Regulation and deregulation of cholesterol homeostasis:
The liver as a metabolic “power station”

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Abstract
Cholesterol plays several structural and metabolic roles that are vital for human biology. It spreads along the entire plasma membrane of the cell, modulating fluidity and concentrating in specialized sphingolipid-rich domains called rafts and caveolae. Cholesterol is also a substrate for steroid hormones. However, too much cholesterol can lead to pathological pictures such as atherosclerosis, which is a consequence of the accumulation of cholesterol into the cells of the artery wall. The liver is considered to be the metabolic power station of mammals, where cholesterol homeostasis relies on an intricate network of cellular processes whose deregulations can lead to several life-threatening pathologies, such as familial and age-related hypercholesterolemia. Cholesterol homeostasis maintenance is carried out by: biosynthesis, via 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity; uptake, through low density lipoprotein receptors (LDLr); lipoprotein release in the blood; storage by esterification; and degradation and conversion into bile acids. Both HMGR and LDLr are transcribed as a function of cellular sterol amount by a family of transcription factors called sterol regulatory element binding proteins that are responsible for the maintenance of cholesterol homeostasis through an intricate mechanism of regulation. Cholesterol obtained by hepatic de novo synthesis can be esterified and incorporated into apolipoprotein B-100-containing very low density lipoproteins, which are then secreted into the bloodstream for transport to peripheral tissues. Moreover, dietary cholesterol is transferred from the intestine to the liver by high density lipoproteins (HDLs); all HDL particles are internalized in the liver, interacting with the hepatic scavenger receptor (SR-B1). Here we provide an updated overview of liver cholesterol metabolism regulation and deregulation and the causes of cholesterol metabolism-related diseases. Moreover, current pharmacological treatment and novel hypocholesterolemic strategies will also be introduced.

Key words: Cholesterol; 3-hydroxy-3-methylglutaryl coenzyme A reductase; Hypercholesterolemia; Low density lipoprotein receptors; Liver

INTRODUCTION
Cholesterol plays several structural and metabolic roles that are vital for human biology. Although cholesterol
spreads along the entire plasma membrane of the cell where it modulates fluidity, it also concentrates in specialized sphingolipid-rich domains called rafts and caveolae. In addition, cholesterol is a substrate for steroid hormones. Too much cholesterol in cells, however, can lead to pathological consequences. This is particularly true for cells of the artery wall, where accumulation of cholesterol initiates atherosclerotic cardiovascular disease. Therefore, the body relies on a complex homeostatic network to modulate the availability of cholesterol for tissues. This network operates on both the cellular level, mainly in the liver, and within the plasma compartment.

This paper reviews the present knowledge of cholesterol metabolism, homeostasis, deregulation and related pathologies. Moreover, standard and alternative therapeutic targets will also be discussed.

**LIVER CHOLESTEROL METABOLISM**

Cholesterol is both synthesized by cells and taken in with food intake. The liver is the principal site for cholesterol homeostasis maintenance carried out in many mechanisms, such as biosynthesis, **via 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, E.C. 1.1.1.34) activity**, uptake through low density lipoprotein receptors (LDLr), lipoprotein release in the blood, storage by esterification and degradation and conversion into bile acids. The major precursor of cholesterol synthesis is acetyl-CoA which gives rise to hydroxyl methylglutaryl-CoA (HMG-CoA). The rate limiting step in the cholesterol biosynthetic pathway is the conversion of HMG-CoA to mevalonic acid (MVA) by HMGR.

The MVA biosynthetic pathway is a sequel of complex reactions that, besides cholesterol, produces several biomolecules involved in RNA transcription (isopentenyl tRNAs), protein N-glycosylation (dolichol), protein prenylation (farnesyl and geranylgeranyl moieties) and mitochondrial electron transport (ubiquinone), all indispensable for cell survival.

In addition to being synthesized, cholesterol can also be taken up through a classic example of receptor-mediated endocytosis by hepatocytes. LDLr plays an important role in cholesterol homeostasis since it binds plasma LDL particles, thus lowering plasma cholesterol levels. This receptor was first discovered in cultured human fibroblasts. Later on, genetically and immunologically identical receptors were also identified in the liver.

LDLr and other proteins involved in cholesterol metabolism regulation, such as HMGR, are transcribed as a function of cellular sterol amount by a family of transcription factors called sterol regulatory element binding proteins (SREBPs). Once synthesised, SREBPs are associated with the endoplasmic reticulum (ER) membrane where they remain transcriptionally inactive. In the ER, the SREBP C-terminus interacts with the cargo protein SCAP (SREBP cleavage activating protein) which functions as a sterol sensor. In sterol-deprived cells, SCAP binds SREBPs and escorts them from the ER to the Golgi apparatus where SREBPs are proteolytically processed to yield active fragments that enter the nucleus and induce the expression of their target genes (e.g., LDLr, HMGR). On the other hand, when intracellular sterol content increases, SCAP binds the insulin induced gene protein, which keeps the SCAP/SREBP complex into the ER, thus blocking the transcription of cholesterogenic genes. This intricate mechanism of regulation is at the root of cholesterol homeostasis maintenance.

The cholesterol pool obtained from *de novo* synthesis by hepatocytes can be enzymatically esterified by Acyl-CoA-sterol Acyl transferase and incorporated into apolipoprotein B (apoB)-100-containing very low density lipoproteins (VLDL), which are then secreted into the bloodstream for transport to peripheral tissues. Cholesterol synthesis in the peripheral tissues also contributes to the hepatic cholesterol pool through the transfer of cholesterol to the liver in a process mediated by high density lipoprotein (HDL) particles (known as reverse cholesterol transport). Dietary cholesterol is also transferred from the intestine to the liver by HDL; all HDL particles are internalized in the liver, interacting with the hepatic scavenger receptor (SR-B1).

Once in the hepatocyte, cholesterol delivered by HDL particles may be utilized for hepatic cholesterol needs, converted into bile acids, or excreted into bile and eliminated through the feces.

**CHOLESTEROL METABOLISM Deregulations**

**Familial hypercholesterolemia (FH)**, defined as the heritable occurrence of severe hypercholesterolemia with cholesterol deposits in tendons and premature heart disease, is caused by mutations in at least four genes whose products are involved in sterol and lipoprotein pathways: the LDLr, apoB, proprotein convertase subtilisin/kexin 9 (PCSK9) and the autosomal recessive hypercholesterolemia (ARH) adaptor protein. All of these disorders have a defective clearance of LDL in common, principally in the liver, within a complex system of lipid and lipoprotein metabolism and regulation.

**Familial hypercholesterolemia**

The primary causative defects in approximately 85% of FH cases are mutations or deletions in the liver plasma membrane LDLr. Over 1000 different mutations in the LDLr gene on the distal short arm of chromosome 19 (p13.1-p13.3) have been described to date, such as large rearrangements, premature stop codons, single amino acid substitutions, mutations in the promoter region that affect gene transcription, and mutations that affect splicing of the pre-messenger RNA (pre-mRNA).

LDLr is a cell-surface glycoprotein that specifically binds extra-cellular lipoprotein particles containing apoB100 such as LDL with high affinity. The receptor:lipoprotein complex is then internalized by endocytosis.
via clathrin-coated pits involving the specific clathrin adaptor ARH, and delivered first to early and then to late endosomes, where the acidic environment promotes dissociation of the receptor and the lipoprotein. The receptor recycles to the cell surface whereas the lipoprotein is degraded in lysosomes to release free cholesterol that regulates transcription of the LDL-receptor gene and genes involved in cholesterol biosynthesis [13].

Owing to mutations in both alleles of the LDLr locus, homozygous LDLr-associated FH patients present markedly elevated total serum cholesterol (>500 mg/dL, 13 mmol/L) and LDL-cholesterol levels (LDL-C, >450 mg/dL, 11.7 mmol/L). The deposition of insoluble cholesterol causes xanthomata on the tendons of the hands and feet and cutaneous planar and corneal arcus in early life. Atheroma of the aortic root and valve can lead to myocardial infarction (MI) and sudden death before the age of 30 years.

Heterozygous patients typically have a lower serum cholesterol level (250-450 mg/dL or 6.5-11.6 mmol/L) and LDL-C (200-400 mg/dL or 5.2-10.4 mmol/L) with positive age correlation. They develop the above clinical features at a less accelerated rate but if untreated most suffer a severe MI and often sudden death or other cardiovascular events in the fourth or fifth decade of life [12].

Autosomal recessive hypercholesterolemia

The recessive rather than dominant pattern of inheritance of severe hypercholesterolemia is referred to as ARH. In patients suffering from ARH, LDLs cannot be taken up into cells, even although the LDL-receptor protein is produced normally. Instead, recessive null mutations in LDLRAP1 (or ARH) are observed [14].

The LDLRAP1 protein seems to work as an accessory adaptor protein which interacts with LDLr, enabling the receptor to engage with clathrin coated pit machinery for endocytosis. The phenotype in ARH is similar to that of patients with homozygous FH but is somewhat milder in terms of serum total cholesterol and LDL cholesterol levels [13].

Mutation in PCSK9

Mutations in PCSK9, a gene that encodes a putative protease subtilisin/kexin type 9 (PCSK9), were observed to cosegregate with severe hypercholesterolemia in a number of families in several countries. PCSK9 undergoes intra-cellular autolysis and the cleaved protein is then secreted from the hepatocyte, together with the cleaved fragment that remains tightly associated. Once in the circulation, PCSK9 binds with high affinity to the extracellular region of the LDLr and is internalized with the receptor. With the acidic pH of the late endosome, the affinity of PCSK9 for the LDLr increases and the complex fails to dissociate. This has the apparent result of directing the LDLr to the lysosome for degradation, thereby preventing it from recycling normally to the cell surface for further rounds of LDL uptake [14].

Some patients suffering from hypercholesterolemia show PCSK9 gain of function mutations: the explanation for the gain of function variants that cause FH seems to be simply that they have a much higher binding affinity for the LDLr, especially at acid pH [15].

Familial ligand-defective apolipoprotein B

In the 1980s, a reduced LDL turnover in some hypercholesterolemic patients without any mutation in LDLr gene was described; a single amino-acid substitution of Arg3500 with glutamine in apoB gene in its proposed LDLr-binding domain was found to cause FH in those patients. The penetrance of the mutant apoB allele is not 100%, thus patients with familial ligand-defective apoB have less severe phenotypes than FH patients with LDLr mutations [11,14]. This mutant apoB allele is common in Europe, where 2%-5% of hypercholesterolemic patients are homozygous for the defective allele.

Despite extensive research, only one other mutation of the apoB gene has been found that affects its receptor-binding function: a substitution of Arg3500 with tryptophan. This mutation is rare in Europe but is relatively common in the Chinese population [17].

Other candidate genes

Variants in genes involved in cholesterol metabolism, such as CYP7A1, SREBP-2 and SCAP, have been found in patients with FH [18], even although the evidence that these variants cause the phenotype is not strong [19].

AGE-RELATED HYPERCHOLESTEROLEMIA

Recent findings have shown that increased plasma cholesterol levels and hepatic cholesterol synthesis are accompanied by full activation of HMGCR in the liver of aged rats, where the mitochondria produce significantly higher levels of superoxide ions and the ability of cells to remove deleterious surpluses of free radicals is strongly reduced, thus leading to a rise in intracellular ROS content [20].

According to the free radical theory of aging, the age-related deregulation of HMGCR is accounted for by the increase of reactive oxygen species (ROS) levels that induce the dephosphorylation and the consequent full activation of HMGCR [21].

Moreover, while many studies have established that susceptibility to coronary artery disease (CAD) increases with age, little is known about the mechanisms underlying the increased incidence of CAD in postmenopausal women compared to men of the same age.

Studies carried out on the liver of 12 mo old estrogen-paulous rats whose estrogen levels are decreased, showed that the animals have higher levels of plasma cholesterol, increased activation of HMGCR, and decreased LDLr membrane exposure than 3 mo old female rats. These changes result in a reduction of cholesterol uptake and an increase of cholesterol synthesis, supporting the correlation between hypercholesterolemia, aging and estropause.
Increased activation of HMGR does not depend on an increase in ROS, as seen in aged-matched male rats\(^{19,24}\).

Different types of hypercholesterolemia are summarized in Table 1.

### PHARMACOLOGICAL TREATMENT OF HYPERCHOLESTEROLEMIA

#### Statins

As described above, the decrease of intracellular cholesterol leads to a homeostatic response which induces the up-regulation of cell-surface receptors that bind atherogenic lipoproteins, which are taken up into cells and degraded. Thus, the reduction of hepatic cholesterol synthesis via HMGR inhibition is an attractive approach for the treatment of dyslipidemia.

Statins, strong HMGR inhibitors, are widely used in therapies against hypercholesterolemia and they are available or in late-stage clinical development. Statin treatment strongly reduces MVA production and, as a consequence, hepatic cholesterol biosynthesis. Although these drugs are generally well tolerated, statins can lead to several side effects, the most frequent is myopathy. Statin-associated myopathy is characterized by a wide spectrum of symptoms, ranging from myalgia up to life-threatening rhabdomyolysis\(^{20,22}\). These adverse effects could be ascribable to the decrease of some HMGR end-products such as prenyls or ubiquinone\(^{29}\).

#### Niacin

Nicotinic acid (niacin) has long been used for the treatment of cholesterol disorders and CAD. Recently, new findings have provided new insights into the molecular mechanisms by which this compound is able to regulate lipid metabolism. For instance, niacin pharmacological doses reduce apoB100-containing lipoproteins. The liver is the most important organ for the synthesis and secretion of apoB100, its associated lipids and, subsequently, lipoprotein particles\(^{24}\). Thus, the hepatic apoB100 processing plays a pivotal role in the modulation of apoB100-rich lipoprotein secretion. It has been demonstrated that niacin increases the intracellular apoB100 degradation and in turn reduces its secretion in HepG2 cell line\(^{21}\). This mechanism of action is at the root of the lower plasma atherogenic lipoprotein levels observed in hyperlipidemic patients who have undergone niacin pharmacological treatment\(^{28}\).

In addition to the intestine, the liver is the major organ for the synthesis and secretion of apoAI and HDL particles. Studies on plasma HDL turnover showed that niacin decreases the fractional catabolic rate of HDL-apoA without altering apoA biosynthesis. In particular, this effect is due to a niacin-dependent inhibition of HDL-apoAI uptake, as demonstrated in HepG2 cells\(^{27}\). This mechanism, by which niacin reduces HDL-apoAI catabolism, is responsible for the increase of HDL half-life, thereby enhancing cholesterol efflux and reverse cholesterol transport\(^{29}\). Although effective in plasma cholesterol lowering at pharmacological doses, niacin is responsible for a wide range of side effects. The most common is the onset of cutaneous flushes that result from the prostaglandin D2-mediated vasodilatation of small subcutaneous blood vessels. Several gastrointestinal adverse effects, such as nausea, vomiting, dyspepsia and abdominal pain, can also occur. However, the most severe niacin-induced toxicity is hepatotoxicity, which is accompanied by an increase in hepatic transaminase levels\(^{29}\).

#### Fibrates

Several studies have shown that fibrate therapies can lead to an overall benefit on plasma cholesterol levels. Fibrates exert their primary effects on the regulation of cholesterol levels by activating peroxisome proliferator-activated receptor alpha (PPAR\(\alpha\)), which modulates several target genes involved in lipid metabolism\(^{29}\). Besides triglycerides (TG) reduction by the decrease of both hepatic apoC\(\Pi\) and apoC\(\III\)\(^{10,31}\), the fundamental hypocholesterolemic action of fibrates is the promotion of apoA1 and apoA1 biosynthesis in the liver, which are the main apolipoproteins present in HDL\(^{15}\). Fibrates, modifying HDL metabolism, are able to regulate the reverse cholesterol transport pathway. Specifically, these PPAR\(\alpha\) agonists increase pre-\(\beta\)-1-HDL levels in patients with metabolic syndrome, induce the activity of adenosine triphosphate-binding cassette transporter (ABCA1) and decrease total plasma cholesteryl ester transfer protein activity\(^{15}\). Fenofibrate also reduces apoB100 levels as a result of reduced hepatic synthesis and secretion of TG, not by a direct influence on apoB100 production. Moreover, fibrates have shown the ability to reduce cholesterol biosynthesis.

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**Table 1 Summary of the different causes of hypercholesterolemia**

| Disease | Cause | References |
|---------|-------|------------|
| LDLr-related familial hypercholesterolemia | Over 1000 different mutations in LDLr gene | [12,13] |
| PCSK9-related hypercholesterolemia | Mutations in PCSK9 gene | [13,14] |
| Other mutation-related hypercholesterolemia | Mutations in CYP7A1, SREBP-2, SCAP genes | [14,18] |
| Autosomal recessive hypercholesterolemia | Recessive null mutations in LDLRAP1 gene | [13,14] |
| Familial ligand-defective apolipoprotein B | Mutations in apol gene | [15-17] |
| Age-related hypercholesterolemia | Increased activation of HMGR | [4,19,20] |

LDLR: Low density lipoprotein receptors; PCSK9: Proprotein convertase subtilisin/kexin 9; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase.
through HMGR inhibition and by increasing cholesterol excretion into bile[34]. Despite the efficacy of the class of these compounds in modulating cholesterol metabolism, fibrate therapy has at times been discontinued because of adverse effects, such as myopathy, cholelithiasis and venous thrombosis[35].

NOVEL HYPOCHOLESTEROLEMIC STRATEGIES: FUTURE PERSPECTIVES

Non-statin enzyme inhibitors
Owing to the side effects of statin treatment, new molecules for the prevention of hypercholesterolemia should be considered. In particular, the development of new compounds that are able to inhibit cholesterol synthesis by blocking enzymes downstream of HMGR are interesting (Figure 1). Indeed, cholesterol-lowering agents targeting enzymes below the farnesyl pyrophosphate branch point of the cholesterol biosynthetic pathway might offer the possibility of removing the adverse effects of statins and be beneficial to patients suspected to be at risk of muscular damage.

Squalene synthase (SQS) is one of the most known enzymes of the MVA pathway since it catalyzes the first committed step of the de novo cholesterol biosynthesis. Inhibitors of this enzyme could be good candidates to be hypocholesterolemic drugs. Indeed, SQS inhibitors reduce plasma LDL levels and, as a consequence, increase the hepatic expression and membrane exposure of LDLr, as reported for statins[36]. Furthermore, since SQS, unlike HMGR, is not the major regulatory enzyme of the cholesterol biosynthetic pathway, it is less subject to feedback regulation. This important feature limits the induction of upstream and downstream enzymes of the MVA pathway that could participate to increase the rate of atherogenic lipoprotein production[37]. The compound EP2306 is one of the most promising SQS inhibitors in hypercholesterolemia treatment if it is considered that 2 mg/kg EP2306 significantly reduces total cholesterol and atherosclerotic lesions in a cholesterol-fed rabbits. Moreover, treatment with EP2306 does not affect liver transaminases or induce any histopathological change in several organs[38], thus indicating that this SQS inhibitor could prevent atherosclerosis-related disorders without inducing side effects.

Squalene epoxidase (SQLE), is a FAD containing enzyme located in the endoplasmatic reticulum catalyzing the epoxidation of squalene and producing 2,3-oxidosqualene[39,40]. Only recently, SQLE inhibitors have received attention as drugs for hypercholesterolemia treatment if it is considered that 2,3-monoepoxysqualene is a potent inhibitor of HMGR and is reported as less toxic than statins[41]. Several OSC inhibitors have been reported to show in vitro and in vivo potency, exerting deep lipid-lowering effects[42]. Furthermore, OSC downregulation stimulates HMGR degradation. OSC inhibition does not induce the overexpression of HMGR because of an indirect and negative feedback regulatory mechanism involving the production of 24(S),25-epoxycholesterol. This negative feedback potentiates synergistically the primary inhibitory effect with an indirect inhibition of HMGR[43,44]. The treatment with OSC inhibitors is not associated with the development of the range of severe side effects that are commonly reported for statins.

Microsomal triglyceride transport protein inhibitors
Microsomal Triglyceride Transport Protein (MTP) is an endosomal protein, mainly expressed in gut and liver, which catalyzes the assembly of cholesterol, triglycerides and apoB to form VLDL or chylomicrons. Given the importance of this protein in atherogenic lipoprotein production, MTP inhibitors are good candidates to lower plasma cholesterol levels. Indeed, MTP inhibitors block fat intestinal absorption and reduce hepatic secretion of VLDL[45]. Phase 2 clinical trials have shown that MTP inhibitor AEGR-733 monotherapy led to a significant dose-dependent decrease in LDL cholesterol. On the other hand, MTP treatment can also cause hepatic steatosis, elevated transaminase plasma levels and other gastrointestinal side effects[46].

ApoB100 antisense oligonucleotides
Antisense Oligonucleotides (ASOs) are single-stranded DNA sequences that correspond to a specific mRNA.
ASOs, binding to mRNA by Watson-Crick hybridation, are able to induce the degradation of specific mRNAs. ApoB100 appears to be a good target for ASO therapy. Moreover, as far as we know, ASO is the only oral small molecule able to inhibit this protein production in the liver\[49\]. Phase 2 clinical trials demonstrated that the second-generation ASO apoB100 ( mipomersen) induces a significant dose-response decrease in LDL cholesterol and apoB100 levels\[49\]. A LDL reduction ranging from 30% to 50% has been also reached in small trials but dose limitations have to be taken into account because of transaminase elevation and injection site reactions\[47\].

**PCSK9 inhibition**

As described above, PCSK9 plays a major regulatory role in cholesterol homeostasis. Given the important function of PCSK9 in regulating cholesterol plasma levels, pharmacological strategies that result in the inhibition of PCSK9 biosynthesis or in the inhibition of the binding of this protein to the LDLr could be useful in hypercholesterolemia treatment. Chan et al demonstrated that infusion of a humanized murine monoclonal antibody that binds to PCSK9, thus preventing LDLr internalization and the subsequent degradation, is able to reduce LDL levels by 80% at 10 d. Moreover, new studies have demonstrated that the decrease in hepatic PCSK9 production through ASOs administration is associated with lower circulating LDL levels\[50\].

**Antioxidants**

Mevalonate pathway deregulation by ROS clearly suggests that antioxidant compounds could play a significant role in restoring HMGR activity. For instance, \( \omega-3 \) fatty acids completely prevented the ROS-induced age-related hypercholesterolemia in 24 mo old rats by exerting a powerful reduction of hepatic intracellular ROS\[49\].

Other antioxidants, such as naringenin and tocochromanol, have shown the ability, not only to block the ROS-induced HMGR activation, but also to modulate the hepatic enzyme protein levels independently from their antioxidant activity\[51\].

In addition, it was recently demonstrated in the HepG2 hepatocarcinoma cell line that a novel synthetic 4-methylcoumarin (4-methylesculetin) led to the reduction of a ROS-induced HMGR activation state through the increase of the enzyme phosphorylation. Moreover this compound modulates the protein amount of HMGR, affecting the enzyme long-term regulation\[52\].

**CONCLUSION**

Cholesterol homeostasis results from the network of complex processes mainly occurring in the liver. It is plain that even an impairment in one of the factors involved in cholesterol metabolism can cause deep alterations and, in turn, diseases such as FH and age-related hypercholesterolemia.

Considering that plasma cholesterol increase is the main cause of cardiovascular disease, cholesterol biosynthesis via HMGR and uptake via LDLr were the targets of hypercholesterolemia treatment: thus statins, able to inhibit intracellular cholesterol synthesis and, as a consequence, to increase LDLr membrane exposure, have been considered the golden standard against hypercholesterolemia. Nevertheless, since disruption of cholesterol homeostasis can be ascribable to other factors in addition to HMGR and LDLr deregulation, the current editorial highlights how hypercholesterolemia treatment should be supported by a specific diagnosis and, in turn, adapted to the identified causes of plasma cholesterol increase.

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