression. Confirmed by [30].
Interleukins (various)	IL-1Ra ($-/-$) mice/high cytokine levels in IL-1Ra ($-/-$) mice reduce mRNA expression of CYP7A1 with concurrent upregulation of SHP mRNA expression [31]. SHP significantly expressed in IFN-γ/CH11-resistant HepG2 cells [32].
PGC-1α (gene expression inducer)	COS-7 cell lines/PGC-1α mediates the ligand-dependent activation of FXR and transcription of Shp gene. Relevance in mitochondrial oxidative metabolism in brown fat, skeletal muscle, and liver gluconeogenesis [33].
PMRT1 (group of protein arginine methyltransferases)	Hepatic cell lines/PRMT1 functions as FXR coactivator and has a role in chromatin remodeling. PRMT1 induces BSEP and SHP and downregulation of NTCP and CYP7A1 (targets of SHP) [30].
Procyanidins (polyphenols)	Grape seed procyanidin extract is given orally in male Wistar rats. Increase of liver mRNA levels of small heterodimer partner (SHP) (2.4-fold), cholesterol 7α-hydroxylase (CYP7A1), and cholesterol biosynthetic enzymes with improved lipidogenic profile and atherosclerotic risk [34].
(5) *Factors/conditions associated with SHP repression*	
β Klotho (type I membrane protein)	In βKlotho ($-/-$) mice: enhanced bile acid synthesis with attenuation of bile acid-mediated induction of *Shp*. βKlotho involved in CYP7A1 selective regulation [35].
IL-1β (interleukin)	SHP downregulation [36].
oxLDL (oxidized low density lipoprotein)	Murine macrophage cell line RAW 264.7, HeLa, and CV-1 cells/oxLDL decreased SHP expression. SHP transcription coactivator of NF-κB which became progressively inert in oxLDL-treated RAW 264.7 cells (see also Table 3). Relevance for differentiation mechanism of resting macrophage cells into foam cells and resulting atherogenesis [44].

AP-1: adaptor protein-1; bHLH: basic helix-loop-helix; DAX1: dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1; E2A: E2A2 gene products belonging to the basic helix-loop-helix (bHLH) family of transcriptor factors; ERα: estrogen receptorα; ERRγ: estrogen receptor-related receptor-γ; FXR: farnesoid X receptor; HGF: Hepatocyte growth factor; HNF-1α: hepatocyte nuclear factor-1α; HNF4α: hepatocyte nuclear factor-4α; Id: inhibitor of differentiation; IL-1Ra ($-/-$): interleukin-1 receptor antagonist; JNK: Jun N-terminal kinase; LRH-1: liver receptor homologue-1; LXRα: liver X receptorα; NFκB: nuclear factor-κB; NR: nuclear receptor; NTCP: Na^+-taurocholate cotransport peptide; oxLDL: oxidized low-density lipoprotein; PGC-1: PPARγ (peroxisome-proliferator-activated receptor γ) coactivator-1α; PMRT1: protein arginine methyltransferase type 1; PPRE: PPAR response element; RNF31: member of the ring-between-ring (RBR) family of E3 ubiquitin ligases; RXR α: retinoid X receptor; SF-1: steroidogenic factor-1; SHP: small (short) heterodimer partner; hSHP: human small (short) heterodimer partner; SMILE: SHP-interacting leucine zipper protein; SRC-1: steroid receptor coactivator-1; SREBP-1: sterol regulatory element binding protein-1; USF-1: upstream stimulatory factor-1.

expression of the two major pathway enzymes cholesterol 7hydroxylase (CYP7A1) and sterol 12 hydroxylase (CYP8B1).

The BA feedback regulation primarily occurs since BAs act as transcriptional regulators for the expression of the gene encoding CYP7A1. Both cholic acid and chenodeoxycholic acid function as endogenous ligands for the nuclear bile acid receptor FXR [62]. FXR expression is high in the intestine and liver, the two sites where BAs reach high concentrations to activate FXR. The transcription by FXR includes heterodimerization with retinoid X receptors (RXRs) in the cytoplasm, translocation into the nucleus, and binding to DNA response elements in the regulatory regions of target genes [63]. When the bind of BAs to FXR, SHP transcription is increased [60, 64, 65], this alteration leads to the inhibition of LRH-1 activity or HNF4α on the BA response elements (BAREs) of CYP7A1 and CYP8B1 promoters [64, 65]. In this scenario, BA synthesis is downregulated by a precise feedback regulatory mechanism, which represents the major pathway under normal physiological conditions [64–66] (Figure 3). LRH1 is also a well-known activator of *Shp* gene transcription [64, 65], and this step leads to an autoregulatory loop of gene expression by SHP [42]. This step also includes the G protein pathway suppressor 2 (GPS2) interacting with FXR, LRH-1, and HNF4α to regulate CYP7A1 and CYP8B1 expression in human hepatocytes [67] (Table 3). A critical role in maintaining cholesterol homeostasis for CYP7A1 has been recently advocated in a model of in Cyp7a1-tg mice [68].

The hepatocyte nuclear factor-1α (HNF1α), which haploinsufficiency causes the Maturity-onset diabetes of the young type 3 (MODY3), also appears to modulate SHP expression

Table 3: SHP targets [12, 39–43].

(1) *Nuclear receptors*	
Protein	Model(s)/putative function
AR	The AR/SHP interaction leads to >95% inhibition of AR via the LXXLL motifs. Mechanisms involve inhibition of AR ligand-binding domain and AR N-terminal domain-dependent transactivation and competing with AR coactivators [23].
CAR, RAR, TR	HepG2 and JEG-3 cells/early evidence that SHP interacts with several receptor superfamily members and inhibits transactivation. CAR is an NR-inducing CYP2 and CYP3 genes involved in the metabolism of xenobiotics [10, 24].
DAX-1	Human embryonic kidney 293 cells/beside individual homodimerization of DAX1 and SHP, this is the first evidence of DAX1-SHP heterodimerization in the nucleus of mammalian cells. Involvement of the LXXLL motifs and AF-2 domain of DAX1 in this interaction. Distinct functions for SHP (different from transcriptional repressor) are anticipated [141, 142].
ER	293 human embryo kidney cells, Cos7 kidney cells/direct inhibitory binding of SHP to ERs via LXXLL-related motifs to the AF-2 domain [21]. RL95-2 human endometrial carcinoma cells/SHP inhibits the agonist activity of 4-hydroxytamoxifen displaying a potent inhibitory effect for ERα >ERβ. Direct interaction of SHP with ER and inhibition of ER transcriptional activity [143]. Prevention of tamoxifen-induced estrogen agonistic effects and neoplastic changes in the endometrium in women with breast cancer taking tamoxifen?
ERRγ	HeLa (human cervical carcinoma), CV-1 (green monkey kidney), and HEK 293 (human embryonic kidney) cell lines/SHP inhibits ERRγ transactivation by physical interaction with the 3 members of the ERR subfamily. Interaction is dependent on N-terminal receptor interaction domain of SHP and AF-2 surface of ERRγ. Part of the autoregulatory mechanism of gene expression going through ERRγ/SHP/ERRγ. A potential role in some forms of moderate human obesity during SHP mutations [133].
GR	293 human embryo kidney cells and COS-7 monkey kidney/SHP inhibits the transcriptional activity of GR via the LXXLL motif. Physiological role of SHP in glucocorticoid signaling and gluconeogenesis [22]. See also HNF4 [90] and Foxo1 [115].
HNF4	Human ANG transgenic mice and HepG2 cells treated with bile acids/evidence that bile acids negatively regulate the human ANG gene through the FXR/SHP-mediated process (inhibition of the binding of HNF4 to the ANG promoter) [90]. Mechanisms: SHP binds the AF-2 region and the N-terminal region of HNF4 and inhibits the binding of HNF4 to DNA. Also, modulation of HNF4 activity by SHP has important metabolic effects and interacts with the pathway of gluconeogenesis [47](see text and Foxo1) [115].
LRH-1	HepG2 cells/SHP interacts directly with the orphan receptor LRH-1 (AF-2 surface) and competes with other coactivators, leading to repression of LRH-1 transcriptional activity [48]. Demonstration that repression of CYP7A1 and bile acid synthesis requires coordinate interaction/transcription of FXR/LRH-1/SHP autoregulatory cascade, essential for maintenance of bile acid-induced negative feedback, and therefore hepatic cholesterol metabolism [65] (see also Figure 2).
LXRα	*In vitro* experiments and *in vivo* human colon Caco-2 cells/SHP directly inhibits the transcriptional activity of LXRα via the AF-2 domain. Relevance for direct downregulation of specific LXR target genes (controlling CYP7A1, ABCA1, ABCG1, ABCG5, ABCG8, CETP, ApoE, SREBP-1c) and therefore cholesterol-bile acid homeostasis [144].
Nur77 (NGFI-B)	HepG2 cells/Nur77 plays a key role in apoptosis of many cell types and cancer cells. Evidence that SHP functions to repress the transcriptional function of Nur77 (binding coactivator CBP, see elsewhere in this table). SHP plays a protective role in the Nur77-mediated apoptosis in liver. Mutations in SHP: a role also for affect initiation and progression of inflammatory liver diseases such as alcoholic hepatitis and hepatic viral infections? [32].
PPARα	*In vitro* binding assays and *in vivo* experiments/the promoter regions of the genes encoding the first two enzymes of the peroxisomal beta-oxidation pathway (AOx, HD), contain transcriptional regulatory sequences (PPRE) bound by the PPARα/RXRα heterodimeric complex. SHP-inhibited transcription by PPARα/RXRα heterodimers from the AOx-PPRE. SHP potentiated transcription by PPARα/RXRα heterodimers from the HD-PPRE (evidence of SHP-dependent upregulation PPARα-mediated gene transcription) [46].
PPARγ	*In vitro* experiments, COS-7 cells/*Shp* gene expressed also in adipose tissue. SHP induces PPAR activation via C terminus (direct binding to the DBD/hinge region of PPARγ) and inhibition of the repressor activity of NCoR. SHP may act as an endogenous enhancer of PPARγ by competing with NCoR [45]. Mutant SHP proteins display less enhancing activity for PPARy compared with wild-type SHP, and a human model leading to mild obesity and insulin resistance has been described in Japanese during naturally occurring mutations [111] (see also text and Table 3).

Table 3: Continued.

PXR	*In vitro* experiments, human hepatocytes, mouse model on cholic acid-supplemented diet/SHP act as potent repressor of PXR transactivation. Upon sensing xenobiotics and bile acid precursors, PXR controls CYP3A gene induction and inhibits CYP7α, acting on both bile acid synthesis and catabolism. PXR function might be also inhibited in the presence of cholic acid, chenodeoxycholic acid-dependent SHP upregulation [1].
RXR	HepG2 cells/demonstration that SHP acts as a transcriptional repressor for RXR. Full inhibition by SHP requires its direct repressor activity [47].
SHP	Human embryonic kidney 293 cells/LXXLL motifs and AF-2 domain are involved in SHP homodimerization in the nucleus (similarly to DAX1-SHP heterodimerization). NR0B family members use similar mechanisms for homodimerization as well as heterodimerization. Distinct functions for SHP (different from transcriptional repressor) are anticipated [141, 142].
(2) *Transcription factors*	
Protein	Model(s)/putative function
ARNT	RL95-2 human endometrial carcinoma cells/TCDD binds to AHR (a member of bHLH-PAS family of transcription factors). Studies on physical and functional interaction of SHP with the ligand AHR/ARNT heterodimer showed that SHP inhibits the transcriptional activity of ARNT (not AHR) *in vitro*. Consequent inhibition of binding of AHR/ARNT to XREs. [41]. Relevance for expression of several genes involved in drug and hormone metabolism [145].
BETA2/NeuroD	293T, COS-7, CV-1 cells/BETA2/NeuroD is a member of tissue-specific class B bHLH proteins and cats as a positive regulator of insulin gene expression [146] and neuronal differentiation [147]. SHP physically interacts and inhibits helix-loop-helix transcription factor BETA2/NeuroD transactivation of an E-box reporter in mouse pancreas islets. The inhibitory effect of SHP requires its C-terminal repression domain, interference with coactivator p300 for binding to BETA2/NeuroD, and direct transcriptional repression function. Relevance for development of the nervous system and the maintenance and formation of pancreatic and enteroendocrine cells [148].
C/EBPα	HepG2 hepatoma cells/SHP interacts directly with C/EBPα and represses C/EBPα-driven PEPCK gene transcription. Overall, a role for SHP in regulation of hepatic gluconeogenes is driven by C/EBPα activation in the liver [149].
Foxo1	C57BL/6J mice and HepG2 and HEK293T cells/treatment with chenodeoxycholic acid was associated with FXR-dependent SHP induction, downregulation of gluconeogenic gene expression (G6Pase, PEPCK, FBP1), interaction of the forkhead transcription factor Foxo1 with SHP, and repression of Foxo1-mediated G6Pase transcription (competition with CBP). A similar mechanism is postulated for SHP-driven HNF-4 repression of PEPCK, FBP1 transcription. A mechanism by which bile acids metabolism is linked to gluconeogenic gene expression via an SHP-dependent regulatory pathway [115].
HNF3 (Foxa)	HepG2, 293T, NIH3T3, and HeLa cells, primary hepatocytes/SHP physically interacts and inhibits the transcriptional activity of the forkhead transcription factor HNF3 (isoforms α, β, γ). Relevance for SHP-driven regulation of gluconeogenic genes encoding G6Pase, PEPCK, and bile acid synthesis (CYP7A1), via inhibition of DNA-binding of HNF3 [51].
Jun D	Two rat models of liver fibrosis and Hepatic Stellate cells (HSC)/promoting the ligand-induced FXR-SHP cascade (by the FXR ligand 6-EDCA, in rat models) and overexpressing SHP in HSC prevented fibrogenic changes in the liver. SHP binds JunD and inhibits DNA binding of adaptor protein (AP)-1 induced by thrombin. FXR ligands as therapeutic agents to treat liver fibrosis? [52].
NF-κB	Murine macrophage cell line RAW 264.7/SHP acts as a positive transcription coactivator of NF-κB and essential for NF-κB transactivation by palmitoyl lysophosphatidylcholine (one of the oxLDL constituents). Relevance for differentiation mechanism of resting macrophage cells into foam cells and resulting atherogenesis (see also [44]).
Smad	HepG2, CV-1, and HeLa cells/SHP represses Smad3-induced transcription by competing for the coactivator p300. SHP therefore represses TGF-β-induced gene expression. Relevance for TGF-β-dependent regulation of cell growth, apoptosis, carcinogenesis, and regeneration following liver injury [40]. SHP-Smad3 interaction similar to SHP-BETA2/NeuroD [148].
TRAF6, p65	Macrophages/a novel function of SHP in innate immunity involving Toll-like receptors (TLRs). SHP negatively regulates TLR signaling to NF-κB. Likely, SHP negatively regulates immune responses initiated by various pathogen-recognition receptors by forming a complex with TRAF6 and effect on TRAF6 ubiquitination. In the cytosol of LPS-stimulated cells. SHP also acts as specific transrepressor of the transcription factor p65 (part of the p50/p65 heterodimer found in NF-κB). An additional role for SHP in sepsis and inflammatory disease? [128, 129].

Table 3: Continued.

(3) *Transcriptional coregulators*	
Protein	Model(s)/putative function
Brm, BAF155, BAF47, mSin3A, Swi/Snf	HepG2 cells/The *CYP7A1* gene was used as a model system. SHP has direct interaction with corepressors at the level of native chromatin. SHP directly interacted and mediated the recruitment of mSin3A-Swi/Snf-Brm chromatin remodelling complex to the *CYP7A1* promoter (TATA and BARE II region of the promoter). Also, the mSinA3/HDAC1 corepressor complex is inhibiting transcription by histone deacetylation. SHP also interacted with known proteins belonging to the Swi/Snf complex (BAF155, BAF47). This mechanism explains the complex and subtle SHP-driven inhibition of hepatic bile acid synthesis [50].
CBP	HepG2 cells, CV-1 cells/SHP binds coactivator CBP and competes with Nur77. The mechanism explains the repression of the transcriptional function of Nur77, which is fundamental in apoptosis in the liver [32].
EID-1	Cos-7 cells/SHP specifically interacts with EID-1 providing inhibitory mechanisms. EID-1 (a non-HDAC cofactor) acts as inhibitor of the coregulator complex EID1–p300–CBP. Results clarify essential repression mechanisms of SHP involving coinhibitory factors (upstream targets) distinct from NRs corepressor [12, 150].
G9a, HDAC-1	Caco-2, HepG2, HeLa, Cos-1 cells/SHP localized exclusively in nuclease-sensitive euchromatin regions. SHP can functionally interact with HDAC-1 (HDAC of class I) and the euchromatic histone 3 methylase G9a, and the unmodified K9-methylated histone 3 [151]. Additional data on mechanisms involved SHP-driven repressive activity, involving also target genes regulated by G9a and SHP-mediated inhibition of hepatic bile acid synthesis via coordinated chromatin modification at target genes [152].
GPS2	Cos-7, HepG2, Huh7 cells/SHP negatively interacts with GPS2 (a stoichiometric subunit of the NR corepressor, N-Cor) complex, involved in bile acid synthesis and differential coregulation of CYP7A1 and CYP8B1 expression [67].
SIRT1	HepG2, HEK293T (293T), and HeLa cells/SIRT1 is a HDAC of class III. SHP recruits SIRT1 (activating deacetylase activity of SIRT1) to repress LRH1 transcriptional activity as well as inhibition LRH1 target gene promoter activity and mRNA levels. A novel mechanism is described for SHP repressive action and control of bile acid homeostasis. SIRT1 in working concertedly with NRs and affecting chromatin remodeling in target gene promoters [42].
SMRT/NcoR	Hepatoma cell lines/studies on the role of SHP in CAR-mediated transactivation of the CYP2B gene. SHP might interact with subunits of functionally distinct coregulator complexes, including HDAC3-N-CoR-SMRT [24, 120].
(4) *Others*	
Factor	Model(s)/putative function
miRNA-206	SHP $^{-/-}$ mice/SHP as an important transcriptional activator of miRNA-206 gene expression via a cascade dual inhibitory mechanism involving AP1 but also YY1 and ERRγ. Relevance for multiple steps involving cellular development, proliferation, and differentiation [153].
RNA Pol II	Caco-2 cells/within the pathway of SHP-LXR interaction, it is shown that SHP can interact *in vitro* with RNA polymerase II but not with TFIID and TFIIE transcription initiation factor II D (TFIID), general transcription factor II E (TFIIE) (components of the basal transcription machinery). A further mechanism by which SHP could inhibit both basal and induced transactivation [144].

ABCA1, ABCG1, ABCG5, and ABCG8: ATP-binding cassette transporters; AP1: transcription factor activator protein 1; AHR: aryl hydrocarbon receptor (AHR); ARNT: aryl hydrocarbon receptor (AHR)/AHR nuclear translocator protein; ANG: angiotensin; AOx, acyl-CoA oxidase; ApoE: apolipoprotein E; bHLH-PAS: basic helix–loop–helix–PAS; AR: androgen receptor; BAFs: Brm- or Brg-1-associated factors; BARE: bile acid response element; Brm: human Brahma; CAR: constitutive androstane receptor; CBP: CREB-binding protein; C/EBPα: CCAAT/enhancer-binding protein α; CETP: cholesteryl ester transfer protein; CREB: coactivator cAMP-response element-binding protein; CYP7A1: cholesterol-7-α-hydroxylase; DAX1: dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome: gene 1; DBD: DNA-binding domain; 6-ECDCA, 6-ethylchenodeoxycholic acid; EID1: E1A-like inhibitor of differentiation 1; ER: estrogen receptor; ERRγ: estrogen receptor-related receptor-γ; FBP1: fructose-1,6-bisphosphatase; FXR: farnesoid X receptor; G6Pase: glucose-6-phosphase; GR: glucocorticoid receptor; GPS2: G protein pathway suppressor 2; HD: enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; HDACs: histone deacetylases; HDAC-1: histone deacetylase-1; HDAC-1: histone deacetylase-3; JunD: predominat Jun family protein; HNF3/Foxa: hepatocyte nuclear factor-3; HNF4: hepatocyte nuclear factor-4; LPS: lipopolysaccharides; LXRα: liver X receptorα; LRH-1: liver receptor homologue-1; miRNAs (miR): microRNAs; NcoR: nuclear receptor corepressor; NF-κB: nuclear factor-κB; Nur77: nuclear growth factor I-B; PEPCK: phosphoenolpyruvate carboxykinase; PPRE: peroxisome proliferator-response elements; PXR: pregnane X receptors RAR: retinoid acid receptor; RNA Pol II: RNA polymerase II; RXR: retinoid X receptor; SIRT1: sirtuin1; SREBP-1c: sterol regulatory element-binding protein-1c; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TFIID: transcription initiation factor II D (TFIID); TFIIE: transcription factor II E; TGF-β: transforming growth factor-β; TLRs: Toll-like receptors; TR: thyroid receptor; TRAF6: TNF-receptor-associated factor-6; XRE, xenobiotic response element; YY1: Ying Yang 1.

via the FXR pathway. In this respect, HNF1α $(-/-)$ mice displayed a defect in bile acid transport, increased bile acid and liver cholesterol synthesis, and impaired HDL metabolism [69].

A role for SHP in mediating the recruitment of mSin3A-Swi/Snf to the CYP7A1 promoter, with chromatin remodeling and gene repression, has been described. In HepG2 cells, Kemper et al. [50] have shown that bile acid treatment

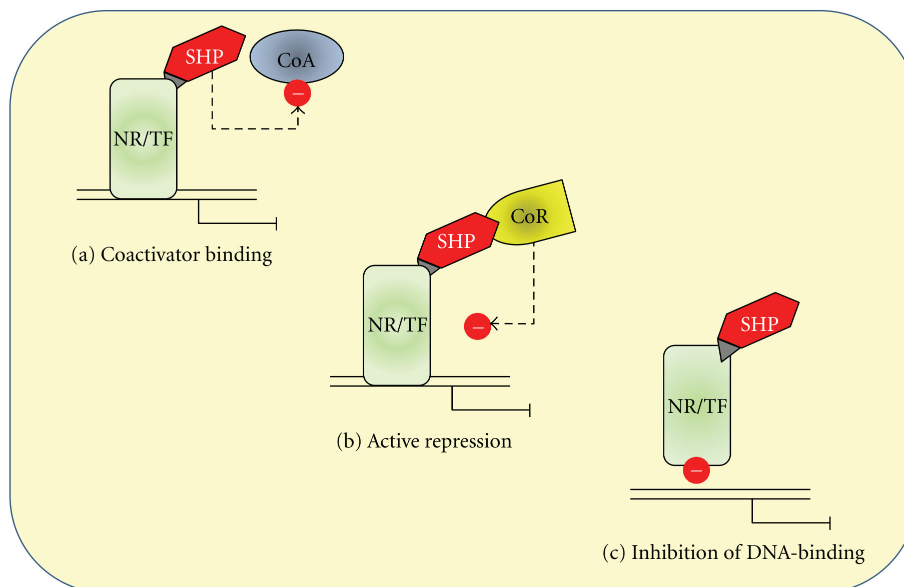

Figure 2: The SHP-mediated repression of target transcription factors occurs by at least three distinct transcriptional repression mechanism: (a) direct interference with the AF-2 coactivator domain of NRs (competition for coactivator binding, leading to the repression of NR-mediated transcriptional activity); (b) recruitment of corepressors, resulting in active repression; (c) direct interaction with the surface of NR or transcription factor, resulting in the blockade of DNA binding and the consequent inhibition of its transcriptional activity. See text for details. The dotted arrows and (-) symbols indicate inhibition. CoA: coactivator; CoR: corepressor; NR: nuclear receptor; SHP: small heterodimer factor; TF: transcription factor. Modified after [12, 15, 131].

resulted in SHP-mediated recruitment of transcriptional coregulators mSin3A and Swi/Snf complex to the promoter, chromatin remodeling, and gene repression (Table 3). This is an additional mechanism involving transformation of nucleosome conformation for the repression by SHP of genes activated by various NRs. In line with such results, increased synthesis and accumulation of BAs occurs in SHP (−/−) mice, due to the loss of SHP repression and consequent derepression of the rate-limiting CYP7A1 and cholesterol 12α-hydroxylase (CYP8B1) (the rate-determining enzyme of the alternative but minor BA synthesis pathway) in the biosynthetic pathway [70–72].

Mechanisms independent of the FXR/SHP/LRH pathway might also exist, since BAs feeding to SHP (−/−) mice reduced the levels of CYP7A1 mRNA to similar levels of control mice [70, 71]. Such SHP-independent and alternative pathways include the protein kinase C/Jun N-terminal kinase (PKC/JNK) pathway [73], the FXR/FGFR4 (FGF receptor 4) pathway [57, 74], the cytokine/JNK pathway [75], the pregnane X receptor (PXR) mediated pathway [76], and the JNK/c-Jun signaling pathway [77].

Another study demonstrated, in SHP (−/−) mice on a background of 129 strain, the protection against hypercholesterolemia in three different models: an atherogenic diet, hypothyroidism, and SHP (−/−) mice intercrossed with LDLR (−/−) mice (to generate SHP/LDLR double (−/−) mice in a mixed 129-C57BL/6 background). When fed an atherogenic diet, the latter strain was almost completely resistant to diet-mediated increases in triglyceride, very low-density lipoprotein (VLDL) cholesterol, and low-density lipoprotein (LDL) cholesterol but had an increase in high-density lipoprotein (HDL) cholesterol as compared with LDLR (−/−) mice. Such results point to the protection against dyslipidemia following the inhibition of hepatic SHP expression, although no antagonist ligands have yet been identified for SHP [78]. We have recently examined biliary lipid secretion and cholesterol gallstone formation in male SHP (−/−) and (+/+) mice before and during the feeding of a lithogenic diet for 56 days [79]. Deletion of the *Shp* gene significantly increased hepatic bile salt synthesis, and doubled the increase of biliary bile salt outputs in SHP (−/−) mice than in (+/+) mice. The intestinal bile acid pool size was significantly greater in SHP (−/−) mice than in (+/+) mice. These increased BAs are efficacious ligands of FXR and can stimulate the expression of intestinal fibroblast growth factor 15 (FGF15) in mice through the FXR signaling pathway, which is consistent with the expanded bile acid pool size in SHP (−/−) mice. At 14 days on the lithogenic diet, fasting gallbladder volume was significantly larger in SHP (+/+) mice than in (−/−) mice [80].

Indeed, FGF15/19 (mouse and human orthologs, resp.) is another FXR gene target in the intestine and appears to contribute to the fine tuning of bile acid synthesis in the liver. Thus, a model for FXR-mediated repression of bile acid synthesis should also take into account the bile acid-mediated activation of intestinal FXR and FGF15 in the small intestine (while the FXR-SHP pathway is activated in the liver). According to the most plausible view, FGF15 acts as a hormone to signal between intestine and liver. The secreted FGF15 by the intestine circulates to the liver, likely through

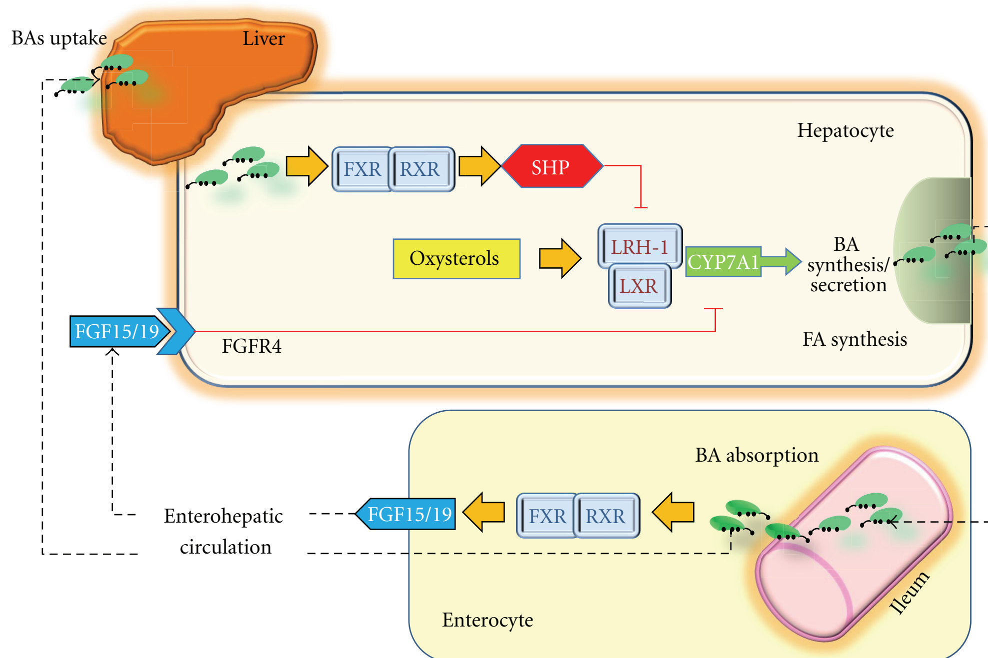

Figure 3: The potential molecular mechanisms of crosstalk between nuclear receptors LXR and FXR–SHP–LRH-1 regulatory cascade in the liver and intestine. Bile acids act as ligands for FXR, which regulates transcription by binding as a heterodimer with RXRs. This step results in increased SHP expression. SHP in turn inhibits LRH-1, preventing the activation of target genes that participate in bile acid and fatty acid synthesis. In the absence of bile acids, LRH-1 acts together with LXR to stimulate bile acid synthesis [64, 65, 132]. The important pathways in the intestine that contribute to modulation of bile acid synthesis are also depicted (see text for details). There is a bile-acid-mediated activation of intestinal FXR and, as a result, the release of FGF15 in the small intestine. The secreted FGF15 by the intestine circulates to the liver, likely through the portal circulation or lymph flow [81] and induces the activation of FGFR4 in the liver. The FGF15/FGFR4 pathway synergizes with SHP *in vivo* to repress CYP7A1 expression [57]. Bas: bile acids; FGF: fibroblast growth factor; FGFR4: FGF receptor; FXR: farnesoid X receptor; LRH-1: liver receptor homologue-1; LXR: liver X receptor; RXR: retinoid X receptors; SHP: short heterodimer partner. Adapted from Ory [66] and Inagaki et al. [57].

the portal circulation or lymph flow [81], and induces the activation of FGFR4 in the liver. As shown in Figure 3, the FGF15/FGFR4 pathway synergizes with SHP *in vivo* to repress CYP7A1 expression [57]. In humans, a similar mechanism should involve the FGF19. Of note, activation of FXR transcription in the intestine protected the liver from cholestasis in mice by inducing FGF15 expression and reducing the hepatic pool of BA. This suggests a potential approach to reverse cholestasis in patients [82]. Hepatic fatty acid homeostasis is also regulated by SHP since regulating these genes involves in fatty acid uptake, synthesis, and export [83–87]. In a study exploring global gene expression profiling combined with chromatin immunoprecipitation assays in transgenic mice constitutively expressing SHP in the liver, overexpression of *SHP* in the liver was associated with the depletion of the hepatic bile acid pool and a concomitant accumulation of triglycerides in the liver [84]. By contrast, fat accumulation induced by a high-cholesterol or high-fat diet is prevented by the deletion of *SHP* [88, 89]. The pleiotropic role of SHP can also be found in the case of nonalcoholic liver steatosis since *OB*/*SHP* double (−/−) mice (a model of severe obesity and insulin resistance) became resistant to liver steatosis and showed improved insulin sensitivity [86].

Another interesting role for SHP emerged after it was found that BAs negatively regulate the human angiotensinogen (ANG) gene. ANG is the precursor of vasoactive octapeptide angiotensin II, and BAs act through the SHP pathway by preventing hepatocyte nuclear factor-4 (HNF4) from binding to the human ANG promoter [90].

3. Fat Mass, Adipocytes, and Obesity

SHP appears to play a central role in obesity. Human obesity is considered a polygenic disorder characterized by partly known abnormal molecular mechanisms resulting in increased fat mass, with an imbalance between the energy acquired from nutrients that dissipated as heat (i.e., thermogenesis). In this respect, weight stability requires a balance between calories consumed and calories expended [91]. In adipose tissue depots, two main types of adipocytes exist, that is, brown adipocytes and white adipocytes. In several animal species, some adipose tissue sites mainly include brown adipocytes (BATs) and the other contains mainly white adipocytes (WATs). BAT dissipates chemical energy to produce heat either as a defense against cold [92] or as energy expenditure to compensate food intake [93, 94]. The unusual

function of BAT might be better understood by considering that they share a common origin with myocytes [95, 96], and BAT was indeed considered something in between muscle and adipose tissue [95]. BAT is deemed as the major site for sympathetic (adrenergic) mediated adaptive thermogenesis; this pathway involves the uncoupling protein-1 (UCP1). WAT is mainly implicated in the regulation of lipid storage and catabolism but also in the synthesis and secretion of adipokines [97–100]. While the percentage of young men with BAT is high, the activity of BAT is reduced in men who are overweight or obese [101]. Thermogenesis unequivocally exists in both humans and animals, and BAT is the major site of thermogenesis which can be increased by environmental factors (i.e., adaptive thermogenesis). In both human and animal species, dietary composition, chronic cold exposure, and exercise may increase thermogenesis [102]. As far as adipose tissue biology is concerned, SHP seems to play a distinct regulatory function in WAT, as compared with BAT. A number of experiments have focused on animal models of obesity and subtle molecular changes. SHP-deficient mice are protected against high-fat-diet-induced obesity [89].

Peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 (PGC-1) family members are multifunctional transcriptional coregulators. PGC-1 acts as a molecular switch in several metabolic pathways. In particular, PGC-1α and PGC-1β regulate mitochondrial biogenesis, adaptive thermogenesis, fatty acid and glucose metabolism, fiber-type switching in skeletal muscle, peripheral circadian clock, and development of the heart [103]. In particular, SHP functions as a negative regulator of energy production in BAT [89] because SHP is a negative regulator of PGC-1α expression in BAT. In turn, PCG-1α is a coactivator of uncoupling protein 1 (UCP1) which plays a major role in energy dissipation as heat in multilocular BAT of different animal species and humans [104–106]. Fat-specific (BAT) SHP-overexpressed transgenic mice had increased body weight and adiposity. Energy metabolism, however, was increased, and BAT cold exposure function was enhanced with activation of thermogenic genes and mitochondrial biogenesis (enhanced β1-AR gene expression and PGC1α). Compared with wild-type mice on a high-fat diet, SHP overexpression was associated with enhanced diet-induced obesity phenotype with weight gain, increased adiposity, and severe glucose intolerance. An additional feature of SHP transgenic mice was a decreased diet-induced adaptive thermogenesis, increased intake of food, and decreased physical activity [107]. This leads to the conclusion that, although expressed at low levels in fat, activation of SHP in adipocytes has a strong effect on weight gain and diet-induced obesity [107]. Moreover, if mechanisms linked to energy metabolism and the development of obesity are considered, SHP has distinct roles in WAT and BAT. As previously mentioned, while *SHP* deletion in obese leptin-deficient mice (*ob/ob*) prevented the development of nonalcoholic fatty liver and improved peripheral insulin sensitivity [86], *SHP* deletion did not overcome the severe obesity caused by leptin deficiency. A significant protective effect from obesity by *SHP* deficiency was likely associated with the low basal level of SHP expressed in fat. Adipogenesis appears to be influenced by SHP: when SHP was overexpressed in 3T3-L1 preadipocytes, cell differentiation was inhibited, as well as the accumulation of neutral lipids within the cells. Thus, SHP may act as a molecular switch governing adipogenesis. In particular, SHP appears to be a potent adipogenic suppressor, and preadipocytes are kept in an undifferentiated state through the inhibition of the adipogenic transcription factors and stimulators [108]. Further studies will address whether the loss of *SHP* function results in inhibition of lipid accumulation in adipocytes, similar to what is observed in hepatocytes. In a future clinical setting, treatment of obesity might also include drugs able to mimic or stimulate the effects of SHP. Mutations in the *Shp* gene have also been reported in patients with lipodystrophy carrying four different polymorphisms [109].

SHP mutations may not be considered a common cause of severe obesity. A number of important clinical studies have examined this issue (Table 4); however, Hung et al. [110] in UK examined the relationships between genetic variation in *SHP* and weight at birth, adiposity, and insulin levels in three different populations (the Genetics of Obesity Study) GOOS, the Avon Longitudinal Study of Parents and Children (ALSPAC), and the Ely studies). In the 329 cases of severe early-onset obesity (GOOS study), two novel and rare missense mutations (R34G and R36G) were identified which might in part contribute to obesity in the probands. Furthermore, two common polymorphisms, namely, G171A (12% of subjects with higher birth weight) and −195CTGAdel (16% of subjects with lower birth weight) were found. In the ALSPAC cohort of 1,079 children, the G171A variant was associated with increased body mass index and waist circumference together with higher insulin secretion 30 minutes after glucose load. Thus, whereas mutations in the *Shp* gene cannot be seen as a common cause of severe human obesity, genetic variation in the *Shp* gene locus may influence birth weight and have effects on body size. The effect might ultimately involve insulin secretion by the negative regulation between SHP and the hepatocyte nuclear factor-4α (HFN-4α), a transcription factor involved in differentiation and function of pancreatic β-cells [110].

A possibility is that decreased *SHP* expression or function results in increased HFN-4α activity with a cascade of events, including fetal hyperinsulinemia, and increased birth weight. At a later stage, sustained hyperinsulinemia might be responsible of insulin resistance and obesity of the adult [110].

Mutations in the *Shp* gene were also associated with influence on birth weight, mild obesity, and insulin levels in the study by Nishigori et al. on 274 Japanese subjects [111]. Mutations in several genes encoding transcription factors of the hepatocyte nuclear factor (HNF) cascade are associated with maturity-onset diabetes of the young (MODY). MODY is a monogenic form of early-onset diabetes mellitus (defective insulin secretion with normal body weight), and SHP is deemed as a plausible candidate MODY gene; this is because SHP is able to inhibit the transcriptional activity of the hepatocyte nuclear factor-4α (HFN-4α), a key member of the MODY regulatory network. Thus, further studies have looked for segregation of *SHP* mutations with MODY in a cohort of Japanese patients with early-onset diabetes. In this context, variants in *SHP* appeared to cosegregate with

Table 4: Studies on the association between *SHP (NR0B2)* genetic variation and birth weight, high BMI obesity, and fasting insulin diabetes.

Author	Country	Study populations/ mutation	Subjects number	Mutation(s)	Association with birth weight increase	Association with BMI/obesity	Association with increased insulin levels	Association with diabetes	Conclusions
Nishigori et al. [111]	Japan	Young-onset type 2 diabetes	274	In 7 subjects, 5 different mutations (H53fsdel10, L98fsdel9insAC, R34X, A195S, R213C) and 1 apparent polymorphism (R216H) (all in a heterozygous state)	Yes	Yes	—	No	*Shp* genetic variation: most common monogenic determinant of obesity and increased birth weight in Japanese
Hung et al. [110]	UK	GOOS (severe early-onset obesity)	329	R34G and R36C Missense mutations	Yes	No (selection of extreme obesity: stronger effect from other major gene?)	Yes	—	Genetic variation in the SHP locus may influence birth weight and have effects on BMI, possibly through effects on insulin secretion
				G171A (12%)	Yes		—	—	
				-195CTGAdel (16%) common polymorphisms	No (lower birth weight)		No (lower fasting levels)		Subtle effects in heterozygosity, stronger effects in homozygosity
	UK	ALSPAC (cohort of children)	1,079	G171A	No	Yes (higher BMI and waist circumference at 7 yrs)	Yes (higher fasting levels and 30-min response)	—	
				-195CTGAdel		No (lower BMI)			
	UK	Ely Study (Caucasian adults)	600	G171A	Data not available	Yes (BMI increased)	No	—	
				-195CTGAdel		Yes (female: higher BMI), No (male: lower BMI)			

Table 4: Continued.

Author	Country	Study populations/ mutation	Subjects number	Mutation(s)	Association with birth weight increase	Association with BMI/obesity	Association with increased insulin levels	Association with diabetes	Conclusions
Mitchell et al. [113]	UK	Young-onset type 2 diabetes, obesity, birth weight	1,927	Birth weight: the only child homozygous for the A allele had a birth weight ≥4 kg Obesity: no association if G/A genotype; yes (?) (if A/A homozygotes)	No	No Yes (?)	—	No	Mutations in SHP < UK than in Japanese obese type 2 subjects G171A coding polymorphism in 14.1% of UK subjects The A allele (G/A genotype) not associated with obesity or increased birth weight Homozygous for the rare A allele: predisposed to moderate obesity and possibly increased birth weight
Echwald et al. [114]	Denmark	Early-onset obesity (men)	750	2 silent variants c.65C4T [p. Y22Y], c.339G4A [p. P113P] 3 missense variants c.100C4G [p. R34G], c.278G4A [p. G93D], c.415C4A [p. P139H] G171A polymorphism (8.9%)	—	Yes (only among obese) No ($P = 0.07$ versus obese)	—	—	Very low prevalence of functional SHP variants associated with obesity among Danes A role for G171A polymorphism low penetrance SHP variants) for obesity risk in Europe? Major differences in prevalence and impact of SHP variants between Danish and Japanese obese
		Nonobese controls	795	No variants G171A polymorphism (7.1)					
		Functional analyses in MIN6-m9 and HepG2 cell lines				93D mutant protein: reduced *in vitro* inhibition of the HNF4α transactivation of the HNF-1α promoter expression			

Note: SHP is expressed in the liver, pancreas, spleen, small intestine, and adrenal gland in humans [18] and inhibits the transcriptional activity of hepatocyte nuclear factor-4 α (HNF4α). ALSPAC: Avon Longitudinal Study of Parents and Children; GOOS: Genetics of Obesity Study; HNF4α: hepatocyte nuclear factor-4α.

increased body mass index in families, thus contributing to obesity among Japanese subjects. Also, increased risk of morbidity was observed in another study from Japan, examining patients with type 2 diabetes and *SHP* mutations [112].

Major differences, however, might exist in the prevalence and function of *SHP* variants in different populations. Of note, the results from other Caucasian cohorts did not confirm the association between *SHP* mutation and obesity [113, 114]. Echwald et al. conducted an elegant study on the prevalence of *SHP* variants by single-strand conformational polymorphism and heteroduplex analysis among 750 Danish obese men with early-onset obesity [114]. As control, a cohort of 795 nonobese control subjects was genotyped using PCR-RFLP. Functional analyses of the identified coding region variants were performed in both MIN6-m9 and HepG2 cell lines. Five novel variants were identified (including 3 missense variants (c.100C>G [p.R34G], c.278G>A [p.G93D], and c.415C>A [p.P139H]) and 2 silent variants (c.65C>T [p.Y22Y] and c.339G>A [p.P113P])). The previously reported [111] c.512G>C [p.G171A] common polymorphism was identified; however, the prevalence of functional *SHP* variants associated with obesity was considerably lower among Danish subjects (1 out of 750 obese, none of control subjects), compared to the prevalence observed in Japan by Nishigori et al. [111]. Mitchell at al. [113] investigated SHP variants in 1927 UK subjects according to type 2 diabetes, obesity, and birth weight. Although reporting a raised body mass index among homozygous carriers of the 171A variant (<1%), this polymorphism was unlikely to be associated with all three conditions in Caucasians. Taken together, the above-mentioned studies suggest that the 171A variant might contribute only to subsets of polygenic obesity.

4. Other Functions of SHP

The existence of multiple interactions of SHP with NRs, transcription factors and transcriptional cofactors (Tables 2 and 3) points to the pleiotropic and central role of SHP in the body.

SHP has been hypothesized to act in glucose homeostasis via complex pathways involving the inhibition of glucocorticoid receptors (GR) in mammalian cells and the inhibition of PGC-1 gene, a coactivator of NRs important for gluconeogenic gene expression and the PGC-1-regulated phospho(enol)pyruvate carboxykinase (PEPCK) promoter. Such steps underscore a physiologically relevant role for SHP in modulating hepatic glucocorticoid action [22]. Following the bile acid-induced induction, SHP inhibited a number of other pathways, including the HNF4α-mediated transactivation of the PEPCK and fructose biphosphate (FBP) promoters, as well as the transactivation of the glucose-6-phosphatase (G6Pase) promoter mediated by Foxo1 [115]. The interaction between SHP inhibitory function and the 3 isoforms (α, β, and γ) of the hepatocyte nuclear factor-3 (HNF4) points to the regulatory role of SHP on gluconeogenesis [51]. A role for SHP in insulin secretion pathway has also been reported. Mutations in hepatocyte nuclear factor 1α (HNF-1α) is associated with maturity-onset diabetes of the young type 3. This condition depends on impaired insulin secretory response in pancreatic beta cells.

Indeed, loss of HNF-1α function in HNF-1α (−/−) mice resulted in altered expression of genes involved in glucose-stimulated insulin secretion, but also insulin synthesis, and beta-cell differentiation. Pancreatic islets of HNF-1α (−/−) mice showed a distinctive reduction of SHP expression and a downregulation of the HNF4α gene expression. Since SHP appears to repress its own transcriptional activation following heterodimerization with HNF4α, a feedback autoregulatory loop between SHP and HNF4α has been hypothesized [116]. Also, SHP likely functions as a negative regulator of pancreatic islet insulin secretion. SHP (−/−) mice were characterized by hypoinsulinemia, increased glucose-dependent response of islets, increased peripheral insulin sensitivity, and increased glycogen stores [117]. The role played by SHP in the regulation of hepatic gluconeogenesis has also emerged in a number of additional experiments. For example, the liver of SHP (−/−) mice showed increased glycogen stores [117], while hepatic *Shp* gene expression (induced by the antidiabetic biguanide drug metformin) was associated with inhibition of hepatic gluconeogenesis. Induction of SHP was achieved via AMP-activated protein kinase (AMPK) and associated with downregulation of essential gluconeogenic enzyme genes, that is, phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) [118], and fructose-1,6-bisphosphatase (FBP1) [119].

PGC-1 gene is a coactivator of NRs, and this step is relevant for gluconeogenic gene expression. Yamagata et al. [119] showed that bile acid (chenodeoxycholic acid) was able to induce the downregulation of PGC-1 gene, and this mechanism involved forkhead transcription factors (Foxo1, Foxo3a, Foxo4) via a SHP-dependent manner.

Drug metabolism and detoxification might be regulated by SHP. This is also the case for excess BAs: the pregnane X receptor (PXR) induces CYP3A and inhibits CYP7α, both involved in biochemical pathways leading to the conversion of cholesterol into primary BAs, whereas CYP3A is also involved in the detoxification of toxic secondary bile acid derivatives. SHP acts as a potent repressor of PXR transactivation, and this finding suggests that PXR can act on both bile acid synthesis and elimination detoxification [1]. Additional mechanisms involved in the SHP-dependent control of pathways of drug metabolism have been identified. The expression of genes involved with the metabolism of xenobiotics might be regulated by SHP in the spleen acting on (aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT)) AHR/ARNT heterodimers which, in turn, bind to xenobiotic response elements (XREs) at the level of specific DNA sequences [41]. A number of genes involved in hormone and drug metabolism would be expressed (i.e., UGT16, ALDH3, CYP1A1, CYP1A2, CYP1B1, etc.). SHP also appears to downregulate the constitutive-androstane-receptor- (CAR-) mediated CYP2B1 gene expression, induced by phenobarbital to form the CAR/RXR heterodimer which, in turn, binds to 2 DR-4 sites to form the phenobarbital responsive unit in the CYP2B gene [120] (Table 3). One role of SHP in steroidogenesis has been identified in the testes

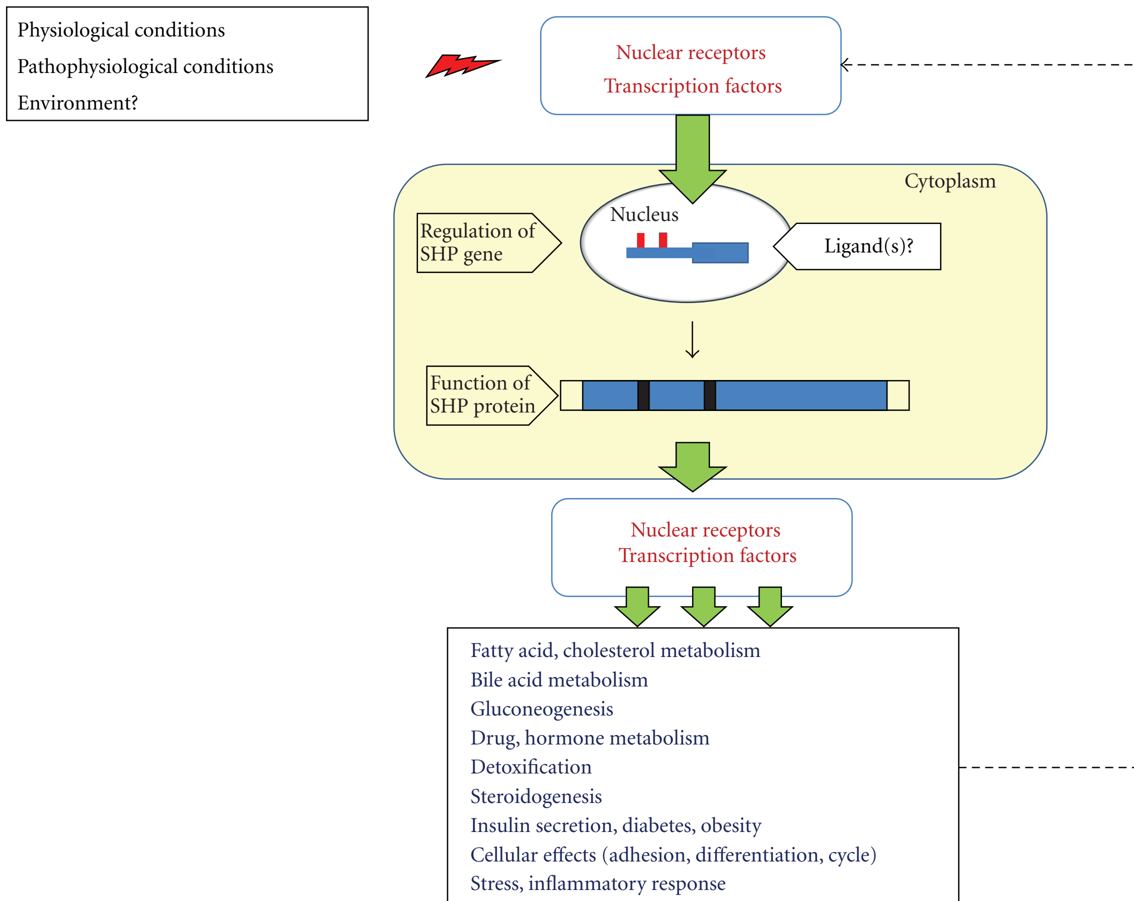

FIGURE 4: Schematic diagram of the function and gene regulation of SHP. Different conditions will lead to activation of nuclear receptors and/or transcription factors able to regulate *Shp* gene expression in the nucleus and protein synthesis in the cytoplasm. The protein acts as a transcriptional corepressor of a number of other nuclear receptors and transcription factors involved in a wide series of regulatory pathways. The potential role of a feedback mechanism and of ligand(s) is hypothesized.

with influence on testosterone synthesis and germ cell differentiation [121] and in the intestine for glucocorticoid synthesis [122].

A role for SHP in cell proliferation and apoptosis signaling is emerging. Depending on the cell type, SHP seems to have both inhibitory and stimulatory effects on apoptosis. However, the manipulation of SHP through the synthetic ligands adamantyl-substituted retinoid-related (ARR) compounds 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437/AHPN) and 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3-Cl-AHPC) induces apoptosis of a number of malignant cells (i.e., leukemia and breast carcinoma) both *in vitro* and *in vivo* [123, 124]. The complex mechanism implies binding of ARR and 3-Cl-AHPC to SHP with formation of a corepressor complex containing Sin3A and nuclear receptor corepressor (*N*-CoR) which activate local control of mitochondrial function and apoptosis, with a limiting function on tumorigenesis [17, 123] (Table 3). SHP appears to be also involved in DNA methylation and acting as a tumor suppressor, at least in the human and mouse livers [125–127]. Whether manipulation of SHP will be helpful in the treatment of hepatic and other gastrointestinal cancers is still a matter of research. The recent finding that SHP negatively regulates TLR signaling to NF-κB has raised the interest for the role of SHP in mechanisms governing innate immunity. SHP appears to negatively regulate the expression of genes encoding inflammatory molecules. Of note, direct binding of NF-κB seems to occur in resting cells, while binding of SHP to TRAF6 occurs in LPS-stimulated cells [128, 129].

5. Conclusions and Perspectives

A remarkable number of metabolic functions in the body appear to be regulated by the orphan unique NR, small heterodimer partner SHP, which targets a complex set of genes in multiple pathways as a transcriptional corepressor (Figure 4). Pathways include fatty acid metabolism, glucose homeostasis, and drug-hormone detoxification. When looking at complex mechanisms leading to some important *lipidopathies,* that is, obesity and liver steatosis, enlightening data about the regulatory function of SHP are provided by studies using *Shp*-deleted and *Shp*-overexpressed animal models. Most likely, a condition of *Shp* deficiency might

counteract lipid accumulation and improve plasma lipoprotein profiles. Further studies are urgently needed to confirm that such an important metabolic regulatory mechanism of SHP is true and has high translational value. To date, however, no synthetic antagonists or agonists for SHP are available, and one should keep in mind that rather divergent and somewhat elusive data have been observed regarding the loss of SHP function in humans and rodents. Thus, careful examination of subtle SHP intrinsic functions is essential to dissect potential modulatory pathways of SHP for a variety of metabolic abnormalities but also in tumorigenesis. Moreover, identifying specific endogenous ligands and synthetic agonists of SHP will pave the way to for therapeutic intervention. The effect of synthetic ligands on SHP modulation in hepatocytes and adipocytes, for example, might represent therapeutic tools for the treatment of constituents of the metabolic syndrome, namely, hypercholesterolemia, overweight obesity, and liver steatosis.

Abbreviations

AF1: Activation function 1
AF2: Activation function 2
AHR/ARNT: Aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT)
ALDH3: Human aldehyde dehydrogenase 3 gene
AMPK: AMP-activated protein kinase
ARR: Adamantyl-substituted retinoid-related molecules
β1-AR: β1-adrenergic receptor
BAREs: Bile acids response elements
BAT: Brown adipose tissue
Brm: Human Brahma (a catalytic component of the SWI/SNF-related chromatin-remodeling complex)
CAR: Constitutive androstane receptor
CD437/AHPN: 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid
3-Cl-AHPC: 4-[3-(1-Adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid
CYP3A: Hepatic cytochrome P-4503A
CYP1A1: Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2: Cytochrome P450, family 1, subfamily A, polypeptide 2
CYP1B1: Cytochrome P450, family 1, subfamily B, polypeptide 1
CYP2B1: Cytochrome P450, subfamily IIB
CYP7A1: Cholesterol 7 alpha-hydroxylase
CYP8B1: Cholesterol 12alpha-hydroxylase
DBD: DNA-binding domain
DAX-1: Dosage-sensitive sex reversal adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1
DR-4: Nuclear receptor half-site repeat
ER: Estrogen receptor
FBP1: Fructose-1,6-bisphosphatase
FGF: Fibroblast growth factor
FGFR: Fibroblast growth factor receptor
FISH: Fluorescence *in situ* hybridization
Foxo: Forkhead transcription factors
FXR: Farnesoid X receptor
G6Pase: Glucose-6-phosphatase
GPCR: G-protein-coupled Receptor
GPS2: G protein pathway suppressor 2
GR: Glucocorticoid receptors
HDL: High-density lipoprotein
HNF: Hepatocyte nuclear factor
JNK: Jun N-terminal kinase
JUN-D: Jun family of the activator protein 1 (AP-1) transcription factor complex
LBD: Ligand-binding domain
LDL: Low-density lipoprotein
LDLR: Low-density lipoprotein receptor
LRH1: Liver receptor homologue 1
3T3-L1: Mouse embryonic fibroblast adipose-like cell line.
LXXLL: Leu-Xaa-Xaa-Leu-Leu
mSin3A: Mammalian homolog of the Saccharomyces cerevisiae transcriptional corepressor Sin3p
N-CoR: Nuclear receptor corepressor
NRs: Nuclear receptors
PEPCK: Phosphoenolpyruvate carboxykinase
PGC-1: Peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1
PKC: Protein kinase C
PXR: Pregnane X receptor
RAR: Retinoid acid receptor
RNA Pol II: RNA polymerase II
RNF31 Member of the ring-between-ring (RBR) family of E3 ubiquitin ligases
RXR: Retinoid X receptor
SHP: Small heterodimer partner
SMILE: SHP-interacting leucine zipper protein
SMRT: Silencing mediator of retinoid and thyroid hormone receptor
SRC-1: Steroid receptor coactivator-1
SWI/SNF: SWItch/Sucrose NonFermentable (yeast nucleosome remodeling complex composed of several proteins which are products of the SWI and SNF genes)
UCP1: Uncoupling protein-1
USF-1: Upstream stimulatory factor-1
TFIID: Transcription initiation factor II D
TFIIE: Transcription factor II E
UGT-16: Uridine 5′-diphospho (UDP) glucuronosyl transferase family member
VLDL: Very-low-density lipoprotein
XREs: Xenobiotic response elements
WAT: White adipose tissue.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Nuclear Receptor Variants in Liver Disease

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This review aims to provide a snapshot of the actual state of knowledge on genetic variants of nuclear receptors (NR) involved in regulating important aspects of liver metabolism. It recapitulates recent evidence for the application of NR in genetic diagnosis of monogenic ("Mendelian") liver disease and their use in clinical diagnosis. Genetic analysis of multifactorial liver diseases such as viral hepatitis or fatty liver disease identifies key players in disease predisposition and progression. Evidence from these analyses points towards a role of NR polymorphisms in common diseases, linking regulatory networks to complex and variable phenotypes. The new insights into NR variants also offer perspectives and cautionary advice for their use as handles towards diagnosis and treatment.

1. Introduction

Systematically, genetic analysis with regard to disease onset and progression can be separated into pre- and post-hoc examination of monogenic or polygenic diseases. Monogenic ("Mendelian") diseases are caused by a single gene defect and follow relatively straightforward inheritance patterns. The most prominent of these disorders are rather rare, often severe, and characterized by early onset. Genetic testing for monogenic liver disease in symptomatic patients is based on known disease-associated gene variants, thereby confirming the genetic etiology and sometimes allowing prediction of disease progression [1].

In contrast, polygenic diseases such as fatty liver disease and gallstones result from combinations of multiple gene variants and environmental factors, all of which play a role in disease initiation and progression [2]. The assessment of predisposition towards polygenic disease is based on sequence analysis of known contributory genes and construction of "polygenic risk scores" from variants of these genes [1]. Still in its infancy, personal genome information might eventually be able to predict a variety of risks associated with an individual's lifestyle such as fatty food and alcohol consumption, as well as susceptibility to infectious diseases such as infection with hepatitis B or C virus.

2. Nuclear Receptors

Nuclear receptors (NRs) are a subclass of regulatory molecules that orchestrate gene transcription in response to the presence or absence of specific ligands. Due to these functional requirements, they are characterized by the presence of a ligand-binding and a DNA-binding domain. NRs represent a central point of interaction between environment and gene regulation. They are the "hinge" connecting endogenous and environmental stimuli, that is, ligands, with the cells' transcriptional response (Figure 1).

This position makes them a prime target for medical intervention by agonistic or antagonistic binding of synthetic compounds. However, the regulatory orchestra of molecules conducted by NR is highly complex and abounds with redundancy and crosstalk, hence any impact might be potentially difficult to predict as well as disappointingly diffuse. Even identical variations in a single NR can result in a wide variety of phenotypes due to genetic differences in the cofactors involved and higher order networks based on mutual regulatory interaction [3].

The core position in maintaining cellular equilibrium should render NR susceptible to the impact of natural sequence variation. On the other hand, it has been suggested that the phenotypic effects of NR gene variation are bound to

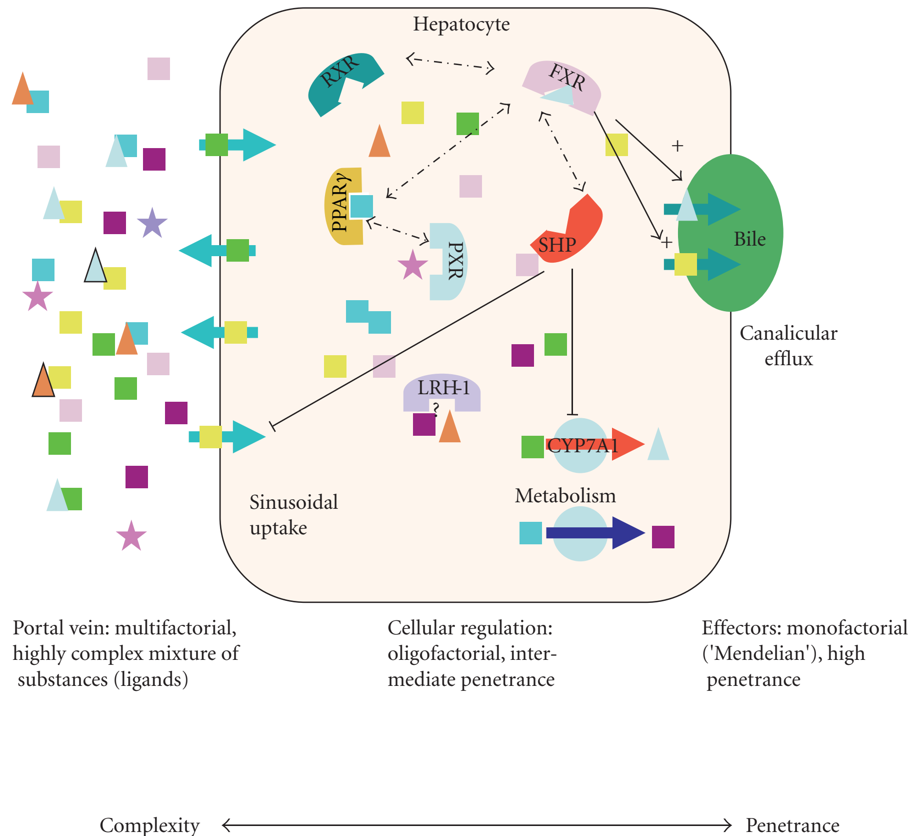

Figure 1: Schematic depiction of NR action in hepatocytes demonstrating a reduction in complexity and an increase in penetrance of genetic variants from the sinus to the canaliculus. Squares represent metabolic compounds such as triglycerides, cholesterol, fatty acids, and phospholipids; triangles represent bile salts; stars represent toxins; large semi-circles symbolise nuclear receptors, and circles stand for metabolic enzymes.

be less severe than a variation in functionally defined effector molecules such as a single-substrate transmembrane transporter. The transcriptional regulation directed by NR is kept in tight check and fine-tuned by a set of co-regulators (co-activators or corepressors) [4, 5]. Consequently, relatively few congenital, "Mendelian" diseases have been identified to date, which are caused by genetic variations in NR. One example is maturity-onset diabetes of the young (MODY1), caused by mutations affecting the gene encoding the hepatic nuclear factor (HNF) 4-alpha (*NR2A1*) [6] (Table 1).

While the denominator of "monogenic disease" seems to imply a straightforward genotype-to-phenotype correlation, the reality in even those seemingly "simple" diseases is anything but simple: Tirona et al. [7] showed that HNF4A is critically involved in the PXR (NR1I2)- and CAR (NR1I3)-mediated transcriptional activation of cytochrome P450 (CYP) 3A4, hence involving two more NRs even in its basal regulatory function. Whereas Hani et al. [8] identified *HNF4A* mutations as being causative in maturity-onset diabetes of the young (MODY type 1, OMIM #125850) based on a nonsense mutation (p.Q268X) in an extended pedigree, many other seemingly functional variants have turned out to be either innocent bystanders or of relatively low penetrance. Pearson et al. [9] were able to show that functional *HNF4A* variants are associated with a considerable increase in birth weight and macrosomia, and a novel cause of neonatal hypoglycemia. Results from the investigation of 108 members of 15 families with MODY1 show how genotype-phenotype correlation is far from clearcut, with both described phenotypes being found only in 15–56% of mutation carriers. Ek et al. [10] examined the impact of two disease-associated variants (p.T130I and p.V255M, Table 1) on various aspects of HNF4-alpha function. Both variants showed decreased transactivation. Only the p.T130I polymorphism was associated with T2D, whereas the p.V255M variant was associated with a decrease in fasting serum C-peptide levels. Array analyses revealed that HNF4-alpha bound to the promoters of 12% of hepatocyte islet genes represented on a microarray and hence can be considered a "master regulator" of hepatocyte and beta-cell genes [11]. But even in a complex and occasionally ambiguous setting, where the detection of a functional variant does not necessarily predict disease phenotypes, genetic testing appears to be helpful, if only to identify at-risk relatives and motivate affected individuals towards lifestyle changes [12]. Evidently, there is no clear delineation between the "Mendelian" diseases and contribution towards complex phenotypes modulated by NR variants (Figure 1). A meta-analysis of polymorphisms in

Table 1: Single nucleotide polymorphisms (SNPs) associated with liver disease.

Gene	SNP	*rs number*	Disease	*OR* (95% CI)	*P*-value	Cohort (controls)	Population	Reference
HNF4a (NR2A1)	Q268X	*rs6093980*	MODY-1	N/A	N/A	>360	R-W pedigree	[6]
	Y16X	N/A		N/A	N/A	108	UK	[9]
	S34X							
	R127W							
	D206Y							
	E276Q							
	R303H							
	I314F							
	L332P							
	M364R							
	c. IVS5nt+1G>A							
	c.IVS4nt-2A>G							
	T(3;20)							
	V393I	N/A	NIDDM	N/A	N/A	N/A	F-40 pedigree	[8]
	T130I	N/A	T2D	1.26 (1.01–1.57)	0.04	1,466 (4,520)	Danish	[10]
	V255M	N/A	Decreasing fasting serum C-peptide levels	1.0 (0.28–3.65)	1.0			
FXR (NR1H4)	−20,647T>G	N/A	Gallstones	0.42 (0.17–1.01)	0.053	77 (74)	Mexican	[34]
	−1G>T	*rs56163822*		0.25 (0.07–0.95)	0.042	75 (70)		
	IVS7-31A>T	*rs7138843*		0.47 (0.22–1.01)	0.053	77 (88)		
	−1G>T	*rs56163822*	ICP	0.92 (0.35–2.44)	0.96	342 (349)	British/Swedish	[35]
	M173T	N/A		3.2 (1.1–11.2)	0.02			
VDR (NR1I1)	c.1025-49G>T (ApaI)	*rs7975232*	AIH	0.72 (0.40–1.30)	0.27	123 (214)	Caucasian	[22]
	Intron 8 (BsmI)	*rs1544410*		0.63 (0.37–1.06)	0.08			
	Exon 2 (FokI)	*rs1073581*		0.5 (0.28–0.92)	0.02			
	I352I (TaqI)	*rs731236*		1.27 (0.69–2.33)	0.43			
	c.1025-49G>T (ApaI)	*rs7975232*	PBC	1.85 (1.02–3.35)	0.04	74 (214)		
	Intron 8 (BsmI)	*rs1544410*		2.1 (1.22–3.62)	0.006			
	Exon 2 (FokI)	*rs1073581*		0.55 (0.27–1.12)	0.09			
	I352I (TaqI)	*rs731236*		1.16 (0.56–2.39)	0.69			
	c.1025-49G>T (ApaI)	*rs7975232*	AIH	0.82 (0.42–1.58)	0,55	49	Chinese	[20]
	Intron 8 (BsmI)	*rs1544410*		1.44 (0.59–3.51)	0.42			
	Exon 2 (FokI)	*rs1073581*		2.18 (1.07–4.43)	0.019			
	I352I (TaqI)	*rs731236*		0.00 (0.00)	0.28			

Table 1: Continued.

Gene	SNP	*rs number*	Disease	*OR* (95% CI)	*P*-value	Cohort (controls)	Population	Reference
	c.1025-49G>T (ApaI)	*rs7975232*	PBC	0.90 (0.49–1.64)	0.727	58		
	Intron 8 (BsmI)	*rs1544410*		4.41 (1.29–15.02)	0.01			
	Exon 2 (FokI)	*rs1073581*		1.30 (0.63–2.68)	0.05			
	I352I (TaqI)	*rs731236*		0.00 (0.00)	0.224			
	c.1025-49G>T (ApaI)	*rs7975232*	PBC	0.71 (0.47–1.08)	0.133	195 (179)	Japanese	[21]
	Intron 8 (BsmI)	*rs1544410*		0.71 (0.44–1.16)	0.179			
	I352I (TaqI)	*rs731236*		1.02 (1.00–1.04)	0.109			
	c.1025-49G>T (ApaI)	*rs7975232*		1.02 (0.52–1.98)	1.000	139 (156)	Italian	
	Intron 8 (BsmI)	*rs1544410*		0.33 (0.12–0.92)	0.039			
	I352I (TaqI)	*rs731236*		0.94 (0.51–1.75)	0.876			
	c.1025-49G>T (ApaI)	*rs7975232*	HBV	3.3 (1–11)	0.05	214 (408)		[23]
	c.1025-49G>T (ApaI)	*rs7975232*	HCC	0.852 (0.345–2.113)	n.s.	80 (160)	Caucasian	[30]
	Intron 8 (BsmI)	*rs1544410*		1.711 (0.766–3.813)	n.s.			
	Exon 2 (FokI)	*rs1073581*		1.338 (0.605–2.968)	n.s.			
	I352I (TaqI)	*rs731236*		0.491 (0.212–1.141)	0.09			
PPARγ (NR1C3)	P12A	*rs1805192*	T2D	0.78 (0.59–1.05)	0.045	333	Scandinavian	[40]
				1.37	0.04	2,126 (1,124)	French	[38]
				0.12 (0.03–0.52)	0.005	532 (386)	Asian Sikh	[39]
	C161T	*rs121909245*	Obesity	2.33 (1.03–5.29)	0.042	292 (371)	Australian	[37]
			NAFLD	4.606 (3.744–10.263)	0.003	96 (96)	Chinese	[46]
LXRα (NR1H3)	N/A	*rs2167079*	HDL cholesterol level	N/A	5.13×10^{-8}	4763	Northern Finland Birth cohort 1966	[78]
		rs7120118			3.57×10^{-8}			
AR (NR3C4)	N/A	*rs5031002*	LDL cholesterol level	N/A	2.37×10^{-7}			
PXR (NR1I2)	Intronic	*rs7643645*	NAFLD	3.48 (1.25–10.62)	0.008	188	Argentine	[54]
		rs2461823		N/A	0.039			
	−25385	*rs3814055*	DILI	3.37 (1.55–7.30)	0.0023	51 (64)	European	[74]

Abbreviations: OR: odds ratio; N/A: not annotated; n.s.: not significant.

the promoter and along the entire coding region of the *HNF4A* gene and type 2 diabetes in 49.577 individuals revealed significant associations for more than one locus [13].

Nuclear transcription factors are known to undergo posttranslational modifications modulating their regulatory activity, which obviously makes the interpretation of genetic tests more difficult. Recent findings of epigenetic modification of the *HNF4A* promoter add an additional layer of uncertainty and environmental impact. Maternal diet and aging alter the epigenetic control of a promoter-enhancer interaction at the *Hnf4a* gene in rat pancreatic islets [14]. Environmentally induced changes in promoter-enhancer interactions might represent a key epigenetic mechanism by which nutrition can influence NR signaling.

3. Candidate Receptor Studies

3.1. Vitamin D Receptor (VDR/NR1I1). The human vitamin D receptor (VDR/NR1I1) has been in the focus of research for over a decade, a main reason being a wide spectrum of known effects of vitamin D deficiency. A second, more mundane reason might be the availability of four frequent variants (rs7975232, *Apa*I; rs1544410, *Bsm*I; rs10735810, *Fok*I; rs731236, *Taq*I) amenable to relatively quick and easy analysis using simple technology that has been available in every genetics laboratory, restriction fragment length polymorphism (RFLP) analysis. A Pubmed search (as of June 7, 2011) on "vitamin D receptor polymorphism" resulted in 1,200 articles, covering a wide-range of associated biochemical processes and diseases ranging from the more obvious bone density in various species at various ages [15, 16] to Parkinson disease [17] within the first 20 hits, ulcerative colitis [18], and inflammatory bowel disease [19]. Limiting the search to the liver results in a more manageable set of less than 30 publications, with a detectable focus on inflammatory and autoimmune liver diseases, in particular autoimmune hepatitis and primary biliary cirrhosis [20–22], but also hepatitis B virus (HBV) infection [23] (Table 1).

In contrast to the mouse liver, which showed no VDR expression [3], VDR was detected mainly in the nonparenchymal cells of rat liver, whereas hepatocytes expressed barely any VDR in murine livers [24]. Human hepatocytes express VDR, albeit at very low abundance [25]. One of the potential ligands of hepatic VDR in humans is the secondary bile acid lithocholic acid, resulting in a repression of bile salt synthesis by transcriptional repression of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in bile salt biosynthesis [26]. The effect is achieved by competing for promoter binding with HNF4-alpha. This example shows that results from animal models have to be treated with caution, and once again illustrates the complex interaction between NR-regulated pathways in human liver.

Association studies between gene variants and diseases provide signposts towards genes underlying functional effects, but do not elucidate how these effects are achieved. Investigations into the detailed effects of the respective polymorphisms in NR are harder to interpret than similar investigations in other molecules of clearer functional delineation [27]. Cell-type specific splicing events might modulate transcriptional activation or ligand binding and cause effects in a substrate-dependent manner. As an example, a functional effect of the 3′*Bsm*I polymorphism in intron 8 of the *VDR/NR1I1* gene was shown to have a modulatory function on epithelial cell proliferation when combined with the effects of calcium [28]. The *Fok*I polymorphism of *VDR/NR1I1* results in distinct translation initiation sites and was shown to have an effect on cell growth inhibition, possibly through estrogen receptor-α protein repression in a cancer cell line [29]. These pleiotropic and highly variable functions go some way towards explaining the miscellany of associations that have been detected for *VDR* polymorphisms, among others with the occurrence of hepatocellular carcinoma (HCC) in patients with liver cirrhosis, particularly in patients with an alcoholic etiology [30]. They also show why the idea of using NR as a handle towards personalized treatment of patients is not straightforward, due to the high number of unspecific side effects.

3.2. FXR: The Central Bile Salt Sensor. FXR/NR1H4 is the hepatic nuclear bile salt receptor, regulating bile salt synthesis and transport in hepatocytes, the central hub of cholesterol synthesis and conversion. Bile salts are direct FXR ligands and bind to the ligand binding domain of the molecule at low concentrations as dimers with the retinoid X receptor (RXR/NR2B1). Upon binding of the heterodimer, conformational change causes FXR activation. FXR also controls enterohepatic circulation through regulation of intestinal bile salt uptake via expression of the intestinal bile acid binding protein (I-BABP) in enterocytes. At the same time, FXR increases expression and release of fibroblast growth factor 19 (FGF19, mouse orthologue FGF15), which provides a feedback regulation loop from the intestine to the liver via association with β-klotho and activation of its dedicated receptor FGFR4 (Figure 2). In the liver, dimerization with RXR induces the expression of various genes involved in bile salt transport from the hepatocyte into the bile canaliculus such as the phosphatidylcholine floppase *ABCB4* and the bile salt export pump *ABCB11*. Interaction of FXR with the short heterodimer partner (SHP/NR0B2) decreases bile salt synthesis by repression of CYP7A1 and CYP8B1. Hence, FXR occupies a key role and is a prime target for manipulating the balance of bile salts in multiple parts of the enterohepatic circulation. However, results from a pilot experiment assessing the metabolic impact of a synthetic FXR agonist indicate that caution is warranted. The administration of GW4064-induced obesity and diabetes in mice fed a high-fat diet and worsened the metabolic effects in liver and adipose tissue [31].

A potential role of FXR dysregulation in gallstone formation could be shown in the FXR deficient mouse. Due to the lack of positive feedback via FXR, the hepatocanalicular transporters *ABCB4* and *ABCB11* are not induced by bile salts (Figure 2) [32]. Under normal circumstances, biliary cholesterol is solubilized in mixed micelles, consisting of cholesterol, phosphatidylcholine, and bile salts. Lack of the latter two constituents causes supersaturation of cholesterol and the precipitation of crystals in the FXR-knockout mouse.

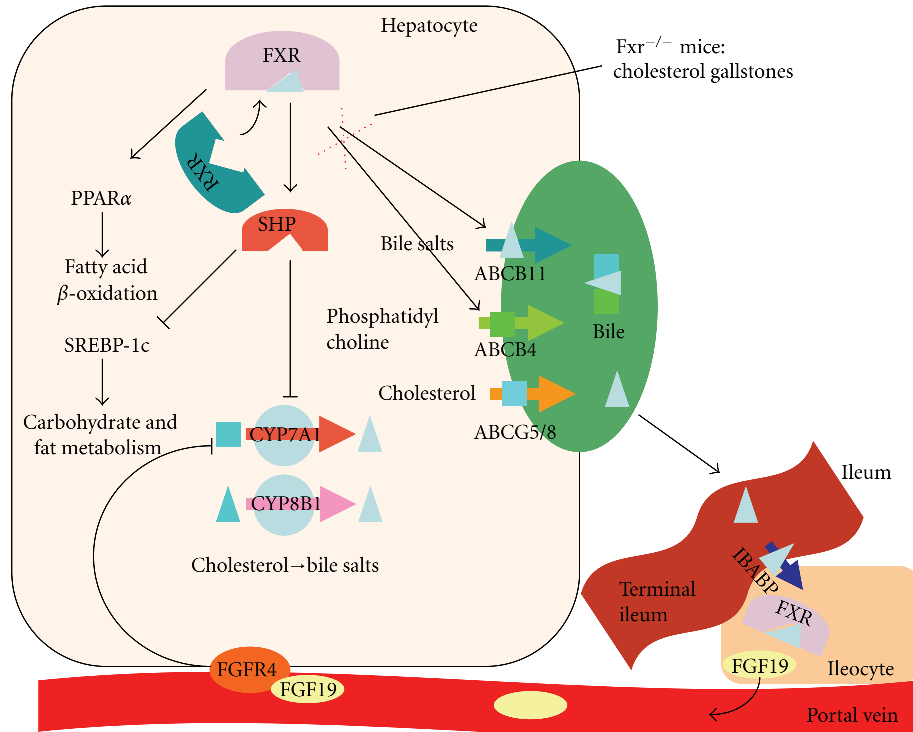

Figure 2: Schematic diagramme showing various aspects of FXR function as an example of complex nuclear receptor regulation and interaction. IBABP: intestinal bile acid binding protein, SREBP-1c: sterol regulatory element binding protein 1c, FGF: fibroblast growth factor, and FGFR: fibroblast growth factor receptor.

When administered to gallstone-susceptible wild-type mice, the FXR agonist GW43456 reinstated the biliary balance of cholesterol, phospholipids, and bile salts by induction of hepatocanalicular transporter expression [32]. This makes FXR a potential target for the treatment of cholesterol gallstones.

3.2.1. FXR Variation and Functional Conservation. A survey of genetic variation in 13 NR that control the expression of drug metabolizing enzymes revealed an intriguing paucity of known functional variants in the coding region of *FXR/NR1H4*, comparable only in numbers to the androgen receptor (*AR/NR3C4*) and the aryl hydrocarbon receptor (*AHR*) [33]. This scarcity has been speculated to be indicative of considerable evolutionary selective pressures that conserve the functional domains in these receptors. However, compared to the both aforementioned receptors with low frequency of functional polymorphisms in the coding region, *FXR* revealed a relatively high number of base substitutions in the regulatory sequence. Thus, protein abundance of the molecule appears to be more variable than its conformation. Comparison of the frequency of variants in the sequences of drug metabolizing CYP enzymes with the frequency in essential enzymes in protein biosynthesis (ribosomal genes) and NR genes revealed similar patterns. A high level of variation in the regulatory sequence and a high conservation in coding areas of NR genes was juxtaposed by a reverse distribution in ribosomal genes [33]. No difference in variation frequency was observed in the noncoding, intronic areas.

3.2.2. FXR Variation in Complex Disease. Quantitative trait locus mapping in inbred mice identified the *Nr1h4* gene encoding murine Fxr as a candidate gene for a gallstone susceptibility (lithogenic) locus (*Lith7*). Sequencing, genotyping, and haplotype analysis in humans revealed no more than three frequent haplotypes accounting for >95% of the variability. Kovacs et al. [34] described an association of a common risk haplotype *NR1H4_1* (−20,647T; −1G; IVS7-31A) with gallstones in Mexican patients (OR = 2.1, P = 0.02) (Table 1). The association was inconsistent between different populations, pointing to a minor contributory role of FXR in overall gallstone susceptibility. Sequence variants of *FXR* have also been investigated in other pathological liver conditions, such as intrahepatic cholestasis of pregnancy (ICP). ICP is an interesting model disease as it illustrates the step up in complexity from monogenic diseases like severe familial cholestasis in children to complex cholestatic syndromes. The central role of FXR in balancing bile salt concentrations throughout the enterohepatic circulation makes it a good candidate for investigations into the causes of bile salt imbalances during pregnancy. Van Mil et al. [35] used an elegant and convincing experimental setup to prove the molecular impact of the few variants found in or near the transcribed sequence of *FXR*: They could show how two disease-associated single nucleotide polymorphisms (SNPs) at the methionine start codon or its immediate vicinity resulted in decreased translation. Expanding beyond the mere quantitative change, van Mil et al. [35] went on and proved that decreased translation diminished or abolished transactivation

of FXR target genes in response to bile salt stimulation. A similar effect could be shown for the only nonconservative sequence variant that was found in both ICP patients and unaffected controls [35] (Table 1). Marzolini et al. [36] were able to confirm an effect of this variant *in vivo* by examining samples from a human liver bank. The expression levels of the *FXR* target genes short heterodimer partner (*SHP*/*NR0B2*) and organic anion transporting polypeptide 1B3 (*OATP1B3*) were reduced in livers harboring the rare [T] allele at position −1 of the *FXR*-coding sequence.

3.3. PPAR-Gamma and Diabetes. The peroxisome proliferator-activated receptor gamma (PPARG/NR1C3) is a fatty acid-activated member of the PPAR subfamily of NR. These receptors play important roles in lipid and glucose metabolism. Members of the family have been implicated in obesity-related metabolic diseases such as hyperlipidemia, insulin resistance, and coronary artery disease. Like FXR, PPARs form heterodimers with RXR, and these heterodimers regulate the transcription of various genes in liver. Polymorphisms in *PPARG*, particularly the proline-to-alanine substitution at aminoacid position 12, have been associated with diabetes, insulin levels, insulin sensitivity, body mass index, and dyslipidemia [37–40] (Table 1).

Disease prediction for population subgroups based on a combined "diabetes risk matrix" including *PPARG* p.P12A has been proposed to be informative and might, if accompanied by lifestyle intervention, prove a worthwhile path for prevention [41, 42]. Genome-wide association studies (GWAS) of type 2 diabetes in 2,335 Finns confirmed a contributory role of *PPARG* [43]. In a comprehensive example of complementarity between human and animal model research, Heikkinen et al. [44] were able to show that the p.P12A variant exerts its impact on various aspects of metabolism and human longevity in a diet-dependent manner. Hence, this prominent member of the NR family of molecules is a leading example of gene × environment interaction and "nature via nurture".

3.4. PPAR-Gamma and NAFLD. Meirhaeghe et al. [45] described an association of a silent SNP in exon 6 of the *PPARG* gene (c.C161T) and the level of circulating leptin in obesity. Obese subjects carrying at least one [T] allele displayed higher plasma leptin levels than homozygous carriers of the common allele. The [T] allele was also associated with lower BMI at a given leptin level, indicating a complex interaction between PPAR-gamma and leptin signaling. These findings could be confirmed and extended by a study in 96 Chinese patients with nonalcoholic fatty liver disease (NAFLD), which reported an association of this variant with adiponectin levels and the development of NAFLD [46]. Zhou et al. [47] replicated this finding in an independent cohort, demonstrating that *PPARG* c.C161T and other polymorphisms are associated with the levels of tumor necrosis factor (TNF)-alpha, leptin, and adiponectin in NAFLD. When patients in Germany (NAFLD and AFLD, $n = 363$) [48] and Italy (NAFLD, $n = 202$) [49] were analyzed for the p.P12A variant, the results were less conclusive: German patients with fatty liver disease of either etiology were more likely to carry the rare minor allele, but no association was detected between p.P12A and the severity of steatosis, necroinflammation, or fibrosis.

3.5. Interactions between FXR and PPAR-Gamma. Bile salts are intimately entwined with lipid metabolism, and besides their well-known role in dietary lipid absorption and cholesterol homeostasis, evidence is accumulating that the body uses blood levels of bile salts as sensor for metabolic processes (reviewed comprehensively by Thomas et al. [50]). Hence, bile salts have been considered as metabolic signaling molecules. The decrease in energy expenditure following reduction of the bile salt pool by treatment of mice with the FXR-agonist GW4064 is proof of this concept, although the resulting weight gain and insulin resistance are not a desirable outcome [31]. The observation that PPAR-gamma might be induced by FXR in hepatic stellate cells underscores and highlights the complex interaction between bile salts, metabolism, inflammation, and fibrogenesis [51]. It also adds weight to the argument that NRs are involved in most aspects of metabolic regulation and liver cells' response to both internal and external stimuli. Further complexity is added to the spectrum of NR interactions in the liver by a recent report on the effect of GW4064 on the expression of the PPAR-gamma coactivator-1alpha (PGC1α/*PPARGC1*). The synthetic agonist GW4064 enhances expression of PGC1α and thus mitochondrial function, however enhanced expression of FXR increases *PPARGC1* RNA not directly, but via protection from repression by the atypical corepressor SHP [52].

3.6. PXR and NAFLD. The pregnane X receptor (PXR/NR1I2) is known to be involved in the regulation of hepatic detoxification processes. Using PXR knockout and humanized mouse models, PXR was found to influence drug × drug interactions, hepatic steatosis, and the homeostasis of vitamin D, bile salts, and steroid hormones [53, 54]. Investigations into the genetic contribution of the *PXR* locus in 188 patients with NAFLD showed an association of two variants (rs7643645 and rs2461823) with several phenotypes of the disease, among others ALT levels [54] (Table 1). The combined analysis of both loci provided information with regards to disease progression. One of the associated SNPs (rs7643645) is localized within a potential binding site for HNF4-alpha, once again illustrating the interactions between NR pathways.

3.7. NR and Liver Cancer. Liver receptor homologue 1 (LRH-1, NR5A2) is an orphan member of the NR superfamily, that is, it has no known endogenous ligand. LRH-1 is involved in the regulation of genes that participate in steroid, bile salt, and cholesterol homeostasis [55]. Knockout of *Nr5a2* in mice results in compromised intestinal lipid absorption as well as defective embryogenesis and cholesterol homeostasis [56, 57]. Application of dilauroyl-phosphatidylcholine, a specific LRH-1 agonist, increased bile salt levels and lowered hepatic triglyceride and serum glucose concentrations [58].

In mouse models of type 2 diabetes, the LRH-1 agonist also decreased hepatic steatosis and improved glucose homeostasis, pointing towards a new intervention target for the treatment of type 2 diabetes. LRH-1 has also been implicated in the growth of liver tumors via reversal of repression by SHP: *In vitro* methylation of the *SHP* promoter reversibly decreased transactivation and LRH-1 binding; overexpression of *SHP* inhibited HCC foci formation, arrested HCC tumor growth in xenografted nude mice, and increased the sensitivity of HCC cells to apoptotic stimuli [59].

3.8. NR and Viral Hepatitis. The replication of hepatitis C virus (HCV) is linked to lipid droplets, and a combined genomic/metabolomic analysis of HCV-infected HUH-7.5 cells by RNA sequencing, microarray, and proteomics revealed profound changes in, among others, PPAR signaling and PXR/RXR activation [60]. Viral replication efficiency has been linked to variations in cellular bile salt concentrations using the HCV replicon system [61]. Of note, variants of the human bile salt that export pump *ABCB11* might be associated with sustained virological response [62] and progression towards liver cirrhosis [63]. *In vitro* experiments using HCV replicon-harboring cells have shown that the impact of bile salts on HCV replication might be through the action of FXR rather than a direct effect of bile salts themselves [64]. FXR antagonization by guggulsterone blocked the bile salt-mediated induction of HCV replication, and guggulsterone alone inhibited basal HCV replication by tenfold [64]. Hence, it seems feasible that HCV uses transcriptional activation via FXR. It would certainly be of interest to analyze the HCV-binding of natural *FXR* variants implicated in ICP [35]. We speculate that the increased susceptibility for cholestatic disease might be counterbalanced by decreased susceptibility to hepatotropic viruses.

Differential regulation of the pre-C and pregenomic promoters of HBV by members of the NR superfamily (HNF4-alpha and PPAR-gamma) has been known for some time [65]. Ramière et al. [66] were able to show that FXR-RXR-heterodimers bind to two motifs on the HBV enhancer II and core promoter regions, which are characterized by high homology to the consensus inverted repeat FXR response elements [66]. The tight connection between hepatotropic viruses, liver nutrition, and metabolism by means of NR is intriguing and might reveal new therapeutic targets or even dietary recommendations to optimize the efficacy of antiviral treatment for HBV or HCV infected patients.

3.9. NR and Drug-Induced Liver Injury (DILI). While diet-induced metabolic overload, alcohol and HCV are the most common insults to the liver, drug-induced injury (DILI) is gaining in prominence due to increasing age and multimorbidity of the general population. The likelihood of medication-induced liver damage increases substantially beyond the threshold of 50 mg per day cumulative ingestion [67]. The involvement of the bile salt transporter system in estrogen-induced cholestasis has been observed for a long time in patients with ICP [35, 68, 69]. Functional variants of *ABCB11* are known to be associated with cholestasis induced by oral contraceptives and other drugs [70], as reviewed elsewhere [71].

The role of gene polymorphisms in predisposition towards DILI has been reviewed comprehensively [72, 73]. Suffice to say that next to drug-metabolising enzymes, drug transporters and genes for the immune response to injury, variants in the NR genes *PXR* [74] and *CAR* [58, 75, 76] are the most prominent contributors, for example, towards acetaminophen (APAP) toxicity. Evidence comes from knockout or knockdown of these genes, conferring resistance to the toxic effects of APAP.

4. The Search for New Targets

4.1. NR and Metabolic Traits. Identifying and quantifying genes associated with metabolic traits is one of the prime challenges when devising means to deal with the epidemics of diabetes and metabolic syndrome. This information might be used as cost-efficient leverage to identify and motivate at-risk individuals towards lifestyle changes, but to date there is no evidence for clinical impact [77]. A long standing question with regard to the usefulness of genome wide association studies (GWAS) has been how to detect the impact of other than high-frequency low-risk variants. While the setup of GWAS is a case-control scenario in an otherwise unselected population, more in-depth information is expected from longitudinal studies on large cohorts of individuals with defined but limited genetic heterogeneity. A combination of results from both approaches might be required: since GWAS needs to accommodate higher levels of heterogeneity, and hence a greater variety of factors that influence the trait under examination, only factors that reach the threshold across the total population are identified. Studies on smaller cohorts, possibly with less genetic variety due to a relatively small common founder population, enable researchers to identify the impact of comparatively rare variants that have been enriched in the respective population due to a lack of admixture, while contributory genetic factors that are not present in the founder population might be missed. In fact, using large cohorts with relatively small founder populations have enabled researchers to identify metabolic risk factors by GWAS and association mapping. Typing 4,763 individuals from the Northern Finland birth cohort 1966 for 329,091 SNPs identified 21 genomic loci that were associated with metabolic traits such as HDL and LDL cholesterol levels [78]. Besides the "usual suspects" such as a frequent polymorphism in the *NR1H3* gene encoding LXR-alpha, which affects HDL cholesterol level with an effect size of only 4% but shows a risk allele frequency of 42%, the study also detected some higher impact rare alleles. A variation in the gene for the androgen receptor AR (*NR3C4*) exerted a strong influence on LDL cholesterol levels, with a considerable effect size of 30% set aside a risk allele frequency of only 2% [78] (Table 1).

4.2. Complex NR Genetics. At our present state of knowledge, the variation of transcript abundance is probably a more important mechanism underlying disease susceptibility than

structural protein alterations by nonsynonymous SNPs [79]. The view of health as a balanced state or equilibrium of interrelated gene expressions holds a lot of attraction for liver homeostasis, with diseases representing "network perturbations" [80]. This view also explains why transcription factors are more likely to be detected in GWAS studies of quantitative metabolic traits, since they might be master regulators of multiple other genes. Hence, NR variants impact not just on their own expression but can rather, via multiple effectors, exert stronger effects on a complex phenotype.

A GWAS with more than 2.5 million SNPs in 19,840 individuals, with replication in up to 20,632 individuals, identified more than 30 loci that had an impact on serum LDL cholesterol, HDL cholesterol, or triglyceride levels. A rare variant (risk allele frequency 3%) in *HNF4A* (p.T130I) was identified as a new contributory factor for HDL cholesterol levels, with an effect size of 19% [81].

4.3. Metabolic Traits as Quantitative Trait Loci (QTLs). Technical progress, particularly the combination of increased speed and decreased cost of genotyping and expression quantification, is ushering in systematic approaches to metabolic traits. Once sufficiently large numbers of individuals from any population (humans, animals, or plants) have been genotyped for markers covering the entire genome, statistics can be used to calculate the likelihood of correlation between the inheritance of genotypes and the expression values of quantifiable phenotypes, including the abundance of transcripts assayed by expression arrays. This methodology, denoted as "expression quantitative trait loci (eQTL) mapping" can be used to unravel gene regulation by association and identify regulatory networks involving multiple loci. To extend the information from these large datasets, Schadt et al. [82] proposed mathematical models and tools to infer relationships between genes and groups of genes as well as between gene expression and disease phenotypes. Liu et al. [83] used data from the mouse phenome database covering 173 mouse phenotypes to map 937 quantitative trait loci *in silico*. Phenotypes examined included various metabolic traits such as fat mass at different diets, cholesterol, and triglyceride levels under normal and atherogenic diet. Ten of the QTL regions identified in this study contained candidate genes that had previously been characterized and shown to cause metabolic phenotypes in agreement with the trait used for mapping, serving as a proof of principle for the application of this methodology.

A note of caution is obviously due when transferring data and results from mouse models into the human genomic context. Transcriptional regulation, particularly organ-specific mechanisms and binding sites, has diverged significantly between man and mouse, probably more so than NR function [84]. Nevertheless, results from animal models, particularly knockouts, still provide valuable clues towards unexpected mechanisms of disease [85, 86].

4.4. Combining the Power of GWAS and eQTL. The identification and functional characterization of regulatory SNPs have encouraged the use of eQTL data for the interpretation of GWAS results. These genome-wide scans using anonymous SNP markers usually detect associations of disease with polymorphic markers, often in regions without known candidate genes [87]. For an example we refer to Kathiresan et al. [81], who identified 30 loci that contributed towards the regulation of three dyslipidemia traits. Increasing knowledge about *trans*regulatory effects by eQTL might help to pinpoint functional mechanisms for these disease-associated variants. eQTL data enables us to identify a mechanism by which a SNP controls expression of a remote locus, hence causing predisposition to disease by allelic variation across long genomic distances [88].

5. Bringing It Together: Future Perspectives

Technical advances increase our knowledge regarding the biochemical and gene-regulatory mechanisms underlying metabolic diseases. Transgenic animals inform us about gene function, eQTL studies in human samples and model systems provide us with information about genetic loci that are associated with the inheritance of multiple metabolic parameters, and GWAS in genetically well-characterized cohorts yield candidate genes for metabolic disturbances at ever increasing resolution and depth. Considering all this amassed knowledge, our understanding of disease pathobiology is improving constantly. Considering the reality of how little lifestyle modification is achieved by risk information, as illustrated by antismoking campaigns, it remains a challenging task to employ this knowledge to combat the epidemics of the metabolic syndrome and its associated burden of disease.

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Review Article

Fatty Acid Oxidation and Cardiovascular Risk during Menopause: A Mitochondrial Connection?

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Menopause is a consequence of the normal aging process in women. This fact implies that the physiological and biochemical alterations resulting from menopause often blur with those from the aging process. It is thought that menopause in women presents a higher risk for cardiovascular disease although the precise mechanism is still under discussion. The postmenopause lipid profile is clearly altered, which can present a risk factor for cardiovascular disease. Due to the role of mitochondria in fatty acid oxidation, alterations of the lipid profile in the menopausal women will also influence mitochondrial fatty acid oxidation fluxes in several organs. In this paper, we propose that alterations of mitochondrial bioenergetics in the heart, consequence from normal aging and/or from the menopausal process, result in decreased fatty acid oxidation and accumulation of fatty acid intermediates in the cardiomyocyte cytosol, resulting in lipotoxicity and increasing the cardiovascular risk in the menopausal women.

1. Menopause: A Burden for Aging Women

Menopause is one of the most critical periods in women's life. Although being a natural biological process that occurs with aging, physiological alterations observed during this period can be challenging. Caused by a reduced secretion of ovarian hormones estrogen and progesterone after depletion of the storage of ovarian follicles, menopause defines the end of women menstrual cycle and their natural fertility. On average, spontaneous or natural menopause occurs around the early 50s and is confirmed after 12 months of nonpathological amenorrhoea. However, when premature ovarian failure (POF) occurs before the 40s due to pathological causes, an early or premature menopause can be induced, which is thus disconnected from the aging process properly said. When a bilateral oophorectomy is necessary, menopause occurs immediately without women experiencing the gradual transition of perimenopause. Chemotherapy can also provoke a permanent damage in ovaries and induces menopause *per se* [1]. Women who experience an early menopause are more susceptible to certain health problems, such as osteoporosis and heart diseases, since they spend more time in their lives without the benefits of estrogens. POF can also be temporary (temporary menopause) induced by high levels of stress, excessive exercising and/or dieting, and by medications used to treat fibroids [2] and endometriosis [3]. However, as soon as women adopt a healthier life style or stop medication, the ovaries may resume normal production of hormones. Normally, menopausal transition or perimenopause starts around mid-to-late 40s and persists several years before the last menstrual period, normally for 4-5 years (Figure 1). Smoking and genetic background are two factors that can influence the timing of spontaneous menopause. Normally, smokers can reach menopause earlier than nonsmokers [4]. During perimenopause, levels of estrogen and progesterone start gradually to decline and menstrual periods become irregular. Since sex hormones are physiologically important to maintain the health and normal functioning of several

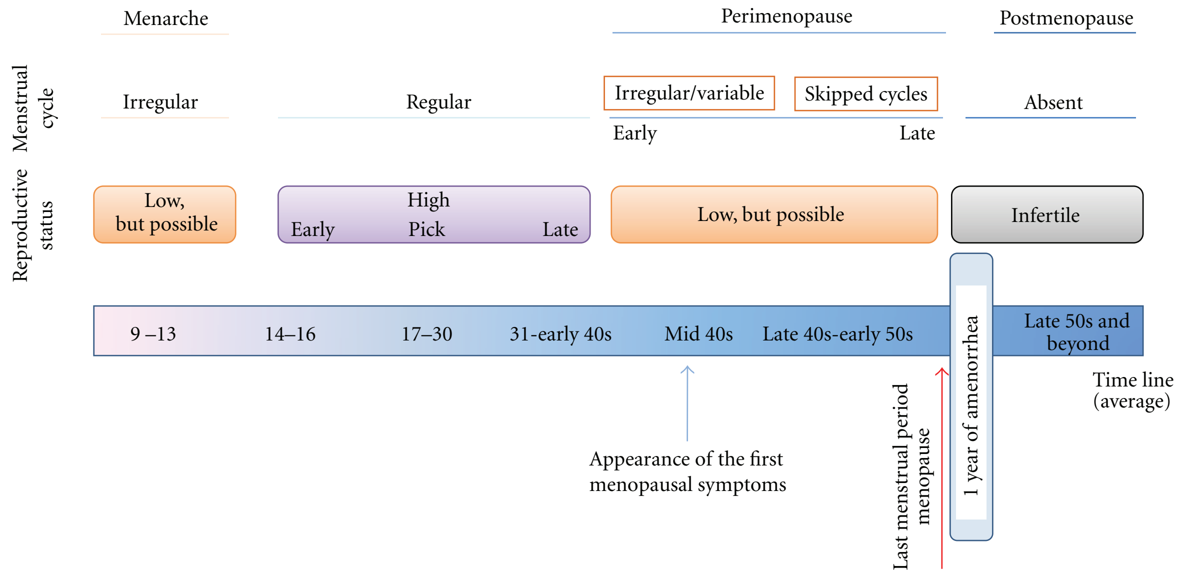

Figure 1: Women reproductive stages during aging: from menarche to postmenopausal. Time line represents only an average for the normal age. More details can be found in the text.

organs, such as the heart, liver, brain, and bone, hormonal changes observed during this menopausal transition may induce several chronic medical conditions [5]. All women experience menopause, but different women may cope with different symptoms. The variation of menopause phenotypes around the world and in different ethnic groups suggests both cultural and genetic influences [6, 7]. Menstrual irregularities, vaginal atrophy, and vasomotor instability are the most frequent menopausal symptoms that have been directly related with the decreased levels of female sex hormones [8].

Menopause-associated vasomotor symptoms (also known as hot flashes) include spontaneous feeling of warmth, usually on face, neck, and chest and are usually associated with perspiration, palpitations, and anxiety, being variable in frequency, duration, and severity, and can be the cause for fatigue, difficulty concentrating, and memory lapses, symptoms that have also been observed during menopause transition. The cause for menopause-associated vasomotor symptoms is not completely understood, although some theories have been proposed [8, 9].

Vaginal atrophy is also a common symptom during menopause transition. Due to loss of estrogens, vagina lining may become thinner and dryer, and the pH also changes, making the vagina more susceptible to infections. Those alterations can affect sexual function and quality of life [10].

Others menopause-associated complications include increased cardiovascular risk (see below), osteoporosis [11] and body weight gain, which can all be a combination of changes in hormone levels and aging.

Increase in body weight is another characteristic associated with menopause. Although it is known that the metabolic rate decreases with aging, the increase in body weight and visceral adipose tissue accumulation after menopause have been associated with ovarian hormone withdrawal [12]. It has been shown that, in abdominal adipocytes, estrogen regulates the expression of lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) [13]. In hepatocytes, estrogen regulates the synthesis of structural apolipoproteins for very low-density lipoproteins (VLDLs) and high-density lipoproteins (HDLs) and decreases the synthesis of hepatic lipases [14]. By regulating lipidogenesis in adipocytes and hepatocytes, estrogen modulates lipid concentration in plasma. The withdrawal of estrogens during induced or natural menopause leads to several lipid metabolism disorders. For example, dyslipidemia was also observed in bilateral oophorectomized in women [15]. Abdominal accumulation of adipose tissue and associated dyslipidemia are important components of a group of metabolic irregularities strongly related with increased cardiovascular risk in the menopausal woman.

2. Cardiovascular Disease in Women during Menopause: The Role of Hormone Replacement Therapy

2.1. Clinical Data: What Do We Know? Cardiovascular disease (CVD) is a multifactorial disease. Both bad lifestyle including inappropriate diet, sedentary life, smoking and drinking, and determined factors (e.g., aging, sex, genotype, and menopause) influence CVD [16, 17]. The impact of CVD on overall mortality in westernized countries is enormous, accounting for up to 30% of all deaths worldwide. The definition of CVD includes four major groups of diseases: coronary heart disease (CHD) disclosed by angina pectoris, myocardial infarction, heart failure, and coronary death, cerebrovascular disease such as stroke or transient ischemic attack, clinically evident peripheral artery disease, aortic atherosclerosis, and thoracic or abdominal aortic aneurysm. What is less known is that CVD is the leading cause of death in women, with more deaths than all other causes combined yearly [18]. Various studies showed a growing risk for CVD in menopausal women due to negative changes in

metabolism and hemodynamic parameters [16]. According to the guidelines of the National Cholesterol Education Program (NCEP) [19], the American Heart Association (AHA), and the American College of Cardiology (ACC) [18, 20], evaluation of CVD risk factors in women must include a personal CHD history, age over 55, family history of premature CHD, diabetes mellitus, dyslipidemia, hypertension, personal history of peripheral artery disease, and smoking.

Guidelines for prevention of CVD in women were first published in 1999 by the American Heart Association (AHA) [21]. One consequence of such increased attention to gender-related health problems, is awareness of CVD as the leading cause of death among women has nearly doubled since 1997 [22]. The impact of menopause should be taken into account when discussing CVD, and this aspect has been the matter of debate [23].

Premenopausal women have a lower incidence of CVD when compared to men with the same age-range. Whereas CHD is sporadic in premenopausal women [24], the incidence of myocardial infarction increases with age in both sexes, but occurs later and after menopause [24]. Estrogen loss during menopause causes negative effects on metabolism and cardiovascular function [25], and the progression to menopause with the changes in estrogen levels decreases or cancels the women advantage versus men [26–29].

Postmenopausal women have a higher risk of coronary artery disease, atherosclerosis, and all causes of mortality [29]. A consequence of this gender-related trend is that the postmenopausal state is acknowledged as a risk factor for CHD, with a weight similar to that of male sex [30]. Furthermore, an early natural menopause appears to be associated with increased risk of CVD [31, 32], even in non-smokers.

Indeed, menopause is associated with increased total serum cholesterol, triglycerides, and fibrinogen, as well as with a decrease in high-density lipoprotein (HDL) cholesterol. A plausible explanation is that menopause is believed to be a result of fluctuations in hormonal status, primarily a deficiency in estrogen [33]. Whether other contributing factors may have a role on CVD after menopause, is less clear and difficult to demonstrate. The transition from premenopausal phase to menopause, for example, may induce a weight gain responsible for increased in blood pressure, total cholesterol, low-density lipoprotein (LDL), triglycerides, and fasting insulin [33]. What should be mentioned is that aging *per se* can be more important than menopause itself for a number of CHD risk factors. In the SWAN study (Study of Women's Health Across the Nation) [34], changes in traditional risk markers of CHD were evaluated in three different stages: before, within a year, and after the final menstrual period within a multiethnic group (African, American, Hispanic, Japanese, or Chinese and Caucasian women). Changes due to menopause were only represented by total cholesterol, low-density lipoprotein cholesterol, and apolipoprotein B. By contrast, chronological aging was responsible for changes in the other risk factors with a linear model. Many other potential factors might be also implicated in the sex differences in coronary heart disease [35]. The possibility that heart disease risk determines menopausal age rather than the inverse has already been proposed [36].

Oxidative stress plays a role in hypertension, hypercholesterolemia, diabetes, and promoting CVD [37]. The formation of free radicals leads to cellular oxidative stress with a contribution to the first step of endothelial damage and the progression to atherosclerotic lesion. The perpetuation of the process induces the final events of CVD, which appears to be linked to some oxidative stress biomarkers [38, 39]. Oxidative stress appears to be an emerging factor also in the pathophysiology of CVD in menopausal women. Studies have shown that during menopause the risk of CVD increases at the same time of a rise in oxidative status [40, 41].

It is still unclear if the type of menopause (surgical or natural) can have a role on cardiovascular risk. The Nurses' Health Study (1987) demonstrated that the risk of CHD was higher in patients undergoing bilateral oophorectomy compared with natural menopause. An estrogen-replacement therapy could prevent this effect [42]. In a later study, carotid artery intima-media thickness showed a positively association with years elapsed since menopause; however, according to this marker of subclinical atherosclerosis, women with natural menopause presented no difference compared with those who had surgical menopause [43]. Indeed, men with the common estrogen receptor alpha (ESR1) c.454-397CC genotype have a major risk of myocardial infarction, suggesting the potential linkage between estrogen receptors and CVD susceptibility. In this respect, a variation in estrogen receptor could clarify the contrasting results of hormone therapy on CVD susceptibility in women [44]. The apparent protective effect of hormone replacement therapy (HRT) has been a matter of debate for several years [45–47]. Prevention of CHD and osteoporosis in menopausal women was originally achieved by exogenous estrogen plus progestin, assuming a protective effect of estrogen on the heart. Additional effects included a protective effect on the bone and on colon cancer [48–52], despite increasing incidence of breast cancer [53, 54]. Two landmark studies, however, changed this view. The Women's Health Initiative (WHI) Estrogen plus Progestin (E+P) trial in 2002 showed no protection for CHD and confirmed the increased risk in breast cancer and thromboembolic disease [55].

Two years later the WHI Estrogen Alone trial confirmed the lack of effect on CHD while suggesting a trend for decreased breast cancer, with a rise in stroke and venous thromboembolic disease. A nonsignificant protective effect on CHD was seen in the younger women (ages 50 to 59) [56]. The public consequence was that hormone therapy was abandoned or was conducted with lower doses [57].

The possibility that CHD risk is lowered by earlier hormone therapy after menopause should also be considered, although results are not conclusive [58]. Whether hormone replacement therapy results in either increased or unchanged risk for stroke, is also a matter of debate [56]. Of note, recent guidelines do not identify estrogen therapy for the primary or secondary prevention of CHD [59, 60].

2.2. Animal Models: Helping to Define the Role of Estrogens. Although the WHI and the Heart and Estrogen/progestin Replacement Studies (HERS) showed no CVD protection resulting from HRT, several animal studies have suggested

an important cardioprotective role for estrogens against heart failure [61], mediated by a genomic or a nongenomic estrogen-receptor-mediated signaling pathway (see [62] for a review).

Tumor necrosis factor-alpha (TNF-α) has been reported as an important factor during I/R injury and ischemia preconditioning. In a Langendorff-perfused rat heart model, estrogen reversed the deterioration of heart hemodynamics induced by TNF-α treatment [63]. Several evidences have been demonstrated that stromal cell-derived factor 1 (SDF-1) is increased in ischemic hearts and induced cardioprotection [64]. A higher expression of myocardial SDF-1 was observed in female rats in response to I/R and the increased myocardial SDF-1 production in female hearts was due to estrogen-estrogen Receptor α (ERα) interactions [65]. In C57BL/6J male mice, estrogen also induced cardioprotection after acute myocardial infarction through a decreased activity of matrix metalloproteinase-9 and increased Akt-Bcl-2 antiapoptotic signaling [66]. In a Langendorff isolated perfused rat heart model, estrogen increased the perfusion pressure and coronary resistance through activation of L-type calcium channels [67].

Estrogen-related receptor alpha (ERRα) is a transcription factor for some myocardial mitochondrial enzymes, essential to maintain cardiac energy reserves. A decrease in myocardial ERRα, regulated by the metabolic sensor AMP-activated protein kinase alpha 2 (AMPKα2), was recently reported during congestive heart failure [68]. Proteins from the intracellular lipin family are also involved in metabolism regulation. It was reported that lipin 1 is the principal protein of this family in myocardium and is also regulated by ERRα [69].

The lack of CVD protection observed during HRT has been proposed to be related with alterations in sex hormone synthesis and metabolism that can occur during aging, and can affect the hormone environment in postmenopausal women. Also age-related changes in vascular estrogen receptors (ERs) subtype, structure, expression, distribution, and the signaling pathway in the endothelium and vascular smooth muscle, preexisting CVD conditions, and structural changes in blood vessels architecture have been suggested as possible causes for the failure of HRT in CVD [70]. It also should be noticed that HRT is not only composed by estrogens, but also by a combination of estrogen and progesterone. A recent study demonstrated that a combination of 17-α-estradiol and medroxyprogesterone acetate aggravates chronic heart failure after experimental myocardial infarction, which can also explain the results from previous studies including WHI and HERS [71].

3. Cardiac Mitochondrial Fatty Acid Beta-Oxidation in Health and Disease: Where Does Menopause Stand?

The heart is one of the organs with the highest energy demand in the body, which is hardly surprising due to high energetic input required by the contractile apparatus. Although the heart is considered an omnivorous organ due to the fact that it can use several substrates for energy generation, including glucose, amino acids, lactate, and ketone bodies, fatty acids are the favored fuel for the cardiac muscle [72, 73]. In fact, the adult heart generates between 50–70% of its ATP from fatty acid beta-oxidation, which occurs mainly in mitochondria [72], possesses an elaborate system to import and process fatty acids of different lengths [72, 74]. In fact, in itself, mitochondrial function is one among different factors that impact the flux of fatty acid beta-oxidation. Others include the fatty acid supply itself, which is modulated among other factors by diet, competing substrates for the cardiac tissue, the energy demand and oxygen availability, and the regulation at a nuclear or allosteric level of enzymes which are involved in all steps of fatty acid uptake, esterification, and metabolism [72].

Fatty acids can be transported in the plasma as free fatty acids (FFAs) conjugated with albumin or as part of triacylglycerol (TAG) contained in chylomicrons or very-low density lipoproteins (VLDLs) [75, 76]. FFA concentration in the plasma is highly variable, depending not only on the diet, but also on the developmental state of the organism and if any pathology is present. For example, the amount of FFA in the plasma is known to greatly increase during myocardial infarction [77] and diabetes [78], which leads to an augmented cardiomyocyte FFA uptake and accumulation, since the concentration of FFA in the plasma is a major determinant for these two events [72]. Regardless of the mechanism underlying an acute or chronic accumulation of FFA in the plasma (reviewed in [72]), the end result of cardiomyocyte cytosolic accumulation of fatty acids can differ, depending on a wide range of factors.

The first step after entering the cardiomyocyte is conversion to CoA esters, through the action of fatty acyl CoA synthase (FACS). Fatty acid uptake by cells is made by membrane proteins with high affinity for fatty acids [79, 80], namely, the fatty acid translocase (FAT/CD36), the fatty acid binding protein (FABPpm) and a variety of fatty acid transport proteins (FATPs), as well as by simple diffusion of fatty acids through either the phospholipid bilayer or a pore or channel formed by one or more of the referred fatty acid transporter proteins [81]. Upon entering the cell, the rate of utilization is governed by a variety of factors, including malonyl-CoA, the ratio acetyl-CoA/CoA and the availability of other substrates, namely, glucose, lactate, and ketone bodies that can compete with free fatty acids as a source of acetyl-CoA [79]. Long-term regulation of uptake and utilization requires alterations in expression rates of genes encoding for fatty acid handling proteins [82]. Free fatty acids can also by themselves modulate the expression of such genes via nuclear transcription factors such as peroxisome proliferator-activated receptors (PPARs) [83].

Mitochondrial beta-oxidation of long-chain fatty acids starts with its association with CoA, forming acyl-CoA esters that are transported into mitochondria by carnitine palmitoyl transferase I (CPT-I). Beta-oxidation produces in each round one NADH, one $FADH_2$ (as part of an enzymatic complex), and one acetyl-CoA, which is further oxidized in the Krebs cycle to CO_2, with the concomitant further generation of three NADH, reduced FAD co-factor in succinate dehydrogenase complex, and one GTP. NADH, via NADH

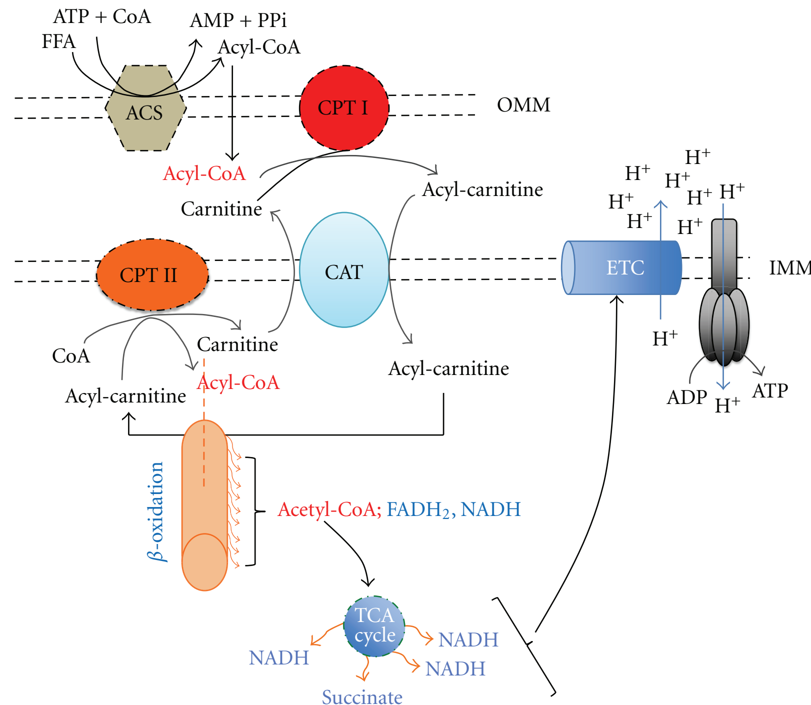

FIGURE 2: Transport of fatty acids from the cytoplasm to the mitochondrial matrix for oxidation. Following activation to acyl-CoA, CoA is exchanged for carnitine by carnitine palmityl transferase (CPT-I), which is then transported to the inside of the mitochondria where a reversal exchange takes place through the action of carnitine acylcarnitine translocase (CPT-II), and beta-oxidation machinery initiates its activity, producing reducing equivalents that feed the electron transport chain. More details are available in the text. CAT: Carnitine Acylcarnitine Translocase, FFA: free fatty acid, ACS: Acyl-coA synthase, ETC: electron transport chain, IMM: inner mitochondrial membrane, OMM: outer mitochondrial membrane, coA: coenzyme A, ATP: adenosine triphosphate, ADP: adenosine diphosphate, and AMP: adenosine monophosphate.

dehydrogenase, and succinate dehydrogenase deliver electrons to the remaining electron transport chain complexes which contribute to the generation of a proton gradient used to synthesize ATP (Figure 2). Throughout this whole process, several regulation mechanisms can operate, starting with the transport of the acyl chain to the mitochondrial matrix and ending at the accumulation of end products of the oxidation process, namely, reducing equivalents and ultimately ATP levels. The transport process is considered a major player in the control of the flux through beta-oxidation [84], mostly in intact muscle, since levels of malonyl-CoA are kept considerably high. With this type of control, it is possible for the tissues to rapidly adapt to different metabolic demands, such as in muscles [84]. An inhibition of fatty acid beta-oxidation, which as mentioned can occur at several stages, will ultimately result in free fatty acid intracellular accumulation which subsequently will be responsible for poor removal of fatty acids from plasma in any of their forms of transportation. In fact, a possible role has been attributed to female sex hormones in the development of fatty liver pregnancy on the basis of their effect in the reduction of mitochondrial fatty acid oxidation [85] and in regulating cellular energy balance *in vivo* by regulating the expression of the medium chain acyl coenzyme A dehydrogenase (MCAD) gene [86].

Besides mitochondrial oxidation, long-chain fatty acyl coA can also be used for the synthesis of intermediates, including TAG, diacylglycerol (DAG), and ceramide [72, 87]. Under normal intracellular concentrations, these intermediates are stored and/or channeled to different biosynthetic pathways, including biomembrane synthesis. If alterations in normal fatty acid homeostasis occur, which can originate from excessive plasma FFA content or from enhanced FACS expression and/or activity, long-chain fatty acyl coA derivatives can accumulate in cells. Depending on the tissue, accumulation of some of these intermediates can have distinct effects. For example, it is known that excessive accumulation of TAG in nonadipocyte tissues can result in different negative outcomes including impaired insulin signaling in the liver and skeletal muscle [88] and apoptosis and other metabolic disturbances in the heart [87, 89, 90]. DAG has also been determined to cause similar effects in the same tissues [88], including increased insulin resistance observed in a model of rodent high-fat diet [91]. It is interesting to note that both increases in TAG and ceramide intracardiac content did not correlate with the increased insulin resistance [91].

Ceramide, by its turn, has been demonstrated in different biological models to increase apoptotic signaling in several tissues [92–94], although evidence is scarcer for the heart [95]. It is interesting to note that ceramide derivatives have been involved in the triggering of the mitochondrial permeability transition pore (MPT pore) and outer-membrane permeabilization [96, 97], conditions closely linked with mitochondrial dysfunction and cell death [98]. In

opposition, long-chain ceramide species have been shown to inhibit the MPT pore [99]. The discrepancy of results regarding ceramide implicates this lipid species in the control of mitochondrial cell death pathways.

From the short description above, it is clear that a balance between FFA cell uptake and metabolism must be reached in order to avoid the accumulation of undesired fatty acid metabolites. Also, increased reliance of fatty acids as fuel for cardiac cells has undesired effects, one of them being decreased ATP synthesis, resulting from increased ATP hydrolysis for noncontractile purposes, increased mitochondrial uncoupling due to increased activity/expression of uncoupling proteins and greater proton futile cycling, creating the so-called oxygen wasting and resulting in several physiological complications [100–102]. Interestingly, inhibition of fatty acid metabolism is proposed to be beneficial for some forms of heart failure [103].

The important question is now where the menopausal heart stands. As described above, menopause is a normal consequence of the aging process in women and is accompanied of important physiological and biochemical alterations. There are several evidences in the literature that the content in FFA in the plasma tends to increase during menopause. One particular study performed with 4-vinylcyclohexene-diepoxide- (VCD-) treated rats indicated that progressive loss of ovarian function induced by VCD results in an increase of plasma FFA, which initiated several alterations leading to the development of the metabolic syndrome [104]. This important piece of evidence mimics what is observed in the menopausal women, where an increase in circulating FFA was measured [105]. It is also known that women experience a characteristic increase in circulating lipids at the time of the final menstruation period [34], although it is difficult to evaluate the component resulting from hormonal alterations and what is the result of the normal aging process [34, 106]. The increased FFA was partly reverted by hormone-replacement therapy, showing that, at least in part, it is a hormone-dependent effect [105]. The role of estrogens in fatty acid metabolism is well described and involves different mechanisms [107–109]. One important effect is that estradiol promotes the channeling of FFA toward oxidation and away from triglyceride storage (Figure 3) by upregulating the expression of peroxisome proliferation activator receptor delta and its targets and also by directly and rapidly activating AMP-activated protein kinase (AMPK). AMPK acts as a fuel sensor that increased fatty acid beta-oxidation during higher metabolic demands [110].

The data, although still scarce and largely spread out, indicates that during menopause, fatty acid metabolism is altered. The decrease in estradiol levels may result in decreased fatty acid oxidation and increased accumulation in the adipose tissue, with hormone replacement therapies recovering the pre-menopausal fatty acid status quo. But is this so straightforward? Maybe not, one important player in fatty acid metabolism is, as described, the mitochondrion. A proper channeling of fatty acyl-CoA and subsequent beta-oxidation is necessary for the energy-generating process. It is clear that a failure of mitochondrial bioenergetics causes an unbalance in fatty acid metabolism, which may result in the accumulation of fatty acyl-CoA esters in the cytosol of cardiomyocytes. This phenomenon could result in a larger channeling of fatty acyl-CoA esters to the synthesis of the intermediates described above, including TAG, DAG, and ceramide. It is interesting to recapitulate here that ceramide has been involved in the induction of apoptosis in a variety of biological models [92–94]. Although the relationship between increased ceramide intracellular levels in the menopausal heart and increased apoptotic signaling is still to be determined, several endpoints for increased cardiac Fas-dependent and mitochondrial-dependent apoptosis were identified in the hearts of bilateral ovariectomized Wistar rats [111, 112]. A logical question would be if there is a possible relationship between intracellular lipid metabolism alterations resulting from ovariectomy and enhanced apoptotic signaling in the heart.

Decreased fatty acid oxidation by mitochondria occurs in a variety of situations, ranging from xenobiotic-induced toxicity to several pathologies. There are many fatty acid oxidation disorders identified in humans, and which affect organs as different as muscle [113] and brain [114], which result in altered fat deposition and mitochondrial beta-oxidation. Defects are commonly present in the mitochondrial machinery that shuttles long-chain fatty acid metabolites to mitochondria, resulting in decreased beta-oxidation [113]. Several xenobiotics also alter fatty acid metabolism in different organs [115], examples are fluorochemicals [116] and the antibiotic tetracycline [117] in the liver. As for the heart, it is now becoming increasingly recognized that alterations in fatty acid uptake and/or beta-oxidation can result in the so-called fatty heart, a largely unrecognized entity for a long time, and which, as described has important cardiovascular complications [89, 118]. This subject will deserve more attention in the future.

It has been proposed that mitochondrial function in the heart decreases with the progression of aging. Alterations include loss or oxidation of cardiolipin, a tetra-acyl phospholipid involved in the activity of many oxidative phosphorylation enzymes including complex I [119–121]. This presents a clear determinant of loss of mitochondrial function and also represents a phenotype of mitochondrial membrane aging which impacts both the bioenergetics and several signaling pathways to and from mitochondria.

It is also known that aging-dependent cardiac mitochondrial effects are more specific to interfibrillar mitochondria, which is the subpopulation responsible for the majority of energy supply to the myocardium [122, 123]. Such alterations include decrease respiratory complex activity and increased oxidative stress, while a decreased capacity for beta-oxidation has also been demonstrated in an animal model for aging due to alterations in carnitine palmitoyltransferase I which were suspected to originate from a decrease in cardiolipin content [123]. Mitochondrial "power" in the heart is thus affected with aging [124], which is further illustrated by a decrease in the nuclear control of mitochondrial biogenesis and function [125] and by increased mtDNA deletions frequency found in the aged heart [126].

Adding to mitochondrial aging, *per se*, one has to have in mind that other factors may be operating in the menopausal

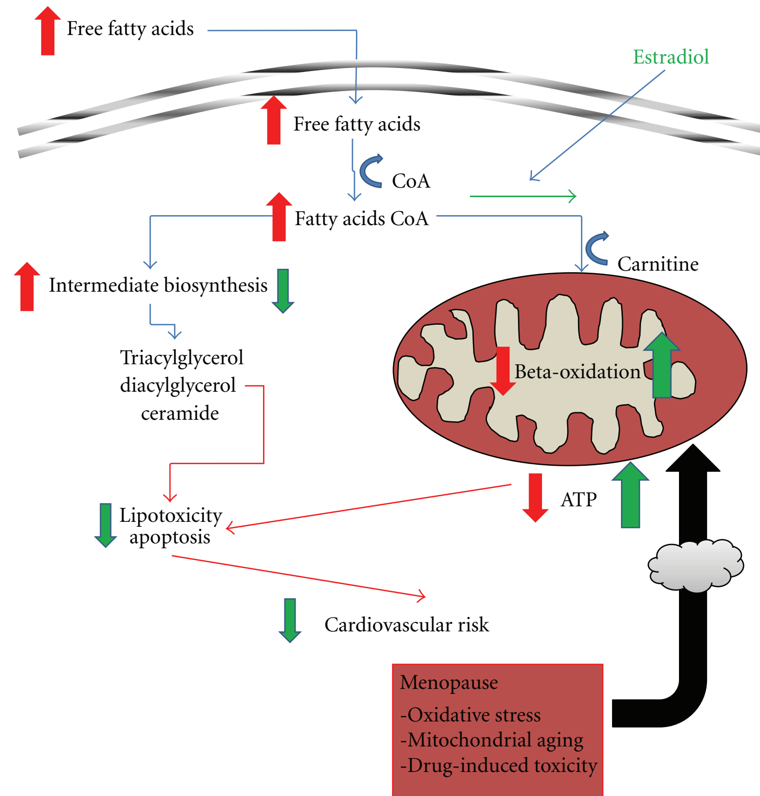

Figure 3: General scheme of the hypothesis raised by the present paper. It is proposed that menopause, as a condition natural to the normal aging process, is accompanied by specific mitochondrial alterations (bottom red box, arrow with a dark cloud) which decrease their ability to cope with an increased flux of long-chain fatty acyl CoA, resulting from augmented plasma levels. Inability to process fatty acyl CoA may result in accumulation of fatty acid intermediates including tri- and diacylglycerol, as well as ceramide, which causes myocardial lipotoxicity and may even result into activation of apoptotic signaling. The cardiovascular risk increases under these circumstances, which is fueled by other coexisting pathological conditions or by pharmacological interventions that present toxicity to the cardiovascular system. Estradiol (represented by green arrows) has been proposed to increase fatty acid oxidation by mitochondria, decreasing the flux through other biosynthetic pathways, preventing the potential accumulation of deleterious metabolites and increasing fatty acid-derived mitochondrial ATP production.

woman that can contribute to altered mitochondrial function and result in disrupted fatty acid metabolism. For example, the incidence of diabetes, and obesity increases during menopause [127], which also contributes to accelerate mitochondrial dysfunction [128–130]. By its turn, the menopausal woman may be under treatment with different medications which may also affect the bioenergetic efficacy of cardiac mitochondria [131, 132], especially if other conditions occur at the same time.

To summarize, ageing results into a progressive degradation of mitochondrial capacity in the heart, which, in combination with hormonal alterations resulting from menopause and its associated alterations in lipid profile, may result into a progressive decrease in lipid oxidation in mitochondria and increased lipid storage in adipocytes and formation of fatty acyl intermediates in the cytosol of cardiomyocytes (Figure 3). The development of insulin resistance, diabetes and obesity can be several faces of the same coin, the increased lipotoxicity in the cardiomyocyte of the menopausal woman. This is a clear avenue for research that still is largely unexplored and deserves attention since menopause is a condition that affects an increasingly number of women, as the general population is progressively aging.

If the hypothesis put together in this paper is correct, then prophylactic measures that improve mitochondrial capacity in menopausal women would contribute to decrease cardiovascular risk. In fact, besides hormone replacement therapy, which replenishes estrogens and reequilibrates lipid homeostasis, other cotherapies may help improve the lipid profile in the menopausal woman through different mechanisms. For example, endurance exercise has been demonstrated to increase mitochondrial capacity in the heart [133, 134]. In a menopausal setting, twelve weeks of endurance exercise have been demonstrated to provide some benefits in increasing lipid oxidation, besides improving other cardiorespiratory parameters [135, 136]. Carnitine, which is essential to long-chain fatty acid beta-oxidation, has been shown to recover some of skeletal muscle function and inhibit alterations in ovariectomized rats [137]. Nevertheless, to the best of our knowledge, no work on the impact of carnitine on lipid profile and oxidation in the menopausal heart has been provided.

Cardiac oxidative stress after ovariectomy has also been observed in animal models [138] although evidence for increased oxidative stress in the cardiovascular system is scarce. Estrogens *per se* act as antioxidants, although it is still unclear if estrogen supplementation during menopause is completely without risks for the cardiovascular system [139, 140]. Also, it is unclear so far if antioxidant supplementations would improve mitochondrial fitness in menopausal women. Finally, an interesting alternative was proposed by Zern et al. [141]. Lyophilized grape powder was given to a group of postmenopausal women for 4 weeks. The powder was enriched in phytochemicals such as flavans, anthocyanins, quercetin, myricetin, kaempferol, and resveratrol. The results showed alterations in lipoprotein metabolism, oxidative stress, and inflammatory markers, which were all decreased in the treated group. Although the heart was not specifically targeted in the study, the results may suggest a positive impact in this organ as well. Interestingly, resveratrol is considered an activator of mitochondrial biogenesis in different model systems, acting through sirtuin-1-dependent and independent mechanisms [142–144]. The future will tell if this is a trail worth exploring.

4. Concluding Remarks

Although there are many loose ends in the story, it appears logical to consider that progressive deterioration of mitochondrial function in the aging woman with menopause contributes to the metabolic alterations observed in the heart, including a decreased capacity for lipid oxidation. A decreased mitochondrial flux of fatty acid beta-oxidation, can result in most cases in the accumulation of toxic intermediates in the cytosol and also of nonmetabolized fatty acids in mitochondria, which leads to further deterioration of mitochondrial function and progressive metabolic changes that can increase cardiovascular risk. Not only this line of thought needs to be demonstrated in animal models and humans, but if true, pharmacological, or nonpharmacological strategies must be devised to counteract this metabolic remodeling.

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Review Article

Nuclear Receptors in Nonalcoholic Fatty Liver Disease

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Nuclear receptors comprise a superfamily of ligand-activated transcription factors that are involved in important aspects of hepatic physiology and pathophysiology. There are about 48 nuclear receptors in the human. These nuclear receptors are regulators of many hepatic processes including hepatic lipid and glucose metabolism, bile acid homeostasis, drug detoxification, inflammation, regeneration, fibrosis, and tumor formation. Some of these receptors are sensitive to the levels of molecules that control lipid metabolism including fatty acids, oxysterols, and lipophilic molecules. These receptors direct such molecules to the transcriptional networks and may play roles in the pathogenesis and treatment of nonalcoholic fatty liver disease. Understanding the mechanisms underlying the involvement of nuclear receptors in the pathogenesis of nonalcoholic fatty liver disease may offer targets for the development of new treatments for this liver disease.

1. Introduction

Liver diseases are a serious problem throughout the world. In Mexico, since 2000, cirrhosis and other chronic liver diseases have become among the main causes of mortality [1]. The incidence and prevalence of liver diseases are increasing along with changes in lifestyle and population aging, and these diseases were responsible for 20,941 deaths in 2007 [2].

In Mexico, the incidence of metabolic syndrome is also increasing. The metabolic syndrome has recently been associated with nonalcoholic fatty liver disease (NAFLD), and about 90% of patients with NAFLD have more than one feature of the metabolic syndrome [3]. The severity of NAFLD is one factor contributing to the development of nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [4, 5]. The growing obesity epidemic requires a better understanding of the genetic networks and signal transduction pathways that regulate the pathogenesis of these conditions. A clear definition of the mechanisms responsible for metabolic control may provide new knowledge for the development of new drugs, with novel mechanisms of action, for the treatment of chronic liver diseases.

The ability of individual nuclear receptors (NRs) to regulate multiple genetic networks in different tissues and their own ligands may represent a new class of potential drugs targets. To elucidate the challenges involved in developing such drugs, this paper focuses on the role of hepatic NRs in lipid metabolism and the possible effects on the physiopathology of NAFLD.

2. Nonalcoholic Fatty Liver Disease

NAFLD is defined by the accumulation of triglycerides in the form of droplets (micro- and macrovesicles) within hepatocytes [6]. The mechanism involves impaired insulin regulation, which affects fat and glucose metabolism (intermediary metabolism) in the liver, skeletal muscle, and adipose tissue, a condition known as insulin resistance. Insulin resistance increases free fatty acids and hepatic *de novo* lipogenesis, causes dysfunction in fatty acid oxidation, and alters very-low-density lipoprotein (VLDL) triglyceride export [7].

NAFLD is associated with insulin resistance, obesity, and a lifestyle characterized by physical inactivity and an unlimited supply of high-fat foods. However, more recent studies

Table 1: Nuclear receptors in hepatic lipid metabolism.

RXR partner	Ligands	Official name	Role in hepatic lipid metabolism
LXRα	Oxysterols (22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, 27-hydroxycholesterol) and fatty acids	NR1H3	(i) Increases fatty acid synthesis, TG level, HDL level, cholesterol secretion (ii) Upregulation of SREBP-c {FAS, ACC, SCD1} (iii) Upregulation of ChREBP, Angptl3 (iv) Downregulation of ApoA-V
PPARα	Fatty acids, fibrates, statins, eicosanoids, and leukotrienes	NR1C1	(i) Promotes fatty acid oxidation (by lipoprotein lipase activation) (ii) Improves insulin resistance (iii) Suppression: acyl CoA oxidase (ACO-OX), acyl CoA synthase (ACS), enoyl-CoA hydratase, malic enzyme, HMG-CoA synthase, mitochondrial enzymes, APOA1 and APOCIII
FXR	Bile acids, pregnadiene, and fexaramine	NR1H4	(i) Induces lipoprotein metabolism genes/clearance represses hepatic genes involved in the synthesis of TG (ii) Induces human PPARα (iii) Increases hepatic expression of receptors VLDL (iv) Reduces: hepatic lipogenesis and plasma triglyceride and cholesterol levels (v) Decreases expression of proteins apoC-III and Angptl3 (inhibitors of LPL)
PXR	Pregnanes, progesterone, and glucocorticoids, LCA, xenobiotics/drugs, rifampicin	NR1I2	(i) Induces lipogenesis by increasing expression of the fatty acid translocase CD36, SCD-1, and long-chain free fatty acid elongase (ii) Suppression of several genes involved in fatty acid β-oxidation (PPARα, thiolase, carnitine palmitoyltransferase 1a (Cpt1a), and mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 (Hmgcs2))
CAR	Androstane metabolites, estrogens, progesterone, and xenobiotics	NR1I3	(i) Induction of Insig-1, a protein with antilipogenic properties (ii) Interacts with PPARα during fasting (iii) Suppresses lipid metabolism and lowers serum triglyceride level by reducing SREBP-1 level

have proposed that not all individuals with NAFLD develop insulin resistance before the presence of a fatty liver [3, 8].

NAFLD is a cluster of metabolic, histological, and molecular disorders characterized by liver injury [9]. The purpose of this paper is to describe the complex working of NRs and their role in the hepatic accumulation of fat independent of excessive alcohol consumption.

NRs are ligand-activated transcription factors that have a broad range of metabolic, detoxifying, and regulatory functions. NRs are sensitive to the levels of many natural and synthetic ligands including hormones, biomolecules (lipids), vitamins, bile acids, metabolites, drugs, and xenobiotic toxins. In addition to their functions at the hepatic level, NRs also control hepatic inflammation, regeneration, fibrosis, and tumor formation [10]. These functions can be understood through a complex transcriptional network that allows them to maintain cellular nutrient homeostasis, to protect against toxins by limiting their uptake and facilitating their metabolism and excretion, and to play a role in several key steps in inflammation and fibrosis [11].

New knowledge about the functions of NRs helps clarify the pathogenesis and pathophysiology of a wide spectrum of hepatic disorders (see Table 1).

3. Nuclear Receptor Structure

The NRs are characterized by a central DNA-binding domain, which targets the receptor to specific DNA sequences known as hormone-response elements. The DNA-binding domain comprises two highly conserved zinc fingers that isolate the nuclear receptors from other DNA-binding proteins. The C-terminal half of the receptor encompasses the ligand-binding domain, which possesses the essential property of ligand recognition and ensures both specificity and selectivity of the physiological response [12, 13]. The predominant role of these receptors is the transcriptional regulation of enzymes and other proteins involved in energy homeostasis (Figure 1(a)).

4. Action Mode of Nuclear Receptors

NRs act in three steps [14]: repression, derepression, and transcription activation. Repression is characteristic of the apo-NR, which recruits a corepressor complex with histone deacetylase activity. Derepression occurs following ligand binding, which dissociates this complex and recruits the first coactivator complex, with histone acetyltransferase activity,

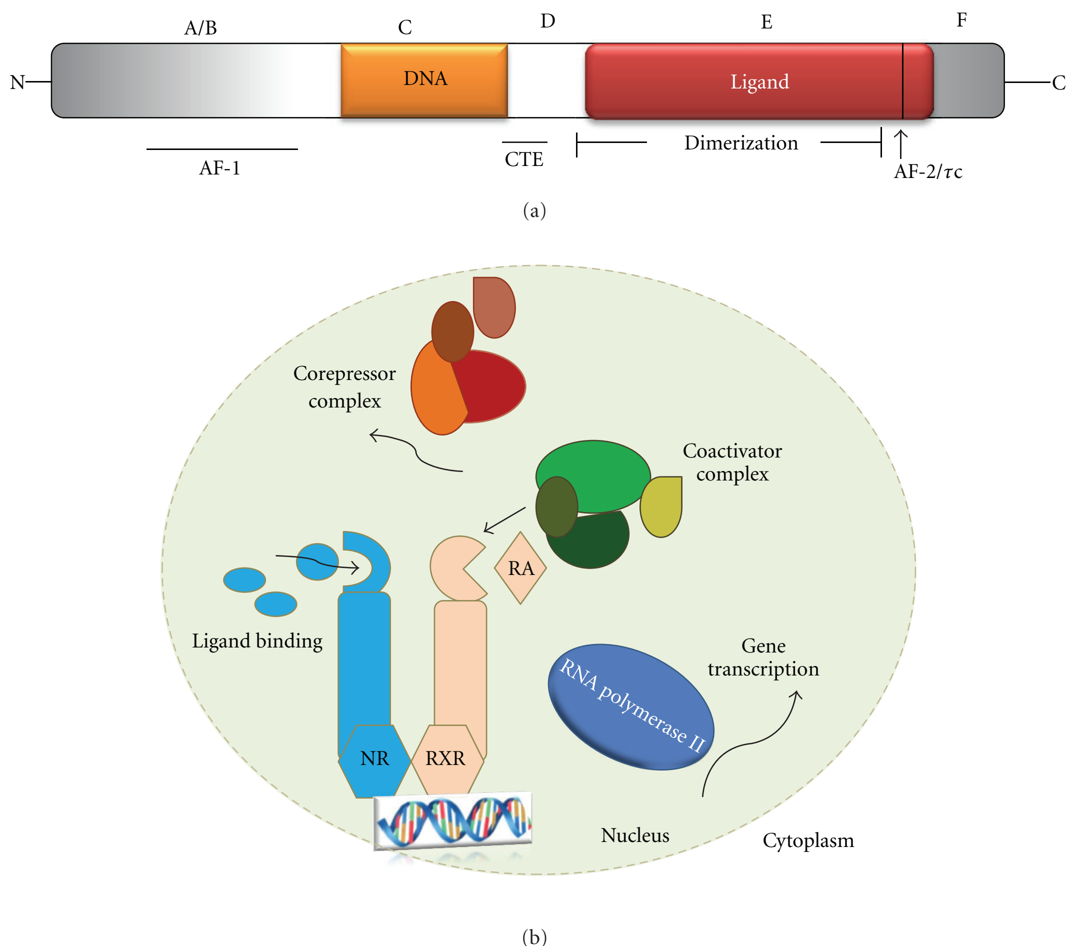

Figure 1: (a) Schematic representation of a typical nuclear receptor. Nuclear receptors may be divided into five regions based on structural and functional similarities (denoted A, B, C, D, E, and F). Regions C and E contain the conserved DNA-binding domains (DBDs) and ligand-binding domains (LBDs) that are the signature of this superfamily. In addition, the constitutive transport element (CTE) is a dimerization region within the LBD and two transactivation domains (denoted AF-1 and AF-2/τc). A second dimerization domain (not shown) exists in the DBD and is required for heterodimerization of receptors on response elements. (b) NR function. Ligand binding to NRs triggers changes in their conformation leading to the dissociation of corepressors and the recruitment of coactivators. After this exchange of coregulators, RNA polymerase II is recruited and mRNA transcription is initiated. Most NRs bind to their DNA response elements in a sequence-specific manner as dimers, functioning either as homodimers or as heterodimers with the RXR. RA: retinoic acid. Modified from [13, 94].

and causes chromatin decondensation, which is believed to be necessary, but not sufficient, for activation of the target gene. In the third step, transcription activation, the histone acetyltransferase complex dissociates to cause the assembly of a second coactivator, which can establish contact with the basal transcriptional machinery to activate the target gene [15] (Figure 1(b)).

Coactivators are molecules recruited by ligand-bound activated NRs (or other DNA-binding transcription factors) that increase gene expression. Coactivators contribute to the transcriptional process through a diverse array of enzymatic activities such as acetylation, methylation, ubiquitination, and phosphorylation, or as chromatin remodelers [16].

The result is the modulation of the expression of a wide array of physiologically important groups of genes involved in diverse pathological processes including cancer, inherited genetic diseases, metabolic disorders, and inflammation.

In contrast to the coactivator function, corepressors interact with NRs that are not bound to the ligand and repress transcription. Corepressor-associated proteins such as histone deacetylases enforce a local chromatin environment that opposes the transcription-promoting activities of coactivators [17].

5. Nuclear Receptors in the Liver

The hepatocyte is responsible for processes involved in providing for many of the body's metabolic needs, including the synthesis and control of the pathways involved in the metabolism of cholesterol, fatty acids, carbohydrates, amino acids, serum proteins, and bile acids, and the detoxification of drugs and xenobiotics.

The hepatocyte employs multiple levels of regulation to perform its functions and possesses self-protective processes

to avoid self-destruction. Some members of the NR superfamily provide hepatic mechanisms for self-regulation in hepatocytes [18].

Gene regulation by NRs is more complex than simply the presence of a potential DNA recognition sequence in a promoter. Rather, it is a complex and multilayered process that involves competition between agonists and antagonists, heterodimerization, coregulator recruitment, and NR protein modification.

The NR family comprises 48 family members and is the largest group of transcriptional regulators in the human. Because some NRs participate in the control of hepatic homeostasis, they may provide a new therapeutic target for the treatment of liver diseases such as NAFLD [19].

5.1. Liver X Receptor. The transcriptional factor liver X receptor (LXR) is involved in cholesterol metabolism. The LXR gene encodes two distinct products, LXRα and LXRβ, each with diverse patterns of expression but similar target DNA-binding elements and ligands. The human LXRα gene is located on chromosome 11p11.2, and the human LXRβ gene is located on chromosome 19q13.3. We will focus on LXRα because of its high expression in the liver, although it is also expressed at lower levels in the kidney, intestine, lung, fat, adrenal, spleen, and macrophages [20, 21]. The ligands for LXR are oxysterols. Once activated, LXR induces the expression of a cluster of genes that function in lipid metabolism; these functions are cholesterol absorption, efflux, transport, and excretion [22–24]. Besides its metabolic role, LXRs also modulate immune and inflammatory responses in macrophages [25].

Like most other nuclear receptors, LXR forms heterodimers with the retinoid X receptor (RXR) within the nucleus. Binding of the RXR to LXR leads to the formation of a complex with corepressors such as silencing mediator of retinoic acid, thyroid hormone receptor, and nuclear corepressor [26].

In the absence of a ligand, these corepressor interactions are maintained and the transcriptional activity of target genes is suppressed. Binding of a ligand to LXR causes a conformational change that facilitates inactivation of the corepressor complex and the transcription of target genes [27].

LXR is a key regulator of whole-body lipid and bile acid metabolism [20, 28] (Figure 2). LXR regulates a cluster of genes that participate in the transport of excess cholesterol in the form of high-density lipoprotein (HDL) from peripheral tissue to the liver—a process called reverse cholesterol transport. *In vivo* activation of LXR with a synthetic, high-affinity ligand increases the HDL level and net cholesterol secretion [29]. LXR positively regulates several enzymes involved in lipoprotein metabolism including lipoprotein lipase (LPL), human cholesteryl ester transport protein, and the phospholipid transfer protein [30]. LXR also regulates the crucial bile acid enzyme CYP7A1. In rodents, this enzyme contains an LXR response element that is upregulated in response to excess cholesterol in the diet. The enzymatic activation and conversion of cholesterol to bile acids is one mechanism for handling excess dietary cholesterol [31–33].

In addition to its ability to modulate cholesterol and bile acid metabolism, LXR is also a key regulator of hepatic lipogenesis. Its lipogenic activity results from the upregulation of the master regulator of hepatic lipogenesis sterol regulatory element-binding protein-c (SREBP-c) and from the induction of fatty acid synthase, acyl coenzyme A carboxylase, and stearoyl CoA desaturase 1, all leading to increased hepatic lipid levels [34, 35], one of the etiological agents in the pathogenesis of NAFLD. Moreover, LXR induces the carbohydrate-response element-binding protein, ChREBP [36]. ChREBP is a target gene of LXR and is a glucose-sensitive transcription factor that promotes the hepatic conversion of carbohydrates into lipids. Several important proteins might mediate the LXR-mediated hypertriglyceridemic effect. These include angiopoietin-like protein 3 (Angptl3) [37], a liver-secreted protein that increases the concentrations of both plasma triglycerides by inhibiting LPL activity in different tissues and free fatty acids by activating lipolysis in adipocytes and/or apoA-V. LXR activation increases Angptl3 expression and downregulates apoA-V expression [38]. The second "hit" in NAFLD is related to the proinflammatory molecules, whose expression is repressed by LXR. These include inducible nitric oxide synthase, cyclooxygenase 2, interleukin-6 (IL-6), IL-1β, chemokine monocyte chemoattractant protein-1, and chemokine monocyte chemoattractant protein-3 [39].

LXR-activated pathways play central roles in whole-body lipid metabolism by regulating multiple pathways in liver cells. Further investigation into the effects of synthetic LXR-specific agonists and/or antagonists may provide new therapeutic tools for the treatment of NAFLD.

5.2. Peroxisome Proliferator-Activated Receptors. NAFLD appears to be a link between insulin resistance and obesity. Several recent studies have shown that a family of transcription factors, named the peroxisome-proliferator-activated receptors (PPARs), improve several of the metabolic abnormalities associated with insulin resistance and impaired fat metabolism [40].

The PPARs are nuclear hormone receptors. Three isotypes have been identified in humans: PPARα, PPARβ/δ, and PPARγ [41]. These receptors exhibit different tissue distribution and functions and, to some extent, different ligand specificities. PPARα is highly expressed in the liver, brown adipose tissue, heart, skeletal muscle, kidney, and at lower levels in other organs. PPARγ is highly expressed in adipose tissues and is present in the colon and lymphoid organs. PPARβ/δ is expressed ubiquitously, but its levels may vary considerably [42, 43].

Mechanistically, the PPARs also form heterodimers with the RXR and activate transcription by binding to a specific DNA element, termed the peroxisome proliferator response element (PPRE), in the regulatory region of several genes encoding proteins that are involved in lipid metabolism and energy balance. Binding of agonists causes a conformational change that promotes the binding to transcriptional coactivators. Conversely, binding of antagonists induces a conformation that favors the binding of corepressors. Physiologically, PPAR-RXR heterodimers may bind to PPREs

in the absence of a ligand, although the transcriptional activation depends on the ligand-bound PPAR-RXR [44, 45]. The predominant role of these receptors is the transcriptional regulation of enzymes and other proteins involved in energy homeostasis, some of which are in the liver. To explain their possible action in the development and treatment of NAFLD, a brief description of each PPAR follows [46, 47].

In the liver, PPARα promotes fatty acid oxidation. It is the target for the hypolipidemic fibrates, such as fenofibrate, clofibrate, and gemfibrozil, which are used in the treatment of hypertriglyceridemia [48].

The role of PPARα in hepatic fatty acid metabolism is especially prominent during fasting. In fasted PPARα-null mice, its absence is associated with pronounced hepatic steatosis, decreased levels of plasma glucose and ketone bodies, and elevated plasma free fatty acids levels, and hypothermia. These severe metabolic disturbances are the result of the decreased expression of many genes involved in hepatic lipid metabolism. The PPARα target genes are those for acyl CoA oxidase (ACO-OX), acyl CoA synthase (ACS), enoyl-CoA hydratase, malic enzyme, HMG CoA synthase, mitochondrial enzymes, liver-fatty-acid-binding protein, and fatty acid transport protein. PPARα can also regulate other genes such as LPL, which is involved in the degradation of triglycerides, and APOA1 and APOCIII, which are both downregulated by PPARα [49–55] (Figure 2).

Whereas PPARα controls lipid catabolism and homeostasis in the liver, PPARγ promotes the storage of lipids in adipose tissues and plays a pivotal role in adipocyte differentiation. It is a target of the insulin-sensitizing thiazolidinediones. Despite its relatively low expression levels in healthy liver, PPARγ is critical for the development of NAFLD [56].

In the liver, PPARβ/δ is protective against liver toxicity induced by environmental chemicals, possibly by downregulating the expression of proinflammatory genes. PPARβ/δ regulates glucose utilization and lipoprotein metabolism by promoting reverse cholesterol transport [57–60]. PPARs appear to be targets for the treatment of metabolic disorders. PPARα and PPARγ are already therapeutic targets for the treatment of hypertriglyceridemia and insulin resistance, respectively, disorders that relate directly to the progress of NAFLD. The discovery of more pathways may provide new treatments for hepatopathies.

5.3. Farnesoid X Receptor. The farnesoid X receptor (FXR), a member of the NR superfamily, has a typical NR structure and contains a hydrophobic pocket that accommodates lipophilic molecules such as bile acids [61]. Its gene is located on chromosome 12, and it is expressed predominantly in the liver, gut, kidneys, and adrenals and at lower levels in white adipose tissue [62, 63]. The FXR binds to specific response elements as a heterodimer with the RXR, although it has also been reported to bind DNA as a monomer [28, 64]. The main physiological role of the FXR is to act as a bile acid sensor in the enterohepatic tissues. FXR activation regulates the expression of various transport proteins and biosynthetic enzymes crucial to the physiological maintenance of bile acids and lipid and carbohydrate metabolism.

Bile acids bind to and activate this NR. The order of potency of FXR binding to bile acids is chenodeoxycholic acid > lithocholic acid = deoxycholic acid > cholic acid [65, 66].

In addition to their well-established roles in bile acid metabolism, recent data have demonstrated that activation of the FXR is also implicated in lipid metabolism. Activation of the FXR reduces both hepatic lipogenesis and plasma triglyceride and cholesterol levels, induces the genes implicated in lipoprotein metabolism/clearance, and represses hepatic genes involved in the synthesis of triglycerides [67]. The FXR promotes reverse transport of cholesterol by increasing hepatic uptake of HDL cholesterol via two independent mechanisms. The first is FXR-mediated suppression of hepatic lipase expression [68]. Hepatic lipase reduces HDL particle size by hydrolyzing its triglycerides and phospholipids in hepatic sinusoids, which facilitates hepatic uptake of HDL cholesterol. The second mechanism is the induction by the FXR of the expression of the gene for scavenger receptor B1, the HDL uptake transporter in the liver [69].

Activation of the FXR also increases the hepatic expression of receptors such as VLDL receptor and syndecan-1, which are involved in lipoprotein clearance, and increases the expression of ApoC-II, which coactivates lipoprotein lipase (LPL). FXR activation also decreases the expression of proteins such as ApoC-III and Angptl3 [70] that normally function as inhibitors of LPL. Finally, the FXR induces human PPARα [71], an NR that functions to promote fatty acid β-oxidation. Taken together, these data suggest that FXR activation lowers plasma triglyceride levels via both repressing SREBP1-c and triglyceride secretion and increasing the clearance of triglyceride-rich lipoproteins from the blood (Figure 2).

In carbohydrate metabolism, activation of the hepatic FXR regulates gluconeogenesis, glycogen synthesis, and insulin sensitivity [72]. The bile acid sensor FXR also has anti-inflammatory properties in the liver and intestine, mainly by interacting with NF-κB signaling. FXR agonists might therefore represent useful agents to reduce inflammation in cells with high FXR expression levels, such as hepatocytes, and to prevent or delay cirrhosis and cancer development in inflammation-driven liver diseases.

These data suggest that FXR activation by its ligands would reduce hepatic steatosis and that such activation may have a beneficial role in NAFLD by decreasing hepatic *de novo* lipogenesis, which constitutes the first "hit" of the disease. Inflammatory processes lead to the development of hepatitis and subsequent liver fibrosis. The hepatic FXR appears to be downregulated during the acute-phase response in rodents in a manner similar to that seen for other NRs such as PPARα and the LXR [73].

5.4. The Pregnant X Receptor and Constitutive Androstane Receptor. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) share some common ligands and have an overlapping target gene pattern. The CAR gene is the product of the NR1I3 gene located on chromosome 1, locus 1q23, whereas hPXR is the product of the NR1I2 gene,

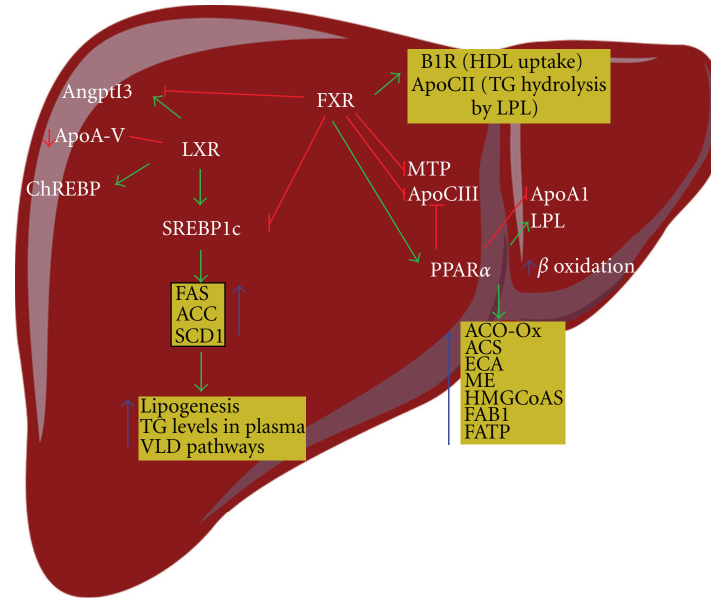

Figure 2: NRs as central regulators of hepatic lipid metabolism. Oxysterols activate the LXR, whereas bile acids (BA) stimulate SHP expression through the FXR (not shown). The LXR activates SREBP-1c and induces *de novo* fatty acid (FA) synthesis and hypertriglyceridemia by activating FAS, ACC, SCD1, and ChREBP (a glucose-sensitive transcription factor that promotes the hepatic conversion of carbohydrates into lipids). Several important proteins that could mediate the LXR-mediated hypertriglyceridemic effect are regulated. One protein is angiopoietin-like protein 3 (Angptl3), a liver-secreted protein that increases both plasma triglyceride level by inhibiting LPL activity in different tissues and free fatty acid level by activating lipolysis in adipocytes. LXR activation increases the expression of Angptl3 and LPL and downregulates apoA-V expression. Activation of the FXR leads to the repression of hepatic lipogenesis by reducing the expression of SREBP-1c. By increasing the expression of PPARα, the FXR also promotes FFA catabolism via β-oxidation, which induces ACO-OX, ACS, ECA, HMG-CoAS, FAB1, and FATP. By repressing the expression of MTP, an enzyme that controls VLDL assembly, the FXR reduces VLDL production. Activation of the FXR increases TG clearance by promoting LPL activity, via induction of ApoC-II and B1R. Activation of the FXR also reduces TG clearance by decreasing the expression of ApoC-III and Angptl3, two LPL inhibitors. PPARα can be activated by FXR and fibrates (not shown). PPAR activation leads to β-oxidation, which induces ACO-Ox, ACS, ECA, HMG-CoAS, FAB1, and FATP. Others genes are regulated. For example, LPL, which is involved in the degradation of TG, is activated, and APOA1 and APOCIII are both downregulated. The activation pathways are shown by green arrows, inhibitory pathways by red lines, and inhibited activation pathways by broken green arrows. Angptl3: angiopoietin-like protein 3; ACC: acetyl-CoA carboxylase; Apo: apolipoprotein; ChREBP: carbohydrate response element-binding protein; FAS: fatty acid synthase; FATP: fatty acid transport protein; FXR: farnesoid X receptor; LPL: lipoprotein lipase; LXR: liver X receptor; MTP: microsomal triglyceride transfer protein; PPAR: peroxisome proliferator-activated receptor; SCD1: stearoyl-coenzyme A desaturase 1; SREBP-1c: sterol regulatory element-binding protein-1c; TG: triglyceride. Arrows and stop bars indicate positive regulation or activation and negative regulation or repression, respectively.

which is located on chromosome 3, locus 3q12–q13.3 [74–76]. Like most other NRs, the PXR and CAR have an N-terminal DNA-binding domain and a C-terminal ligand-binding domain. PXR and CAR regulate gene expression by forming heterodimers with the RXR.

The PXR is located in the nucleus and has a low basal activity and is highly activated upon ligand binding [77, 78]. By contrast, in the noninduced state, the CAR resides in the cytoplasm. Compounds that activate the CAR and PXR are structurally very diverse; most are small and are highly lipophilic [79]. The PXR is activated by pregnanes, progesterone, and glucocorticoids [80, 81], whereas the CAR is affected both positively and negatively by androstane metabolites, estrogens, and progesterone [82, 83]. For this reason, in addition to functioning as xenobiotic receptors, the PXR and CAR are thought to be endobiotic receptors that influence physiology and diseases [84, 85].

For example, several studies have shown that the PXR induces lipogenesis in a SREBP-independent manner. Lipid accumulation and marked hepatic steatosis in PXR-transgenic mice are associated with increased expression of the fatty acid translocase CD36 (also called FAT) and several accessory lipogenic enzymes, such as SCD-1 and long-chain free fatty acid elongase. CD36, a multiligand scavenger receptor present on the surface of a number of cell types, may contribute to hepatic steatosis by facilitating the high-affinity uptake of fatty acids from the circulation [86]. The CD36 level in the liver correlates with hepatic triglyceride storage and secretion, suggesting that CD36 plays a causative role in the pathogenesis of hepatic steatosis [87]. PXR may also promote hepatic steatosis by increasing the expression of CD36 directly or indirectly through the PXR-mediated activation of PPARγ [86].

Interestingly, an independent study showed that hepatic triglyceride level decreases temporarily after short-term (10-hour) activation of the PXR [88]. PXR activation is also associated with upregulation of PPARγ, a positive regulator of CD36 and a master regulator of adipogenesis [89]. PXR

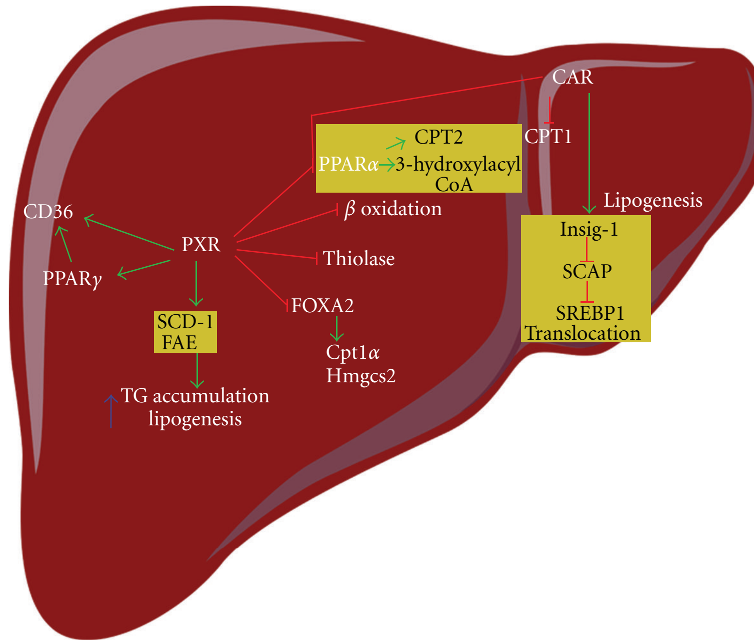

Figure 3: Activation of the PXR induces lipogenesis and inhibits fatty acid β-oxidation. The PXR induces lipogenesis through activation of CD36, PPARγ, SCD1, and FAE gene expression. The PXR inhibits fatty acid β-oxidation through its suppression of PPARα and thiolase gene expression. In addition, PXR binds to FoxA2, a key regulator of β-oxidation, and inhibits FoxA1-mediated activation of Cpt1a and Hmgcs2 gene expression. CAR activation inhibits lipogenesis by inducing Insig-1, a protein that plays a role in SREBP-mediated regulation of lipogenic genes. Insig proteins bind and trap SCAP, retaining it in the ER and preventing it from escorting SREBPs to the site of proteolytic activation in the Golgi complex (not shown). SREBPs are cleaved by two proteases in the Golgi complex, and the bHLH-Zip domain of SREBPs transfers from the membrane to the nucleus to bind the sterol response elements in the promoter region of the target genes (not shown). CAR inhibits fatty acid β-oxidation. CAR competes with PPARα for its binding site in the 3-hydroxyacyl CoA dehydrogenase gene promoter. Activation of CAR also decreases the expression of Cpt1, a rate-limiting enzyme of β-oxidation. Arrows and stop bars indicate positive regulation or activation and negative regulation or repression, respectively. Cpt1a: carnitine palmitoyltransferase 1a; FAE: long-chain free fatty acid elongase; FoxA2: forkhead box factor A2; Hmgcs2: mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2; PPAR: peroxisome proliferator-activated receptor; SCAP: SREBP cleavage-activating protein; SCD1: stearoyl CoA desaturase 1; SREBP: sterol regulatory-element binding protein.

activation is also associated with suppression of several genes involved in fatty acid β-oxidation, such as PPARα and thiolase [90]. A study by Nakamura and colleagues showed that PXR represses β-oxidation-related genes such as carnitine palmitoyltransferase 1a (Cpt1a) and mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 (Hmgcs2) through crosstalk with the insulin-responsive forkhead box factor A2 (FoxA2) (Figure 3).

Activation of the CAR might suppress lipid metabolism and lower serum triglyceride levels by reducing the level of SREBP-1, a master regulator of lipid metabolism. The inhibitory effects of the CAR on lipid metabolism might also be attributed to induction of Insig-1, a protein with antilipogenic properties [88].

The CAR interacts with PPARα during fasting and has been reported to interfere with fatty acid metabolism by binding to DNA elements overlapping with the PPARα-binding site in the promoter region of 3-hydroxyacyl CoA dehydrogenase, an important enzyme in peroxisomal fatty acid β-oxidation [91] (Figure 3).

Finally, other studies indicate that the CAR might be involved in the pathogenesis of NASH [92] by regulating the response of serum triglyceride level to metabolic stress [93]. The overlap of the activation of endogenous lipids by the CAR and PXR suggests a functional connection between these receptors in liver physiology. This knowledge might be useful in the development of new treatments to limit or prevent the pathogenesis of NAFLD by developing agonists or antagonists to prevent or lessen lipid accumulation within the liver parenchyma.

6. Conclusion

NAFLD encompasses a spectrum of conditions characterized histologically by hepatic steatosis ranging from simple fatty liver to NASH cirrhosis and HCC [4].

NRs control fatty acid transport from peripheral adipose tissue to the liver and regulate several critical metabolic steps involved in the pathogenesis of NAFLD, including fat storage, export, uptake, oxidation, and lipolysis [94]. The discovery that many ligands activate the whole family of NRs (FXR, LXR, PPARs, PXR, and CAR) and their possible interconnected mechanisms that control lipid metabolism suggests the possibility of developing novel therapies for the treatment of NAFLD. The LXR and PXR regulate several metabolically relevant pathways and clusters of genes that

lead to hepatic lipogenesis and might be directly related to the pathogenesis of liver diseases. The FXR, PPARα, and CAR are activated by ligands to orchestrate a broad range of lipolytic activities. These might become future candidates for drugs designed to target metabolic liver disorders.

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Review Article

Non-Alcoholic Fatty Liver Disease: The Bile Acid-Activated Farnesoid X Receptor as an Emerging Treatment Target

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Non-alcoholic fatty liver disease (NAFLD) is currently evolving as the most common liver disease worldwide. It may progress to liver cirrhosis and liver cancer and is poised to represent the most common indication for liver transplantation in the near future. The pathogenesis of NAFLD is multifactorial and not fully understood, but it represents an insulin resistance state characterized by a cluster of cardiovascular risk factors including obesity, dyslipidemia, hyperglycemia, and hypertension. Importantly, NAFLD also has evolved as independent risk factor for cardiovascular disease. Unfortunately thus far no established treatment does exist for NAFLD. The bile acid-activated nuclear farnesoid X receptor (FXR) has been shown to play a role not only in bile acid but also in lipid and glucose homeostasis. Specific targeting of FXR may be an elegant and very effective way to readjust dysregulated nuclear receptor-mediated metabolic pathways. This review discusses the body's complex response to the activation of FXR with its beneficial actions but also potential undesirable side effects.

1. Introduction

One characteristic of our modern civilization is the easy and unlimited access to unhealthy and caloric dense food. A typical American diet furnishes the liver with ~20 g of fat each day, equivalent to one-half of the total triglyceride content of the liver. In combination with little need for physical activity due to technological advances, one consequence of our sedentary and excessive lifestyle is non-alcoholic fatty liver disease (NAFLD).

NAFLD is a major health problem affecting up to 60 million Americans and evolving as the most common liver disease worldwide [1, 2]. This is several-fold higher than other common chronic liver diseases such as hepatitis C and alcohol-related liver disease. While the majority of subjects with NAFLD are obese, the condition can occur in the absence of obesity or other features of the metabolic syndrome. In patients with diabetes and morbid obesity the prevalence of NAFLD has been shown to be as high as 62% and 96%, respectively [3, 4].

The earliest stage of NAFLD is fatty liver that is defined as the presence of cytoplasmic triglyceride droplets in more than 5% of hepatocytes [5]. Although often self-limited, in 12–40% it can progress to non-alcoholic steatohepatitis (NASH) [6]. NASH is distinguished from simple fatty liver by the presence of hepatocyte injury such as hepatocyte ballooning and apoptosis, an inflammatory infiltrate, and/or collagen deposition. Over a time period of 10–15 years, 15% of patients with NASH will progress to liver cirrhosis [7]. Once cirrhosis has developed in the absence of viral hepatitis, hepatic decompensation occurs at a rate of 4% annually while the ten-year risk of developing liver cancer is 10% [7, 8]. Although liver cancer secondary to NASH typically develops in the setting of cirrhosis, carcinogenesis can occur in the absence of advanced liver disease. It is thus not surprising that NAFLD is poised to become the primary indication for liver transplantations. Like other causes of chronic liver disease, NASH recurs following liver transplantation almost universally [9].

2. Basic Pathophysiological Concepts and Treatment of NAFLD

The pathogenesis of NAFLD is multifactorial and only partially understood. Fatty liver arises in the setting of

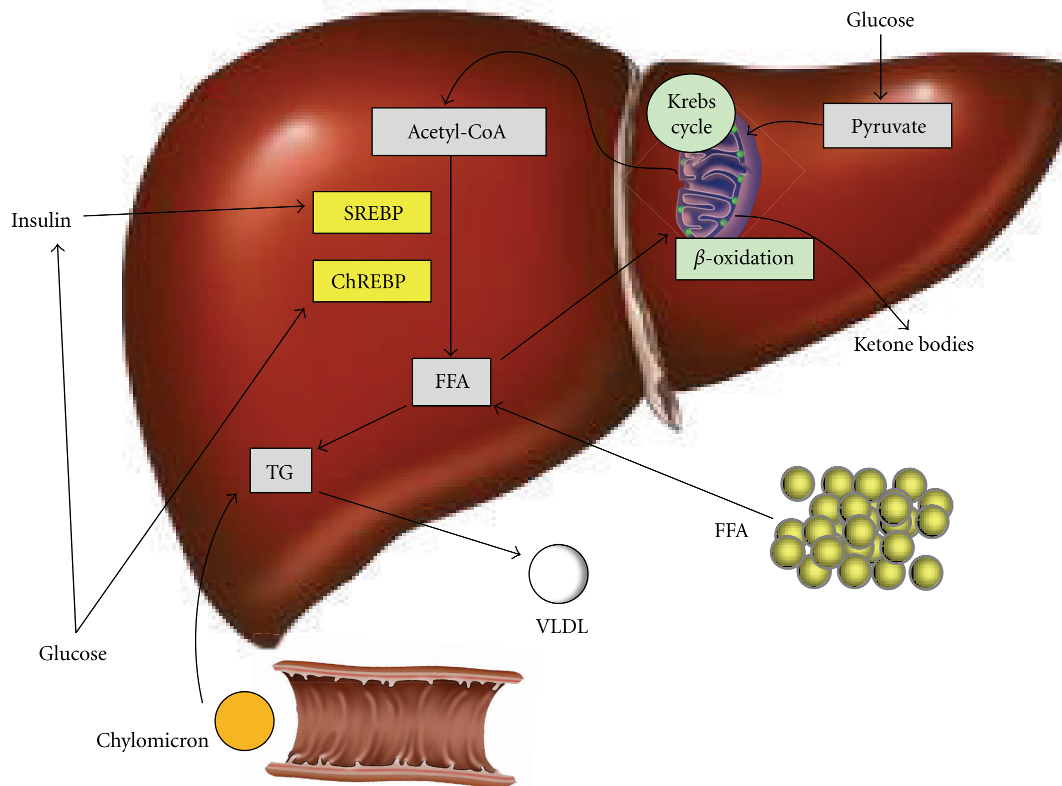

Figure 1: Hepatic triglyceride (TG) formation, acquisition, and removal. Fatty liver is a result of an imbalance between free fatty acids (FFAs), and TG input and FFA and TG output. FFA derives from peripheral tissue, endogenous synthesis or diet in form of chylomicrons. Carbohydrate intake increases glucose and insulin levels thereby promoting lipogenesis through the activation of transcription factors sterol regulatory element-binding protein 1c (SREBP) and carbohydrate responsive element-binding protein (ChREBP). Reducing the FFA burden include β-oxidation in mitochondria, storage as TG, or export as very low-density lipoprotein (VLDL).

an imbalance between triglyceride formation/acquisition and removal (Figure 1). Assembly of triglycerides and lipid droplet formation requires fatty acids that can derive from diet, *de novo* synthesis, or adipose tissue. Dietary fat packed in chylomicrons is hydrolyzed releasing free fatty acids of which approximately 20% are delivered to the liver [8]. Carbohydrate-enriched diets promote *de novo* synthesis of free fatty acids via insulin-stimulated activation of sterol regulatory element-binding protein-1c [10, 11]. In addition, glucose facilitates lipogenesis via activation of carbohydrate responsive element-binding protein [12]. In the fasting state, a decline of insulin levels stimulates adipocyte triglyceride hydrolase thereby releasing free fatty acids that are transported to the liver [13]. In the liver, free fatty acids can be (i) used for energy and ketone body production via mitochondrial β-oxidation, (ii) esterified and stored as triglycerides in lipid droplets, or (iii) packaged with apolipoprotein B into very low-density lipoproteins that are secreted into the circulation. As the liver extracts approximately 20% of free fatty acids from the circulation, the daily input of triglycerides from diet and fatty acids from adipocyte tissue is equivalent to the entire triglyceride content of the liver [14]. Once the capacity of the liver to store fatty acids in form of triglycerides is overwhelmed, NASH, differentiated from a fatty liver by the presence of increased cell injury, apoptosis, inflammation, and fibrosis, starts to develop. A detailed review of the steps involved in the progression of NAFLD to NASH and cirrhosis has been recently published [15].

Treatment of NAFLD should either prevent disease progression to liver cirrhosis or reverse already established NASH, respectively. Despite many advances in our understanding of the pathogenesis of NAFLD, there is currently no established treatment available. Life-style changes and exercise to reduce body weight and treatment of concomitant diabetes and dyslipidemia are accepted first-line therapy but have not been shown to convincingly reduce the risk of disease progression [16]. Therefore exploring new avenues for treatment of this common disease is crucial.

3. The Bile Acid-Activated Nuclear Farnesoid X Receptor (FXR)

Nuclear receptors are a group of transcription factors that consist of 48 members in humans. They have a common

structure consisting of a ligand-independent activation domain for interaction with cofactors, a central DNA binding domain, and a unique ligand binding domain allowing receptor dimerization and coregulator interactions. Most nuclear receptors function either as homodimers or as heterodimers with the retinoid X receptor. Binding of the ligand promotes conformational changes facilitating the release of corepressors and resulting in conformational changes of chromatin enabling access of the transcriptional machinery to the respective promoters. Upon ligand activation, the corepressor complex dissociates and the coactivator complex is recruited allowing start of transcription. Control of nuclear transcriptional activity is also thought to occur by posttranslational modifications [17–19].

In 1995 a protein was discovered that was interacting with the human retinoic X receptor and named retinoic X receptor-interacting protein 14 [20]. Because it was activated by an intermediate of the mevalonate pathway, farnesol, it was renamed to farnesoid X receptor [21]. Another four years later, three independent groups [22–24] discovered bile acids as endogenous ligands for FXR. From an evolutionary point of view the *FXR* gene is highly conserved suggesting that it plays an important role in many species. At the tissue level, FXR is expressed predominantly in the liver, intestine, kidney, and adrenal gland. Expression in heart and adipose tissue is low [25]. The generation of mice with *Fxr* gene ablation identified FXR as a master regulator in bile acid homeostasis [26]. Subsequently novel functions of FXR have been identified including protecting the intestinal barrier and modulating the innate immunity [27, 28] and tumorigenesis [29, 30]. The most important roles of FXR are likely in regulating metabolic processes.

4. FXR as Key Player in Multiple Metabolic Processes

For a long time, physiological effects of bile acids have mainly been attributed to their physicochemical properties [31]. In the last couple of years it has been evident that bile acids act like signaling molecules [32] regulating not only their own homeostasis during the enterohepatic circulation but also triglyceride, cholesterol, and glucose metabolism.

4.1. Bile Acid Metabolism. A major physiological role of FXR in bile acid metabolism is to protect hepatocytes from the deleterious effects of increased bile acid levels by inhibiting endogenous bile acid synthesis and accelerating bile acid biotransformation and excretion. In this regard, FXR-mediated effects occur in a tightly coordinated fashion at the level of the hepatocyte and enterocyte and have been reviewed in detail elsewhere [33].

4.2. Triglyceride and Cholesterol Metabolism. It has been known for years that bile acids can modulate lipid metabolism in humans. Reducing the transhepatic flux of bile acids decreases low-density lipoprotein cholesterol and increases high-density lipoprotein cholesterol and very low-density lipoprotein triglyceride levels. Opposite effects are observed when the bile acid pool is expanded [34–36]. Studies in mice with *Fxr* gene ablation or administering FXR agonists provided key information demonstrating a central role of FXR in lipid homeostasis.

As illustrated in Figure 2, FXR activation of short heterodimer partner is required to suppress sterol regulatory element-binding protein 1c expression [37]. As sterol regulatory element-binding protein 1c is known to regulate several genes involved in fatty acid and triglyceride formation [11], FXR-mediated repression of sterol regulatory element-binding protein 1c inhibits triglyceride and fatty acid synthesis and secretion. Interestingly, recent studies support the concept that FXR-independent mechanisms may also contribute [38]. In addition to decreasing lipogenesis, activation of FXR facilitates the clearance of very low-density lipoproteins and chylomicrons. This is achieved by increasing the expression of the very low-density lipoprotein receptor [39], a protein that enhances lipoprotein lipase-mediated triacylglycerol hydrolysis. Very low-density lipoprotein assembly is controlled by FXR via repressing the expression of microsomal triglyceride transfer protein and apolipoprotein B [38]. FXR also activates syndecan-1, a transmembrane protein that binds remnant particles before their transfer to receptors [40]. Activation of lipoprotein lipase, a key enzyme involved in the lipolysis of triglyceride rich lipoproteins, is also FXR-dependent. This involves activation of apolipoproteins C-II and AIV [41–43] and inhibiting the expression of apolipoprotein C-III [44] and angiopoetin-like 3 [37], respectively. Another effect of FXR activation is the induction of peroxisome proliferator-activated receptor α that promotes fatty acid β-oxidation [45]. Collectively these findings support the concept that FXR activation decreases plasma triglyceride levels by suppressing hepatic lipogenesis and triglyceride secretion and increasing the clearance of triglyceride-rich lipoproteins from blood. These observations therefore support the concept that FXR activation may have a beneficial effect in patients with NAFLD by decreasing hepatic lipogenesis.

Activation of FXR also modulates the reverse cholesterol transport, a pathway that promotes cholesterol delivery from the periphery to the liver for biliary disposal and fecal elimination. In this scenario, the selective uptake of high-density lipoprotein cholesteryl ester via scavenger receptor BI [46], intracellular cholesteryl ester hydrolysis facilitated by neutral cholesteryl ester hydrolase [47], as well as the canalicular routing of cholesterol by sterol carrier protein 2 [48] for biliary excretion via adenosine triphosphate binding cassette subfamily G member 5/8 [49] are positively regulated by FXR [50]. In addition but controversial, FXR appears to suppress apolipoprotein A-I expression [46, 50, 51], the primary protein constituent of high-density lipoprotein defining its size and shape. This may be of particular importance as it could influence the capability of high-density lipoprotein to remove cholesterol from peripheral cells, activating the lecithin-cholesterol acyl transferase enzyme and delivering the resulting cholesteryl ester to the liver. Another target of FXR is paraoxonase 1, a protein produced in the liver with phospholipase A2 activity that may be important for inactivation of proatherogenic lipids produced

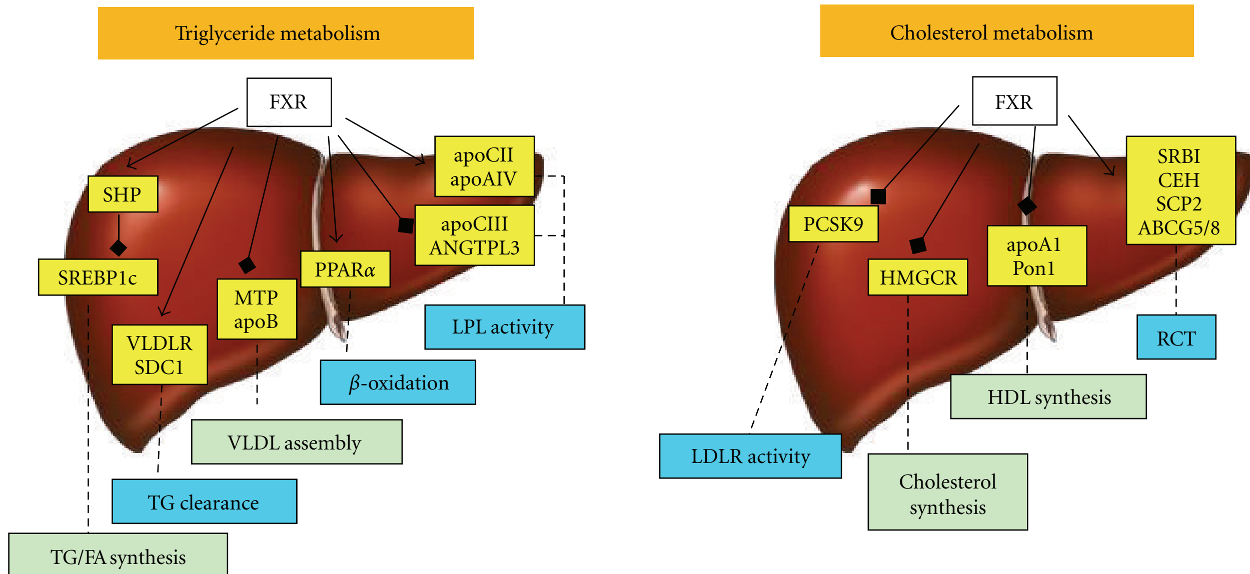

Figure 2: Effect of FXR activation on triglyceride and cholesterol metabolism in the liver. FXR agonists result in a variety of responses modulating triglyceride (TG) and cholesterol metabolism. Activation of FXR inhibits triglyceride (TG)/fatty acid (FA) synthesis facilitated by suppressing sterol regulatory element-binding protein 1c (SREBP1c) via activation of short heterodimer partner (SHP). FXR controls assembly of very low-density lipoprotein (VLDL). FXR may increase the clearance of TG by stimulating lipoprotein lipase (LPL) activity as well as the hepatic uptake of remnants and low-density lipoprotein by inducing syndecan 1 (SDC1) and the VLDL receptor (VLDLR). FXR agonists may modulate LDL receptor activity via inhibition of proprotein convertase subtilisin/kexin 9 (PCSK9) and activate the reverse cholesterol transport pathway (RTC). FXR activation also impairs high-density lipoprotein (HDL) formation and suppresses cholesterol synthesis. apoA1, apoB, apoCII, apoCIII, apoAIV: apolipoprotein A1, B, CII, CIII, AIV; ANGTPL3: angiopoetin like 3; ABCG5/8: adenosine triphosphate binding cassette subfamily G member 5/8; CEH: cholesterylester hydrolase; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; MTP: microsomal triglyceride transfer protein; PON1: paraoxonase 1; SRBI: scavenger receptor B1; SCP2: sterol carrier protein 2.

by oxidative modification of low-density lipoprotein. FXR-mediated repression of paraoxonase 1 involves the induction of fibroblast growth factor 19, its subsequent binding to the fibroblast growth factor receptor 4, and activation of the c-Jun N-terminal kinase pathway [52, 53]. FXR also regulates the expression of phospholipid transfer protein [54] that is responsible for the transfer of phospholipids and cholesterol from low to high-density lipoprotein and suppresses 3-hydroxy-3-methyl-glutaryl-CoA reductase likely involving sterol regulatory element-binding protein 2 [55]. Finally, FXR represses proprotein convertase subtilisin/kexin 9 [56], a protein that promotes the intracellular degradation of the low-density lipoprotein receptor by interfering with its recycling to the plasma membrane. In summary, these findings raise concern that activation of FXR may alter the cholesterol metabolism in a way that increases the susceptibility to atherosclerosis and thus limit its application in patients with NAFLD.

4.3. Glucose Homeostasis. In addition to their pleiotropic effects on lipid metabolism, bile acids also affect glucose homeostasis. This is supported by an improved glycemic control in patients with diabetes mellitus response to cholestyramine [57]. Several studies addressed the role of bile acids and FXR activation in glucose metabolism, but the underlying mechanisms are far from being understood. It appears clear that FXR exerts a role in glucose homeostasis [58]. In the state of *Fxr* gene ablation, the failure to suppress gluconeogenesis and a reduced peripheral glucose disposal led to glucose intolerance [59–61]. A potential molecular basis for these observations is the suppression of hepatic phosphenoyl-pyruvate carboxykinase and glucose 6-phosphatase [60, 62]. Reduced plasma levels of free fatty acids in response to FXR activation (see above) may explain the increased insulin sensitivity in the liver. Of note, FXR activation was shown to enhance insulin-stimulated glucose uptake as well as insulin signaling in adipocytes [61]. It should be noted that bile acids also modulate glucose homeostasis in an FXR-independent fashion through cell signaling pathways [63]. Collectively these findings suggest that FXR activation might prove useful in the treatment of hyperglycemia and hyperlipidemia that are present in patients with NAFLD.

4.4. Hepatic Inflammation and Fibrogenesis. Inflammation and collagen deposition in the liver are key histopathological features of NASH. FXR appears to antagonize hepatic inflammatory processes by antagonizing the nuclear factor kappa B pathway [64]. Another protective FXR mechanism involves induction of antimicrobial factors in the intestine [65]. As FXR is expressed in rodent hepatic stellate cells that play a critical role in hepatic fibrosis, it is not surprising that FXR agonists protect against liver fibrosis [66]. This appears to be mediated by a decreased hepatic expression of various profibrotic growth factors including transforming growth factor $\beta 1$, tissue inhibitor of metalloproteinase 1, $\alpha 1$(I)

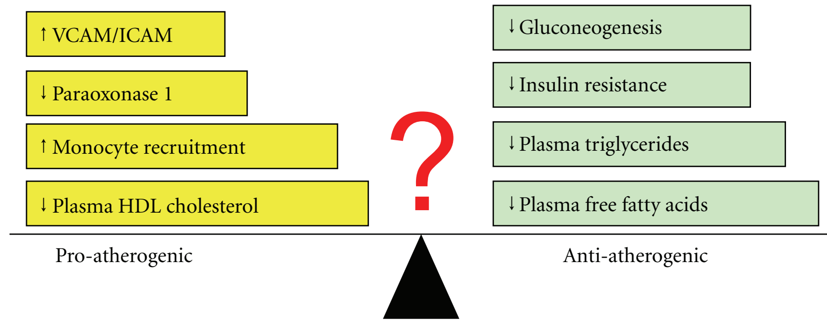

Figure 3: Pro- and antiatherogenic effects of FXR activation. With regard to atherosclerosis, activation of FXR may be associated with beneficial and potential negative effects. Unless tested in humans, one cannot predict with certainty whether pro- or antiatherogenic effects are dominant and development of specific FXR modulators may help to avoid some or most of the negative effects. VCAM: vascular cell adhesion protein. ICAM: intracellular adhesion molecule.

collagen, α smooth muscle actin, matrix metalloproteinase 2 and α2(I) collagen, and microRNA-29a [67–69]. However, if this mechanism is also operational in humans with a lower expression level of FXR remains to be determined [70]. These data suggest that targeting FXR may impact progression from NAFLD to NASH.

5. FXR and Atherosclerosis

As demonstrated earlier in this article and illustrated in Figure 3, activation of FXR seems to be associated with both anti- and proatherogenic properties. In addition to its impact on dyslipidemia and hyperglycemia, FXR may also directly act at the levels of the arterial wall. Potential beneficial effects of FXR activation against atherosclerosis include suppressing the vasoconstrictive peptide endothelin-1 [71]. Induced expression of intracellular adhesion molecule 1 and vascular cell adhesion molecule 1, however, promotes atherosclerosis by recruiting macrophages to the endothelium [72]. The role of FXR in the initiation and progression of atherosclerosis has been studied in mice with *Fxr* gene ablation that were backcrossed into atherosclerosis-susceptible strains with either deletion of the low-density lipoprotein receptor or apolipoprotein E, respectively [73, 74]. These studies produced discrepant results whereas more recent experimentations employing an FXR agonist uniformly demonstrated protection against diet-induced aortic plaque formation [75, 76]. Translating these findings to humans is not straightforward as humans carry most cholesterol in LDL compared to the mouse that lacks cholesteryl ester transfer protein activity and thus transports most cholesterol in high-density lipoprotein [77]. In knowledge of these limitations, it would be most logical to carry out future studies in low-density lipoprotein receptor deficient mice that overexpress human cholesteryl ester transfer protein [78].

6. Summary and Perspective

FXR plays a key role in the transcriptional control of a myriad of target genes that control metabolic pathways relevant to NAFLD. By virtue of that role FXR is critically involved in the development and progression of NAFLD. Targeting FXR therefore offers exciting new perspectives for the treatment of NAFLD. However, when interpreting data obtained in cell culture and rodent models of human disease, attention needs to be paid to differences between these models and humans. One particular challenge in designing FXR agonists is separating the desired therapeutic effects from the undesirable side effects. The design of organ- or gene-specific FXR ligands may enhance the specificity and reduce side effects of this therapeutic approach. An increased understanding of the effect of cellular signaling of FXR and its coregulator proteins has the potential to aid in discovering novel selective therapeutic modulators and the development of new and more effective therapeutics. Finally one also needs to consider that the response to modulation of the FXR receptor may differ among patient with NAFLD and NASH.

Despite all the concerns raised, it is anticipated that targeting FXR will result in a more specific and individually tailored therapy that could revolutionize the management of NAFLD. Support comes from studies in rats with diabetes mellitus and fatty liver disease that received the FXR agonist INT-747 for two months [79]. This intervention decreased glucose levels and dyslipidemia, protected against body weight gain, and improved insulin resistance. It is thus very encouraging that INT-747 also has shown to improve insulin resistance in patients with diabetes mellitus and NAFLD [80]. Based on this study with a limited number of patients, an ongoing large multicenter trial enrolling 280 patients at eight U.S. centers comprising the NIDDK-sponsored NASH Clinical Research Network is under way, the results of which are eagerly awaited.

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Review Article

Ezetimibe: Its Novel Effects on the Prevention and the Treatment of Cholesterol Gallstones and Nonalcoholic Fatty Liver Disease

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The cholesterol absorption inhibitor ezetimibe can significantly reduce plasma cholesterol concentrations by inhibiting the Niemann-Pick C1-like 1 protein (NPC1L1), an intestinal sterol influx transporter that can actively facilitate the uptake of cholesterol for intestinal absorption. Unexpectedly, ezetimibe treatment also induces a complete resistance to cholesterol gallstone formation and nonalcoholic fatty liver disease (NAFLD) in addition to preventing hypercholesterolemia in mice on a Western diet. Because chylomicrons are the vehicles with which the enterocytes transport cholesterol and fatty acids into the body, ezetimibe could prevent these two most prevalent hepatobiliary diseases possibly through the regulation of chylomicron-derived cholesterol and fatty acid metabolism in the liver. It is highly likely that there is an intestinal and hepatic cross-talk through the chylomicron pathway. Therefore, understanding the molecular mechanisms whereby cholesterol and fatty acids are absorbed from the intestine could offer an efficacious novel approach to the prevention and the treatment of cholesterol gallstones and NAFLD.

1. Introduction

The small intestine is a unique organ providing dietary and reabsorbed biliary cholesterol to the body [1–3]. High plasma total and low-density lipoprotein (LDL) cholesterol concentrations are an important risk factor for cardiovascular diseases. The restriction of dietary calories, cholesterol, and saturated fat has been used as the primary initial therapeutic modality for the treatment of patients with dyslipidemia [4]. However, the reduction of dietary cholesterol is frequently not associated with a significant decrease in circulating LDL cholesterol levels, despite significant restrictions in dietary intake. Therefore, pharmacological intervention aimed to reduce intestinal cholesterol absorption is potentially an effective way of lowering plasma total and LDL cholesterol concentrations [2]. The use of cholesterol absorption inhibitors for treating hypercholesterolemia has a long history, and several classes of compounds such as hydrophilic bile acid ursodeoxycholic acid (UDCA) [2], the bile acid sequestrants, specific lipase inhibitors, the intestinal acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors [5, 6], and cholesterol ester transfer protein inhibitors [7] have been developed, and some of them are currently being evaluated in clinical trials. Recently, the discovery and development of ezetimibe, a novel, selective, and potent inhibitor that effectively blocks intestinal absorption of dietary and biliary cholesterol, opened a new door to the treatment of hypercholesterolemia [2, 8–11]. Ezetimibe, which can be administered either as monotherapy or in combination with statins, has been shown to be a safe and efficacious treatment for hypercholesterolemia, potentially enabling more patients to reach recommended LDL cholesterol standards set by the National Cholesterol Education Program Adult Treatment Panel III guidelines [12].

Unexpectedly, it was found that ezetimibe treatment can induce a complete resistance to cholesterol gallstone

formation [13] and nonalcoholic fatty liver disease (NAFLD) in addition to its effect on hypercholesterolemia in mice on a Western diet [14]. Furthermore, ezetimibe can prevent gallstones by effectively reducing intestinal cholesterol absorption and biliary cholesterol secretion and protecting gallbladder motility function by desaturating bile in mice. Treatment with ezetimibe also promotes the dissolution of gallstones by forming an abundance of unsaturated micelles in bile. Furthermore, ezetimibe significantly reduces biliary cholesterol saturation and retards cholesterol crystallization in biles of patients with gallstones [15]. It is also found that ezetimibe could prevent fatty liver by reducing hepatic lipogenesis in mice on a high-fat diet and attenuating diet-induced insulin resistance, a state known to drive hepatic lipogenesis through elevated circulating insulin levels [16]. Therefore, it is highly likely that ezetimibe could be a novel approach to reduce biliary cholesterol content and hepatic triglyceride accumulation, and thus a promising strategy for preventing or treating cholesterol gallstones and NAFLD, by inhibiting intestinal cholesterol absorption [15].

In this paper, we will review recent progress in understanding the biochemical and physical-chemical mechanisms, whereby ezetimibe could prevent or treat cholesterol gallstones and NAFLD, the two most prevalent hepatobiliary diseases that constitute a considerable health care burden in the USA.

2. Chemistry and Pharmacology of Ezetimibe

Ezetimibe (SCH 58235), 1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone, and an analog, SCH 48461, (3*R*)-(3-Phenylpropyl)-1, (4*S*)-bis(4-methoxyphenyl)-2-azetidinone, are highly selective intestinal cholesterol absorption inhibitors. They can effectively and potently prevent the absorption of cholesterol by inhibiting the uptake and transport of dietary and biliary cholesterol across the apical membrane of enterocytes [17]. It has been found that the 2-azetidinones are able to inhibit cholesterol absorption at very low doses and induce significant reductions in plasma cholesterol concentrations in humans and in a series of different animal models [2, 18–20]. After oral administration, ezetimibe undergoes rapid monoglucuronidation in enterocytes and the liver during its first pass. Because ezetimibe and its glucuronide are enterohepatically recirculated, it is most likely that they could repeatedly produce an inhibitory action on the Niemann-Pick C1-like 1 protein (NPC1L1) on the apical membrane of enterocytes, exhibiting multiple peaks of serum drug concentrations with an elimination half-life of approximately 22 hours [21]. This may explain why ezetimibe has a longer duration of action and why its therapeutic effects persist for several days after its cessation. These observations support the notion that once-daily dosing should be sufficient for an adequate therapeutic effect. It has also been demonstrated that 12 hours after oral administration of the glucuronide (SCH-60663), more than 95% of the compound still can be found in the intestine. Because the glucuronide is more potent in inhibiting cholesterol absorption than ezetimibe, it confirms that ezetimibe acts directly in the intestine as glucuronide [6]. Studies with [^{125}I]-labeled ezetimibe glucuronide and [^{14}C]-labeled cholesterol have found that the glucuronide could block the cholesterol uptake into the enterocytes [2] because it is often detected in the brush border membrane, a site predominantly associated with cholesterol uptake and transepithelial transport. Furthermore, ezetimibe and its analogs are relatively small molecules that may not be able to change the physical-chemical nature of the intraluminal environment, nor affect the enterohepatic flux of bile acids [2].

A careful analysis of 399 patients receiving either placebo- or ezetimibe-doses of 0.25, 1, 5, or 10 mg once daily found a median percentage reduction of plasma LDL cholesterol levels of 0%, 12.7%, 14.7%, 15.8%, and 19.4%, respectively [22]. Ten milligrams of ezetimibe daily reduces intestinal cholesterol absorption by 54% compared with placebo. This effect is accompanied by a decrease in plasma LDL cholesterol levels of 20%, a compensatory increase of 89% in hepatic cholesterol biosynthesis (versus placebo) and is also associated with a decrease in the absorption of plant sterols that are highly structurally related to cholesterol [22]. During ezetimibe treatment, there is a marked compensatory increase in cholesterol biosynthesis in the liver, but not in the extrahepatic organs, and an accelerated loss of cholesterol in the feces with little or no change in the rate of conversion of cholesterol to bile acids. Therefore, the combined administration of the cholesterol absorption inhibitor (ezetimibe) and the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (e.g., atorvastatin or simvastatin) produces an enhanced reduction in plasma total and LDL cholesterol levels, as well as provides a complementary treatment strategy for high-risk patients, including patients with homozygous familial hypercholesterolemia [23]. These results showed that ezetimibe in combination with HMG-CoA reductase inhibitors would be particularly effective at reducing plasma cholesterol levels in humans.

In the early studies, it was reported that ezetimibe may not affect intestinal absorption of triglycerides, fatty acids, bile acids, or fat-soluble vitamins, including vitamins A, D, E, and α- and β-carotenes [24]. More recently, intestinal fatty acid absorption was carefully reexamined by a sensitive and physiologically accurate method, the sucrose polybehenate technique in mice [25, 26]. Instead of monitoring the appearance in plasma of digestion products from an acutely delivered bolus of oil, fecal excretion of dietary fat is measured by this technique and normalized to the excretion of a nonabsorbable fat, sucrose polybehenate, incorporated into the diet [26]. It is observed that on the chow diet, dietary fatty acid absorption is significantly reduced from approximately 95% in control mice to about 87% in ezetimibe-treated mice. Moreover, ezetimibe treatment can significantly reduce intestinal absorption of saturated fatty acids in a graded manner that correlates with chain length. Thus, intestinal absorption of palmitate (16 : 0) and stearate (18 : 0) is reduced from approximately 90% and 70% in control mice to 80% and 50% in ezetimibe-treated mice, respectively.

Intestinal absorption efficiency of medium-chain saturated fatty acids is more moderately affected, possibly because medium-chain fatty acid absorption is less dependent on the chylomicron pathway. Myristate (14 : 0) absorption is reduced by 7–10% and laurate (12 : 0) absorption by 4% in ezetimibe-treated mice as compared with control mice [26]. These experiments strongly indicate that ezetimibe can reduce intestinal absorption efficiency of not only cholesterol but also long-chain fatty acids in mice. Additionally, it has been found that besides plasma total and LDL cholesterol concentrations, ezetimibe reduces liver cholesteryl ester levels in a dose-dependent fashion in cholesterol-fed hamsters, rats, and monkeys by inhibiting intestinal cholesterol absorption. However, ezetimibe does not significantly affect plasma HDL cholesterol or triglyceride levels. It was found from an indirect measurement of chylomicrons from plasma, but not lymph, that cynomolgus monkeys fed a single high-cholesterol meal and treated with an ezetimibe analogue displayed a significant reduction in the chylomicron cholesterol content, but not in the triglyceride content [27]. These results suggest that it may be important to carefully investigate the absorption and lymphatic transfer of cholesterol and fatty acids in lymph-fistula animal models. Because chylomicrons and chylomicron remnants may be atherogenic [28], further investigation of this phenomenon might shed more light on the mechanism of the antiatherogenic effect of ezetimibe. It will also be important to investigate whether ezetimibe could influence the lipid and lipoprotein composition of chylomicrons and their physical structure, as well as their assembly and secretion by the enterocytes into the lymph in animals and humans. Of note is that while statins may increase the clearance of chylomicron remnants, they do not reduce the cholesterol content of chylomicrons. Therefore, the combination of a statin and ezetimibe could be highly effective in reducing the atherogenic potential of chylomicrons [29].

3. Mechanisms of Ezetimibe Action on Intestinal Absorption of Cholesterol and Fatty Acids

Although ezetimibe reduces intestinal cholesterol absorption, it does not influence intestinal gene expression levels of *Abcg5*, *Abcg8*, *Sr-b1,* and *Abca1* in mice [2, 30]. Employing a genomic-bioinformatics approach, Altmann et al. [31] identified transcripts containing expression patterns and structural characteristics anticipated in cholesterol transporters (e.g., sterol-sensing and transmembrane domains, extracellular signal peptides) and established a strong candidate for the ezetimibe-sensitive cholesterol transporter, the awkwardly named Niemann-Pick C1-like protein 1 (NPC1L1). NPC1L1 has 50% amino acid homology to NPC1 [31], which is defective in the cholesterol storage disease Niemann-Pick type C and functions in intracellular cholesterol trafficking [32]. However, in contrast to *NPC1* that is expressed in many tissues [33, 34], *NPC1L1* is expressed predominantly in the gastrointestinal tract with peak expression in the proximal jejunum. Subfractionation of brush border membranes suggests that NPC1L1 is associated with the apical membrane fraction of enterocytes. Moreover, NPC1L1 deficient mice show a ~65% reduction in intestinal cholesterol absorption (16%) compared with wild-type mice (45%). The cholesterol absorption efficiency in NPC1L1 deficient mice is unaffected by ezetimibe or cholic acid, supporting the presence of redundant alternative pathways [31]. These studies strongly suggest that NPC1L1 could be an ezetimibe-sensitive target protein and is responsible for cholesterol uptake by the enterocyte for intestinal absorption (Figure 1) [31].

Many of the details of how ezetimibe prevents cholesterol absorption have been elucidated, and recently a molecular mechanism for cholesterol uptake mediated by the NPC1L1 has been proposed. The NPC1L1 protein recycles between the plasma membrane facing the extracellular space and the endocytic recycling compartment [35]. If the cholesterol concentration in the intestinal lumen is high, it is incorporated into the plasma membrane and is sensed by NPC1L1 that is localized on the surface of apical membrane of the enterocytes [34]. Both NPC1L1 and cholesterol are then internalized together through clathrin/AP2-mediated endocytosis [36]. The clathrin-coated globular vesicles are transported along microfilaments to the endocytic recycling compartment where large quantities of cholesterol and NPC1L1 are subsequently stored [36, 37]. If the intracellular cholesterol level is low, endocytic recycling compartment-localized NPC1L1 free of cholesterol moves back to the plasma membrane along microfilaments to transfer new cholesterol as it is absorbed by the enterocytes. The key role of the NPC1L1 inhibitor ezetimibe is to prevent NPC1L1 from entering the AP2-mediated clathrin-coated vesicles. At this stage, the endocytosis of NPC1L1 is inhibited and cholesterol absorption is decreased [36].

Although it has been observed that ezetimibe can reduce intestinal fatty acid absorption in mice, the molecular mechanism of this action is still unclear. As reviewed above, the deletion of the *Npc1l1* gene also reduces intestinal absorption of fatty acids, especially long-chain fatty acids. A potential mechanism may be that inhibition of intestinal cholesterol absorption by ezetimibe could somehow influence intestinal expression of genes involved in fatty acid uptake and transport. It is well known that hydrolysis and absorption of dietary fat (mainly triglycerides) are extremely efficient processes (>90%). However, it remains a matter of debate whether intestinal fatty acid absorption occurs solely by passive diffusion or also by protein-facilitated transport. Some studies have suggested that fatty acid transporter/cluster determinant 36 (FAT/CD36) may play a role in intestinal fatty acid absorption [38, 39]. Thus, it has been hypothesized that ezetimibe may have potential inhibitory effects on "protein-facilitated" absorption of fatty acids by enterocytes [26]. As found by Western blot analysis, protein concentrations of fatty acid transport protein 4 (FATP4) in the small intestine are significantly reduced by approximately 50% in ezetimibe-treated mice compared with control mice (Figure 1), which is associated with reduced intestinal absorption of long-chain saturated fatty

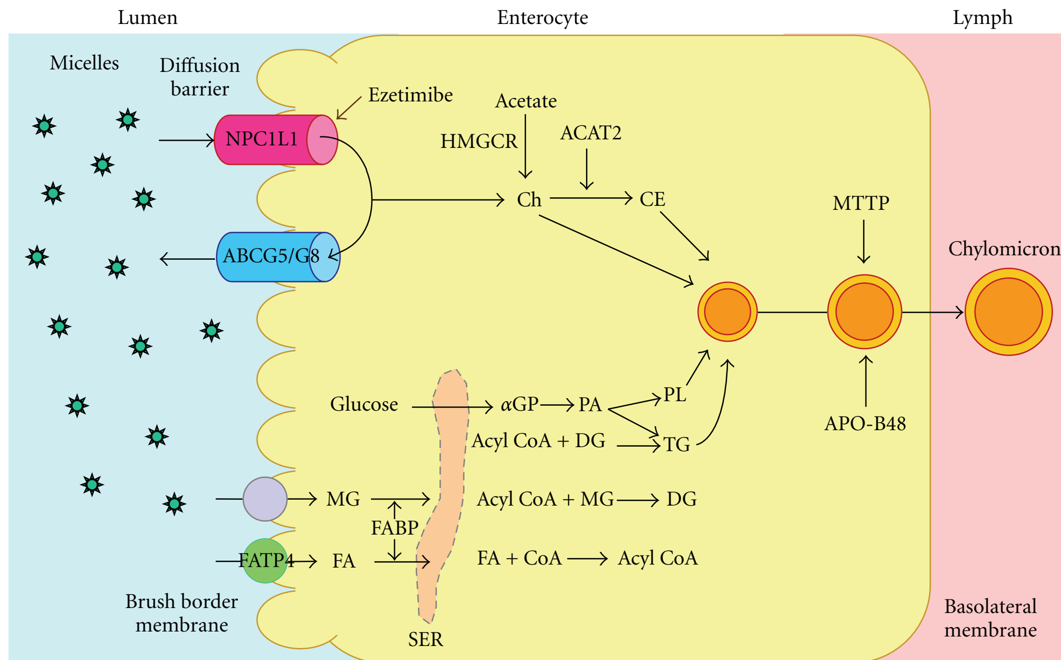


Figure 1: Within the intestinal lumen, the micellar solubilization of sterols facilitates movement through the diffusion barrier overlying the surface of the absorptive cells. In the presence of bile acids, large amounts of the sterol molecules are delivered to the aqueous-membrane interface so that their uptake rate is greatly increased. The Niemann-Pick C1-like 1 protein (NPC1L1), a newly identified sterol influx transporter, is located at the apical membrane of the enterocyte and may actively facilitate the uptake of cholesterol and plant sterols by promoting the passage of these molecules across the brush border membrane of the enterocyte. In contrast, ABCG5/G8 promote active efflux of cholesterol and plant sterols from the enterocyte into the intestinal lumen for excretion. The combined regulatory effects of NPC1L1 and ABCG5/G8 play a critical role in modulating the amount of cholesterol that reaches the lymph from the intestinal lumen. Ezetimibe may reduce cholesterol uptake by the enterocytes through the NPC1L1 pathway, possibly a transporter-facilitated mechanism. Absorbed cholesterol as well as some that is newly synthesized from acetate by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) within the enterocyte is esterified by acyl-CoA:cholesterol acyltransferase isoform 2 (ACAT2) to form cholesteryl esters. It is likely that fatty acids (FA) and monoacylglycerol (MG) could be taken up into enterocytes by facilitated transport. With the assistance of fatty acid binding protein 4 (FABP4), fatty acids and monoacylglycerol are transported into the smooth endoplasmic reticulum (SER) where they are used for the synthesis of diacylglycerol (DG) and then triacylglycerol (TG). Glucose is transported into the SER for the synthesis of phospholipids (PL) through the phosphatidic acid (PA) pathway (abbreviation: α-GP, α-glycerophosphate). All of these lipids participate in the formation of chylomicrons, a process which also requires the synthesis of apolipoprotein (APO)-B48 and the activity of microsomal triglyceride transfer protein (MTTP). As observed in lymph, the core of the secreted chylomicrons contains triglycerides and cholesteryl esters and the surface of the particles is a monolayer containing phospholipids, mainly phosphatidylcholine, unesterified cholesterol and apolipoproteins including APO-B48, APO-AI, and APO-AIV. Therefore, intestinal cholesterol absorption is a multistep process that is regulated by multiple genes. Reproduced with modifications and with permission from [50].

acids [26, 40]. It is unclear whether this inhibitory effect on intestinal FATP4 is induced by ezetimibe through a direct or indirect action pathway. Another explanation is that ezetimibe treatment significantly reduces cholesterol absorption so that the physical structure of chylomicrons may be modified and their assembly, and/or secretion into the lymphatics may be impaired. Because chylomicrons are a crucial vehicle for the transfer of cholesterol and fatty acids as triglyceride from the intestinal lumen to the lymph, impairing their formation by reducing cholesterol availability may induce a secondary action on fatty acid absorption. Because of this possible mechanism of action, it will be important to examine the physical structure of chylomicrons and their assembly and secretion into lymph to prove this hypothesis.

4. Physical-Chemistry of Bile, Physical Forms of Cholesterol Carrier, and Pathophysiology of Cholesterol Gallstones

Cholesterol, phospholipids, and bile salts are three major lipid components of bile in animals and humans [41]. Because cholesterol is virtually insoluble in an aqueous medium such as bile, specialized transport mechanisms are required to maintain it in solution and the mechanism for its solubilization in bile is complex. Similarly, phospholipids are insoluble in water and require carrier vehicles in bile. Bile salts have the property of amphiphilicity with both hydrophilic and hydrophobic areas of the molecules and are soluble in aqueous solutions to varying degrees, depending on the number and characteristics of hydroxyl

groups and side chains, as well as the composition of the particular aqueous solution. Bile salt monomers can aggregate spontaneously to form simple micelles when their concentration exceeds the critical micellar concentration [42]. As defined, a micelle is a colloidal aggregation of molecules of an amphipathic compound (e.g., bile salt) in which the hydrophobic portion of each molecule faces inward and the hydrophilic groups point outward [41]. The formation of simple micelles of bile salts alone depends primarily on the concentration of bile salts. Thus, micelles are formed at, but not below, a critical micellar concentration of bile salts in bile, which is approximately 2 mmol/L [41]. The formation of micelles is also influenced by the concentrations of biliary solids and counterions, by the type of bile salt (i.e., by its degree of hydroxylation and whether it is conjugated with taurine or glycine or not), and by the temperature and pH of the bile. These simple micelles (~3 nm in diameter) are small, thermodynamically stable aggregates that are principally composed of bile salts [43]. The cholesterol can be solubilized within the hydrophobic center of the micelle. Also, simple micelles of bile salts are capable of solubilizing and incorporating phospholipids. This enables the micelles—then referred to as mixed micelles—to solubilize at least three times the amount of cholesterol solubilized by simple micelles. The solubility of cholesterol in mixed micelles is enhanced when the concentration of total lipids (bile salts, phospholipids, and cholesterol) in bile is high. Moreover, maximal solubility occurs when the molar ratio of phospholipids to bile salts is between 0.2 and 0.3 [41]. Mixed micelles (~4–8 nm in diameter) are large, thermodynamically stable aggregates that are composed of bile salts, cholesterol, and phospholipids. Their size varies depending on the relative proportion of bile salts and phospholipids. The shape of a mixed micelle is that of a lipid bilayer with the hydrophilic groups of the bile salts and phospholipids aligned on the "outside" of the bilayer, interfacing with the aqueous bile, and the hydrophobic groups on the "inside." Cholesterol molecules can, therefore, be solubilized on the inside of the bilayer away from the aqueous areas on the outside. The amount of cholesterol that can be solubilized in micelles depends on the relative proportions of bile salts and phospholipids, with additional phospholipids aiding in cholesterol solubilization [41].

Studies using techniques such as quasielastic light-scattering spectroscopy (QLS) and electron microscopy to investigate the physical-chemistry of model and native bile samples have defined more complex mechanisms of cholesterol solubilization in bile [41, 44, 45]. Beside simple and mixed micelles, biliary vesicles, nonmicellar carriers of cholesterol, do exist in bile for the solubilization of cholesterol. Vesicles are unilamellar spherical structures and contain phospholipids, cholesterol, and little, if any, bile salts. Thus, vesicles (~40 to 100 nm in diameter) are substantially larger than either simple or mixed micelles, but much smaller than liquid crystals (~500 nm in diameter) that are composed of multilamellar spherical structures. Vesicles are present in large quantities in hepatic bile and are presumably secreted by the hepatocyte [41].

Vesicles in bile have one of two distinct origins. Those formed at the canalicular membrane of hepatocytes are unilamellar and rich in phosphatidylcholines compared with cholesterol (i.e., contain one cholesterol molecule per three phosphatidylcholine molecules). Because of increasing bile salt concentrations in the biliary tree, these vesicles rapidly undergo structural rearrangements and are therefore detectable only in bile specimens analyzed immediately after collection. A second type of vesicle forms spontaneously in bile when the capacity of mixed and simple micelles to solubilize cholesterol is exceeded. These unilamellar or multilamellar vesicles are cholesterol-rich, with cholesterol content reaching as high as two cholesterol molecules per phosphatidylcholine molecule.

When concentrations of bile salts are relatively low, vesicles are relatively stable, especially in dilute hepatic bile. Moreover, vesicles may transform or convert completely to mixed micelles when bile salt concentrations in concentrated gallbladder bile are increased. When the bile salt concentration is not high enough, only some vesicles convert to micelles. Because relatively more phospholipids than cholesterol can be transferred from vesicles to mixed micelles, the residual vesicles, now remodeled, may be rich in cholesterol relative to the phospholipids. If the remaining vesicles have a relatively low cholesterol/phospholipid ratio (<1), they are relatively stable. However, if the cholesterol/phospholipid ratio in vesicles is >1, vesicles become increasingly unstable [46]. These cholesterol-rich vesicles may transfer some cholesterol to less cholesterol-rich vesicles or to micelles, or may fuse or aggregate to form larger (~500 nm in diameter) multilamellar vesicles that may now be termed liposomes or liquid crystals [41]. Liquid crystals are visible by polarizing light microscopy as lipid droplets with birefringence in the shape of a Maltese cross. Liquid crystals are inherently unstable and may form solid cholesterol monohydrate crystals, which is termed cholesterol nucleation. As a result, the nucleation of cholesterol monohydrate crystal induces a decrease in the amount of cholesterol contained in vesicles but not of cholesterol in micelles, supporting the concept that vesicles could serve as the primary source of cholesterol during cholesterol nucleation and crystallization [41].

It is well known that cholesterol cholelithiasis is a multifactorial disease influenced by a complex interaction of genetic and environmental factors [15, 42, 47]. Based on recent studies on humans and mouse models, a novel concept has been proposed that interactions of five defects could play an important role in determining the formation of cholesterol gallstones (Figure 2), which are considered in terms of *LITH* genes (genetic defect), thermodynamics (solubility defect), kinetics (nucleation defect), stasis (residence time defect), and lipid sources (metabolic defect) [48]. Furthermore, cholesterol gallstone formation represents a failure of biliary cholesterol homeostasis in which the physical-chemical balance of cholesterol solubility in bile is disturbed [41, 42, 47]. The liver is the source of cholesterol-supersaturated bile in the gallbladder with cholesterol gallstones. Thus, gallstones can be viewed in one sense as a liver disease because some metabolic defects or a combination of defects within the liver result in hypersecretion of biliary

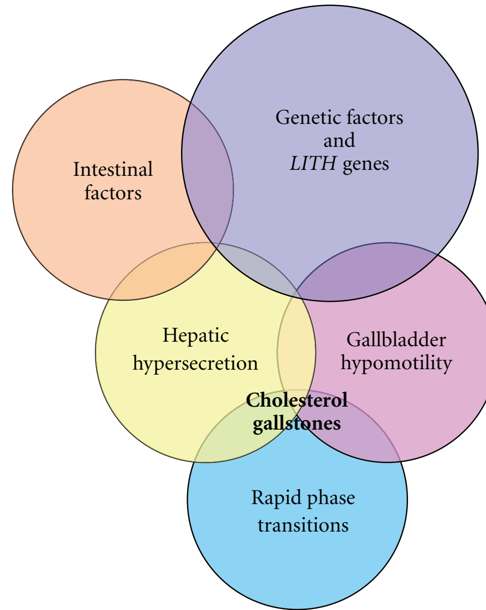


Figure 2: Venn diagram of five primary defects: genetic factors and *LITH* genes, hepatic hypersecretion, gallbladder hypomotility, rapid phase transitions, and intestinal factors. The hypothesis proposed is that hepatic cholesterol hypersecretion into bile is the primary defect and is the outcome in part of a complex genetic predisposition. The downstream effects include gallbladder hypomotility and rapid phase transitions. A major result of gallbladder hypomotility is alteration in the kinetics of the enterohepatic circulation of bile salts, resulting in increased cholesterol absorption and reduced bile salt absorption that lead to abnormal enterohepatic circulation of bile salts and diminished biliary bile salt pool size. Not only does gallbladder hypomotility facilitate cholesterol nucleation/crystallization, but also it allows the gallbladder to retain cholesterol monohydrate crystals. Although a large number of candidate *Lith* genes have been identified in mouse models, the identification of human *LITH* genes and their contributions to gallstones require further investigation. Reproduced with modifications and with permission from [48].

cholesterol. As noted, supersaturated bile is a prerequisite for cholesterol gallstone formation, and hypersecretion of biliary cholesterol is the primary metabolic abnormality responsible for initiating cholelithiasis. However, the gallbladder and intestine also conspire as part of a "vicious cycle" that creates physical-chemical instabilities in bile and culminates in the formation of cholesterol gallstones. Therefore, the formation of cholesterol gallstones is the final consequence of excess secretion of cholesterol from the liver into bile [42, 49]. It has been hypothesized that reducing cholesterol bioavailability in the liver for biliary secretion can prevent the formation of cholesterol gallstones and promote the dissolution of cholesterol crystals and gallstones. This information on the physical-chemistry of bile and the physical forms of cholesterol carriers can help us in understanding why ezetimibe could have a potential therapeutic effect on cholesterol gallstones.

5. Effects of Ezetimibe on the Prevention and the Treatment of Cholesterol Gallstones

Although some, but not all, studies found that high dietary cholesterol is associated with increased hepatic secretion of biliary cholesterol, epidemiological investigations have clearly demonstrated that cholesterol cholelithiasis is prevalent in cultures consuming a "Western" diet consisting of high total calories, cholesterol, saturated fatty acids, refined carbohydrates, proteins, and salt, as well as low fiber content. Many studies have found that the gallstone incidence in North and South American as well as European populations is significantly higher than that in Asian and African populations [15, 53, 54]. Furthermore, several clinical studies have found an association between the increased incidence of cholesterol gallstones in China and a "westernization" of the traditional Chinese diet. In Japan, cholesterol cholelithiasis once was rare, but over the past 40 years with the adoption of Western-type dietary habits, the incidence has increased markedly [15, 55, 56]. Moreover, it has been observed that there is a significant and positive correlation between the efficiency of intestinal cholesterol absorption and the prevalence of cholesterol gallstone formation in mice, suggesting that high efficiency of intestinal cholesterol absorption and high dietary cholesterol are two independent risk factors in the formation of cholesterol gallstones [57]. In addition, in mouse studies, targeted deletion of the acyl-CoA:cholesterol acyltransferase gene 2 (*Acat2*) resulted in the lack of cholesterol ester synthesis in the small intestine. This causes a marked reduction in intestinal cholesterol absorption and a complete resistance to diet-induced cholesterol gallstones [13, 15]. Furthermore, the absence of expression of intestinal APO-B48, but not APO-B100, reduces biliary cholesterol secretion and cholelithogenesis, possibly by decreasing intestinal absorption and hepatic bioavailability [15, 58]. Reduced gallstone prevalence in

lithogenic diet-fed apolipoprotein E knockout mice may be explained by decreased availability of chylomicron-derived cholesterol in the liver for biliary secretion [15, 59]. These studies support the notion that high dietary cholesterol through the chylomicron pathway could provide an important source of excess cholesterol molecules for secretion into bile, thereby inducing cholesterol-supersaturated bile and enhancing cholelithogenesis [15].

Indeed, because biliary cholesterol hypersecretion is an important prerequisite for cholesterol gallstone formation [15, 42, 47], inhibition of cholesterol absorption in the intestine, or hepatic uptake of chylomicron remnants has become an attractive alternative to decrease biliary cholesterol secretion and saturation [15]. Since ezetimibe significantly suppresses intestinal cholesterol absorption via the NPC1L1 pathway [15, 60], possibly a transporter-facilitated mechanism [15, 34], this should reduce the cholesterol content of the liver, which in turn decreases bioavailability of cholesterol for biliary secretion [15].

It has been found that ezetimibe induces a significant dose-dependent reduction in intestinal cholesterol absorption efficiency, coupled with a significant dose-dependent decrease in biliary cholesterol outputs and gallstone prevalence rates (Figure 3). In particular, even under high dietary cholesterol loads, cholesterol gallstones can be prevented by ezetimibe in C57L mice carrying the *Lith1* and *Lith2* genes that predispose to cholesterol stone formation [15]. Although ezetimibe substantially reduces cholesterol concentrations and to some extent phospholipid concentrations, but not bile acid concentrations in gallbladder bile, all crystallization pathways and phase boundaries on the bile phase diagram are not influenced by ezetimibe [15]. Furthermore, in company with increased doses of ezetimibe, the relative lipid compositions of pooled gallbladder bile samples are progressively shifted down and to the left of the phase diagram, entering the one-phase micellar zone where there is an abundance of unsaturated micelles, but never solid cholesterol crystals or liquid crystals. Because the micellar cholesterol solubility is dramatically increased in gallbladder bile, the cholesterol molecules can be transferred from the cholesterol monohydrate surface into unsaturated micelles. As a result, gallstones become smaller and eventually dissolved (Figure 4) [15]. This excellent physical-chemical mechanism could explain, in part, how ezetimibe treatment prevents cholesterol gallstone formation in mice.

Enlarged fasting gallbladder volume, together with impaired postprandial and interdigestive gallbladder emptying, is a frequent and distinctive feature in gallstone patients [15, 61, 62], indicating that the gallbladder is another key player in cholelithogenesis. This type of “gallbladder stasis” provides time for nucleation of cholesterol crystals and their aggregation into macroscopic stones [15, 42, 47, 62]. Under conditions of cholesterol-supersaturated bile, the gallbladder absorbs a large amount of cholesterol, thereby resulting in the accumulation of excess cholesterol in the gallbladder wall. Because gallbladder absorptive cells apparently cannot assemble lipoproteins for lipid transport into plasma, the absorbed cholesterol is converted to cholesteryl ester and stored in the mucosa and lamina propria. As a result, excess cholesterol in smooth muscle cells could stiffen sarcolemmal membranes and decouple the G-protein-mediated signal transduction that usually occurs when CCK binds to its receptor, thereby further paralyzing gallbladder contractile function and consequently impairing gallbladder emptying function. These lithogenic effects on gallbladder motility function can be completely inhibited by ezetimibe [15, 63]. This effect of ezetimibe on protecting gallbladder motility can mostly be attributed to the desaturation of bile.

Ursodeoxycholic acid (UDCA) is currently used as a first-line pharmacological therapy to treat only a subgroup of symptomatic patients with small, radiolucent cholesterol gallstones [15, 47, 64]. Extensive clinical studies have shown that long-term administration of UDCA promotes the dissolution of cholesterol gallstones and prevents the recurrence of gallstones after extracorporeal shock wave lithotripsy [15, 65]. However, because of a failure to titrate the dose adequately, optimal use of UDCA is not always achieved in clinical practice [15]. It should be pointed out that the hydrophilic bile acid UDCA can greatly favor the formation of vesicles in bile, which can enhance the growth of liquid crystals on the cholesterol monohydrate surface and their subsequent dispersion might occur during gallstone dissolution. Consequently, liquid crystalline dissolution allows the transport of a great amount of cholesterol from stones [15]. Because the cholelitholytic mechanism of ezetimibe is totally different from that of hydrophilic bile acids such as UDCA, it has been proposed that a combined therapy of ezetimibe and UDCA could be a faster means to promote the dissolution of cholesterol gallstones, because of the two distinct mechanisms via the formation of unsaturated micelles by ezetimibe and a liquid crystalline mesophase by UDCA [15], respectively.

A clinical study has been performed to examine whether ezetimibe would reduce biliary cholesterol concentrations in gallstone patients compared to overweight subjects without gallstones [15]. It was observed that 30 days after starting the medication, ezetimibe at 20 mg/day significantly reduced cholesterol concentrations and cholesterol saturation indexes (CSIs) of gallbladder biles in gallstone patients (Table 1), similar to the results as observed in mouse studies [15]. Consequently, cholesterol crystallization was retarded and detection time of cholesterol monohydrate crystals was significantly delayed as analyzed by polarized light microscopy. Although similar results between mice and gallstone patients have been observed regarding the effect of ezetimibe on the reduction in bile cholesterol concentrations and cholesterol crystallization, a long-term human study is needed to observe whether ezetimibe can reduce gallstone prevalence and completely dissolve gallstones [15].

It should be emphasized that there is a difference in tissue distribution of NPC1L1 between mice and humans. In mice, NPC1L1 is expressed only in the intestine, while in humans, it can be detected in both the intestine and liver [34]. Because of this, it has been hypothesized that ezetimibe may have different effects on biliary cholesterol output in humans than in mice. It has been found that the secretion efficiency of biliary cholesterol is most likely determined by the net effect between efflux and influx of

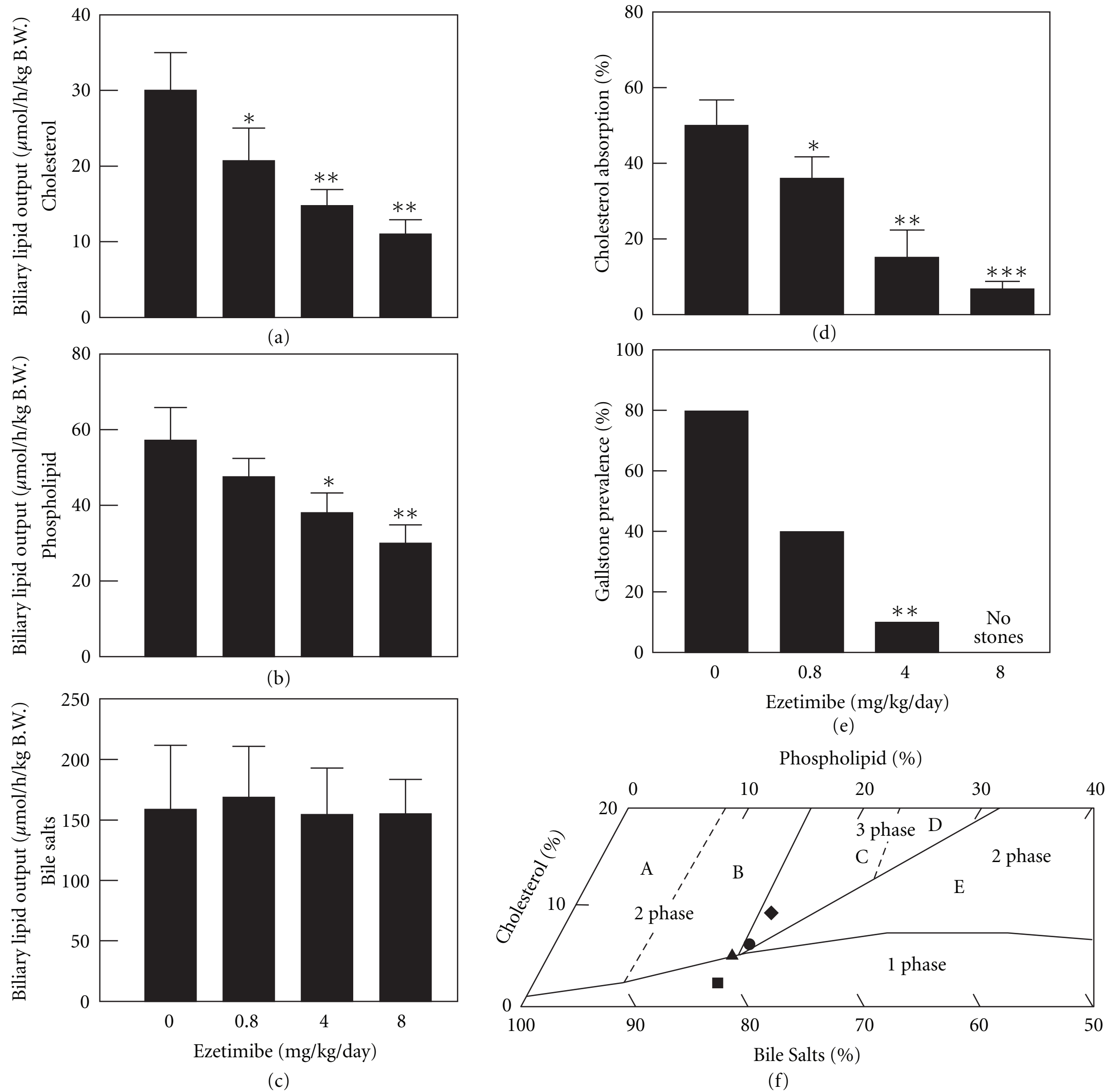
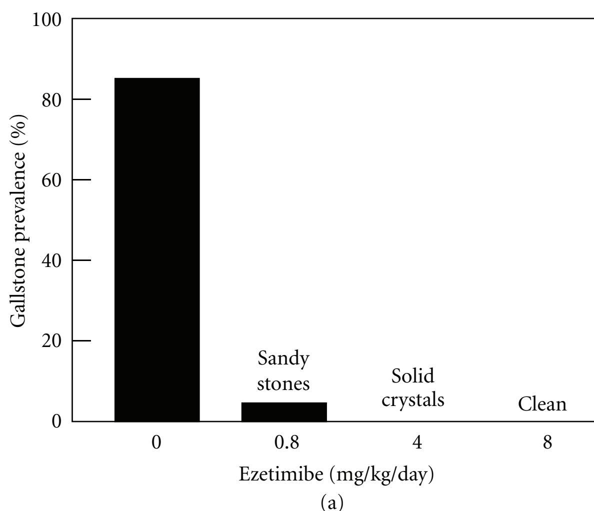
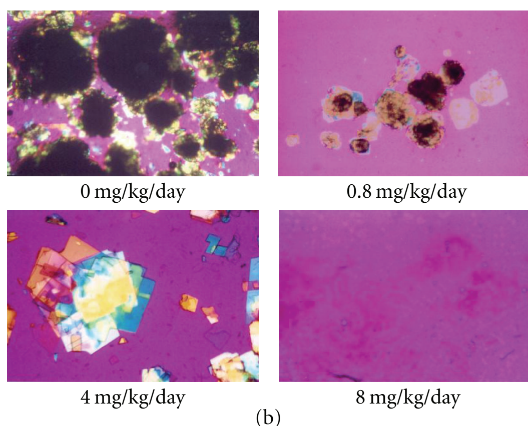


Figure 3: Effect of ezetimibe on the prevention of cholesterol gallstones. Ezetimibe significantly reduced, in a dose-dependent fashion, hepatic output of (a) biliary cholesterol and (b) phospholipid, but not (c) bile salts. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with mice fed the lithogenic diet and receiving no ezetimibe. (d) There is a clear dose-dependent reduction in intestinal cholesterol absorption efficiency from 50 ± 6% to 4 ± 2% in chow-fed with mice, as measured by the fecal dual-isotope ratio method. (e) When doses of ezetimibe are increased from 0 to 4 mg/kg/day, gallstone prevalence rates are reduced from 80% to 10% in mice fed with the lithogenic diet for 8 weeks. No gallstones are found in mice treated with ezetimibe at 8 mg/kg/day. (f) The relative lipid composition of pooled gallbladder bile from mice fed with the lithogenic diet and receiving no ezetimibe are located in the central three-phase zone, where bile is composed of solid cholesterol monohydrate crystals, liquid crystals, and saturated micelles at equilibrium. In contrast, administration of the highest dose (8 mg/kg/day) of ezetimibe resulted in the relative biliary lipid composition of pooled gallbladder bile plotted in the one-phase micellar zone, even upon the lithogenic diet feeding for 8 weeks. By phase analysis, these bile samples are composed of unsaturated micelles at equilibrium. A symbol ♦ represents relative lipid composition of pooled gallbladder bile at 8 weeks on the lithogenic diet supplemented with ezetimibe at 0; • 0.8; ▲ 4; ■ 8 mg/kg/day. Reproduced with modifications and with permission from [15].

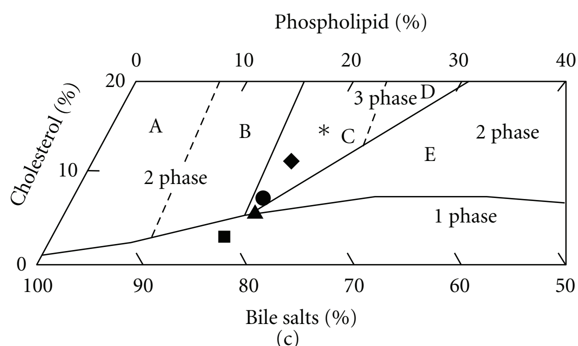
cholesterol molecules across the canalicular membrane of the hepatocyte, which could be regulated by the ABCG5/G8-dependent and independent pathways as well as the NPC1L1 pathway [15, 58]. Indeed, ezetimibe treatment can reduce bile cholesterol content and CSIs and prolong detection times of cholesterol monohydrate crystals in humans. One possible reason for these results in humans is that because biliary cholesterol secretion is a unique path for excretion of cholesterol from the body in humans and animals, hepatic ABCG5/G8 may play a stronger role in the regulation of biliary cholesterol secretion than NPC1L1. Another possible explanation is that in the gut-liver axis, the intestinal



(a)



(b)



(c)

Figure 4: Effect of ezetimibe on the dissolution of cholesterol gallstones. (a) For gallstone dissolution experiments, mice with preexisting gallstones were fed a chow diet alone for 8 weeks, which does not result in a spontaneous dissolution of gallstones. In contrast, treatment with ezetimibe at 0.8 to 8 mg/kg/day induces rapid dissolution of gallstones. Gallstones were completely dissolved by the highest (8 mg/kg/day) dose of ezetimibe. (b) Representative photomicrographs of mucin gel, liquid crystals, cholesterol monohydrate crystals, and gallstones as observed in gallbladder biles at week 8 after ezetimibe treatment. All magnifications are ×800, except for ezetimibe treatment at 0 and 0.8 mg/kg/day, which are ×400, by polarizing light microscopy. (c) The relative lipid composition of pooled gallbladder bile from mice fed 8 weeks with the chow diet supplemented with varying doses of ezetimibe is plotted on a condensed phase diagram. Because of a 12-week feeding period of the lithogenic diet, the relative lipid composition of pooled gallbladder bile from mice that have formed cholesterol gallstones is located in the central three-phase zone. Although the lithogenic diet is replaced with the chow diet for 8 weeks, the relative biliary lipid composition of bile is still in region C, where at equilibrium the bile is composed of solid cholesterol crystals, liquid crystals, and saturated micelles. By feeding varying doses of ezetimibe, the relative lipid composition of pooled gallbladder bile gradually shifts down and, finally, enters the one-phase micellar zone. These alterations explain that gallstones are dissolved through an unsaturated micelle mechanism. A symbol ∗ represents relative lipid composition of pooled gallbladder bile from mice that have preexisting gallstones and before ezetimibe treatment; ⧫ relative lipid composition of pooled gallbladder bile at the end of the gallstone dissolution study at week 8 of feeding the chow diet only (control); • 0.8; ▲ 4; ■ 8 mg/kg/day of ezetimibe. Reproduced with modifications and with permission from [15].

Table 1: Plasma and biliary lipids before (day 0) and at day 30 after ezetimibe treatment in humans (20 mg/day)[a].

Parameter	Overweight subjects without gallstones		Gallstone patients	
	Before	After	Before	After
BMI (kg/m^2)	31.5 ± 3.8	31.4 ± 3.4	27.0 ± 2.8	27.1 ± 2.3
Plasma lipid concentrations				
Total Ch (mg/dL)	220 ± 41	168 ± 29[b]	223 ± 32	193 ± 26
LDL Ch (mg/dL)	144 ± 53	99 ± 36	145 ± 26	115 ± 23[b]
HDL Ch (mg/dL)	44 ± 13	37 ± 13	45 ± 11	45 ± 11
TG (mg/dL)	164 ± 88	160 ± 104	166 ± 64	165 ± 76
Biliary lipid compositions of gallbladder biles				
Ch (mole%)	7.4 ± 0.7	6.8 ± 1.9	9.3 ± 1.9	7.2 ± 1.2[b]
PL (mole%)	20.2 ± 2.4	21.8 ± 2.5	19.3 ± 2.8	20.0 ± 3.5
BS (mole%)	72.4 ± 2.9	71.4 ± 3.9	71.4 ± 4.3	72.8 ± 4.2
Ch/PL ratio	0.37 ± 0.03	0.31 ± 0.08	0.48 ± 0.05	0.37 ± 0.06[c]
Ch/BS ratio	0.10 ± 0.01	0.10 ± 0.03	0.13 ± 0.03	0.10 ± 0.02
[TL] (g/dL)	5.3 ± 0.4	5.0 ± 0.9	5.5 ± 0.7	5.3 ± 0.8
CSI	1.2 ± 0.1	1.0 ± 0.2	1.6 ± 0.2	1.3 ± 0.2[b]
CDT (days)	6.4 ± 1.1	10.4 ± 1.1[c]	4.0 ± 1.2	7.0 ± 1.3[c]

[a]Values were determined from overweight subjects without gallstones ($n = 5$) and gallstone patients ($n = 7$).
[b]$P < 0.05$ and [c]$P < 0.01$, compared with before ezetimibe treatment (paired t test).
BMI: body mass index; TG: triglycerides; Ch: cholesterol; PL: phospholipids; BS: bile salts; [TL]: total lipid concentrations; CSI: cholesterol saturation index; CDT: crystal detection time.
Reproduced with slightly modifications and with permission from [15].

NPC1L1 may play a significant role in providing dietary and reabsorbed biliary cholesterol to the body, and the inhibition of its functions by ezetimibe significantly reduces cholesterol absorption. So, the bioavailability of cholesterol from intestinal sources for biliary secretion is decreased markedly. In contrast, inhibition of the hepatic NPC1L1 by ezetimibe has a weak effect on biliary cholesterol secretion and CSI values [15]. More interestingly, similar to humans, the Golden Syrian hamster displays the abundance of NPC1L1 in the small intestine that far exceeds that in other regions of the gastrointestinal tract such as liver and gallbladder [66]. The tissue distribution pattern of NPC1L1 is nearly similar between hamsters and humans. It was found that the ezetimibe-induced reduction in intestinal cholesterol absorption is coupled with a decrease in the absolute and relative cholesterol levels in bile in hamsters fed a high-cholesterol diet [66]. These results are consistent with a recent finding that ezetimibe treatment significantly reduces biliary cholesterol saturation in patients with gallstones.

Overall, ezetimibe treatment can prevent cholesterol gallstones mainly through inhibiting intestinal cholesterol absorption so that hepatic secretion of biliary cholesterol is reduced, and gallbladder motility function is preserved by desaturating bile (Figure 5). Also, ezetimibe promotes the dissolution of cholesterol gallstones through a greater capacity to form an abundance of unsaturated micelles. Therefore, ezetimibe is a novel and potential cholelitholytic agent for both preventing and treating cholesterol gallstones [15].

6. Pathophysiology of Nonalcoholic Fatty Liver Disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease, which includes a spectrum of hepatic pathology ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis in the absence of alcohol abuse and other causes [67–69]. NAFLD is characterized pathologically by macrovesicular steatosis, mild diffuse lobular mixed acute and chronic inflammation, perivenular and zone 3 perisinusoidal collagen deposition, hepatocyte ballooning, poorly formed Mallory-Denk bodies, glycogen nuclei in periportal hepatocytes, lobular lipogranulomas, and PAS-diastase-resistant Kupffer cells [70, 71].

NAFLD was once proposed to be the result of two distinct but related "hits" to the hepatocyte [72, 73]. The first "hit" is the development of lipid accumulation and hepatic steatosis because of an imbalance of hepatic lipid metabolism, which leads to either excessive lipid influx, decreased lipid clearance, or both [70]. At this point, steatosis is potentially reversible and does not necessarily induce permanent hepatic injury. Although it is less common and occurs in approximately 5% of individuals with steatosis, the second "hit" is more virulent, being an inflammatory process that is induced probably by oxidative stress, lipid peroxidation, and cytokine action [74]. The resulting lobular inflammation causes ballooning degeneration and perisinusoidal fibrosis, which promote apoptosis, and hepatocellular death. These alterations eventually induce scarring and

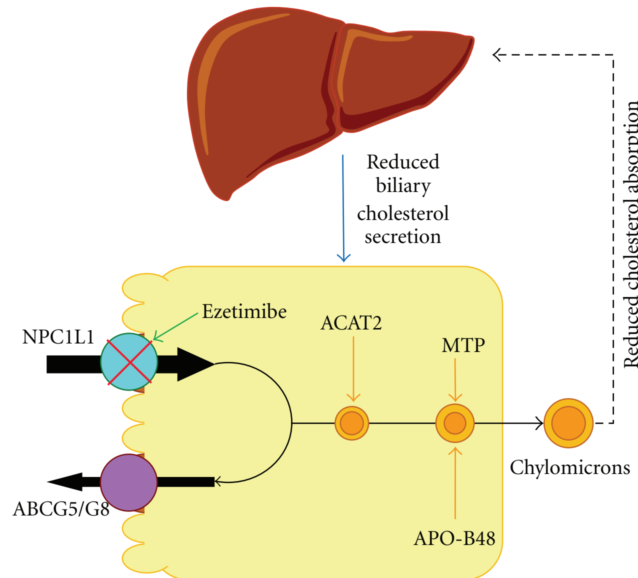


Figure 5: Pathways underlying the absorption of cholesterol from the intestinal lumen and its delivery to the liver. High dietary cholesterol delivery through the chylomicron pathway could provide an important source of excess cholesterol molecules for hepatic secretion into bile, thereby inducing cholesterol-supersaturated bile and enhancing cholesterol gallstone formation. Ezetimibe significantly suppresses cholesterol absorption from the small intestine via the Niemann-Pick C1-like 1 (NPC1L1) pathway, possibly by a transporter-facilitated mechanism. This effect of ezetimibe could significantly diminish the cholesterol content of the liver, which in turn remarkably decreases bioavailability of cholesterol for hepatic secretion into bile. ABCG5/G8: ATP-binding cassette (transporters) G5 and G8; ACAT2: acyl-CoA:cholesterol acyltransferase isoform 2; APO-B48: apolipoprotein B48; MTTP: microsomal triglyceride transfer protein. See text for details.

progression to nonalcoholic steatohepatitis (NASH) [75]. However, many studies have been unable to prove that either oxidant stress or lipid peroxidation is necessary for the development of steatohepatitis in humans.

Recently, the lipotoxicity model of NASH pathogenesis has emerged based on evidence showing that triglyceride often accumulates in the liver as a parallel rather than pathogenic process during lipotoxic hepatocellular injury (Figure 6) [51]. Thus, it has been hypothesized that metabolites of unesterified fatty acids play a critical role in inducing lipotoxic injury in the liver. The generation of lipotoxic metabolites of fatty acids typically occurs in parallel with the accumulation of triglyceride droplets (steatosis), resulting in a phenotype recognized as NASH, where steatosis and features of cellular injury are present together [51]. Metabolic abnormalities predisposing to lipotoxic injury include an increased supply or impaired disposal of unesterified fatty acids. More importantly, insulin resistance could play a central role in these processes by allowing unsuppressed lipolysis in adipocytes resulting in an excessive flow of fatty acids from adipose tissues and also impairing peripheral glucose disposal [51]. *De novo* lipogenesis in the liver using excessive dietary carbohydrate as a substrate for fatty acid synthesis is also a significant contributor to the burden of saturated fatty acids in the liver. Fatty acid disposal in the liver occurs through oxidative pathways and through the formation of triglyceride which is either stored temporarily as lipid droplets or secreted as VLDL [51]. Additional factors, including oxidative stress, mitochondrial dysfunction, gut-derived lipopolysaccharide and adipocytokines, may promote further hepatocellular damage [76, 77]. These processes can lead to inflammation, necrosis, apoptosis and fibrogenesis, which may ultimately lead to cirrhosis, liver failure, hepatocellular carcinoma and death [78].

7. Potential Therapeutic Effects of Ezetimibe on NAFLD

Although the role of dietary fat in the pathogenesis of NAFLD continues to be investigated, evidence from animal studies supports the concept that fat overconsumption plays an important role in the etiology of hepatic steatosis [79]. It has been found that feeding a high-fat diet can induce a significant accumulation of lipids in the liver of animals such as mice and rats [80]. In humans, a large amount of dietary fat could result in the accumulation of triglyceride in the liver, but stable isotope studies found that up to only 15% of lipids accumulated in the liver are derived directly from dietary fat [81, 82]. In contrast, a low-carbohydrate diet, which is otherwise rich in protein and fat, has been used as treatment for NAFLD [83]. Furthermore, long-term overconsumption of fat could increase risk for obesity and insulin resistance, which enhances susceptible to NAFLD [84].

Indeed, mice and rats develop hepatic steatosis in response to a high-fat diet and their livers are enlarged and appear grossly pale. Histopathological studies from these livers reveal that hepatocytes are filled with multilocular droplets of varying sizes (Figure 7) [52]. Strikingly,

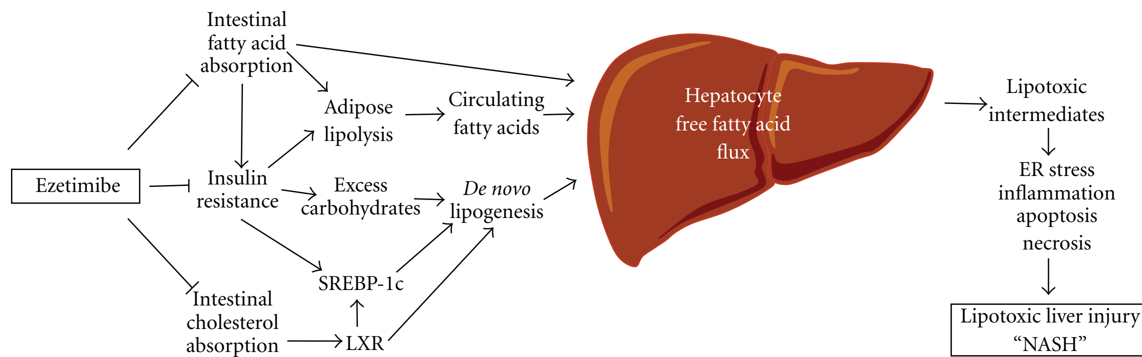


Figure 6: Potential therapeutic effects of ezetimibe on nonalcoholic fatty liver disease and steatohepatitis (NAFLD and NASH). On the basis of the lipotoxicity model of NAFLD and NASH [51], it has been proposed that metabolites of unesterified fatty acids may induce lipotoxic hepatocellular injury manifested as ER stress, inflammation, apoptosis, necrosis, and dysmorphic features such as ballooning and Mallory-Denk body formation. The generation of lipotoxic metabolites of fatty acids often takes place in parallel with the accumulation of triglyceride droplets (steatosis) in the liver. A high-fat diet often causes insulin resistance, a state that is associated with hyperinsulinemia and hyperglycemia. Because insulin resistance promotes an excessive flow of fatty acids from adipose tissue and also impairs peripheral glucose disposal, these alterations increase the need for fatty acid disposal in the liver through oxidative pathways and through the formation of triglyceride which is then either stored temporarily as lipid droplets or secreted as VLDL. Furthermore, elevated blood insulin and glucose activate transcription factors SREBP-1c to increase hepatic lipogenic gene expression. In addition, intestinal cholesterol absorption promotes hepatic lipogenesis via cholesterol-dependent activation of LXR. Ezetimibe treatment could block (i) intestinal fatty acid absorption, which could reduce a delivery of fatty acids from the gut to the adipose tissue through the chylomicron pathway; (ii) diet-induced insulin resistance in part by reducing intestinal fatty acid absorption; (iii) cholesterol-driven lipogenesis by inhibiting intestinal cholesterol absorption, which together may substantially reduce the burden of fatty acids on the liver. ER: endoplasmic reticulum; LXR: liver X receptor; SREBP-1c: sterol regulatory element-binding protein-1c.

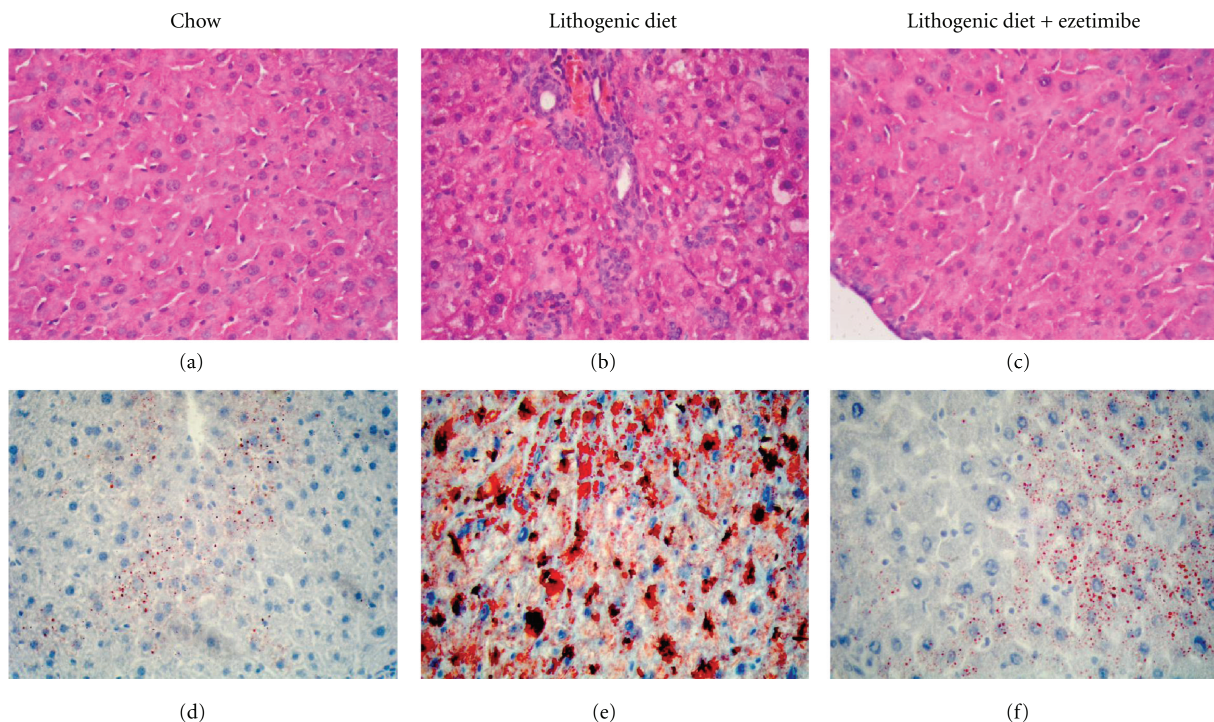


Figure 7: Histological characterization of the hepatic response to ezetimibe in mice fed a chow versus a lithogenic diet for 4 weeks. The liver samples from mice fed with different diets and treated with or without ezetimibe were isolated and subjected to histological analysis. Panels (a)–(c) show representative liver histology with hematoxylin-eosin staining and panels (d)–(f) show Oil Red O staining. (a) and (d) Mice fed with the chow diet. (b) and (e) Mice fed with the lithogenic diet without ezetimibe. (c) and (f) Mice fed eith the lithogenic diet with ezetimibe. The lithogenic diet induced a significant accumulation of triglyceride and cholesteryl ester in the liver as well as hepatocyte damage and inflammation. Interestingly, ezetimibe treatment markedly reduced the accumulation of lipids and prevented hepatic inflammation. Reproduced with modifications and with permission from [52].

these diet-induced pathological abnormalities are completely absent in livers not only from ezetimibe-treated mice, but also from NPC1L1 deficient mice [79]. In addition, no signs of inflammatory cell infiltration are found in these livers. Hepatic concentrations of both triglyceride and cholesteryl ester are significantly reduced in ezetimibe-treated mice compared with chow-fed control mice [79].

Although a high-fat diet may promote fat accumulation in the liver by simply providing more substrate for triglyceride synthesis, an important mechanism whereby a high-fat diet may drive hepatic steatosis is by causing selective insulin resistance [79, 85]. The increased circulating insulin fails to suppress hepatic gluconeogenesis but can promote hepatic lipogenesis. In contrast, ezetimibe treatment could prevent diet-induced hepatic steatosis, weight gain, and insulin resistance [79]. These alterations are associated with reduced circulating insulin levels, hepatic *de novo* fatty acid synthesis, and hepatic levels of mRNAs for lipogenic genes including glucokinase, an enzyme critical in conversion of glucose to fat. Because elevated blood insulin increases hepatic lipogenic gene expression via transcription factors such as SREBP-1c [16, 86, 87] and glucokinase is an important mediator in this lipogenic pathway [16, 88], ezetimibe treatment may protect against diet-induced hepatic steatosis by reducing hepatic lipogenesis, mostly through preventing diet-induced insulin resistance and the associated hyperinsulinemia.

Because excessive amounts of cholesterol are lipogenic through activation of LXR by its metabolites [16, 89, 90], reduced intestinal cholesterol absorption by ezetimibe could significantly decrease cholesterol content in the liver. This may prevent diet-induced hepatic steatosis in part by reducing cholesterol-dependent LXR activation in the liver [16].

Nevertheless, ezetimibe treatment indeed plays a significant role in preventing diet-induced fatty liver in animals such as mice and rats; however, its therapeutic effect on NAFLD needs to be further investigated and proven in humans.

8. Future Research Directions and Clinical Applications

Ezetimibe is a highly potential and selective cholesterol absorption inhibitor that prevents absorption of cholesterol from dietary and biliary sources by suppressing uptake and transport of cholesterol through the enterocytes. Although there is clear evidence showing that ezetimibe can inhibit cholesterol absorption through the NPC1L1 pathway, careful and systematic studies are needed to confirm whether ezetimibe could reduce intestinal absorption of fatty acids in animal models by direct measurement of their absorption and lymphatic transport and studies need to be undertaken in humans by a balance method of intestinal fatty acid absorption. Because of significantly reduced absorption of intestinal cholesterol and fatty acids, the physical structure of chylomicrons and their metabolism in adipose tissues and liver could be influenced by ezetimibe treatment. To evaluate treatment duration, clinical response rates and the overall cost-benefit analysis on cholesterol gallstones and NAFLD, long-term human studies are needed. Similar to atherosclerosis, the risk for cholesterol gallstone formation and NAFLD increases with dyslipidemia, hyperinsulinemia, obesity, diabetes, sedentary lifestyle, and aging. It is highly likely that the long-term administration of ezetimibe may benefit this group of subjects who could have a high predisposition to cholesterol gallstones and NAFLD.

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Review Article

Molecular Mechanisms Underlying the Link between Nuclear Receptor Function and Cholesterol Gallstone Formation

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Cholesterol gallstone disease is highly prevalent in western countries, particularly in women and some specific ethnic groups. The formation of water-insoluble cholesterol crystals is due to a misbalance between the three major lipids present in the bile: cholesterol, bile salts, and phospholipids. Many proteins implicated in biliary lipid secretion in the liver are regulated by several transcription factors, including nuclear receptors LXR and FXR. Human and murine genetic, physiological, pathophysiological, and pharmacological evidence is consistent with the relevance of these nuclear receptors in gallstone formation. In addition, there is emerging data that also suggests a role for estrogen receptor ESR1 in abnormal cholesterol metabolism leading to gallstone disease. A better comprehension of the role of nuclear receptor function in gallstone formation may help to design new and more effective therapeutic strategies for this highly prevalent disease condition.

1. Introduction

Cholesterol gallstone disease (CGD) is one of the most common digestive disease conditions in both industrialized and develop-ing western countries. Worldwide CGD prevalence ranges between 5% and 20% [1], being more common in women than men in every population that has been studied [2]. It is particularly prevalent in some specific ethnic groups including Mapuche and North American Indians as well as Chilean and Mexican Hispanics. Among these populations, CGD has an earlier onset and reaches prevalence rates over 50% and 70% in middle age male and women, respectively. CGD is also a key risk factor for gallbladder cancer. Therefore, CGD represents a serious burden for healthcare systems [3, 4].

Some of the pathogenic hallmarks of CGD are increased biliary cholesterol secretion, in-creased bile acid hydrophobicity, cholesterol microcrys-tal formation, growth, and aggregation with the formation of macroscopic stones in the gallbladder, and gall-bladder inflammation [5–7]. The primary pathogenic mechanism associated with CGD is a disrupted balance between the three major lipids present in bile: cholesterol, bile salts, and phospholipids [8]. Under physiological conditions, bile cholesterol is kept in solution by its incorporation into mixed micelles together with phospholipids and bile salts. When either too much cholesterol or not enough solubilizing bile salt and phospholipid molecules are secreted, cholesterol comes out of solution and then crystallizes [9]. In addition, several biliary proteins have been described as nucleating factors that may promote cholesterol crystallization. Among them, there are immunoglobulins M and G, haptoglobin, α1-acid glycoprotein, aminopeptidase-N, α1-antichymotrypsin, and mucin. Despite correlative evidence between biliary levels and/or activity of these proteins and cholesterol precipitation in *in vitro* and animal models, only mucin seems to have a potential pathogenic role in human CGD [9]. Finally, impaired gallbladder motility is another important factor that contributes to further growth and aggregation of cholesterol microcrystals into macroscopic gallstones [7, 10].

In the hepatocyte, several types of proteins mediate the trafficking of lipids towards the canalicular pole for biliary secretion. These include multiple lipid transport-related gene products, lipoprotein receptors, basolateral lipid transporters, and intracellular lipid binding proteins as well as canalicular lipid transporters. Especially relevant for biliary lipid secretion and composition is the activity of ATP-binding-cassette- (ABC-) transport proteins expressed at the canalicular membrane. Among them, we can highlight the following ones: ABCB4, the transporter for phosphatidylcholine [11]; ABCB11, the bile salt export pump [12]; ABCG5/ABCG8, the obligate heterodimer that induces biliary cholesterol secretion [13].

Thus, biliary lipid secretion is controlled by a variety of proteins that mediate lipid uptake, transport, and metabolism in the liver. Furthermore, the expression of the genes encoding these proteins is coordinated by a series of transcriptional factors, including members of the nuclear receptors family, such as liver X receptor (LXR) and farnesoid X receptor (FXR) as well as the sterol regulatory element binding proteins (SREBPs) [14].

2. The Nuclear Receptors

Nuclear receptors (NRs) are a major component of signal transduction in animals. They are metabolite- and hormone-sensing transcription factors that translate dietary or endocrine signals into changes in gene expression. They have been described as modulators of not only many hormone activities, but also important nutrients and metabolites involved in the homeostasis and physiology of cells and tissues [15].

The NR superfamily contains transcriptional regulators that are conserved throughout metazoans, including nematodes, insects, and vertebrates [16]. For example, there are 48 and 49 NR members encoded in the human and mouse genome, respectively. NRs can bind their DNA target sites as a monomer (e.g., steroidogenic factor (SF-1)), homodimer (e.g., estrogen receptor (ESR)), or heterodimer (e.g., FXR and LXR form heterodimers with the retinoid X receptor RXR). NRs can be ligand-dependent or ligand-independent transcription factors that activate or repress gene expression [17]. They play important roles in diverse functions such as homeostasis, reproduction, development, inflammation, toxicology, and metabolism [18]. NRs are thus key players in the regulation of complex gene networks.

The known endogenous ligands for NRs consist of a wide range of chemical structures, such as bile acids, phospholipids, steroid hormones, thyroid hormone, retinoids, and vitamin D [19]. It is interesting to note that many of these ligands are derived from cholesterol, suggesting that NRs have an important role in cholesterol-related metabolism and pathology. Additionally, it has been suggested that one and the same NR may have distinct endogenous ligands in different tissues or cell types [20]. This could be particularly relevant to design therapeutic interventions selectively targeting the availability of one ligand without interfering with the desired effects of another.

This paper summarizes some recent progress in understanding the role of some NRs, including heterodimeric LXR and FXR and homodimeric ESR, on biliary lipid secretion and their potential clinical implications for CGD. The principal features of mechanisms underlying the effect of NRs on liver and intestine lipid metabolism and transport and CGD are depicted in Figures 1(a) and 1(b).

3. The Liver X Receptor

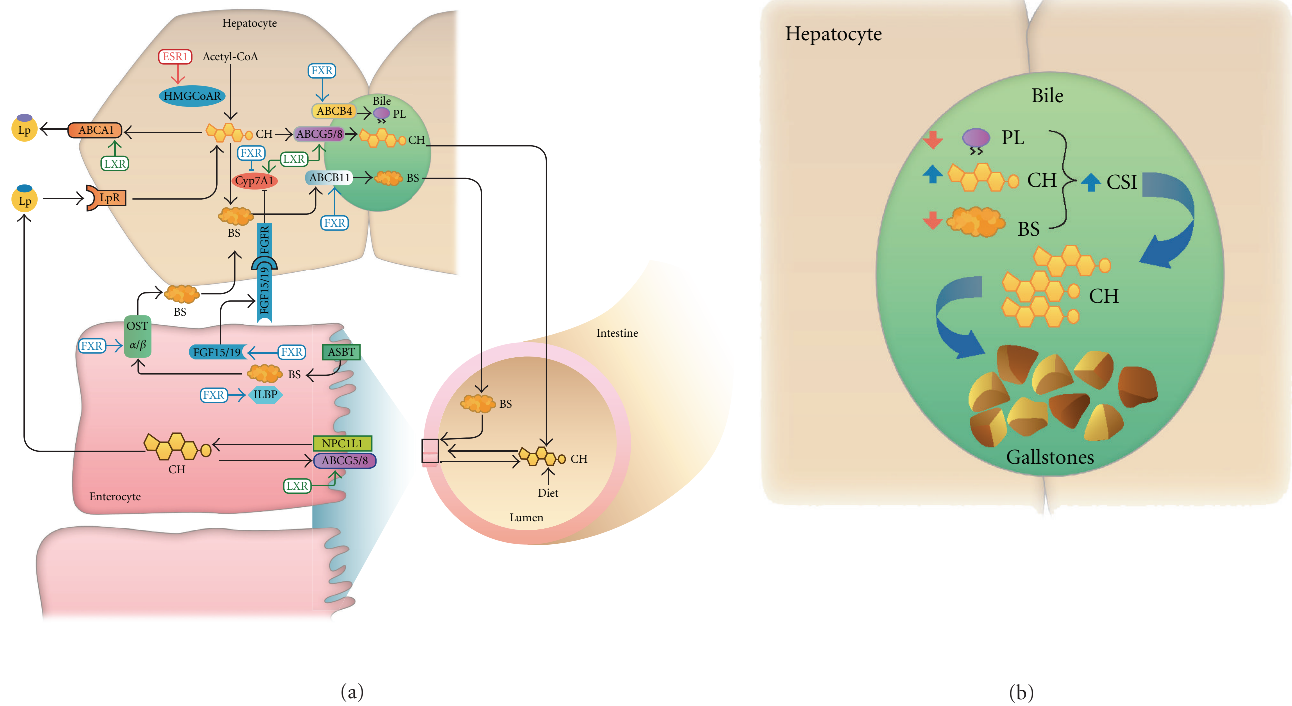
The liver X receptors (LXRs), LXRα and LXRβ, are oxysterol intracellular sensors that regulate key genes related to sterol, bile acid, and lipid homeostasis [21, 22]. In rodents, but not in humans, LXR promotes bile acid synthesis by activating the expression of *Cyp7A1,* the limiting enzyme of the neutral bile acid synthesis pathway [23–25]. LXRs are also known to induce the hepatic expression of cholesterol and phospholipid efflux transporters, including canalicular ABCG5/ABCG8 [26] as well as ABCA1, a basolateral ABC transporter of cholesterol and phospholipids [27].

Uppal et al. evaluated the effect of hepatic LXR activation on lithogenic-diet-fed transgenic mice with constitutively active expression of LXR [28]. They found an increased susceptibility of these mice to gallstone disease that correlated with increased biliary concentrations of cholesterol and phospholipids and decreased biliary bile salt concentrations, leading to a high cholesterol saturation index in bile. As expected, hepatic expression of the canalicular transporters *Abcg5/Abcg8* was induced, as well as *Abca1* and *Cyp7A1*, by administration of LXR agonists in lithogenic-diet-fed LXR transgenic mice. Moreover, the prolithogenic effect of LXR activation was abolished in low-density-receptor-deficient mice. On the other hand, ezetimibe, a cholesterol-lowering agent that blocks intestinal cholesterol absorption, had the same effect. These results confirm that hepatic LDL cholesterol uptake and intestinal cholesterol absorption are relevant for gallstone disease in this specific diet-induced gallstone disease mouse model.

In humans, increased expression of LXR, ABCG5, and ABCG8 was found in livers of nonobese Chinese gallstone patients. Moreover, increases in mRNA levels of these genes significantly correlated with biliary cholesterol levels and saturation [29], suggesting a potential pathogenic role of LXR activation in human gallstone disease.

Genomewide analysis of gallstone traits in inbred mouse strains has yielded a susceptibility map of lithogenic (Lith) loci [30–33]. Interestingly, the Lith1 locus harbors LXRα as a candidate gene in addition to ABCA11 [33]. However, no evidence of association between single nucleotide polymorphisms (SNPs) for the LXR gene and gallstone susceptibility was detected in a German population sample [34]. Clearly, further studies are required to elucidate the relevance of this hepatic nuclear receptor in the pathogenesis of this disease in humans.

Although studies evaluating the relevance of intestinal LXR in gallstone disease are lacking, intestine-specific LXR activation decreased cholesterol absorption in transgenic mice with intestinal expression of constitutively active



(a)

(b)

Figure 1: (a) Possible molecular mechanisms of action of nuclear receptors at the liver and the small intestine. Cholesterol derived from the diet as well as from the bile enters the intestine and is absorbed by the enterocytes through NPC1L1 and can be secreted back to the intestinal lumen by ABCG5/G8. After absorption, cholesterol is incorporated into lipoproteins (Lps), secreted into lymph and blood, and transported to the liver after triglyceride uptake in peripheral tissues. Bile salts (BSs) are absorbed in the intestine by the ASBT transporter and exit into the basolateral surface through OSTα/β transporters, among others, reaching the liver via the systemic blood circulation. The hepatic pool of cholesterol originates from *de novo* synthesis from acetyl-CoA as well as receptor-mediated endocytosis and/or selective lipid uptake from Lp. Cholesterol can be secreted into plasma HDL through ABCA1 transporter or by formation and secretion of VLDL (not shown) or into the bile through the heterodimeric ABCG5/8 transporter. Bile is constituted by cholesterol (CH), phospholipids (PLs), and BSs. PL enters the biliary canaliculi through the ABCB4 transporter. BSs, obtained by neosynthesis from cholesterol or by uptake from plasma, are secreted into the bile by the ABCB11 transporter. The NRs control metabolism and secretion of lipids at different levels: LXR promotes cholesterol efflux from the intestine and from the liver by activation of ABG5/8 and ABCA1 transporters. Also, LXR activates Cyp7A1 leading to an increase in BS synthesis in the liver. The FXR receptor regulates BS concentration at two different levels: promoting the expression of FGF15/19, ILBP, and OSTα/β transporters in the intestine as well as increasing the expression of ABCB4 and ABC11 transporters and repressing Cyp7A1 expression in the liver. ESRs increase *de novo* cholesterol synthesis by regulation of HMGCoAR.ASBT: apical sodium bile acid transporter. OSTα/β: organic solute transporter alpha/beta (b) Cholesterol gallstone formation. An increase in cholesterol and/or a decrease in BS or PL contents in the bile lead to an increase in the biliary cholesterol saturation index (CSI) triggering cholesterol precipitation into crystals and ultimately the formation of cholesterol stones within the gallbladder.

LXR [35]. This phenotype correlated with upregulation of the *Abcg5/Abcg8* transporters, which are localized in the apical membrane in the intestine and mediate cholesterol efflux [26]. Indeed, these transgenic mice fed with ahigh-cholesterol diet were protected against hepatic cholesterol accumulation. Thus, in contrast to hepatic LXR activation, it could be speculated that intestinal LXR activation would protect from CGD. This opens a window for future therapeutic interventions, directed to selective LXR activation in the intestine, avoiding the side effects of hepatic LXR stimulation, such as increased liver and plasma triglyceride levels.

4. The Farnesoid X Receptor

The farnesoid X receptor (FXR) acts as an intracellular bile salt sensor [36, 37], induces the expression of ABCB11 and ABCB4, and represses bile salt synthesis by small-heterodimer-partner-(SHP-) mediated *Cyp7A1* inhibition [36–38]. FXR was also identified as an attractive candidate gene for gallstone disease in mice by genomewide investigation studies [32]. Moreover, lower expression of *Fxr* was observed in a mouse strain susceptible for gallstone formation in comparison with a resistant strain [32]. In addition, mice with isolated hepatic insulin resistance and increased gallstone susceptibility exhibited increased bile salt hydrophobicity in bile and partial resistance to FXR activation by GW4064, a synthetic FXR agonist [39]. More striking, Moschetta et al. [40] found that FXR deficiency in mice conferred a higher susceptibility to CGD when fed a lithogenic diet. This increased susceptibility correlated with a higher bile salt hydrophobicity index and gallbladder mucosal inflammation. Also, they found

a decreased expression of the ABCB4 and ABCB11 transporters involved in biliary phosphatidylcholine and bile salt secretion. In addition, treatment of lithogenic-diet-fed gallstone-susceptible mice with FXR agonist GW4064 prevented cholesterol gallstone formation and increased the expression of ABCB11 and ABCB4 transporters, resulting in substantially higher bile salt and phospholipid bile concentrations in gallbladder bile. These results suggest that modulation of FXR and their downstream targets may be a good strategy for drug therapy in human CGD; as well as the modulation of other nuclear receptors has been used in several other human pathologies [41]. Pharmacological activation of FXR can selectively increase the secretion of bile salts and phospholipids, by increasing expression of the ABCB11 and ABCB4 transporters, allowing the solubilization of cholesterol in bile.

Some studies in humans have also supported a role of FXR in gallstone disease. Kovacs et al. showed an association of a sequence variant in the FXR gene with gallstone prevalence in a Mexican cohort [42]. However, no relationship of this SNP with gallstones was detected in a German cohort, whereas a trend toward a protective effect of the same SNP was found in a Chilean population. Interestingly, FXR variants have been found in Caucasian patients with intrahepatic cholestasis of pregnancy, a condition known to be associated with gallstones [43]. In addition, a small study described the association between reduced hepatic expression of the PPAR-γ coactivator-1 (PGC-I) and decreased FXR levels in gallstone patients [44]. Based on this finding as well as the role of PGC-1 as a positive activator of FXR expression [45], the authors speculated that PGC-1 may function as a protective gene for gallstone disease by increasing FXR activity. In summary, current data strongly suggest a relevance of FXR in human gallstone disease point, but more studies are still required to fully validate this hypothesis.

Besides its role in hepatic lipid homeostasis, FXR activity should also be considered as a regulator of lipid genes expressed in the intestine. In this regard, decreased intestinal expression of FXR and its target genes, ileal lipid-binding protein (ILBP) and OSTα–OSTβ (all involved in bile acid transport), has been described in a subgroup of nonobese gallstone female patients [46, 47]. These findings suggest a FXR-dependent defect in the intestine leading to decreased bile acid absorption and subsequently diminished bile acid pool. Accordingly, increased bile acid and cholesterol synthesis have been reported in a subgroup of Chilean patients [48], suggesting that increased intestinal loss of bile acids may precede gallstone formation.

Another interesting FXR gene target is the fibroblast growth factor (FGF) 15/19 (mouse and human ortholog, resp.). FXR induced the expression of FGF15, which activated a negative feedback on hepatic bile acid neosynthesis after binding to FGF receptor 4 and impaired gallbladder emptying after binding to FGF receptor 3 [49]. Interestingly, ileal FGF19 mRNA levels were diminished in nonobese gallstone females compared with controls [47]. Further studies are required to elucidate if FGF19 has a direct role in the pathogenesis of CGD.

5. Estrogen Receptors

As it is well documented by epidemiological and clinical studies, CGD prevalence is higher in women than in men [50–52]. Physiological increase of estrogen levels, in conditions such as human pregnancy, correlates with increased hepatic secretion of biliary cholesterol and the formation of a cholesterol-supersaturated bile [53]. Furthermore, oral contraceptive steroids and conjugated estrogens increase the risk for CGD [54–56]. Interestingly, estrogens exert their biological functions through the modulation of two closely related classical homodimeric nuclear receptors, ESR1 and ESR2, which are widely expressed in tissues, including the liver [57–59]. Together, these data have lead to the hypothesis that estrogens may enhance the risk for CGD by increasing the functions of the hepatic ESRs [2].

Using gonadectomized gallstone-resistant male or female AKR mice fed with a lithogenic diet in the presence of ESR-selective synthetic estrogens has shown a correlation between gallstone formation and hepatic ESR1 upregulation. Furthermore, the prolithogenic action of estrogens was blocked by ESR1-selective antagonists, suggesting that ESR1 is the specific estrogen receptor pathogenically linked to gallstone formation. Increased gallstone formation mediated by estrogen administration in this animal model correlated with higher biliary cholesterol secretion and the presence of cholesterol supersaturated bile [59].

High plasma levels of estrogens have been correlated with augmented activity of the cholesterol biosynthesis rate-limiting enzyme HMG-CoA reductase in humans and animals [60, 61], even under high-cholesterol diets. Wang et al. studied the relevance of hepatic cholesterol neosynthesis for estrogen-induced gallstone formation in AKR ovariectomyzed mice treated with estrogens and fed with chow or high-cholesterol diets [61]. They found that estrogens induced an increase in cholesterol biosynthesis, even in the presence of a high cholesterol diet. These changes correlated with increased expression of SREBP2, the key transcription factor regulator of the HMG-CoA reductase gene, and also its target genes [61]. There was also an augmented biliary cholesterol secretion, with an important increase in the contribution of newly synthesized cholesterol to biliary cholesterol output. Consistent with accelerated gallstone formation, a higher lithogenicity of the bile was found. Moreover, estrogens could also act at the canalicular membrane by increasing ABCG5/ABCG8 activity [2]. These results have led to a model in which estrogen induces cholesterol gallstone formation by promoting cholesterol biosynthesis through SREBP2 and hepatic biliary cholesterol secretion.

On the other hand, estrogens can also regulate lipid and bile salt metabolism through GPR30 receptor activation. This novel estrogen receptor, a member of the rhodopsin-like family of G-protein-coupled receptors, is a multipass membrane protein that has been found in the endoplasmic reticulum and the cell surface. In normal physiological conditions, GPR30 is widely expressed, with particularly high expression reported in heart, lung, liver, intestine, ovary, and brain [62]. This pattern of expression leads us to propose

a possible metabolic role of GPR30 activation not only in the liver, but also in the small intestine as documented for LXR and FXR receptors. In this regard, estrogen activation of GPR30 may influence CGD through nongenomic activation of rapid kinase signalling pathways.

6. Concluding Remarks

In the past few years, significant advances have been made in understanding the possible molecular mechanisms that link some nuclear receptors such as LXR, FXR, and ESRs with CGD. In the liver as well as in the small intestine, these receptors regulate the expression of key genes involved in synthesis and transport of cholesterol, bile salts, and phospholipids. In such a way, nuclear receptors may modulate bile lipid composition and thus the susceptibility to cholesterol gallstone formation. Even though new insights have been obtained using animal models, more studies are needed to establish more definitively their relevance in human CGD.

The knowledge of nuclear-receptor-dependent mechanisms involved in CGD opens a new opportunity for drug therapy of this disease condition based on modulation of hepatic and/or intestinal cholesterol and bile acid metabolism. Modulation of intestinal lipid metabolism by nuclear receptors as well as the role of estrogen receptors must be explored more deeply to offer new targets for drug development on CGD. In this regard, therapeutic approaches to CGD would not be limited to the classically liver-related receptors LXR and FXR.

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Review Article

Bile Acid Signaling in Liver Metabolism and Diseases

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Obesity, diabetes, and metabolic syndromes are increasingly recognized as health concerns worldwide. Overnutrition and insulin resistance are the major causes of diabetic hyperglycemia and hyperlipidemia in humans. Studies in the past decade provide evidence that bile acids are not just biological detergents facilitating gut nutrient absorption, but also important metabolic regulators of glucose and lipid homeostasis. Pharmacological alteration of bile acid metabolism or bile acid signaling pathways such as using bile acid receptor agonists or bile acid binding resins may be a promising therapeutic strategy for the treatment of obesity and diabetes. On the other hand, bile acid signaling is complex, and the molecular mechanisms mediating the bile acid effects are still not completely understood. This paper will summarize recent advances in our understanding of bile acid signaling in regulation of glucose and lipid metabolism, and the potentials of developing novel therapeutic strategies that target bile acid metabolism for the treatment of metabolic disorders.

1. Introduction

Bile acids are produced only in the liver as the end products of cholesterol catabolism [1, 2]. In addition to the classic function of bile acids in facilitating hepatobiliary secretion of endogenous metabolites and xenobiotics and intestine absorption of lipophilic nutrients, bile acids also play equally important roles in controlling the metabolism of glucose and lipids in the enterohepatic system, and energy expenditure in peripheral tissues [3, 4]. Because of such a close association between bile acid signaling and metabolic homeostasis, targeting bile acid metabolism by using bile acid receptor agonists or bile acid-binding resins have proven to be effective in improving lipid and glucose homeostasis in obesity and diabetes [5]. Furthermore, stimulating de novo bile acid synthesis prevented, whereas, disruption of bile acid signaling caused insulin resistance and dyslipidemia in mice, indicating that impaired bile acid homeostasis may likely contribute to the pathogenesis of metabolic disorders [6–9]. This paper will summarize recent advances in our understanding of bile acid signaling regulation of glucose and lipid metabolism and the potentials of developing novel therapeutic strategies that target bile acid metabolism for the treatment of metabolic disorders.

2. Bile Acid Synthesis

Bile acids are the end product of cholesterol catabolism in the liver [1, 10–12]. In humans, the bile acid pool consists of primary bile acids cholic acid (CA), chenodeoxycholic acid (CDCA), secondary bile acids deoxycholic acid (DCA), and lithocholic acid (LCA). Primary bile acids are synthesized from cholesterol through two general pathways, the classic pathway and the alternative pathway. Secondary bile acids are derived from primary bile acids in the intestine by bacterial enzymes. Enzymes that catalyze these multistep reactions are located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes. In humans, the classic pathway is considered as the major bile acid synthetic pathway (accounts for more than 90% of total bile acid production) and produces CA and CDCA in approximately equal amounts. Cholesterol 7α-hydroxylase (CYP7A1), a microsomal cytochrome p450 enzyme, catalyzes the first and rate-limiting step in the classic pathway [13]. The intermediate product in the classic pathway 7α-hydroxy-4-cholestene-3-one serves as the common precursor for both CA and CDCA. 7α-hydroxy-4-cholestene-3-one can be hydroxylated at C-12 position by microsomal sterol 12α-hydroxylase (CYP8B1), followed up by several reactions including mitochondrial 27-hydroxylase

(CYP27A1) to cleave a 3-carbon unit and eventually converted to CA. Without 12α-hydroxylation, 7α-hydroxy-4-cholestene-3-one is converted to CDCA. Thus, CYP7A1 controls the overall rate of bile acid production, while CYP8B1 controls the CA: CDCA ratio in the bile acid pool. The alternative pathway (also known as acidic pathway), which is thought to account for less than 10% of the total bile acid synthesis in humans, mainly produces CDCA. CYP27A1 catalyzes hydroxylation of cholesterol to 27-hydroxycholesterol and then 3β-dihydroxy-5-cholestionic acid [14]. Oxysterol 7α-hydroxylase (CYP7B1) then catalyzes the hydroxylation reaction at C-7 position of these two intermediates, which are subsequently converted to CDCA by the same enzymes in the classic pathway.

3. Bile Acid Transport and Enterohepatic Circulation

Bile acids, once produced in the liver, are transported across the canalicular membrane of the hepatocytes into the bile and stored in the gallbladder. After each meal, gallbladder bile acids are released into the intestinal tract, efficiently reabsorbed in the ileum, and transported back to the liver via portal blood for reexcretion into the bile. This process is referred to as enterohepatic circulation of bile acids [10] (Figure 1). Bile acid transporters play important roles in this transport process [15]. The biliary excretion of bile acids is the major driving force of bile flow. The bile acid pool size is defined as the total amount of bile acids circulating in the enterohepatic circulation. In humans, bile acid pool consists of CA, CDCA, and DCA in an approximate 40 : 40 : 20 ratio. In mice, the majority of the CDCA is converted into muricholic acids (MCAs), which are highly soluble and less toxic.

Hepatocytes are polarized epithelial cells with basolateral (sinusoidal) and apical (canalicular) membrane domains. Hepatocytes take up bile acids through the basolateral membrane, which is in direct contact with the portal blood plasma, and excrete bile acid at the canalicular membrane into the bile [16]. Since the biliary bile acids concentration is about 100- to 1000-fold higher in the bile than in the hepatocytes, canalicular bile acid transport represents the rate-limiting step in bile formation. Several members of the ATP-binding cassette (ABC) transporter family are responsible for transporting bile acids and other organic compounds across the canalicular membrane against their concentration gradients. The bile salt export pump (BSEP, ABCB11), originally identified as the sister of P-glycoprotein (SPGP), is mainly responsible for bile acid transport at the canalicular membrane [17] (Figure 1). Mutations in *BSEP* were first identified in patients with progressive familial intrahepatic cholestasis subtype 2 (PFIC-2). The absence of functional BSEP in the hepatic canalicular membrane and less than 1% of normal biliary bile acid concentration found in these patients suggested that BSEP is the major canalicular bile acid transport system [18]. Phospholipids are excreted via the phospholipid flippase MDR2 (ABCB4) (Figure 1), and the major phospholipid in the bile is phosphatidylcholine [19, 20]. Biliary-free cholesterol secretion mediated by ABCG5/G8 transporters is an important route for hepatic cholesterol elimination. Bile acids, phospholipids, and cholesterol are three major organic solutes of the bile and once secreted, they form mixed micelles to increase cholesterol solubility and reduce their toxicity to the bile duct. Bile acids are conjugated with taurine or glycine in the peroxisomes and present as bile salts. They cannot cross the hepatocyte membrane and need active transport mechanisms for cellular uptake [21]. Two bile acid transporters, Na^+-dependent taurocholate transporter (NTCP) (Figure 1) and organic anion transporters (OATPs) are responsible for basolateral bile acid transport into the hepatocytes.

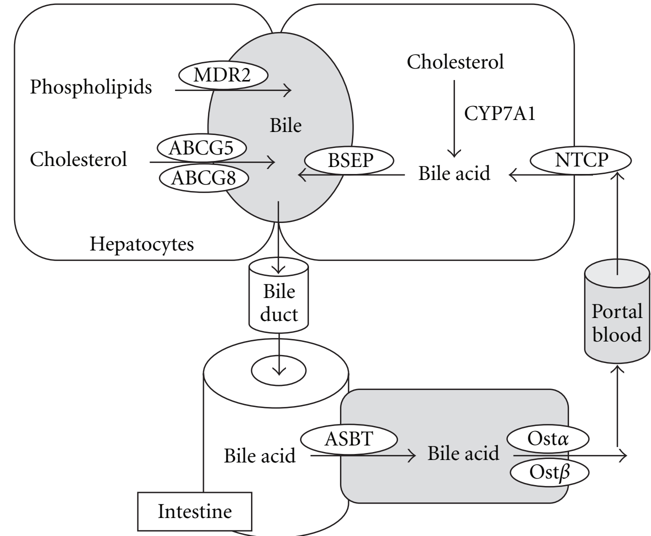

Figure 1: Enterohepatic circulation of the bile. Bile acids are synthesized from cholesterol in the hepatocytes. CYP7A1 regulates the rate-limiting step in the classic bile acid biosynthetic pathway. Bile acids are secreted into the gallbladder via BSEP. Phospholipids are transported via MDR2, and cholesterol is transported by the ABCG5/G8 transporters into the bile. In the gallbladder, bile acids, phospholipids, and cholesterol form mixed micelles to solubilize cholesterol and to reduce bile acid toxicity. After meal intake, gallbladder releases bile into the small intestine where bile acids facilitate the absorption of dietary lipids and vitamins. At the terminal ileum, most of the bile acids are reabsorbed by ASBT into the enterocytes, and secreted into the portal circulation via basolateral bile acid transporters Ostα/Ostβ. At the basolateral membrane of the hepatocytes, bile acids are taken up by the NTCP transporter for resecretion into the gallbladder.

In the intestine, bile salts are deconjugated, and bacterial 7α-dehydroxylase removed a hydroxy group from C-7 and converts CA to DCA and CDCA to LCA. These secondary bile acids are highly toxic. In the intestine lumen, bile acids are reabsorbed mostly at the terminal ileum. Like the hepatic basolateral uptake system, intestinal bile acid uptake is also mainly mediated by the apical sodium-dependent bile salt transporter (ASBT) (Figure 1) [22]. Once absorbed into the enterocytes, bile acids bind the intestinal bile acid binding protein (I-BABP) and are transported to the basolateral membrane for secretion [23]. Recently identified heterodimeric organic solute transporters OSTα and OSTβ

appeared to be the major basolateral bile acid transport system in the intestine and many other epithelial cells (Figure 1) [24]. This is supported by studies showing that over-ex-pression of OSTα and OSTβ in mice enhanced basolateral efflux of taurocholate, while mice lacking *ostα* showed marked decreases in intestinal bile acid absorption, serum bile acid concentration, and bile acid pool size.

4. Regulation of Bile Acid Synthesis

4.1. Bile Acid Feedback Regulation of Its Own Synthesis. It is clear that bile acid synthesis is mainly controlled via the transcriptional regulation of the *CYP7A1* gene [11]. It is well established that the *CYP7A1* gene is repressed by bile acids. This bile acid feedback repression mechanism allows the liver to efficiently increase or decrease bile acid synthesis in response to changes in bile acid levels and thus to maintain a constant bile acid pool. It is thought that bile acid feedback inhibition of *CYP7A1* is mediated by the bile acid-activated nuclear receptor farnesoid X receptor (FXR) in the hepatocytes and the enterocytes (Table 1). It was first discovered that hepatic FXR induced a negative nuclear receptor small heterodimer partner (SHP), which interacts with and represses the transcriptional activator liver-related homologue-1 (LRH-1) that binds to the *CYP7A1* gene promoter and thus inhibit *CYP7A1* transcription [25]. However, the repression of *CYP7A1* by bile acids and FXR agonist in SHP-deficient mice implies that FXR-SHP-LRH-1 cascade is not the only pathway mediating bile acid feedback inhibition of CYP7A1 and redundant pathways also exist [26, 27]. More recently, FXR was shown to induce intestine fibroblast growth factor 15 (FGF15) which may act as an endocrine hormone to repress *CYP7A1* gene transcription via ERK signaling activation [28]. Direct infusion of recombinant FGF15 into mouse blood circulation or overexpression of FGF15 in mouse livers via an adenovirus expression vector caused marked repression of CYP7A1 mRNA expression. The identification of an intestine-initiated endocrine mechanism in mediating bile acid feedback regulation is consistent with the fact of intestine being the major organ for bile acid reabsorption and retention. Such finding also provides an explanation to a long observed phenomenon that intraduodenal, but not intravenous, infusion of taurocholic acid repressed CYP7A1 mRNA expression in rats [29]. In mice lacking functional Ostα/Ostβ, bile acid transport to the liver was reduced and bile acids accumulated in the intestine. Interestingly, these mice showed increased intestine FGF15 expression and reduced liver CYP7A1 mRNA and total bile acid pool [30]. Furthermore, intestine-specific FXR knockout, but not liver-specific FXR knockout, prevented GW4064 repression of liver CYP7A1 gene expression in mice [31]. These studies collectively suggest that intestine FXR plays a dominant role in mediating bile acid feedback repression of bile acid synthesis. Unfortunately, data on detection of the presence of FGF15 protein in the mouse circulation is lacking, and such evidence is needed in order to eventually establish the endocrine mechanism of feedback regulation of bile acid synthesis. Human FGF19 shares ~51% amino acid sequence identity with mouse FGF15 and is considered as the mouse FGF15 orthologue. FGF19 has been shown to repress *CYP7A1* in human hepatocytes [32]. In contrast to FGF15 that is not detectable in mouse livers and circulation, FGF19 mRNA is detectable in human livers and human hepatocytes [32, 33]. FGF19 protein is present in human circulation [34]. In human hepatocytes, FGF19 is highly inducible by bile acids or FXR agonists [32]. Since adenovirus-mediated overexpression of FGF15 in mouse liver has been shown to repress *CYP7A1*, it is likely that bile acid accumulation in human liver may induce FXR/FGF19 pathway to repress CYP7A1 in an autocrine manner [32]. Previous studies from us and others showed that bile acids were able to activate FXR-independent cell signaling pathways to repress the *CYP7A1* gene [35, 36]. We recently showed that feeding FXR knockout mice a cholic acid-containing diet still repressed CYP7A1 despite the absence of FGF15 or SHP induction [6]. These results indicate that in response to super-physiological concentrations of bile acids, redundant pathways are stimulated to repress bile acid synthesis. These pathways ensure prompt repression of de novo bile acid synthesis in response to elevated bile acid levels in the liver and/or the intestine.

4.2. Nutrient Effects on Hepatic Bile Acid Synthesis. Despite the majority of studies being focused on the regulatory roles of bile acids in nutrient metabolism, there is also evidence that nutrition could directly regulate bile acid synthesis. In humans, CYP7A1 activity, as determined by a serum surrogate 7alpha-hydroxycholest-4-en-3-one (C4), increases during postprandial periods during the day and decreases during fasting and at night [37]. A metabolomic study also identified bile acids as the most markedly elevated metabolites in human sera after an oral glucose challenge in patients with normal glucose tolerance, but this response was blunted in patients with impaired glucose tolerance [38]. Since postprandial period is the highly active in metabolism and humans undergo fasting-to-feeding cycles a few times a day, these observations could indicate an important link between bile acid synthesis and postprandial nutrient absorption and metabolism. Nutrient-activated signaling such as those by glucose or insulin is by far the most important signaling that regulates postprandial metabolism. Using primary human hepatocytes, we have demonstrated that insulin rapidly induced while glucagon repressed CYP7A1 mRNA [39, 40]. We also reported that glucose induced *CYP7A1* gene transcription via inducing histone hyperacetylation in *CYP7A1* gene chromatin [41]. Although our in vitro study provide mechanistic support for human observations, studies using in vivo mouse models yielded controversial results. It has been shown that PGC-1α acts as a coactivator of HNF4α and induces CYP7A1 during fasting in mice [42]. Furthermore, CYP7A1 mRNA was induced in STZ-treated type-I diabetic rats lacking insulin secretion [43], which has led to the speculation that insulin may repress *CYP7A1* gene in rats. On the other hand, more recent studies seem to be contradictory to these early observations. First, mouse CYP7A1 mRNA expression peaked during the early dark cycle when food intake was the most active [44, 45]. Furthermore, restricted feeding during light cycles shifted the peak of CYP7A1

Table 1: FXR target genes and their function and lipid and glucose metabolism.

	Gene	Tissue	Regulation	Function
Bile acid metabolism	CYP7A1	liver	down	Encodes the rate-limiting enzyme in classic bile acid synthetic pathway
	BSEP	liver	up	Rate-limiting step in canalicular bile acid transport into the gallbladder
	NTCP	liver	down	Basolateral bile acid uptake into the hepatocytes
	OSTα/β	intestine	up	Enterocyte basolateral bile acid secretion into the portal blood
	I-BABP	intestine	up	Intracellular bile acid transport
	FGF15/19	intestine	up	Bile acid synthesis inhibition
	SHP	liver	up	Bile acid synthesis inhibition
Glucose metabolism	PEPCK	liver	up	Gluconeogenesis
	FGF15/19	intestine	up	Stimulates glycogen synthesis, repress gluconeogenesis
	Insulin	pancreas	up	Glucose metabolism
Cholesterol metabolism	ApoA1	liver	down	HDL metabolism
	LDLR	liver	down	LDL uptake
	ABCG5/G8	liver	up	Biliary-free cholesterol secretion
	SRB1	liver	up	Hepatic HDL uptake, biliary cholesterol secretion
	PCSK9	liver	down	Induces LDL receptor degradation
Fatty acid metabolism	ApoC II	liver	up	LPL activator
	ApoC III	liver	down	LPL inhibitor
	SREBP1	liver	down	Lipogenesis

mRNA expression from dark cycles to light cycles [44]. Such evidence seems to imply that liver bile acid synthesis and liver metabolism are coordinately controlled. Further studies are necessary to determine if nutritional regulation of bile acid synthesis may play a role in metabolic homeostasis during fasting to refeeding cycles.

5. Bile Acid Regulation of Glucose Metabolism

5.1. FXR and Glucose Metabolism. Diabetes is associated with impaired peripheral glucose clearance and increased hepatic glucose production during fasting, which lead to postprandial and fasting hyperglycemia. Initial evidence that bile acids may regulate glucose metabolism came from studies showing that FXR agonist induced phosphoenolpyruvate Carboxykinase (PEPCK) mRNA expression (Table 1) and glucose output in human and rat hepatocytes [46]. Treating mice with an FXR agonist also induced hepatic PEPCK mRNA expression in mice in vivo [46]. A FXR binding site has been identified in the promoter of PEPCK gene. In contrast, later studies carried out in *fxr* knockout mice revealed that FXR-deficient mice had insulin resistance and hyperglycemia phenotypes. Administration of a FXR agonist GW4064 decreased serum glucose, increased liver glycogen, and improved insulin sensitivity in diabetic *db/db* mice [7, 8]. A number of recent studies showed that bile acids and FXR repressed hepatic *PEPCK* and *G6Pase* gene expression and thus liver gluconeogenesis. In this case, it is shown that bile acids may induce the repressor SHP, which inhibits *PEPCK* via inhibiting C/EBP [47], FoxO1 [48], and Glucocorticoid receptor [49]. Although these liver effects of FXR activation may prevent fasting hyperglycemia, it does not sufficiently explain the increased insulin sensitivity and glucose disposal in FXR agonist-treated mice as determined by glucose and insulin tolerance tests. In a similar study, Cariou et al. used hyperinsulinemic euglycemic clamp and demonstrated that FXR-deficiency is associated with decreased whole-body glucose disposal, suggesting a role of FXR in regulating peripheral glucose metabolism [50]. FXR is not expressed in muscle but is expressed in white adipose at a very low level. It is noticed that *fxr*−/− mice had smaller adipocytes, and FXR agonist GW4064 treatment increased adipose differentiation and insulin-dependent glucose uptake in 3T3-L1 cells in vitro. Another study suggests that FXR agonist INT747 induced adipose differentiation via inducing the expression of adipocyte-related genes including C/EBPα and PPARγ [51]. In addition to a role of FXR in adipose, two recent studies provided additional mechanism by which FXR may regulate peripheral glucose homeostasis. These studies revealed that FXR is also expressed in pancreatic β cells and positively regulates glucose-dependent insulin secretion [52]. It is suggested that FXR activation stimulates insulin gene transcription. On the other hand, FXR activation is associated with increased AKT phosphorylation and GLUT2 translocation to the cell membrane and thus enhances glucose uptake into pancreatic β cells and glucose-dependent insulin secretion. Intestine is another major FXR expressing tissue. A recent study showed that FGF15/19, expressed in the intestine and secreted into the blood circulation, acts as a postprandial factor that promotes glycogen synthesis, which may be an important mechanism controlling postprandial glucose metabolism [53]. It has been shown that serum FGF19 increases during postprandial period in humans, presumably due to increased bile acid signaling [34]. Therefore, identification of the regulatory role of FGF15/19 in postprandial glycogen synthesis provides a novel link between bile acid signaling and glucose metabolism. In addition to the nuclear receptor FXR-mediated effects, bile acids have been shown to directly activate hepatic AKT via a Gα_i protein

coupled receptor signaling pathway, which stimulated hepatic glycogen synthesis [54]. Recently, it was further demonstrated that bile acid activation of the Gα_i-AKT signaling cascade was involved in the bile acid induction of FXR and SHP and downregulation of gluconeogenic gene expressions in the liver [55]. In summary, these studies suggest that bile acid regulation of hepatic glucose metabolism involves complex crosstalk between FXR-dependent pathways and FXR-independent signaling pathways.

5.2. TGR5 and Glucose Metabolism. Bile acids also activate a cell surface G-protein coupled receptor TGR5, which is mainly expressed in the intestine, brown adipose, white adipose, and gallbladder. Low levels of TGR5 expression has also been detected in liver and skeletal muscle. Upon activation, TGR5 leads to intracellular cAMP production and PKA activation. Based on the ability to induce cellular cAMP production, taurolithocholic acid (TLCA) and LCA show highest potency in activating TGR5 with EC_{50} of 0.33 and 0.53 μM, respectively, while DCA, CDCA, and CA (in rank order) activate TGR5 at higher 1–8 μM concentrations [56]. It is suggested that in brown adipocytes, bile acid activation of TGR5-cAMP-PKA cascade results in induction of downstream deiodinase, fatty acid oxidation genes, and uncoupling proteins, which increase energy expenditure and promote weight loss [57]. As increased free fatty acid release and cytokine production associated with obesity clearly contribute to the development of insulin resistance, bile acids/TGR5 regulation of weight loss certainly could play a role in regulating glucose homeostasis. However, studies in *tgr*5−/− mice showed that neither under chow condition nor under high fat diet feeding condition did *tgr*5−/− develop obesity or hyperglycemia [58]. The high fat diet feeding effect on insulin sensitivity, as determined by insulin tolerance tests, also seemed to be gender-specific in *tgr*5−/− mice, with male showing impaired, but female showing improved insulin sensitivity [58]. The most potent endogenous ligand for TGR5 is TLCA. TLCA is highly toxic and once synthesized, is rapidly metabolized in the intestine and the liver. Under physiological conditions, liver efficiently extracts bile acids from the portal circulation, and bile acid concentration in the systemic circulation is very low. Because these primary and secondary bile acids activate TGR5 at a higher EC_{50}, it is possible that TGR5 is not activated by physiological concentration of circulating bile acids outside of the enterohepatic system. Thus, opposing to the clear pharmacological benefits of TGR5 activation, the physiological role of TGR5 in mediating bile acid signaling control of metabolic homeostasis needs to be further investigated. In addition to brown adipose, intestine is another major TGR5 expressing tissue. Using an enteroendocrine cell line STC-1, Katsuma et al. first demonstrated that bile acids stimulate glucagon like peptide-1 (GLP-1) production via TGR5 activation [59]. The pharmacological significance of this pathway was then demonstrated by a detailed study carried out by Thomas et al. [60]. These authors showed that administration of a potent TGR5 agonist INT777 raised intracellular ATP/ADP ratio and calcium influx, which leads to enhanced GLP-1 secretion from the intestine. GLP-1 is known to promote insulin secretion and thus regulate glucose homeostasis. Because GLP-1 mimetics and receptor agonists are currently under clinical development and have shown promise in improving glucose homeostasis in diabetes, bile acid-based TGR5 agonists may be a potential therapeutic to stimulate GLP-1 secretion in diabetic patients [61].

In contrast to these studies, bile acid sequestrants, which remove bile acids from the body by binding to bile acids in the intestine and prevent bile acids from being reabsorbed, have been shown to improve insulin sensitivity and lower fasting glucose in both men and several different experimental models [62]. Two studies conducted in rats have so far suggested that bile acid sequestrants may improve insulin sensitivity by increasing GLP-1 release [63, 64]. Although the molecular mechanism is still not clear, both studies suggested that such effect is likely bile acid receptor-independent. This is because both studies showed that administration of bile acid sequestrants significantly lowered serum bile acid levels, which was associated with decreased FXR activation in the liver and the intestine. Furthermore, it is shown that blocking intestine bile acid transport using SC-435, an apical sodium-dependent bile acid transport inhibitor, also lowered serum bile acid levels, but did not modulate insulin sensitivity or GLP-1 secretion. Thus, it is likely that bile acid sequestrants exert its effect by directly modulating cellular signaling in the intestine rather than by altering circulating bile acid levels or modulating bile acid pool.

6. Bile Acids and Lipid Metabolism

6.1. Bile Acids and Cholesterol Metabolism. It has been known for a long time that preventing bile acid reabsorption in the intestine by bile acid sequestration increases hepatic CYP7A1 and bile acid synthesis [65]. The resulting increase in hepatic cholesterol catabolism caused compensatory induction of LDL receptor (LDLR) and LDL cholesterol (LDL-C) uptake. Because of the activation of this liver pathway, cholestyramine has been used to effectively lowering serum cholesterol in human patients. Paradoxically, activation of FXR by its potent agonists, which repress hepatic bile acid synthesis, also decreased serum cholesterol in animal models [7]. In wild-type mice, activation of FXR is mainly associated with a reduction of HDL-C, while in hypercholesterolemic animal models, activation of FXR decreases both LDL-C and HDL-C. In vitro, FXR was shown to induce LDLR expression and repress PCSK9, an LDLR inhibitor [66]. However, activation of FXR still significantly decreased serum non-HDL cholesterol in *ldlr*−/− mice [67]. Furthermore, CDCA administration has been shown to raise serum LDL-C levels in humans. It remains to be determined whether activation of FXR will provide benefits in lowering LDL-C in men.

FXR agonists have been shown to prevent atherosclerosis in various experimental models [68]. Serum HDL transports cholesterol from peripheral tissues to the liver for elimination and thus plays a critical role in reverse cholesterol transport and the development of atherosclerosis. However, the role of FXR in regulating HDL metabolism is still under debate because FXR inhibits the hepatic production of apolipoprotein A1 (ApoAI), a key structural component of HDL, and

activation of FXR is associated with decreased serum HDL [69]. Nevertheless, a recent study showed that activation of FXR promotes reverse cholesterol transport in mice by inducing hepatic expression of scavenger receptor B1 (SR-B1) [70], which is suggested to play a role in both hepatic uptake of HDL-C and biliary secretion of free cholesterol [71]. A FXR binding site has been identified in the *SR-B1* gene promoter [72]. In a recent study, we demonstrated that stimulating de novo bile acid synthesis by transgenic expression of a *CYP7A1* gene in mouse liver prevented diet-induced hypercholesterolemia [6]. Different from CA feeding or FXR agonist administration, *Cyp7a1*-tg mice showed both increased hepatic cholesterol catabolism and bile acid signaling. Using this model, we demonstrated that bile acid activation of FXR induces hepatic expression of ABCG5 and ABCG8 through a common FXRE, which promoted biliary-free cholesterol secretion and fecal cholesterol loss. It is well known that cholesterol activation of LXR only induces mouse, but not human *CYP7A1* gene expression [73]. We also showed that cholesterol/LXR signaling only induced ABCG5 and ABCG8 in mice, but not in primary human hepatocytes [6]. These studies suggest that upon hepatic cholesterol accumulation, LXR may stimulate cholesterol catabolism or biliary cholesterol secretion in mouse livers, but not human livers. Thus, it is possible that bile acid/FXR/ABCG5/G8 pathway plays a more important role in maintaining hepatic cholesterol homeostasis in response to increased cholesterol levels in humans.

6.2. Bile Acids and Fatty Acid Metabolism. It has been known for a long time that serum bile acid and serum triglycerides are inversely correlated, suggesting that bile acid negatively regulates serum triglycerides [74, 75]. Current studies suggest that bile acids may lower serum triglycerides by repressing both hepatic triglyceride production/secretion and stimulating serum triglyceride clearance. In the liver, it is shown that bile acid activation of FXR repressed LXR-induction of SREBP-1 and its target genes in de novo lipogenesis by inducing the repressor SHP, which not only decreased hepatic fat accumulation, but also led to reduced hepatic VLDL secretion [76]. One study showing both LXR-dependent and FXR-dependent induction of hepatic lipogenesis by bile acid sequestrants administration supported this conclusion [68]. The finding that FXR represses microsomal triglyceride transfer protein (MTP) and thus hepatic VLDL secretion seems to provide additional support that the FXR/SHP pathway reduces hepatic triglyceride output [77]. Genetic knockout of *shp* in *ob/ob* mice increased MTP and VLDL secretion [78]. Diabetes and obesity are associated with increased hepatic VLDL output. Both increased fatty acid supply to the liver and hepatic insulin resistance may be involved. On the other hand, there are also studies showing that hepatic VLDL secretion was impaired in diabetic mouse models despite increased triglyceride output [79]. Furthermore, increased VLDL secretion in *shp* knockout mice seemed to be beneficial in reducing hepatic fat accumulation in *ob/ob* mice [78]. Thus, the bile acid regulation of hepatic VLDL secretion and its in vivo significance seem to be complex and may depend on the experimental conditions. Serum triglyceride is cleared after VLDL-triglyceride is hydrolyzed by lipoprotein lipase (LPL) and subsequently taken up by the peripheral tissues. It has been reported that obesity and diabetes are also associated with impaired peripheral triglyceride clearance, contributing to diabetic hypertriglyceridemia. Activation of FXR has been shown to induce apolipoprotein CII (ApoCII), which is an LPL activator, and repress apolipoprotein CIII (ApoCIII), which is an LPL inhibitor in the liver [80, 81]. Increasing ApoCII or decreasing ApoCIII stimulates LPL to hydrolyze triglycerides carried by VLDL, thus accelerates serum VLDL clearance upon FXR activation.

7. Conclusion

Extensive investigations conducted in the past decade have shown that bile acids are important regulators of glucose and lipid metabolism. The identification of bile acid-activated nuclear receptor FXR and cell surface G protein coupled receptor TGR5 has significantly advanced our understanding on how bile acid signaling regulates cellular metabolism in physiological and diseased conditions. The identification of these regulatory mechanisms also provided molecular basis for developing bile acid receptor agonists and receptor antagonists for treating human metabolic diseases. On the other hand, conflicting studies in the field are present, which not only reflects the complex nature of the bile acid signaling in regulation of whole body metabolism, but also implies the difference between physiological role and pharmacological role of bile acid signaling in metabolic control. Furthermore, studies that focus on the regulation of bile acid metabolism in diseased conditions, especially obesity and diabetes, are still insufficient. Future advances in the field are needed to improve our understanding in the bile acid control of metabolism, which is also critical in developing better drug therapies for the treatment of metabolic disorders.

Abbreviations

CA: Cholic acid
CDCA: Chenodeoxycholic acid
DCA: Deoxycholic acid
LCA: Lithocholic acid
CYP7A1: Cholesterol 7α-hydroxylase
CYP8B1: Microsomal sterol 12α-hydroxylase
CYP27A1: Mitochondrial 27-hydroxylase
CYP7B1: Oxysterol 7α-hydroxylase
MCA: Muricholic acids
ABC: ATP-binding cassette
BSEP: Bile salt export pump
SPGP: Sister of P-glycoprotein
PFIC: Progressive familial intrahepatic cholestasis
MDR: Multidrug resistance
NTCP: Na^+-dependent taurocholate transporter
OATP: Organic anion transporter
ASBT: Apical sodium-dependent bile salt transporter
I-BABP: Intestinal bile acid binding protein
OST: Organic solute transporter
FXR: Farnesoid X receptor

SHP: Small heterodimer partner
LRH-1: Liver-related homologue-1
FGF15: Fibroblast growth factor 15
PEPCK: Phosphoenolpyruvate carboxykinase
GLP-1: Glucagon-like peptide-1
LDL: Low density lipoprotein
ApoA1: Apolipoprotein A1
SR-B1: Scavenger receptor B1
MTP: Microsomal triglyceride transfer protein
LPL: Lipoprotein lipase
ApoCII: Apolipoprotein CII
ApoCIII: Apolipoprotein CIII
VLDL: Very low density lipoprotein.

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