Novel pathways in cholesterol metabolism to combat cardiometabolic diseases

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General introduction and outline

1.1 General introduction

Cholesterol is an essential substance for all mammals because of its irreplaceable role in both cell structural organization and many metabolic processes, including the synthesis of steroid hormones, bile acids and vitamin D [1]. Cholesterol can be derived from dietary intake of animal products and *de novo* biosynthesis. Since cholesterol and cholesteryl esters, *i.e.* the storage form of cholesterol, have poor water solubility, cholesterol is mainly transported between cells and tissues in our body via lipoproteins. The widespread utilization of cholesterol implies that its transport and metabolism must be strictly regulated. Both genes and environment can influence circulating cholesterol: *i.e.*, genetic variation as well as improper lifestyle habits, *e.g.* excess dietary cholesterol and lipid intake, may cause high levels of circulating cholesterol. This is also known as hypercholesterolemia, which is a major risk factor of various cardiometabolic diseases, including atherosclerotic cardiovascular disease (CVD) and nonalcoholic fatty liver disease (NAFLD). Thus, cholesterol-lowering management is one of main strategies to prevent these cardiometabolic diseases. Statins are effective cholesterol-lowering agents and are, therefore, widely used in the clinic, but only prevent about 25-45% of all cardiovascular events [2-4], indicating the need for new cholesterol-lowering and other strategies to combat cardiometabolic disease. Liver X receptors (LXR) and farnesoid X receptor (FXR) are key metabolic nuclear receptors that function as intracellular sensors for sterols and bile acids, respectively, to maintain (chole)sterol homeostasis. More insight into their role in cholesterol metabolism may result in therapeutic application of the LXR and FXR pathways to combat cardiometabolic diseases. Recent studies show that brown fat (brown adipose tissue, BAT) is emerging as a promising therapeutic target to regulate glucose and lipid metabolism [5-7]. However, the precise mechanisms how brown fat regulates cholesterol metabolism, and whether brown fat activation may add to current lipid-lowering strategies in combating cardiometabolic disease, is still not fully understood. In this chapter, a general introduction of cholesterol homeostasis is provided, and potential targets and tools that regulate cholesterol metabolism and can potentially be used to combat cardiometabolic diseases are summarized.

1.2 Pathways of cholesterol metabolism

1.2.1 Dietary cholesterol metabolism

The plasma cholesterol level is the result of a delicate balance between dietary cholesterol absorption, endogenous cholesterol synthesis by mainly the liver, and cholesterol excretion via the feces. The absorption of dietary cholesterol, which is solely derived from animal products, mainly occurs in the duodenum and jejunum. Within the intestinal lumen, dietary cholesterol is presented to the brush border of mucosal enterocytes within micelles that also contain bile salts and lipid-derived metabolites including 2-monoacylglycerol and fatty acids (FA). Enterocytes take up cholesterol via Niemann-Pick C1-Like 1 (NPC1L1), FA mainly via cluster of differentiation 36 (CD36) and passive diffusion, and 2-monoacylglycerol solely by passive diffusion. Within enterocytes, cholesterol is mainly converted into its storage form cholesteryl esters (CE) through esterification with FA by the action of acyl coenzyme A: cholesterol acyl transferase (ACAT). Together with triglycerides (TG) that are resynthesized from 2-monoacylglycerol and FA, they form lipid droplets that become surrounded with phospholipids (PL) and free cholesterol, which then fuse with apolipoprotein (Apo) B48 by

the action of microsomal TG transfer protein (MTP), ultimately leading to the formation of chylomicrons (CM) [8].

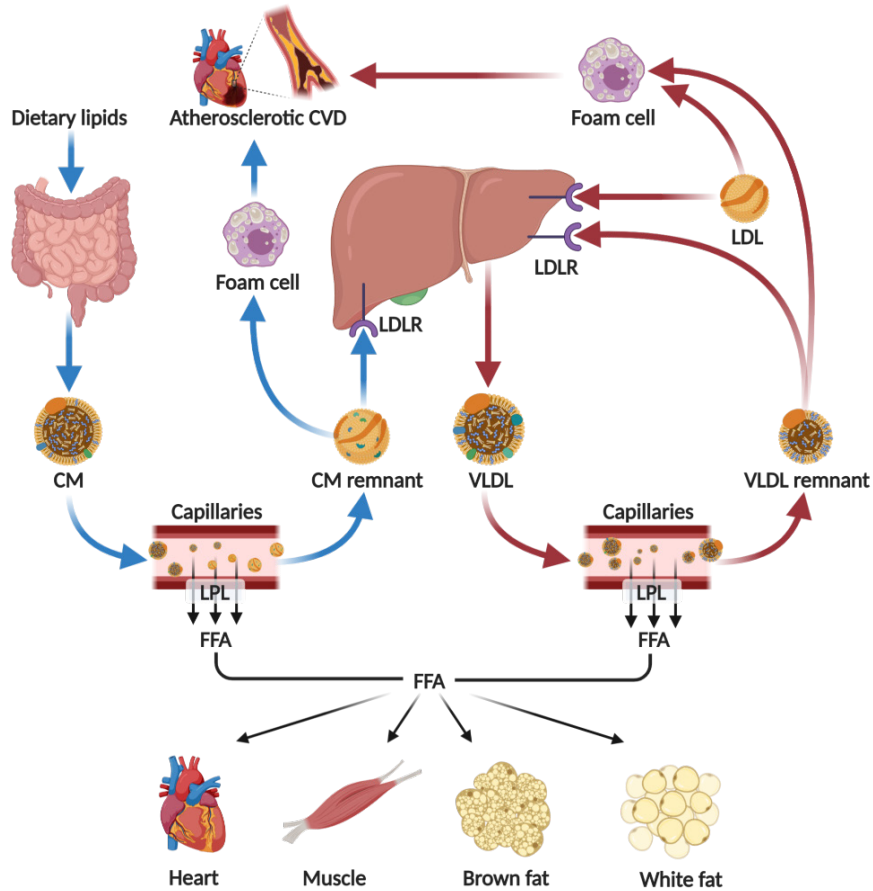

Figure 1. The exogenous (blue) and endogenous (red) lipid pathways. See text for more details. CM, chylomicrons; CVD, cardiovascular disease; FFA, free fatty acids; LDL, low density lipoproteins; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; VLDL, very low density lipoproteins

CM are secreted from enterocytes into lymphatic channels, from which they travel to the peripheral circulation (**blue pathway in Figure 1**). In the circulation, TG are offloaded from the CM particle core via interaction with lipoprotein lipase (LPL) that is expressed on capillaries of metabolically active tissues including white fat, brown fat, heart, and skeletal muscles. LPL hydrolyses TG into free FA that are subsequently taken up by those tissues via mainly CD36, and glycerol that is transported to and taken up by the liver for the generation of glucose. As a result of LPL-mediated lipolysis, CM are catabolized into TG-poor CM remnants that are enriched in CE and acquire ApoE from the circulation, which serves as a ligand for subsequent uptake of the CM remnant by mainly the low-density lipoprotein (LDL) receptor (LDLR) as well as LDLR-related protein-1 (LRP1) to a lesser extent expressed on hepatocytes of the liver. The cholesterol that is delivered from the diet to the liver can either be stored, secreted from the liver within very-low density lipoproteins (VLDL), or metabolized

into bile acids that are secreted via the bile duct into the intestine where they are involved in the emulgation and absorption of dietary lipids. In the clinic, the cholesterol absorption inhibitor Ezetimibe, which is competitive inhibitor of NPC1L1, is being used to lower plasma cholesterol levels in combination with other lipid-lowering medications, *e.g.* in patients with familial hypercholesterolemia (FH) that mainly results from a genetic deficiency for the LDLR [9].

1.1.2 Endogenous cholesterol biosynthesis

Since cholesterol is essential for the human body, a shortage of ingestion of dietary cholesterol can easily be compensated for by upregulation of *de novo* cholesterol biosynthesis. Although most cells can synthesize cholesterol, hepatocytes and enterocytes are predominantly responsible for whole-body cholesterol biosynthesis. Cholesterol synthesis begins in the cytoplasm, where one molecule of acetyl-coenzyme A (CoA) and one molecule of acetoacetyl-CoA are converted into 3-hydroxy-3-methylglutaryl (HMG)-CoA by HMG-CoA synthase (HMGCS) (**Figure 2**). The subsequent steps of cholesterol synthesis occur in endoplasmic reticulum (ER) where HMG-CoA is metabolized to mevalonate by HMG-CoA reductase (HMGCR), the rate-limiting step of the whole process of cholesterol synthesis [10]. Mevalonate is then sequentially converted into isopentenyl pyrophosphate, squalene and lanosterol. Lanosterol can then enter two pathways leading to the synthesis of cholesterol, *i.e.*, the Bloch pathway and the Kandutsch-Russell pathway. In the Bloch pathway, lanosterol is converted via a series of side-chain unsaturated intermediates into desmosterol, which via Δ24-dehydrocholesterol reductase (DHCR24) is finally converted into cholesterol [11] (**blue pathway in Figure 2**). In the Kandutsch-Russell pathway, dihydrolanosterol is converted into cholesterol via a series of side-chain saturated intermediates, by using the same enzymes as in the Bloch pathway [12] (**red pathway in Figure 2**).

DHCR24 not only converts desmosterol into cholesterol, but can also convert any unsaturated intermediate within the Bloch pathway into the corresponding saturated intermediate within the Kandutsch-Russell pathway. The reason why these two pathways are maintained in cholesterol biosynthesis is still not fully clear. Interestingly, the relative use of the two pathways is variable in different tissues. For example, testes, spleen and adrenal glands almost exclusively utilize the Bloch pathway to synthesize cholesterol. In contrast, the Kandutsch-Russell pathway is the preferred pathway in brain, skin and preputial glands to finalize cholesterol biosynthesis [13]. Studies further show that the intracellular cholesterol content selectively regulates the Bloch pathway, without affecting the Kandutsch-Russell pathway [13, 14]. The distinct properties of intermediates of the two pathways in cholesterol homeostasis, FA synthesis, and inflammation may somehow explain why both pathways are maintained [15-17]. As a typical example, desmosterol, the terminal intermediate of cholesterol synthesis, is increased with upregulation of the Bloch pathway and is an endogenous LXR ligand to promote cholesterol efflux, *e.g.* in macrophages [15, 17]. This indicates the importance of the Bloch pathway in the regulation of cellular cholesterol levels. Further studies are required to investigate the specific roles of the other intermediates of cholesterol synthesis, and whether DHCR24 inhibition by small molecules, via increasing desmosterol, can improve cardiometabolic health.

Desmosterol, in addition to some other sterols, can regulate cholesterol biosynthesis by acting on sterol regulatory element binding proteins (SREBPs) [18]. In the presence of high cellular sterol concentrations, a sterol domain within the SREBP cleavage activating protein (SCAP) confines SREBPs to the ER. When cellular concentration of cholesterol is relatively low, SCAP escorts SREBPs to undergo proteolytic cleavage steps and to translocate to the

nucleus, where SREPBs activate target genes involved in the synthesis of both cholesterol and FA [19, 20]. Sterols can also induce HMGCR degradation within the proteasome, which is another important negative regulatory mechanism of cholesterol synthesis [21]. HMGCR contains a sterol-sensing domain (SSD) so that when the flux through the mevalonate synthesis pathway is high, the degradation of HMGCR increases. The primary sterol regulating HMGCR degradation is cholesterol itself, the concentration of which can be affected by both endogenous synthesis and cellular uptake. Being the rate-limiting enzyme in cholesterol synthesis, pharmacological interventions inhibiting HMGCR, such as statins, have been widely applied in the clinic as the main strategy to lower plasma cholesterol.

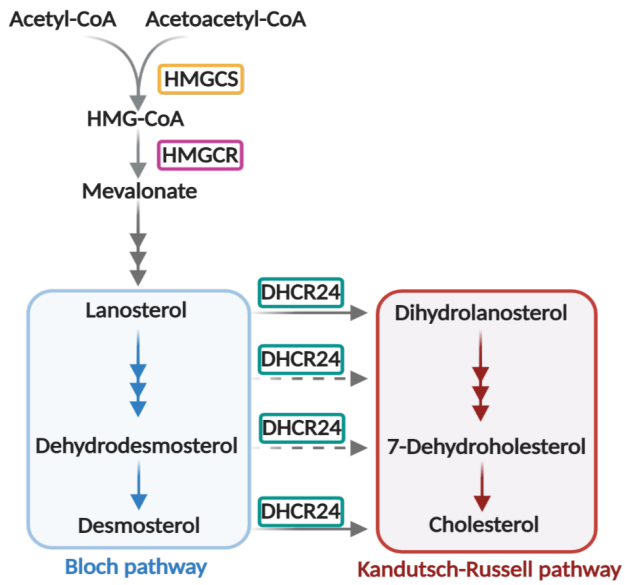

Figure 2. Simplified cholesterol biosynthesis pathways. *De novo* cholesterol biosynthesis begins with acetyl-coenzyme A (CoA) and branches into two pathways: the Bloch pathway (blue) and the Kandutsch-Russell pathway (red). See text for more details. DHCR24, Δ24-dehydrocholesterol reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HMGCS, 3-hydroxy-3-methylglutaryl-coenzyme A synthase

1.2.3 Role of lipoproteins in cholesterol transport

As explained in section 1.2.1 (**Figure 1**), cholesterol circulates within lipoproteins both as free cholesterol within the particle shell, while the majority is carried as CE within the particle core. Lipoproteins are complex particles with a central hydrophobic core of non-polar lipids, primarily CE and TG, which is surrounded by an amphiphilic monolayer consisting of PL and free cholesterol, in which apolipoproteins are embedded. Based on their size, density and composition, lipoproteins are classified into CM, VLDL, intermediate-density lipoproteins (IDL), LDL and high-density lipoproteins (HDL), from largest size and lowest density to smallest size and highest density, respectively.

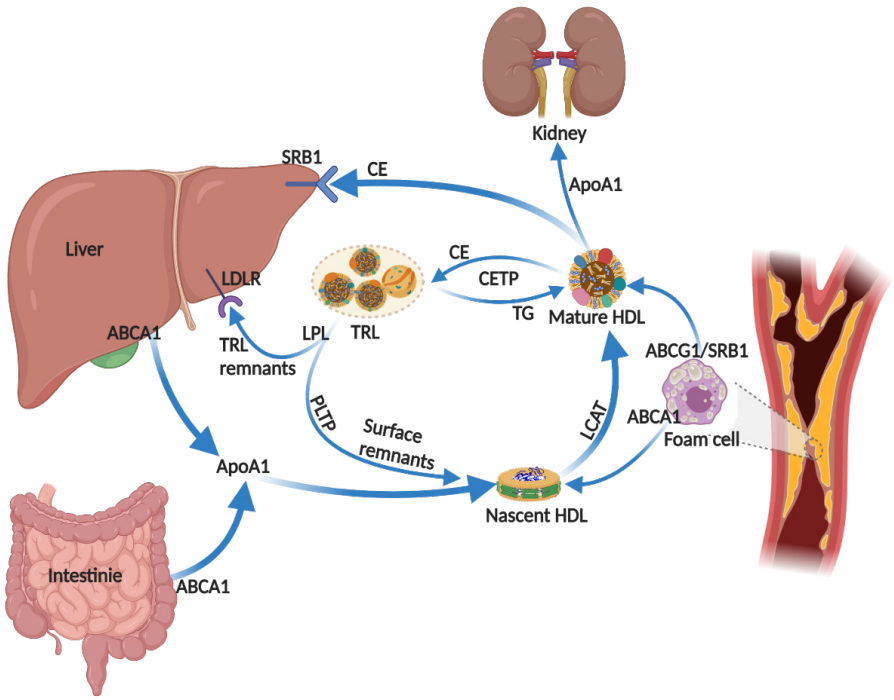

Figure 3. HDL biogenesis and its role in cholesterol metabolism. See text for more details. ABCA1, ATP-binding cassette sub-family A member 1; ABCG1, ATP-binding cassette sub-family G member 1; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; HDL, high density lipoproteins; LCAT, lecithin: cholesterol acyltransferase; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; PLTP, phospholipid transfer protein; SRB1, scavenger receptor class B type 1; TG, triglycerides; TRL, TG-rich lipoproteins

In the exogenous lipid pathway, dietary lipids are incorporated in the small intestine into CM, as explained in section 1.2.1 (**Figure 1**). In the endogenous lipid pathway, lipids within the liver are secreted as VLDL after MTP-induced fusion of intracellular lipid droplets with ApoB100. Both CM and VLDL are TG-rich lipoproteins (TRL), which primarily serve to deliver TG-derived FA mainly to metabolic tissues where they can be stored (white fat) or combusted by beta-oxidation to generate ATP (heart, skeletal muscle) and heat (brown fat) [7, 22]. To this end, TRLs interact with LPL expressed by those tissues to locally release FAs that are taken up mainly via CD36. by these tissues. During LPL-mediated lipolysis, the generated TRL remnants become (further) enriched with ApoE that serves as a recognition signal for subsequent uptake by hepatocytes by receptors and binding sites including the LDLR and LRP1 [23]. Among these, LDLR is the main receptor contributing to the hepatic uptake of TRL remnants. Instead of being taken up by the liver, VLDL remnants, also termed as IDL, can be further metabolized into LDL. As the lipolytic end product of VLDL, LDL has lost all exchangeable apolipoproteins on its surface apart from ApoB100, which is the ligand for the LDLR present on the liver and other tissues. At high circulating concentrations, ApoB-containing TRL remnants and LDL are pro-atherogenic as under inflammatory conditions they can extravasate and accumulate in intima of the arterial wall. Here, they can be modified by oxidation and/or aggregation, upon which they are taken up by macrophages to become lipid-rich foam cells, which is the initial step of atherosclerosis development.

Interestingly, after taking up ApoE-containing TRL remnants and ApoB100-containing LDL, the LDLR partly escapes lysosomal degradation and recycles to the cell membrane to bind and internalize additional lipoproteins. However, hepatocytes also express proprotein convertase subtilisin/kexin type 9 (PCSK9), which is able to bind to LDLR and targets the LDLR for lysosomal degradation [24, 25]. Therefore, reduction of PCSK9 function has been developed as a pharmacological strategy in hypercholesterolemia to increase LDLR levels and consequently lower atherogenic lipoproteins. Thus far, two PCSK9 monoclonal antibodies have been approved for the treatment of heterozygous FH individuals, *i.e.* Alirocumab and Evolocumab [26], although the effects of PCSK9 inhibitors on reducing the risk of CVD have not yet been evaluated.

HDL particles are generated in the liver and intestine through synthesis of its main apolipoprotein ApoA1 (**Figure 3)**. During its release via the ATP binding cassette sub-family A member 1 (ABCA1), ApoAI is lipidated with PL forming nascent cholesterol-poor discoidal HDL particles. Nascent HDL particles acquire additional PL and cholesterol via cellular efflux from peripheral cells and tissues, *e.g.* from resident macrophages in the arterial wall, as well as by transfer of TRL surface components during LPL-mediated lipolysis via phospholipid transfer protein (PLTP) [27]. The enzyme lecithin: cholesterol acyltransferase (LCAT) on HDL esterifies the cholesterol to form CE, which results in CE accumulation in the core of HDL, and formation of larger spherical HDL particles. During the process of HDL maturation, HDL particles facilitate cellular cholesterol efflux from peripheral tissues by sequentially binding ABCA1 and ATP-binding cassette sub-sub-family G1 (ABCG1) or scavenger receptor class B type 1 (SRB1) [28-30]. Subsequently, hepatocytes in the liver can selectively take up CE from HDL via SRB1, after which CE is hydrolyzed and can be secreted into the bile as neutral sterols and after enzymatic modification as bile acids (as detailed in section 1.2.4) [31]. In humans and some other species, *e.g.* rabbits, CE in HDL can be also transferred to ApoB-containing TRLs (chylomicrons and VLDL) directly in exchange for TG via the cholesteryl ester transfer protein (CETP). TG within HDL can be further hydrolyzed by LPL resulting in smaller HDL particles which re-enter the cycle of peripheral cholesterol uptake, or by hepatic lipase (HL), which results in lipid-poor ApoA1 that can be secreted via the kidneys. The process in which HDL delivers cholesterol from peripheral tissues back to liver (**Figure 3**) where cholesterol is secreted into the bile as neutral sterols and bile acids is termed reversed cholesterol transport (RCT). Ever since HDL-cholesterol (HDL-C) levels were found to be inversely correlated with the risk of CVD, HDL has generally been considered to be atheroprotective [32], in contrast to ApoB-containing lipoproteins. However, genetic studies have recently demonstrated that HDL-C is not causal in reducing myocardial infarction [33]. Therefore, HDL is now thought to play an antiatherogenic role mainly by its contribution to RCT, and in addition by its anti-inflammatory, antioxidant, beside other beneficial properties [34-36]. In line with this assumption, HDL cholesterol efflux capacity (CEC) has been demonstrated to be a better measure of HDL atheroprotective capacity than HDL-C levels [37, 38].

1.2.4 Excretion of cholesterol

It should be clear by now that the liver plays a central role in body cholesterol homeostasis through *de novo* cholesterol synthesis (see section 1.2.2), the release of cholesterol into the circulation as VLDL (see section 1.2.3), and the uptake of cholesterol from the circulation contained in TRL remnants, LDL and HDL (see section 1.2.1 and 1.2.3). In addition, the liver is able to metabolize cholesterol into neutral sterols and bile acids, which is a major pathway

of cholesterol catabolism, as will be explained in detail in this section.

The metabolism of cholesterol into bile acids can be initiated by either 7α-hydroxylase (CYP7A1) within the classic pathway, or by mitochondrial sterol 27-hydroxylase (CYP27A1) within the alternative pathway. The classic pathway only exists in hepatocytes, while the alternative pathway is present in all tissues [39]. In humans, cholic acid (CA) and chenodeoxycholic acid (CDCA) are two main products in the classic pathway of bile acid synthesis. In peripheral tissues, cholesterol can also be oxidized to oxysterols, *e.g.* 24-hydroxycholesterol in brain and 27-hydroxycholesterol in lungs, and can be transported to the liver and further converted to CA and CDCA [40]. *De novo* synthesized CA and CDCA are classified as primary bile acids, and can be conjugated in the hepatocyte with glycine and taurine after which they are stored in the gallbladder [39]. Upon food intake, cholecystokinin, a peptide hormone secreted by the duodenum triggers the release of bile acids from the gallbladder into the duodenum to facilitate emulgation and uptake of lipids and fat-soluble vitamins [41]. Under the action of intestinal bacterial flora, some conjugated CA and CDCA are deconjugated and converted by 7α-dehydroxylase into so-called secondary bile acids including deoxycholic acid (DCA) and lithocholic acid (LCA) [39]. In contrast to humans, rodents demonstrate a different composition of bile acids. For instance, in mice CDCA can be further hydroxylated to muricholic acid (MCA) α and β to constitute primary bile acids in mice together with CA and CDCA [42].

Most bile acids that are secreted from the liver into the duodenum (*i.e.* approx. 95%) are reabsorbed in the jejunum and colon through passive diffusion or in the terminal ileum through an active transport process involving the apical sodium-dependent bile salt transporter (ASBT) and intracellular binding to intestinal bile acid-binding protein (IBABP) [43]. After bile acids enter enterocytes across the apical membrane, they exit across the basolateral membrane via the heteromeric organic solute transporter α/β (OSTα/β) [44] and are redirected to the portal vein. At the basolateral membrane of hepatocytes, bile acids are extracted by the Na^+-taurocholate co-transporting polypeptide (NTCP) into hepatocytes for a next cycle [45]. The process in which bile acids circulate between hepatocytes in the liver and enterocytes in the intestine is termed as the enterohepatic circulation of bile acids [46]. The remainder of bile acids (approx. 5%) is excreted via feces, which is compensated for by bile acid synthesis in the liver. This biliary pathway of cholesterol excretion is depicted as the green pathway in **Figure 4**.

Besides acting as biological detergents that facilitate intestinal absorption of lipids and fat-soluble vitamins, bile acids are also ligands for several nuclear hormone receptors, including farnesoid X receptor (FXR) and G-protein-coupled receptors such as Takeda G-coupled receptor 5 (TGR5) to modulate energy metabolism [47-50]. Therefore, bile acids broadly function as metabolic modulators in the body. Therapeutically, inhibiting bile acid reabsorption by using *e.g.* the bile acid sequestrant Colesevelam has been proven as a feasible way to lower LDL-cholesterol and also to improve insulin sensitivity in the clinic [50, 51].

In addition to biliary cholesterol output as neutral sterols and bile acids, transintestinal cholesterol excretion (TICE) is the non-biliary second pathway contributing to total cholesterol output from the body in both mice and humans (~30% under basal conditions) [52, 53] (**red pathway in Figure 4**). In the process of TICE, ApoB-containing lipoproteins recognized by the proximal small intestine though receptors like LDLR cross the enterocyte in a basolateral-to-apical fashion via receptors like ATP-binding cassette sub-family G member 5/8 (ABCG5/8) and ATP-binding cassette sub-family B member 1a/b (ABCB1a/b) [53].

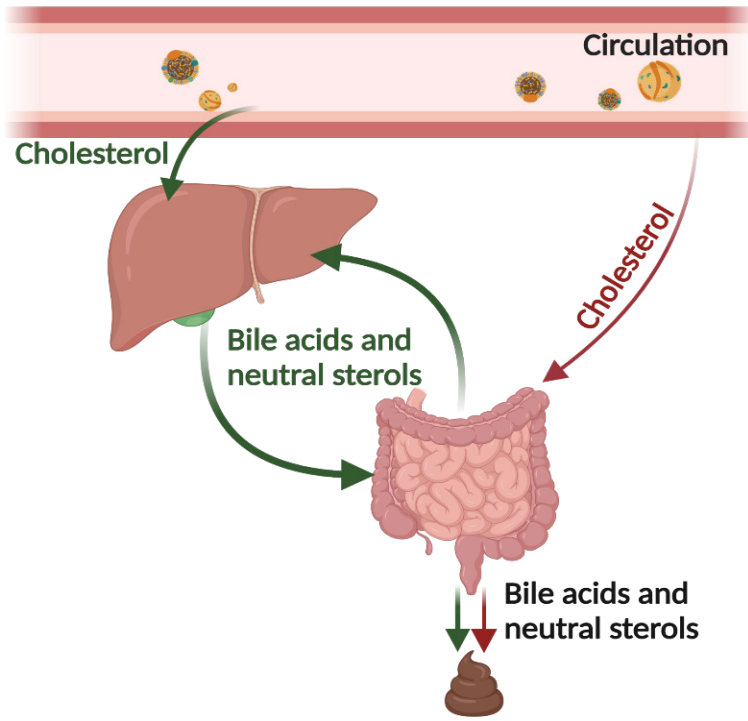

Figure 4. Cholesterol output including a biliary pathway (in green) and a non-biliary pathway, *i.e.* transintestinal cholesterol excretion (TICE) (in red). See text for more details.

1.3 LXR and FXR, the Yin and Yang regulators of cholesterol and bile acid metabolism

To maintain cholesterol homeostasis, a mechanism of negative feedback that senses cholesterol and oxysterols is well-established in the body. LXR, including the LXRα (NR1H3) and LXRβ (NR1H2) isoforms, are nuclear receptors that function as intracellular sensors for (oxy)sterols. LXRα is mainly expressed in the liver, adipose tissues, spleen, kidneys, and lungs, while LXRβ is ubiquitously expressed [54]. LXR forms an obligate heterodimer with the retinoid X receptor (RXR, NR2B1) to bind DNA, and can be activated by either LXR agonists or RXR ligands. In rodents, high cholesterol feeding results in high levels of oxysterols in the liver. In mice, oxysterol-activated LXRα directly induces CYP7A1, the rate-limiting enzyme in the classical pathway of bile acid production, to promote cholesterol conversion into bile acids thus promoting cholesterol secretion from the liver. Therefore, LXRα deficient mice following the consumption of dietary cholesterol accumulate large amounts of cholesteryl esters in the liver [55]. LXR activation also induces RCT by upregulating ABCA1, ABCG1, and ApoE to promote cholesterol efflux from peripheral tissues, particularly in macrophages back to the liver mediated by lipid-poor HDL particles [56-58]. Additionally, LXR promotes cholesterol excretion by increasing ABCG5 and ABCG8 expression in the liver and small intestine [59]. Taken together, the overall effect of LXR activation is to promote cholesterol elimination from the body. On the other hand, LXR activation regulates both innate and adaptive immune responses [60, 61].

The properties of LXR to coordinate metabolic and immune responses constitute an attractive therapeutic target for the treatment of cardiometabolic and inflammatory diseases. However, pharmacological LXR activation by synthetic LXR agonists usually causes hypertriglyceridemia and even hepatic steatosis by inducing lipogenic genes within hepatocytes including *Srebp1c* and *fatty acid synthase* (*Fas*) [62], which hampers the application of pharmacological LXR activation for disease treatment. Notably, a seminal study showed that desmosterol, the terminal intermediate of cholesterol biosynthesis (see **Figure 2**), activates LXR while inhibiting FA and TG synthesis in macrophages [17]. Importantly, desmosterol has no effect on LXR activity in hepatocytes [18]. This strongly suggests therapeutic potential of endogenous LXR ligands for clinical application in cardiometabolic disease.

As described in section 1.2.4, excess cholesterol is prone to be catabolized by hepatocytes into bile acids which can be secreted into the gut via the gallbladder and subsequently be reabsorbed in the gut (**Figure 4**) to maintain a precise balance of cholesterol and bile acids in the body. As bile acids, like cholesterol, are intrinsically toxic, bile acid production and excretion are tightly regulated to prevent excess bile acid accumulation. FXR (NR1H4), a member of metabolic nuclear receptors, is a biosensor for endogenous bile acids to maintain such bile acid homeostasis. CDCA is the most effective activator of FXR, even at physiological levels, while DCA and LCA can also activate FXR but to a much lower extent [63]. In the intestine, activation of FXR induces fibroblast growth factor (FGF) 19 in humans and FGF15 in mice, which can be transported to hepatocytes and inhibit expression of genes within the classical bile acid synthesis pathway, *e.g. Cyp7a1* and *Cyp8b1* via short heterodimer partner (SHP) [64]. Hepatic FXR activation can also directly inhibit bile acid synthesis through the same pathways, albeit to a smaller degree. On the other hand, FXR activation regulates the enterohepatic circulation of bile acids by inducing the gene expressions of BSEP, IBABP, and OSTα/β, and suppressing NTCP and ASBT to finally decrease bile acid levels in the body [65]. In humans, LXR activation directly upregulates SHP to downregulate rather than upregulate CYP7A1, and thus suppresses bile acid synthesis. This results in a relative reduction of bile acid synthesis upon oxysterol-induced LXR activation in humans, which is in contrast to rodents [66]. The overall result of FXR activation is to maintain bile acid homeostasis together with other regulators such as LXR, thereby avoiding bile acid-induced liver toxicity.

Moreover, FXR beneficially regulates lipid metabolism, as FXR deficiency increases hepatic and plasma TG levels and promotes NAFLD progression [67, 68]. A human study showed that FXR is downregulated in NAFLD patients which is associated with increased LXR, SRBEP1c and hepatic TG levels [69]. In line, both hepatic and systemic FXR activation have shown to protect against the development of hepatic steatosis [70, 71]. In addition, FXR deficient mice have lower hepatic expression of hepatic SRB1 [72] and present an atherogenic lipoprotein profile, including increased plasma TG and LDL-C levels [67]. Although these findings suggest atherogenic effects of FXR-deficiency, FXR deficient mice on chow or Western-type diet do not develop atherosclerosis and effects of FXR-deficiency on atherosclerosis development in ApoE and LDLR deficient mice are controversial [73-75]. Therefore, the role of FXR in cholesterol metabolism and atherosclerosis development should still be clarified in future studies.

1.4 Atherosclerosis and nonalcoholic steatohepatitis, two aspects of a shared disease

1.4.1 Atherosclerosis

In 2017, 3.7 – 3.9 million individuals worldwide died from ischemic heart disease and ischemic stroke [76], both of which are mainly caused by rupture of an atherosclerotic plaque. In fact, atherosclerosis is the main pathological process of most CVD. Multiple risk factors are involved in atherosclerosis development, including dyslipidemia, smoking, diabetes, hypertension, inflammation [77].

Generally, atherosclerosis is considered to be a lipid-driven inflammatory disease. Early events in the initiation of atherosclerosis are endothelial dysfunction leading to the deposition and retention of ApoB-containing lipoproteins such as LDL and TRL remnants in the vessel wall. Once trapped, these atherogenic lipoproteins become modified by oxidation and aggregation, which recruits immune cells and further activates endothelial cells [78]. Within the sub-endothelial space, recruited monocytes differentiate into macrophages that take up these modified lipoproteins via scavenger receptors and thereby slowly turn into lipid-laden foam cells. At this stage of initiation, only mild lesions or 'fatty streaks' are formed that do not yet cause clinical symptoms and are still reversible. Since endothelial dysfunction usually occurs at bifurcations, such as those of the common carotid and left coronary arteries, fatty streaks usually start there. Subsequently, the foam cells and activated endothelial cells release inflammatory cytokines, which further increases lipid accumulation and extracellular matrix deposition leading to intimal thickening. The local microenvironment promotes the proliferation of smooth muscle cell (SMC) and migration into the atherosclerotic lesion forming a fibrous cap covering the fatty streak [79]. Foam cells and SMC may eventually die during the development of atherosclerosis, resulting in the formation of a necrotic core consisting of extracellular lipids and debris. Plaque stability is dependent on its composition. Stable plaques are usually composed of solid fibrous cap with no or small amounts of extracellular lipid, and are clinically silent. By contrast, vulnerable plaques are typically characterized by large extracellular lipid deposition and much extracellular debris together with a thin fibrous cap. Vulnerable plaques are prone to rupture, at least in humans, which may lead to life-threatening events like myocardial infarction and ischemic stroke [80].

Nonsurgical treatments of atherosclerotic CVD mainly include lifestyle modulation and medication. Since atherosclerotic plaque formation is generally considered to be irreversible, current therapies mainly target to reduce risk factors to prevent atherosclerosis development. Making lifestyle changes can reduce risk factors for atherosclerosis development, such as cessation of smoking that strongly aggravates the risk for developing atherosclerosis. For that reason, some hospitals offer smoking cessation programs to help patients quit smoking. Other lifestyle managements involve regular exercise and a balanced diet. Statins are the first line drugs for reducing LDL-C levels and can reduce LDL-C levels by as much as 60% [81]. However, since current lipid-lowering strategies only prevent 25-45% of cardiovascular events [2-4], new therapies to prevent atherosclerotic CVD are evidently warranted.

1.4.2 Nonalcoholic steatohepatitis

NAFLD is one of the most common chronic liver diseases worldwide with a prevalence in the general population of ~25% in 2018 [82, 83], and is featured by hepatic fat accumulation

greater than 5% of total liver weight, caused by excessive caloric intake without excessive alcohol consumption. NAFLD has a wide histological spectrum, ranging from 'simple' steatosis to nonalcoholic steatohepatitis (NASH). NASH is a progressed form of NAFLD accompanied with inflammation with or without fibrosis and affects 3-15% of the general population [84]. Over a decade, NASH may eventually develop into cirrhosis and hepatocellular carcinoma [85]. Metabolic risk factors for NAFLD include hypertension, dyslipidemia, obesity and type 2 diabetes mellitus [86].

NAFLD/NASH is a multi-factorial disease and the mechanism underlying the formation of NASH is not fully elucidated. Within the early 'two-hit hypothesis' [87], the 'first hit' involves lipid deposition in hepatocytes, and is present in almost all patients with metabolic syndrome but is not sufficient to cause NASH. However, excess lipid accumulation increases the vulnerability of the liver to many factors, which as the 'second hit' promote liver injury, inflammation and fibrosis. These factors include oxidative stress, lipid peroxidation, cytokines, chemokines, mitochondrial dysfunction [88]. The liver is highly heterogeneous and cellular networks rather than a single cell type modulate NAFLD progression. For example, lipid-laden injured hepatocytes activate liver macrophages, including resident Kupffer cells (KCs) and macrophages derived from circulating monocytes. The activated KCs and recruited macrophages subsequently trigger an inflammatory response and induce hepatic stellate cell activation which eventually causes liver fibrosis [89, 90]. Accumulating evidence shows a key role of KCs in NAFLD progression. Inactivation of KCs via genetic intervention or antibodies reverses liver inflammation [91], and selective depletion of KCs alleviates hepatocellular damage and prevents against diet-induced hepatic steatosis and insulin resistance [92, 93]. Thus, targeting KCs could be a promising therapeutic strategy for the treatment of NAFLD/NASH. Due to advances in understanding the pathogenesis of NAFLD, it has become evident that the 'two-hit hypothesis' is too simplistic and has now been substituted by a 'multiple-hit hypothesis' to recapitulate the complexity of NAFLD progression. Such multiple hits include insulin resistance, genetic and environmental factors, nutritional factors, and gut microbiota, which act together on NAFLD progression [94].

Currently, there are no Food and Drug Administration approved medications for the treatment of NASH. Control of risk factors, such as weight loss through a combination of a healthy diet and exercise is still the only intervention in clinic. Effective and safe treatments for NAFLD/NASH are thus desperately needed.

Interestingly, although hepatocellular carcinoma is the end-stage of NAFLD progression, the first cause of mortality in patients with NAFLD is CVD [95-97], implying a close association between these diseases. In fact, atherosclerosis and NASH are not independent, but interact with each other. NASH-induced oxidative stress, inflammation and the production of hepatokines significantly increase the risk of atherosclerosis development. In addition, NASH is usually associated with obesity, dyslipidemia, diabetes, and other metabolic syndromes. Atherosclerosis is prone to occur in NASH patients. Studies further show that NAFLD is an independent risk factor for atherosclerosis and increases cardiovascular risks as well as mortality [98-100]. Actually, atherosclerosis and NASH are considered to be two aspects of a shared disease with macrophage activation and infiltration central in the progression of both diseases [101]. Therefore, some therapeutic strategies that are designed for the treatment of atherosclerosis are also being tested in NAFLD/NASH patients.

1.5 Brown fat as a potential target for the treatment of cardiometabolic diseases

Brown fat was first discovered in the marmot more than 400 years ago and has been now well studied in mice, rats and hamsters [102]. Brown adipocytes are characterized by multilocular lipid droplets and a high content of mitochondria, which express uncoupling protein 1 (UCP-1) and give rise to the typical brownish color of the tissue. Humans newborns are dependent on brown fat to maintain body temperature. Brown fat activity and mass decrease within a few months after birth, and brown fat was generally thought to be absent in adults. Interestingly, only in 2009, metabolically active brown fat was discovered in adult humans [103], which put brown fat into the spotlights. By using positron emission tomography-computed tomography (PET-CT) scans with the glucose tracer ^{18}F-fluorodeoxyglucose ([^{18}F]FDG), various research groups reported that brown fat was functionally activated by cold exposure in adult humans [104-106]. Subsequent studies showed that brown fat activation reduces body fat mass [107] and that [^{18}F]FDG uptake by brown fat inversely correlates with body mass index and fat mass [104, 105, 108, 109], suggesting the importance of brown fat in energy metabolism in humans.

Physiologically, cold exposure (< 23°C for humans, <30°C for mice) activates brown fat by stimulating the release of norepinephrine which binds β3-adrenergic receptor (AR), at least in mice [7]. Through AR stimulation, adipocytes in white adipose tissue (WAT) can also be induced to express UCP-1, leading to browning/beiging of WAT [110]. Upon activation, UCP-1 in the inner membrane of the mitochondria uncouples oxidative respiration from ATP synthase, which leads to the production of heat (termed thermogenesis) rather than synthesis of ATP [7]. Upon prolonged brown fat activation, intracellular triglycerides are depleted and replenished by TRL-derived FA via hydrolysis by LPL that is produced by brown adipocytes [22, 111]. TRL remnants that are concomitantly generated by brown fat activation are taken up mainly via LDLR by the liver [7, 111, 112]. This process also leads to the generation of TRL surface remnants that intercalate into the HDL pool, which promotes HDL turnover and SRB1-mediated RCT [113]. In humans, long term brown fat activation by cold exposure lowers LDL-C in hypercholesterolemic patients [114], and brown fat activity is associated with a reduced risk of CVD events [115]. In fact, our research group has shown that brown fact activation in APOE*3-Leiden.CETP mice, a well-established mouse model of human-like lipid metabolism and atherosclerosis development, decreases plasma cholesterol and augments RCT, collectively reducing atherosclerosis [112, 113]. Collectively, these findings imply a potential application of brown fat activation in the treatment of atherosclerotic CVD in humans. However, the mechanisms underlying the antiatherogenic effects of brown fat activation are not fully clear and whether brown fat activation is beneficial on top of other antiatherogenic treatments is currently unknown.

1.6 Outline of this thesis

This thesis aimed to elucidate novel targets involved in cholesterol metabolism for the treatment of cardiometabolic diseases including atherosclerotic CVD and NAFLD.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition, by increasing hepatic LDL receptor (LDLR) levels, has recently emerged as a strategy to reduce atherosclerosis by lowering circulating (V)LDL-cholesterol. We hypothesized that the therapeutic effectiveness of PCSK9 inhibition can be increased by accelerating the generation of TRL remnants, which typically have a high affinity for the LDLR. Therefore, in **Chapter 2** we aimed to investigate whether accelerating TRL processing through brown fat activation by β3-AR agonism can

further lower plasma cholesterol and reduce atherosclerosis on top of PCSK9 inhibition, by using APOE*3-Leiden.CETP mice, a well-established model for human-like lipoprotein metabolism and atherosclerosis development.

Brown fat activation has previously been demonstrated to attenuate atherosclerosis development by accelerating TRL turnover and/or stimulation of reverse cholesterol transport via the scavenger receptor class B type 1 (SRB1). To get more insight into the role of hepatic SRB1 in the mechanism underlying the effect of brown fat activation on alleviating atherosclerosis development, in **Chapter 3** we specifically knocked down hepatic SRB1 while activating brown fat by β3-AR agonism, and evaluated their interaction on atherosclerosis development, again using APOE*3-Leiden.CETP mice.

Previously it was observed that prolonged brown fat activation increases plasma bile acid levels accompanied by an increase in liver cholesterol, probably caused by reduced bile acid excretion. Therefore, in **Chapter 4** we hypothesized that bile acid sequestration would enhance the lipid-lowering and antiatherogenic effects of brown fat activation, and therefore studied the combined effect of the bile acid sequestration and β3-AR agonism on cholesterol and bile acid metabolism in the context of atherosclerosis development.

Next, we explored the potency of the metabolic nuclear receptors LXR and FXR in combating cardiometabolic disease. LXR is a promising therapeutic target as LXR activation reduced inflammation, but pharmacological LXR activation usually induces lipogenesis within the liver and consequently causes hyperlipidemia. As desmosterol was reported as an endogenous LXR ligand without unfavorable effects on lipogenesis, in **Chapter 5**, we aimed to evaluate whether increasing desmosterol by pharmacological DHCR24 inhibition could induce LXR activation and thereby induce anti-inflammatory effects in macrophages.

Next, since LXR agonism has theoretical potential for treating NAFLD/NASH, we hypothesized that increasing desmosterol by DHCR24 inhibition would alleviate hepatic steatosis and inflammation without inducing hyperlipidemia. Therefore, the aim of **Chapter 6** was to address this hypothesis experimentally in APOE*3-Leiden.CETP mice fed a NASH-inducing diet.

The bile acid receptor FXR represents another promising target for therapy of cardiometabolic diseases, diseases. Because the effects of pharmacological modulation of bile acid signaling and metabolism on dyslipidemia are incompletely understood, in **Chapter 7** we investigated the effects of FXR activation, again in APOE*3-Leiden.CETP mice.

Finally, the main findings of these studies and their therapeutic implications are discussed in context of current literature in **Chapter 8.**

References

1. Ikonen, E., *Cellular cholesterol trafficking and compartmentalization.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 125-38.
2. Cholesterol Treatment Trialists, C., *Efficacy and safety of statin therapy in older people: a meta-analysis of individual participant data from 28 randomised controlled trials.* Lancet, 2019. **393**(10170): p. 407-415.
3. Collins, R., et al., *Interpretation of the evidence for the efficacy and safety of statin therapy.* Lancet, 2016. **388**(10059): p. 2532-2561.
4. Jukema, J.W., et al., *The controversies of statin therapy: weighing the evidence.* J Am Coll Cardiol, 2012. **60**(10): p. 875-81.
5. Park, H., A. He, and I.J. Lodhi, *Lipid Regulators of Thermogenic Fat Activation.* Trends Endocrinol Metab, 2019. **30**(10): p. 710-723.
6. Carpentier, A.C., et al., *Brown Adipose Tissue Energy Metabolism in Humans.* Front Endocrinol (Lausanne), 2018. **9**: p. 447.
7. Hoeke, G., et al., *Role of Brown Fat in Lipoprotein Metabolism and Atherosclerosis.* Circ Res, 2016. **118**(1): p. 173-82.
8. Chang, T.Y., et al., *Acyl-coenzyme A:cholesterol acyltransferases.* Am J Physiol Endocrinol Metab, 2009. **297**(1): p. E1-9.
9. Mach, F., et al., *2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk.* Eur Heart J, 2020. **41**(1): p. 111-188.
10. Maxfield, F.R. and G. van Meer, *Cholesterol, the central lipid of mammalian cells.* Curr Opin Cell Biol, 2010. **22**(4): p. 422-9.
11. Bloch, K., *The biological synthesis of cholesterol.* Science, 1965. **150**(3692): p. 19-28.
12. Kandutsch, A.A. and A.E. Russell, *Preputial gland tumor sterols. 3. A metabolic pathway from lanosterol to cholesterol.* J Biol Chem, 1960. **235**: p. 2256-61.
13. Mitsche, M.A., et al., *Flux analysis of cholesterol biosynthesis in vivo reveals multiple tissue and cell-type specific pathways.* Elife, 2015. **4**: p. e07999.
14. Rawson, R.B., et al., *Failure to cleave sterol regulatory element-binding proteins (SREBPs) causes cholesterol auxotrophy in Chinese hamster ovary cells with genetic absence of SREBP cleavage-activating protein.* J Biol Chem, 1999. **274**(40): p. 28549-56.
15. Yang, C., et al., *Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands.* J Biol Chem, 2006. **281**(38): p. 27816-26.
16. Lange, Y., et al., *Effectors of rapid homeostatic responses of endoplasmic reticulum cholesterol and 3-hydroxy-3-methylglutaryl-CoA reductase.* J Biol Chem, 2008. **283**(3): p. 1445-55.
17. Spann, N.J., et al., *Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses.* Cell, 2012. **151**(1): p. 138-52.
18. Muse, E.D., et al., *Cell-specific discrimination of desmosterol and desmosterol mimetics confers selective regulation of LXR and SREBP in macrophages.* Proc Natl Acad Sci U S A, 2018. **115**(20): p. E4680-E4689.
19. Brown, M.S. and J.L. Goldstein, *The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor.* Cell, 1997. **89**(3): p. 331-40.
20. Weber, L.W., M. Boll, and A. Stampfl, *Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins.* World J Gastroenterol, 2004. **10**(21): p. 3081-7.
21. Chen, L., et al., *Endogenous sterol intermediates of the mevalonate pathway regulate HMGCR degradation and SREBP-2 processing.* J Lipid Res, 2019. **60**(10): p. 1765-

1775.
22. Khedoe, P.P., et al., *Brown adipose tissue takes up plasma triglycerides mostly after lipolysis.* J Lipid Res, 2015. **56**(1): p. 51-9.
23. Foley, E.M., et al., *Hepatic remnant lipoprotein clearance by heparan sulfate proteoglycans and low-density lipoprotein receptors depend on dietary conditions in mice.* Arterioscler Thromb Vasc Biol, 2013. **33**(9): p. 2065-74.
24. Maxwell, K.N., E.A. Fisher, and J.L. Breslow, *Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment.* Proc Natl Acad Sci U S A, 2005. **102**(6): p. 2069-74.
25. Zhang, D.W., et al., *Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation.* J Biol Chem, 2007. **282**(25): p. 18602-12.
26. Everett, B.M., R.J. Smith, and W.R. Hiatt, *Reducing LDL with PCSK9 Inhibitors--The Clinical Benefit of Lipid Drugs.* N Engl J Med, 2015. **373**(17): p. 1588-91.
27. Lewis, G.F. and D.J. Rader, *New insights into the regulation of HDL metabolism and reverse cholesterol transport.* Circ Res, 2005. **96**(12): p. 1221-32.
28. Hutchins, P.M. and J.W. Heinecke, *Cholesterol efflux capacity, macrophage reverse cholesterol transport and cardioprotective HDL.* Curr Opin Lipidol, 2015. **26**(5): p. 388-93.
29. Wang, N., et al., *ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9774-9.
30. Ji, Y., et al., *Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux.* J Biol Chem, 1997. **272**(34): p. 20982-5.
31. Out, R., et al., *Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice.* J Lipid Res, 2004. **45**(11): p. 2088-95.
32. Despres, J.P., et al., *HDL-cholesterol as a marker of coronary heart disease risk: the Quebec cardiovascular study.* Atherosclerosis, 2000. **153**(2): p. 263-72.
33. Voight, B.F., et al., *Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study.* Lancet, 2012. **380**(9841): p. 572-80.
34. Barter, P.J., et al., *Antiinflammatory properties of HDL.* Circ Res, 2004. **95**(8): p. 764-72.
35. Soran, H., J.D. Schofield, and P.N. Durrington, *Antioxidant properties of HDL.* Front Pharmacol, 2015. **6**: p. 222.
36. Tran-Dinh, A., et al., *HDL and endothelial protection.* Br J Pharmacol, 2013. **169**(3): p. 493-511.
37. Khera, A.V., et al., *Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis.* N Engl J Med, 2011. **364**(2): p. 127-35.
38. Saleheen, D., et al., *Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study.* Lancet Diabetes Endocrinol, 2015. **3**(7): p. 507-13.
39. Chiang, J.Y., *Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms.* J Hepatol, 2004. **40**(3): p. 539-51.
40. Bjorkhem, I., *Do oxysterols control cholesterol homeostasis?* J Clin Invest, 2002. **110**(6): p. 725-30.
41. Gomez, G., et al., *Regulation of the release of cholecystokinin by bile salts in dogs and humans.* Gastroenterology, 1988. **94**(4): p. 1036-46.

42. Botham, K.M. and G.S. Boyd, *The metabolism of chenodeoxycholic acid to beta-muricholic acid in rat liver.* Eur J Biochem, 1983. **134**(1): p. 191-6.
43. Wong, M.H., et al., *Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter.* J Biol Chem, 1994. **269**(2): p. 1340-7.
44. Ballatori, N., *Biology of a novel organic solute and steroid transporter, OSTalpha-OSTbeta.* Exp Biol Med (Maywood), 2005. **230**(10): p. 689-98.
45. Hagenbuch, B. and P.J. Meier, *Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter.* J Clin Invest, 1994. **93**(3): p. 1326-31.
46. Mok, H.Y., K. Von Bergmann, and S.M. Grundy, *Regulation of pool size of bile acids in man.* Gastroenterology, 1977. **73**(4 Pt 1): p. 684-90.
47. Molinaro, A., A. Wahlstrom, and H.U. Marschall, *Role of Bile Acids in Metabolic Control.* Trends Endocrinol Metab, 2018. **29**(1): p. 31-41.
48. Wang, H., et al., *Endogenous bile acids are ligands for the nuclear receptor FXR/BAR.* Mol Cell, 1999. **3**(5): p. 543-53.
49. Parks, D.J., et al., *Bile acids: natural ligands for an orphan nuclear receptor.* Science, 1999. **284**(5418): p. 1365-8.
50. Kuipers, F., V.W. Bloks, and A.K. Groen, *Beyond intestinal soap--bile acids in metabolic control.* Nat Rev Endocrinol, 2014. **10**(8): p. 488-98.
51. Vega, G.L., F.L. Dunn, and S.M. Grundy, *Effect of colesevelam hydrochloride on glycemia and insulin sensitivity in men with the metabolic syndrome.* Am J Cardiol, 2011. **108**(8): p. 1129-35.
52. Jakulj, L., et al., *Transintestinal Cholesterol Transport Is Active in Mice and Humans and Controls Ezetimibe-Induced Fecal Neutral Sterol Excretion.* Cell Metab, 2016. **24**(6): p. 783-794.
53. Temel, R.E. and J.M. Brown, *A new model of reverse cholesterol transport: enTICEing strategies to stimulate intestinal cholesterol excretion.* Trends Pharmacol Sci, 2015. **36**(7): p. 440-51.
54. Lu, T.T., J.J. Repa, and D.J. Mangelsdorf, *Orphan nuclear receptors as LXRs and FXRs of sterol metabolism.* J Biol Chem, 2001. **276**(41): p. 37735-8.
55. Peet, D.J., et al., *Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha.* Cell, 1998. **93**(5): p. 693-704.
56. Repa, J.J., et al., *Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers.* Science, 2000. **289**(5484): p. 1524-9.
57. Venkateswaran, A., et al., *Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12097-102.
58. Laffitte, B.A., et al., *LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes.* Proc Natl Acad Sci U S A, 2001. **98**(2): p. 507-12.
59. Repa, J.J., et al., *Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta.* J Biol Chem, 2002. **277**(21): p. 18793-800.
60. N, A.G. and A. Castrillo, *Liver X receptors as regulators of macrophage inflammatory and metabolic pathways.* Biochim Biophys Acta, 2011. **1812**(8): p. 982-94.
61. Bensinger, S.J. and P. Tontonoz, *Integration of metabolism and inflammation by lipid-activated nuclear receptors.* Nature, 2008. **454**(7203): p. 470-7.
62. Grefhorst, A., et al., *Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles.* J Biol Chem, 2002. **277**(37): p. 34182-90.

63. Makishima, M., et al., *Identification of a nuclear receptor for bile acids.* Science, 1999. **284**(5418): p. 1362-5.
64. Goodwin, B., et al., *A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis.* Mol Cell, 2000. **6**(3): p. 517-26.
65. Pauli-Magnus, C., et al., *Enterohepatic transport of bile salts and genetics of cholestasis.* J Hepatol, 2005. **43**(2): p. 342-57.
66. Goodwin, B., et al., *Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha.* Mol Endocrinol, 2003. **17**(3): p. 386-94.
67. Sinal, C.J., et al., *Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis.* Cell, 2000. **102**(6): p. 731-44.
68. Armstrong, L.E. and G.L. Guo, *Role of FXR in Liver Inflammation during Nonalcoholic Steatohepatitis.* Curr Pharmacol Rep, 2017. **3**(2): p. 92-100.
69. Yang, Z.X., W. Shen, and H. Sun, *Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease.* Hepatol Int, 2010. **4**(4): p. 741-8.
70. Schmitt, J., et al., *Protective effects of farnesoid X receptor (FXR) on hepatic lipid accumulation are mediated by hepatic FXR and independent of intestinal FGF15 signal.* Liver Int, 2015. **35**(4): p. 1133-1144.
71. Zhang, S., et al., *Farnesoid X receptor agonist WAY-362450 attenuates liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis.* J Hepatol, 2009. **51**(2): p. 380-8.
72. Lambert, G., et al., *The farnesoid X-receptor is an essential regulator of cholesterol homeostasis.* J Biol Chem, 2003. **278**(4): p. 2563-70.
73. Zhang, Y., et al., *FXR deficiency causes reduced atherosclerosis in Ldlr-/- mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(10): p. 2316-21.
74. Guo, G.L., et al., *Effects of FXR in foam-cell formation and atherosclerosis development.* Biochim Biophys Acta, 2006. **1761**(12): p. 1401-9.
75. Hanniman, E.A., et al., *Loss of functional farnesoid X receptor increases atherosclerotic lesions in apolipoprotein E-deficient mice.* J Lipid Res, 2005. **46**(12): p. 2595-604.
76. Collaboration, N.C.D.R.F., *Repositioning of the global epicentre of non-optimal cholesterol.* Nature, 2020. **582**(7810): p. 73-77.
77. Libby, P., et al., *Atherosclerosis.* Nat Rev Dis Primers, 2019. **5**(1): p. 56.
78. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
79. Bennett, M.R., S. Sinha, and G.K. Owens, *Vascular Smooth Muscle Cells in Atherosclerosis.* Circ Res, 2016. **118**(4): p. 692-702.
80. Bentzon, J.F., et al., *Mechanisms of plaque formation and rupture.* Circ Res, 2014. **114**(12): p. 1852-66.
81. Feingold, K.R., *Cholesterol Lowering Drugs*, in *Endotext*, K.R. Feingold, et al., Editors. 2000: South Dartmouth (MA).
82. Younossi, Z.M., *Non-alcoholic fatty liver disease - A global public health perspective.* J Hepatol, 2019. **70**(3): p. 531-544.
83. Younossi, Z., et al., *Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention.* Nat Rev Gastroenterol Hepatol, 2018. **15**(1): p. 11-20.
84. Hardy, T., et al., *Nonalcoholic Fatty Liver Disease: Pathogenesis and Disease Spectrum.* Annu Rev Pathol, 2016. **11**: p. 451-96.
85. Calzadilla Bertot, L. and L.A. Adams, *The Natural Course of Non-Alcoholic Fatty Liver Disease.* Int J Mol Sci, 2016. **17**(5).
86. Jarvis, H., et al., *Metabolic risk factors and incident advanced liver disease in non-*

alcoholic fatty liver disease (NAFLD): A systematic review and meta-analysis of population-based observational studies. PLoS Med, 2020. **17**(4): p. e1003100.
87. Day, C.P. and O.F. James, *Steatohepatitis: a tale of two "hits"?* Gastroenterology, 1998. **114**(4): p. 842-5.
88. Soto-Angona, O., et al., *Non-alcoholic fatty liver disease (NAFLD) as a neglected metabolic companion of psychiatric disorders: common pathways and future approaches.* BMC Med, 2020. **18**(1): p. 261.
89. Li, H., et al., *Crosstalk Between Liver Macrophages and Surrounding Cells in Nonalcoholic Steatohepatitis.* Front Immunol, 2020. **11**: p. 1169.
90. Krenkel, O., et al., *Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis.* Hepatology, 2018. **67**(4): p. 1270-1283.
91. Bieghs, V., et al., *Specific immunization strategies against oxidized low-density lipoprotein: a novel way to reduce nonalcoholic steatohepatitis in mice.* Hepatology, 2012. **56**(3): p. 894-903.
92. Huang, W., et al., *Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance.* Diabetes, 2010. **59**(2): p. 347-57.
93. Lanthier, N., et al., *Kupffer cell depletion prevents but has no therapeutic effect on metabolic and inflammatory changes induced by a high-fat diet.* FASEB J, 2011. **25**(12): p. 4301-11.
94. Buzzetti, E., M. Pinzani, and E.A. Tsochatzis, *The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD).* Metabolism, 2016. **65**(8): p. 1038-48.
95. Younossi, Z. and L. Henry, *Contribution of Alcoholic and Nonalcoholic Fatty Liver Disease to the Burden of Liver-Related Morbidity and Mortality.* Gastroenterology, 2016. **150**(8): p. 1778-85.
96. Ong, J.P., A. Pitts, and Z.M. Younossi, *Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease.* J Hepatol, 2008. **49**(4): p. 608-12.
97. Ballestri, S., et al., *Risk of cardiovascular, cardiac and arrhythmic complications in patients with non-alcoholic fatty liver disease.* World J Gastroenterol, 2014. **20**(7): p. 1724-45.
98. Ozturk, K., et al., *Nonalcoholic fatty liver disease is an independent risk factor for atherosclerosis in young adult men.* Atherosclerosis, 2015. **240**(2): p. 380-6.
99. Thakur, M.L., et al., *Nonalcoholic fatty liver disease is associated with subclinical atherosclerosis independent of obesity and metabolic syndrome in Asian Indians.* Atherosclerosis, 2012. **223**(2): p. 507-11.
100. Guleria, A., et al., *Patients with non-alcoholic fatty liver disease (NAFLD) have an increased risk of atherosclerosis and cardiovascular disease.* Trop Gastroenterol, 2013. **34**(2): p. 74-82.
101. Bieghs, V., et al., *NASH and atherosclerosis are two aspects of a shared disease: central role for macrophages.* Atherosclerosis, 2012. **220**(2): p. 287-93.
102. Oelkrug, R., E.T. Polymeropoulos, and M. Jastroch, *Brown adipose tissue: physiological function and evolutionary significance.* J Comp Physiol B, 2015. **185**(6): p. 587-606.
103. Zingaretti, M.C., et al., *The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue.* FASEB J, 2009. **23**(9): p. 3113-20.
104. van Marken Lichtenbelt, W.D., et al., *Cold-activated brown adipose tissue in healthy men.* N Engl J Med, 2009. **360**(15): p. 1500-8.
105. Cypess, A.M., et al., *Identification and importance of brown adipose tissue in adult humans.* N Engl J Med, 2009. **360**(15): p. 1509-17.

106. Virtanen, K.A., et al., *Functional brown adipose tissue in healthy adults.* N Engl J Med, 2009. **360**(15): p. 1518-25.
107. Yoneshiro, T., et al., *Recruited brown adipose tissue as an antiobesity agent in humans.* J Clin Invest, 2013. **123**(8): p. 3404-8.
108. Wang, Q., et al., *Brown adipose tissue activation is inversely related to central obesity and metabolic parameters in adult human.* PLoS One, 2015. **10**(4): p. e0123795.
109. Wang, Q., et al., *Brown adipose tissue in humans is activated by elevated plasma catecholamines levels and is inversely related to central obesity.* PLoS One, 2011. **6**(6): p. e21006.
110. Bertholet, A.M., et al., *Mitochondrial Patch Clamp of Beige Adipocytes Reveals UCP1-Positive and UCP1-Negative Cells Both Exhibiting Futile Creatine Cycling.* Cell Metab, 2017. **25**(4): p. 811-822 e4.
111. Bartelt, A., et al., *Brown adipose tissue activity controls triglyceride clearance.* Nat Med, 2011. **17**(2): p. 200-5.
112. Berbee, J.F., et al., *Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development.* Nat Commun, 2015. **6**: p. 6356.
113. Bartelt, A., et al., *Thermogenic adipocytes promote HDL turnover and reverse cholesterol transport.* Nat Commun, 2017. **8**: p. 15010.
114. De Lorenzo, F., et al., *Central cooling effects in patients with hypercholesterolaemia.* Clin Sci (Lond), 1998. **95**(2): p. 213-7.
115. Takx, R.A., et al., *Supraclavicular Brown Adipose Tissue 18F-FDG Uptake and Cardiovascular Disease.* J Nucl Med, 2016. **57**(8): p. 1221-5.