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Perspective

Cholesterol-induced toxicity: An integrated view of the role of cholesterol in multiple diseases

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SUMMARY

High levels of cholesterol are generally considered to be associated with atherosclerosis. In the past two decades, however, a number of studies have shown that excess cholesterol accumulation in various tissues and organs plays a critical role in the pathogenesis of multiple diseases. Here, we summarize the effects of excess cholesterol on disease pathogenesis, including liver diseases, diabetes, chronic kidney disease, Alzheimer's disease, osteoporosis, osteoarthritis, pituitary-thyroid axis dysfunction, immune disorders, and COVID-19, while proposing that excess cholesterol-induced toxicity is ubiquitous. We believe this concept will help broaden the appreciation of the toxic effect of excess cholesterol, and thus potentially expand the therapeutic use of cholesterol-lowering medications.

INTRODUCTION

Cholesterol is a ubiquitous component of mammalian cell membranes and plays an indispensable role in regulating their fluidity, permeability, and micro-structures. It is also an important precursor for the synthesis of bile acids and steroid hormones (Luo et al., 2020). Cholesterol deficiency is very rare but is thought to have profound consequences. On the other hand, excess cholesterol could decrease membrane fluidity, disrupt membrane micro-domains, alter membrane protein function, and ultimately lead to cell dysfunction and death, indicating that excess cholesterol is also toxic (Musso et al., 2013).

Systemic cholesterol excess has generally been considered to be mainly a hallmark of atherosclerosis—a notion supported by the clear link between hypercholesterolemia and the formation of pathological macrophage foam cells within the arterial walls (Chang et al., 2006). However, in the past two decades, epidemiological studies have reported a positive association between familial hypercholesterolemia and increased risks of chronic kidney disease (CKD) (Emanuelsson et al., 2018), Alzheimer's disease (Zambón et al., 2010) and osteoporosis (Yerges-Armstrong et al., 2013). Also, a number of animal studies have shown that accumulation of excess cholesterol in the liver plays a crucial role in the development of non-alcoholic fatty liver disease (NAFLD) and the subsequent development of non-alcoholic steatohepatitis (NASH) (Gan et al., 2014; Marí et al., 2014; Tirosh, 2018; Yao et al., 2018; Li et al., 2017a). Recently, increasing evidence from our labs and others has shown that excess cholesterol accumulation is also involved in a number of other diseases, including diabetes (Fryirs et al., 2009; Perego et al., 2019), testosterone deficiency (Zhang et al., 2014), hypothyroidism (Li et al., 2017b), and pituitary disorder (Yang et al., 2016a) (Figure 1). However, both cholesterol-induced toxicity and cholesterol-lowering therapies have received scant attention in these diseases. Thus, in this review, we summarize these results, while providing a deeper insight into the nature of the ubiquitous toxicity of excess cholesterol.

THE METABOLISM OF CHOLESTEROL DURING NORMOPHYSIOLOGY

Cholesterol homeostasis includes *de novo* synthesis, transport, absorption, and its conversion into bile acids, as well as steroid hormones (Figure 2). The liver plays a central role in cholesterol homeostasis as it accounts for more than 50% of the biosynthesis of its systemic levels (Luo et al., 2020). It is also a distribution center that delivers both endogenous and exogenous cholesterol to the peripheral tissues by very-low-density lipoproteins (VLDLs) and recycles excess cholesterol from peripheral tissues via high-density lipoprotein (HDL)-mediated delivery (Heeren and Scheja, 2021).

Cholesterol can be synthesized in most mammalian cells from acetyl-CoA. Briefly, three molecules of acetyl-CoA are condensed to create 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), then converted to mevalonate by the rate-limiting enzyme HMG-CoA reductase. Downstream is the formation of isoprenoid, isopentenyl pyrophosphate (PP), geranyl-PP, farnesyl-PP, squalene, and lanosterol, and it is finally converted to cholesterol (Luo et al., 2020).

Besides *de novo* biosynthesis, most mammalian cells take up cholesterol from the bloodstream via low-density lipoprotein (LDL) receptor (LDLR). The LDLs are derived from liver-produced VLDLs through gradual release of its triglyceride content. LDLR-mediated endocytosis of cholesterol-rich LDL, in turn, delivers this complex to the lysosome where it is degraded and the

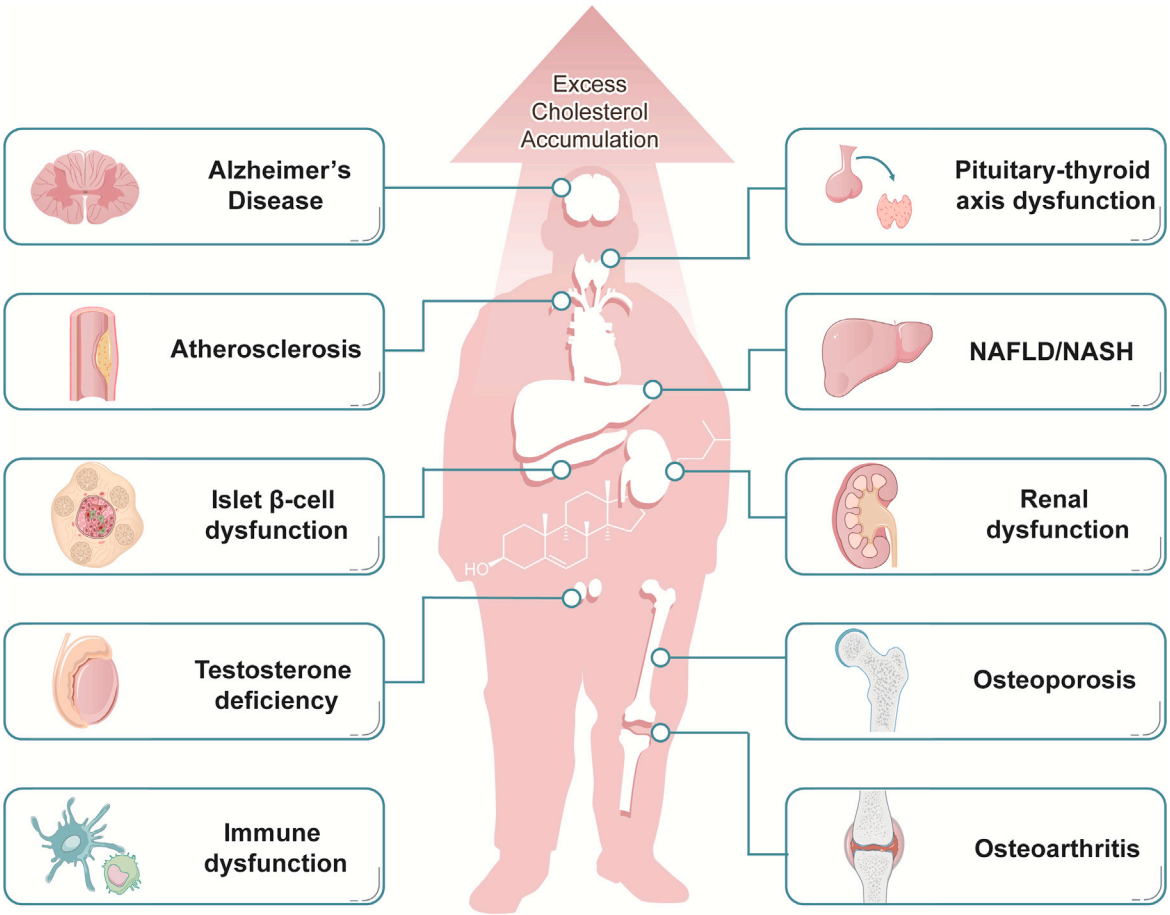

Figure 1. Excess cholesterol accumulation is involved in the pathogenesis of a number of diseases
Evidence shows that excess cholesterol and its derivatives are involved in the pathogenesis of a number of diseases other than atherosclerosis, including Alzheimer's disease, diabetes, testosterone deficiency, immune dysfunction, thyroid disorder, NAFLD and NASH, renal dysfunction, osteoporosis, and osteoarthritis.

cholesterol portion is released. The free cholesterol is then exported from the lysosome by Niemann-Pick type C1 (NPC1) and NPC2 protein to the plasma membrane and other intracellular compartments (Luo et al., 2020). Another mediator of cholesterol uptake is the NPC1-like1 (NPC1L1) on the apical surface of enterocytes, which functions as a gatekeeper for cholesterol absorption from the diet (Luo et al., 2020).

Although almost all mammalian cells are capable of synthesizing cholesterol, very few of them are able to catabolize it, except hepatocytes and some steroid hormone-producing gland cells, in which cholesterol can be converted into bile acid or steroid hormones (Russell, 2009). In other cells, two members of the ATP-binding cassette (ABC) transporter superfamily, ABCA1 and ABCG1 (ABCG5 and 8 for hepatocytes and enterocytes), are responsible for cholesterol efflux. Excess cholesterol is exported out of the cell via these ABC transporters, where it is returned to the liver in the form of an HDL complex (Luo et al., 2020).

MECHANISMS PROTECTING CELLS FROM EXCESS CHOLESTEROL ACCUMULATION

Cholesterol homeostasis is tightly regulated by its abundance, and cells are normally protected from the potential toxicity of excess free cholesterol by multiple mechanisms. For example, excess cholesterol can be rapidly esterified by cholesterol acyltransferase (ACAT) into an inert form, i.e., cholesteryl ester, and stored temporarily in lipid droplets within cells. However, due to limits in the storage capacity of lipid droplets, the esterification strategy cannot be used solely to handle chronic excesses of cholesterol. Rather, an immediate halt to biosynthesis and uptake, along with an efflux of the excess cholesterol from the body into the feces, is the most efficient method to deal with such situations (Tabas, 2002).

Briefly, there are two main crucial players in cholesterol homeostasis: the sterol regulatory element-binding protein 2 (SREBP2)-SREBP cleavage-activating protein (SCAP) complex and the transcription factor liver X receptor (LXR), with the former acting as a cholesterol sensor and the latter as a key transcriptional regulator, that together finely tune cholesterol synthesis (Figure 2). The SREBP2-SCAP complex is localized in the endoplasmic reticulum (ER), and in the absence of sufficient cholesterol, it is released from an inhibitory protein known as INSIG. This release allows the SREBP2-SCAP complex to translocate to the Golgi where SREBP2 is cleaved into an active transcription factor and subsequently translocates to the nucleus. In the nucleus, active

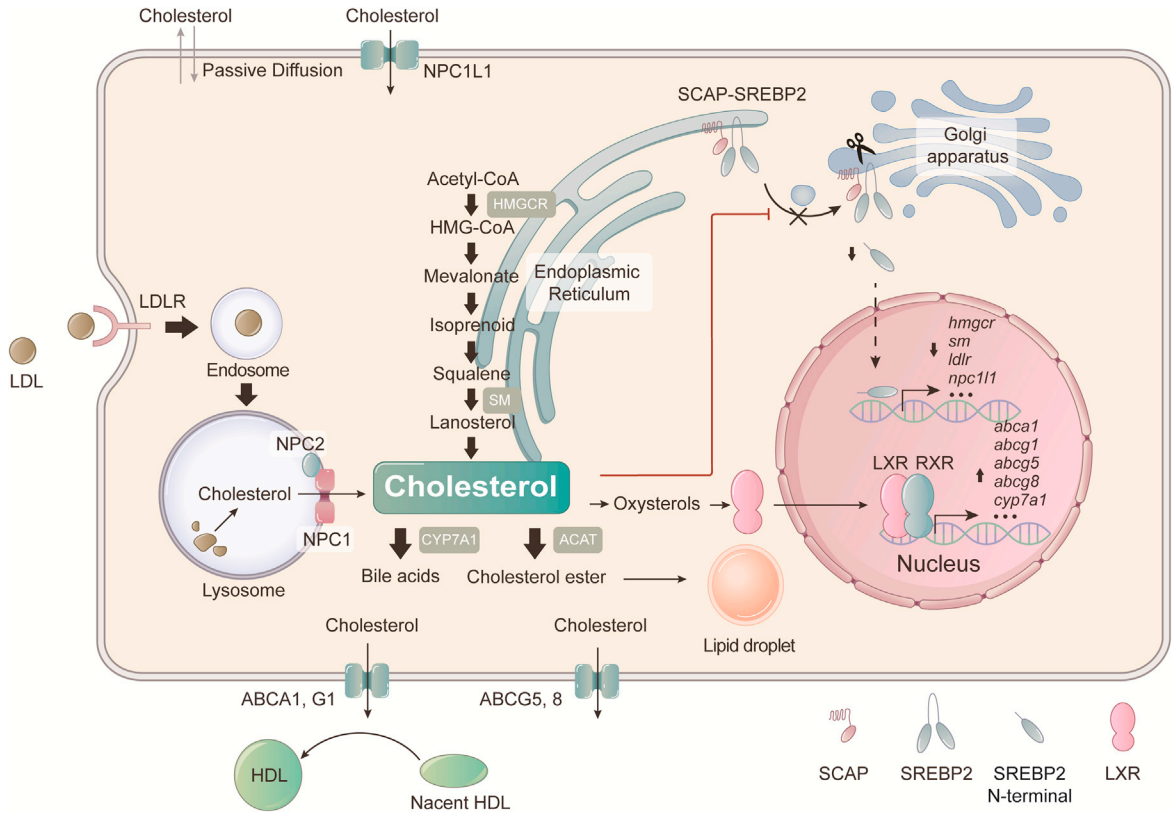

Figure 2. Metabolism of cholesterol and mechanisms to protect cells from excess cholesterol accumulation

Cholesterol metabolism includes, in general, *de novo* synthesis, uptake, and efflux. Cholesterol can be synthesized in almost all mammalian cells from acetyl-CoA through a series of reactions. HMG CoA reductase (HMGCR) and squalene monooxygenase (SM) are the rate-limiting enzymes. Cholesterol can passively penetrate cells. Cells can take up cholesterol carried by low-density lipoprotein (LDL) from the bloodstream by LDL receptor (LDLR)-dependent endocytosis. In addition, enterocytes can absorb free cholesterol from dietary sources by Niemann-Pick type C1-like 1 (NPC1L1). Excess cholesterol is esterified by ACAT or exported to the blood by ATP-binding cassette subfamily A member 1 (ABCA1) and ABCG1 (ABCG5, 8 for hepatocytes and enterocytes). Excess cholesterol triggers the ER retention of SCAP-SREBP2 complex and inhibits cholesterol biosynthesis and uptake by downregulation of HMGCR, SM, LDLR, and NPC1L1 expression. Excess cholesterol also activates LXR, which promotes the efflux of cholesterol by upregulating the expression of ABCA1, ABCG1, 5, and 8.

SREBP2 promotes the transcription of the two rate-limiting enzymes of *de novo* biosynthesis, HMG-CoA reductase and squalene monooxygenase (as well as the expression of LDLR) (Luo et al., 2020). When there is sufficient cholesterol present in the cell, the SREBP2-SCAP complex is retained in the ER by INSIG, which inhibits further cholesterol biosynthesis. Besides biosynthesis, the ER retention of SREBP2 also modulates the absorption of cholesterol by decreasing the expression of NPC1L1 and LDLR and promotes the expression of efflux transporter ABCA1 (Luo et al., 2020).

As mentioned above, another master regulator of cholesterol homeostasis is LXR, which is highly expressed in the liver, adipose tissue, and macrophages. Activation of LXR by high concentrations of cholesterol leads to the upregulation of the expression of ABCA1 and ABCG5/G8, which mediate cholesterol efflux in macrophages, hepatocytes, and intestinal cells, and downregulate NPC1L1 in intestinal cells (Mitro et al., 2007) (Figure 2). However, once these protective mechanisms are disrupted by external risk factors, such as an imbalanced diet, smoking, and obesity, excess free cholesterol accumulates in cells and subsequently contributes to disease progression (Tabas, 2002).

DISEASES ASSOCIATED WITH EXCESS CHOLESTEROL

Hypercholesterolemia leads to atherosclerosis

Atherosclerosis is the best known and most studied cholesterol-induced disease. The disease is typified by the transformation of macrophages into foam cells due to scavenging by the cells of excess modified LDL-cholesterol (Volobueva et al., 2019). Briefly, the pathogenesis of atherosclerosis is initiated by the accumulation of lipoproteins (mainly LDLs) in the subendothelia or intima. As triglycerides are hydrolyzed, the lipoproteins shrink and are subsequently enriched in cholesterol. These smaller lipoprotein particles are capable of crossing the arterial wall, where they are easily modified by oxidation, acetylation, and aggregation and ultimately captured by macrophages or smooth muscle cells. The accumulation of cholesterol will transform the macrophages into foam cells and induce an inflammatory response. Under physiological conditions, HDL prevents the accumulation of cholesterol in macrophages by promoting cholesterol efflux and its return to the liver. However, some risk factors, including hypercholesterolemia, elevated levels of pro-inflammatory cytokines, and high blood pressure, can alter the cholesterol efflux, ultimately leading to the formation of

atherosclerotic plaques inside the arterial wall (Nordestgaard and Varbo, 2014; Varbo et al., 2013). Upon the rupture of the plaques, severe cardiovascular disease occurs.

Excess cholesterol accumulation in the liver may induce liver diseases

NAFLD and NASH are common chronic liver diseases characterized by dysfunction of hepatic lipid metabolism and excess lipid accumulation in the cytoplasm of hepatocytes, as well as liver inflammation and fibrosis (for NASH) (Tiniakos et al., 2010; Zhao et al., 2011). It is well documented that saturated fatty acid-mediated lipotoxicity is a major mechanism leading to NAFLD and NASH (Schuster et al., 2018). However, a recent nested case-control study found that hypercholesterolemia, but not total fat intake, was associated with a higher risk of NAFLD (Sarkar et al., 2020). Also, a nation-wide, large-population follow-up study in the U.S. reported that higher consumption of cholesterol, but not saturated fatty acids, was associated with a higher risk of cirrhosis. This correlation still existed even after adjusting for the confounding factors such as age, gender, ethnicity, alcohol consumption, obesity, and diabetes (Ioannou et al., 2009). Furthermore, a study on ethnic differences reported that African Americans had the highest odds ratio between hypercholesterolemia and steatosis, while Asians, Whites, and Hispanics were more similar (Zhou et al., 2021).

Animal studies show similar results. It is difficult to induce NASH in wild-type mice by merely feeding them a high-fat diet, with certain exceptions, such as housing them at thermoneutrality condition (Giles et al., 2017). Rather, adding excess dietary cholesterol is typically needed to induce NASH in rodent models (Van Rooyen et al., 2011). Also, LDLR knockout (KO) mice fed a high-fat and high-cholesterol diet developed more severe NASH than mice on a high-fat diet only (Subramanian et al., 2011; Wouters et al., 2008). In addition, in some studies, high-cholesterol diet (without high fat) may induce liver fatty acid and triglyceride accumulation, ultimately leading to NASH (Csonka et al., 2017; Hirsch et al., 2016). More importantly, these animal fed with high-cholesterol diets without high-fat diets showed increased blood total cholesterol levels, but they were not associated with some frequently confounding factors that could contribute to the pathogenesis of NASH such as obesity, hypertriglyceridemia, or hyperglycemia (Csonka et al., 2017; Yang et al., 2016a).

Excess cholesterol is preferentially stored in the liver in lipid droplets (Ioannou et al., 2017). Increased hepatic free cholesterol content and hepatic cholesterol synthesis have been detected by biopsies from individuals with NAFLD and NASH (Puri et al., 2007; Min et al., 2012; Ioannou et al., 2013), possibly indicating cholesterol is involved in the development of NAFLD and NASH. In addition, Kim et al. found the ER stress upregulated Caspase-2 expression during onset of steatosis, which in turn triggered SREBP1/2 activation and accumulation of cholesterol in a manner refractory to feedback inhibition (Kim et al., 2018).

Excess cholesterol accumulation may damage liver cells through multiple mechanisms. A key mechanism is cholesterol-induced mitochondria dysfunction. High levels of cholesterol in mitochondrial membrane increase membrane rigidity, which in turn disrupts the function of membrane proteins. The most well-described example is the alteration of mitochondrial glutathione transport, which leads to mitochondrial glutathione depletion and the promotion of reactive oxygen species (ROS) generation (Marí et al., 2020). Moreover, excess cholesterol may also induce ER stress (Hager et al., 2012) and JNK-mediated mitochondrial injury (Gan et al., 2014). Another mechanism is foaming of Kupffer cells, which are endogenous macrophage-like phagocytes of the liver (Ioannou et al., 2013). Excess cholesterol accumulation in the lipid droplets of hepatocytes promotes the formation of cholesterol crystals. Necrotic hepatocytes that are rich in cholesterol crystals attract Kupffer cells and trigger the formation of "crown-like structures." These cholesterol crystals released by dead hepatocytes are then processed by Kupffer cells, transforming the Kupffer cells into foam cells through a mechanism similar to those of macrophage foam cells that arise during atherosclerosis. This efferocytosis by the Kupffer cells triggers a vicious cycle, in which the activation of the NLRP3 inflammasome by cholesterol crystals in the foam cells attracts and activates additional inflammatory Kupffer cells and macrophages, ultimately leading to NASH (Ioannou et al., 2017). In addition, accumulation of free cholesterol in liver stellate cells (Tomita et al., 2014) and macrophages (Marí et al., 2006; Bieghs et al., 2010) promotes the release of the inflammatory cytokine TGF-β, a major driver of organ fibrosis, resulting in liver fibrosis during NASH progression (Figure 3).

Animal studies of cholesterol-lowering drugs support the concept of cholesterol-induced toxicity in the liver. The combination of ezetimibe (an inhibitor of cholesterol absorption in the small intestine) and atorvastatin (a statin, which inhibits HMG-CoA reductase and thus cholesterol synthesis) reverses NASH and liver fibrosis by lowering hepatic cholesterol levels and inhibiting JNK activation in high-cholesterol-diet-fed *foz/foz* mice (Van Rooyen et al., 2013). In another study with high-fat-, high-cholesterol-diet-induced NAFLD rats, 4-week treatment of atorvastatin also decreased hepatic steatosis, inflammation, and fibrosis (Seif El-Din et al., 2015). However, statins also exhibit pleiotropic effects, such as anti-oxidative and anti-inflammatory properties (Zhang et al., 2020a). Ioannou et al. found that either ezetimibe alone or combination with atorvastatin induced the dissolution of cholesterol crystals and the dispersion of coronary structures in the liver, ultimately improving liver steatosis (Ioannou et al., 2015).

Clinical evidence also suggests a beneficial effect of statins on NAFLD and NASH. A post hoc analysis of the IDEAL and GREACE studies reported that atorvastatin therapy improved liver functions in individuals with coronary heart disease (Tikkanen et al., 2013; Athyros et al., 2010). This beneficial effect of statins is further proved in a prospective study by biopsy in individuals with NASH (Kargiotis et al., 2015). A randomized controlled trial found that ezetimibe also significantly decreased serum total cholesterol levels and improved hepatic fibrosis (Takeshita et al., 2014).

In summary, excess cholesterol accumulation in the liver contributes to the pathogenesis of NAFLD and NASH, and cholesterol-lowering treatment has beneficial effects in these diseases. Thus, controlling cholesterol levels should be considered as a potential therapeutic strategy for the treatment of these diseases.

Excess cholesterol accumulation in the pancreatic islets may induce β cell dysfunctions

The accumulation of cholesterol in pancreatic β cells may lead to defects in insulin secretion and β cell apoptosis. Hao et al. found that exposure of β cell lines, βTC3, and INS-1 cells to high-

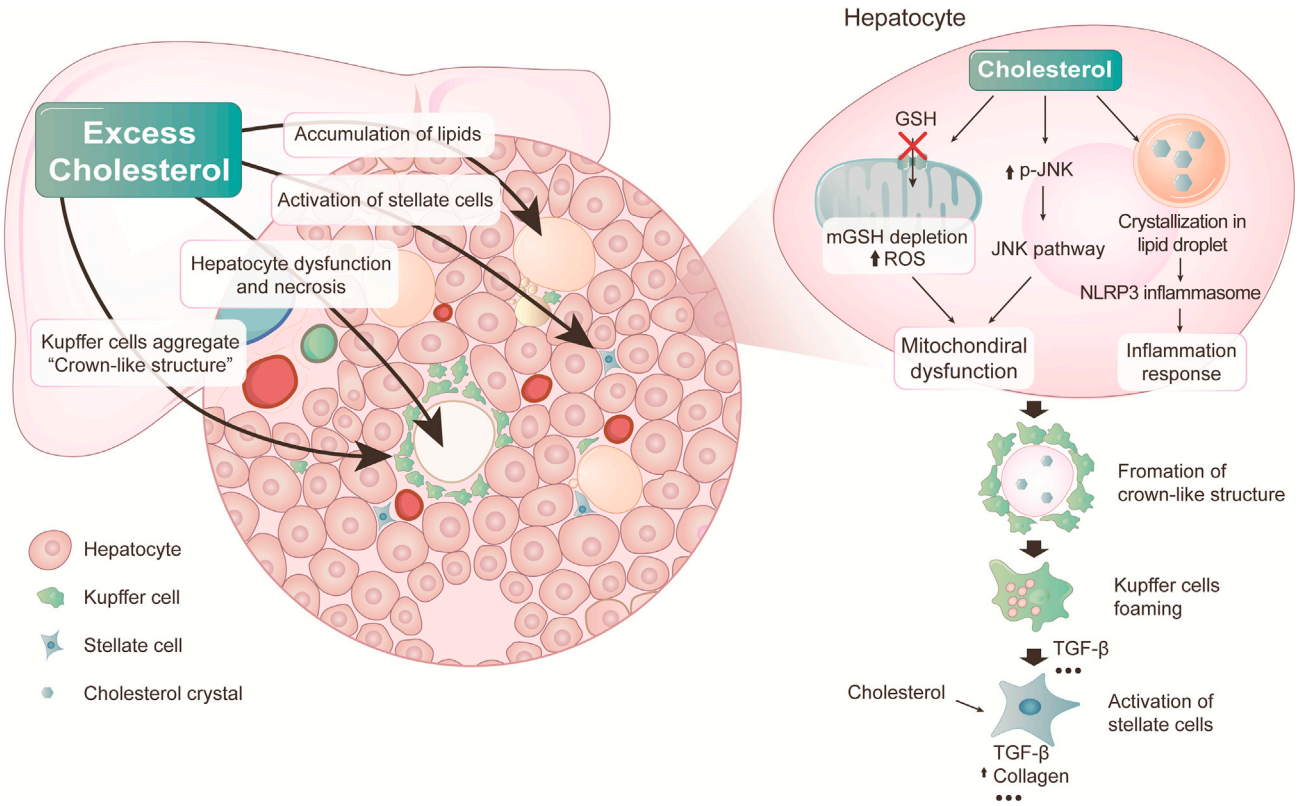

Figure 3. The role of cholesterol toxicity in liver damage in NAFLD and NASH

Free cholesterol accumulation leads to liver injury in NAFLD and NASH through the activation of intracellular signaling pathways that influence the major hepatic cells: Kupffer cells, stellate cells, and hepatocytes. In hepatocytes, cholesterol accumulation in the mitochondria causes mitochondrial dysfunction and induces an increase in ROS; furthermore, cholesterol accumulation causes the activation of NLRP3 inflammasome and the secretion of TGF-β in Kupffer cells that influence neighboring cells and induce inflammation. In addition, high cholesterol levels in stellate cells upregulate Toll-like receptor 4 and sensitize stellate to TGF-β, which induces liver fibrogenesis. These changes result in cellular dysfunction and death. These events cause continual liver damage, inflammation, and steatosis that ultimately lead to the progression to NAFLD and NASH.

cholesterol medium (10 mmol/L for 1 h) significantly reduced glucose-stimulated insulin secretion (GSIS) (Hao et al., 2007). Also, in ApoE KO mice, a model that exhibits elevated cholesterol but normal free fatty acid levels (Hao et al., 2007), they found an increase in islet cholesterol and a dramatic reduction of GSIS compared to wild-type mice. Further study demonstrated that intracellular accumulation of cholesterol inhibited glucose metabolism by affecting glucokinase translocation and activation (Hao et al., 2007).

Besides glucose metabolism, cholesterol also affects insulin granules as the primary site of cholesterol accumulation in β cells is in these granules (Bogan et al., 2012). Cholesterol accumulation increases insulin granule size and alters the fusion dynamics of these vesicles (Xu et al., 2017). A greater number of enlarged insulin granules are observed in the pancreatic β cells of hypercholesterolemia mice (Kruit et al., 2011). Excess cholesterol accumulation in insulin granules affects the architecture of its membrane proteins while also inhibiting its docking and fusion at the plasma membrane. Moreover, high cholesterol levels markedly inhibit the proliferation of β cell lines and promote apoptosis by inducing ER stress (Chen et al., 2014), mitochondrial dysfunction (Zhao et al., 2010), and ROS production (Lu et al., 2011) (Figure 4).

Cholesterol-lowering strategies display beneficial effects on pancreatic β cells. The cholesterol chelator methyl-β-cyclodextrin specifically reduces cholesterol levels in β cells, ameliorates GSIS (Hao et al., 2007), and reduces β cell apoptosis (Lu et al., 2011). Another cholesterol-lowering drug, ezetimibe, is also found to protect the function of β cells in *db/db* diabetic mice (Zhong et al., 2012). It should be noted that statin-based therapy is reported to impair insulin secretion (Agouridis et al., 2015). However, this effect may be due to statin-induced inhibition of isoprenoid synthesis and isoprenylation of small G proteins that are involved in exocytosis (Zúñiga-Hertz et al., 2015).

In summary, excess accumulation of cholesterol in pancreatic islets leads to β cell dysfunction and impairs insulin secretion, accompanied by an increase in β cell apoptosis. However, whether cholesterol-lowering treatments can improve β cell dysfunction remains to be further studied.

Excess cholesterol accumulation in the kidney may induce renal dysfunction

Several decades ago, the lipid deposition in the kidneys of high-cholesterol-diet-fed rabbits was reported (Lee and Ho, 1978). In a recent prospective cohort study, individuals with familial hypercholesterolemia displayed increased risks of CKD even after

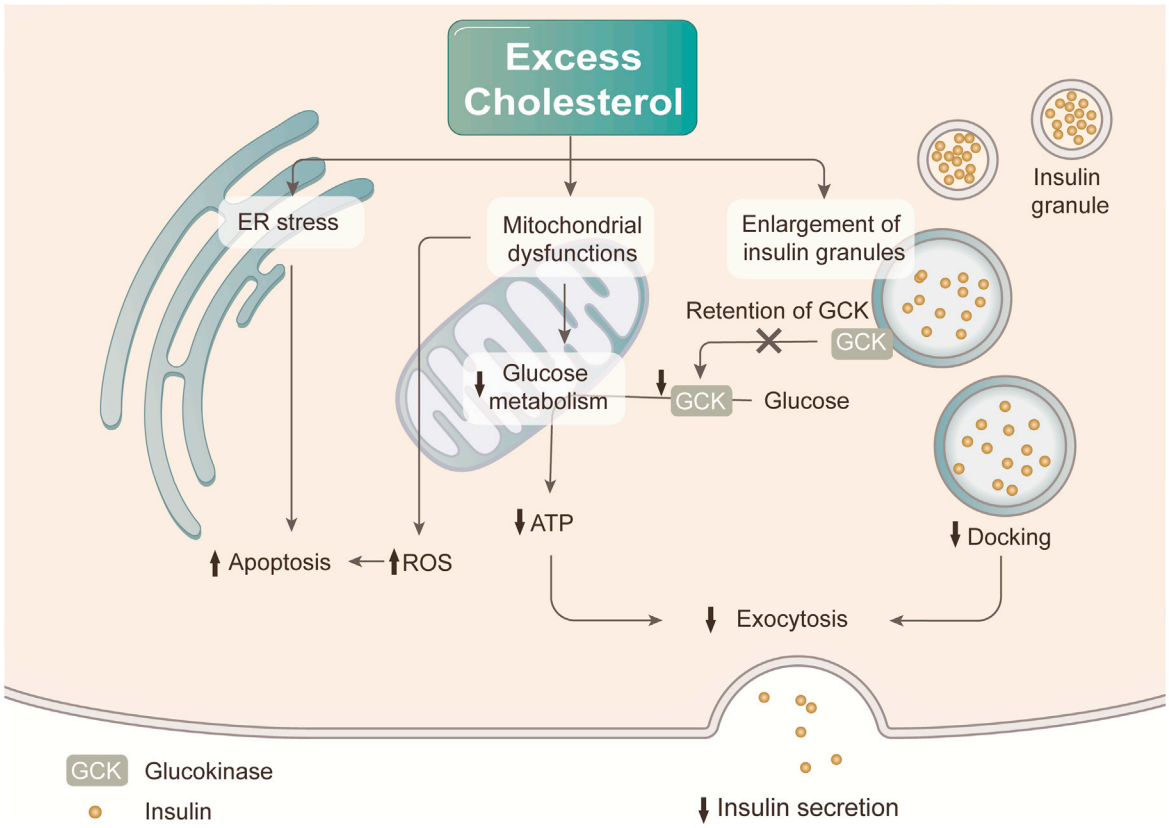

Figure 4. The role of cholesterol toxicity in islet β cell dysfunction

Excess cholesterol accumulation in β cells affects glucokinase translocation and reduces its activity. Excess cholesterol accumulation in insulin granules affects the architecture of its membrane proteins and disturbs its docking and fusion at the plasma membrane. High cholesterol levels also inhibit the proliferation of β cells and promote apoptosis by inducing ER stress and mitochondrial dysfunction.

adjusting for age, sex, BMI, hypertension, and diabetes (Emanuelsson et al., 2018), suggesting that excess cholesterol possibly plays a role in the pathogenesis of CKD. Furthermore, in a study regarding the association between LDL concentration and cystatin C (a readout of renal function), they remarked that the association tended to be of smaller magnitude among Hispanics, while Chinese, Whites, and African Americans were similar (de Boer et al., 2008).

Animal studies suggest that the podocytes in glomerulus are the primary target of excess cholesterol (Pedigo et al., 2016; Ducasa et al., 2019; Yang et al., 2017). Podocytes are crucial for maintaining the glomerular filtration barrier. A minimal alteration in podocyte function may have an important role in the progression of renal disorder (Torban et al., 2019). Podocytes express transmembrane CXCL16 as a scavenger receptor on its surface, which makes them susceptible to oxLDL (Wang et al., 2014). Similar to these animal experimental data, podocyte cholesterol accumulation is also observed in kidney biopsies from individuals with CKD (Herman-Edelstein et al., 2014; Lee and Kruth, 2003). In addition, studies have found that the glomerular expression of LDLR is upregulated, while the expression of ABCA1 and ABCG1 was downregulated in renal biopsies from individuals with diabetic nephropathy (Herman-Edelstein et al., 2014; Tsun et al., 2014) and in human podocytes treated with the sera from an individual with diabetic nephropathy (Merscher-Gomez et al., 2013), suggesting a possible mechanism for cholesterol accumulation in glomerulus podocytes.

Besides the alterations in glomerulus, Honzumi et al. found that the cholesterol content of proximal renal tubular epithelial cells was increased in high-cholesterol-diet-fed mice, which was associated with reduced megalin expression in tubule epithelial cells (Honzumi et al., 2018). Another study has shown that direct exposure of human kidney proximal tubular HK-2 cells to excess cholesterol (10 μg/mL for 6 days) induced ER stress, increased the ratio of Bax/Bcl-2, and promoted cell apoptosis (Qiu et al., 2018).

Studies on cholesterol-lowering drugs support the hypothesis that cholesterol induces toxicity in kidney. A meta-analysis of 59 randomized controlled trials on the efficacy of statins on CKD outcomes demonstrated a potential benefit of statins as they slowed the decline in the estimated glomerular filtration rate and reduced LDL-cholesterol and triglyceride levels in individuals with CKD (Su et al., 2016). Another cholesterol-lowering strategy, LDL apheresis, which physically removes LDL-cholesterol from bloodstream, has been reported to be an effective therapy to reduce albuminuria and podocyte loss in individuals with renal disorder (Sato et al., 2014; Muso et al., 2015). In an animal study, subcutaneous administration of cyclodextrin was found to prevent podocyte injury and protect BTBR *ob/ob* mice from the development of diabetic kidney disease (Merscher-Gomez et al., 2013).

Additionally, as individuals with CKD are highly susceptible to further kidney injury from medications, future trials should provide more definitive proof of the safety and benefits of cholesterol-lowering drugs in this population.

Excess cholesterol accumulation may induce pituitary-thyroid axis dysfunction

The link between hypothyroidism and hypercholesterolemia is a well-accepted clinical finding (Shin and Osborne, 2003). The classic mechanism for hypercholesterolemia in hypothyroidism is a downregulation of LDLR expression due to a thyroxine or triiodothyronine deficiency (Shin and Osborne, 2003). However, an adverse effect of cholesterol on thyroid function has also been suggested in recent studies. Ayuob et al. found a significant accumulation of lipid droplets, mitochondrial degeneration, and cytoplasm loss in thyroid follicular cells by electron microscopy in high-cholesterol-diet-fed mice (Ayuob et al., 2019). Meanwhile, in our prospective cohort study in a Chinese population, we found the risk of developing overt hypothyroidism in subclinical hypothyroidism individuals with higher baseline total cholesterol >200 and >240 mg/dL increases 6 and 15 times, respectively, compared to those with normocholesterolemic during a 3-year follow-up. This correlation still existed even after adjusting for confounding factors such as age, sex, BMI, diabetes, and hypertension (Li et al., 2017b). More importantly, further study in our team has shown that statin treatment can significantly increase the remission rate of subclinical hypothyroidism compared to the control group (50.00% versus 15.38%) (Wang et al., 2021). These observations suggested that hypercholesterolemia may be an important risk factor for hypothyroidism.

Besides the impact on the thyroid gland, a cross-sectional epidemiological study by our team has shown that individuals with "isolated" hypercholesterolemia are correlated with increased thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels (Yang et al., 2016a). Also, in an animal study, high-cholesterol-diet-fed rats developed "isolated" hypercholesterolemia (without hypertriglyceridemia) and increased serum TSH, FSH, and LH levels compared to those with standard diet (Yang et al., 2016a). More importantly, apart from rising serum cholesterol levels, the content of cholesterol is also significantly increased in the pituitary gland of high-cholesterol-diet-fed rats. Meanwhile, increased cell size and number of granules of TSH and FSH are found in the anterior pituitary of high-cholesterol-diet-fed rats. This excess cholesterol-induced increase in serum TSH levels is a slow and time-dependent process, and this is not correlated with increased or decreased levels of triiodothyronine or thyroxine (Yang et al., 2016a). These observations suggest that abnormal accumulation of cholesterol has an adverse impact on the pituitary-thyroid axis, but this hypothesis remains to be further studied.

Excess cholesterol accumulation may induce testosterone deficiency

Testosterone is an important steroid hormone derived from cholesterol. The biosynthesis is initialed in the testicular Leydig cells by P450scc located on mitochondria to produce progesterone, which is subsequently converted to testosterone by 3β-HSD, P450c17, and 17P-HSD located on the ER (Payne and Hales, 2004). The rate-limiting step is the translocation of cholesterol by StAR protein to the inner membrane of the mitochondria. Interestingly, epidemiological studies in both Chinese and U.S. populations found serum testosterone levels were negatively correlated with total cholesterol and triglycerides levels and positively with HDL-cholesterol levels after adjusting for traditional confounding factors such as age, BMI, fasting blood glucose, thyroid hormone, and TSH (Zhang et al., 2014; Page et al., 2008).

Recent animal studies suggest that excess cholesterol accumulation in the testis may directly disrupt the testosterone synthesis pathway. Yu et al. used high-cholesterol-diet-fed rats as a model, in which rats developed "isolated" hypercholesterolemia without alteration in body weight, serum triglycerides, and LH levels. They found that a high-cholesterol diet induced increased cholesterol content in the testicular Leydig cells and decreased serum testosterone levels (Yu et al., 2019). Similar results are observed in high-cholesterol-diet-fed mice: they found the expression of StAR, MnSOD, and GPX4 in testicular tissue is downregulated in these mice compared to those with normal diet (Wang et al., 2015). However, there is a lack of cholesterol-lowering drug studies on this topic, and further studies are needed to clarify the toxicity of excess cholesterol.

Excess cholesterol accumulation may induce osteoporosis

Cholesterol is also involved in bone metabolism as one of its important metabolites, vitamin D, plays a crucial role in maintaining bone calcification. Interestingly, high levels of cholesterol calcify arteries but tend to decalcify bones (Awan et al., 2010). A cross-sectional study has found a negative correlation between total serum cholesterol levels and 25(OH) vitamin D levels in a Spanish population, while no significant association existed between 25(OH) vitamin D and confounding factors such as BMI or serum triglyceride levels (Cutillas-Marco et al., 2013). Individuals with familial hypercholesterolemia present a decreased bone mineral density even after adjusting for age and BMI (Yerges-Armstrong et al., 2013). Furthermore, the magnitude of correlation between decreased whole-body bone mineral content and serum total cholesterol levels in men is higher than pre- and postmenopausal women (Hsu et al., 2006). Additionally, animal studies have also shown that a high-cholesterol diet caused an increase in the number of osteoclasts and bone resorption (Prieto-Potín et al., 2013; Sanbe et al., 2007, 2009).

Cholesterol also affects the bone-forming cells, osteoblasts. A high concentration of cholesterol inhibits the proliferation rate of osteoblast MC3T3-E cells. It also decreases the expression of markers for osteoblast RUNX2, alkaline phosphatase, and collagen alpha-1(I) (You et al., 2011). Li et al. also found that accumulation of cholesterol in ST2 cells inhibited alkaline phosphatase activity and reduced the expression of several osteoblast gene markers (Li et al., 2019). In addition, an important metabolite of cholesterol, 27-hydroxycholesterol, inhibits the differentiation of osteoblasts and enhances osteoclast differentiation and bone resorption by activating estrogen receptor and LXR (Nelson et al., 2011).

The clinical use of statins in the prevention and treatment of osteoporosis has been proposed for decades (Vigna et al.,

2002). A recent meta-analysis has demonstrated that the use of statins was associated with decreased risk of overall fractures and increased bone mineral density (An et al., 2017). Statin treatment has also been found to increase 25(OH) vitamin D levels (Cutillas-Marco et al., 2013). But a recent cross-sectional retrospective study of all Austrians with health claims reported the high-statin doses were associated with an increased risk of osteoporosis (Leutner et al., 2019). This observation may be due to some unknown confounding factors because high statin doses are usually prescribed to individuals with severe hypercholesterolemia who are likely to have high risk of osteoporosis (Burden and Weiler, 2019). In addition, in some large-population clinical trials, use of non-statin cholesterol-lowering drugs, such as cholestyramine and colestipol, is not associated with a significant protective effect on osteoporosis risk (Chan et al., 2000; Wang et al., 2000). Whether these beneficial effects of statin use on osteoporosis incidence are due to its cholesterol-lowering effect remains unclear. Thus, the use of cholesterol-lowering drugs in the treatment of osteoporosis requires further study.

Excess cholesterol accumulation may induce osteoarthritis

High cholesterol levels may also be a risk factor for osteoarthritis. It has been well documented that the synovial fluid of individuals with osteoarthritis contains high concentrations of cholesterol and cholesterol crystals (Oliviero et al., 2012). APOE*3 Leiden mice on a prolonged high-cholesterol diet develop worse osteoarthritis than mice on normal diet in a dose-dependent manner (Gierman et al., 2014). Individuals with osteoarthritis present decreased expression of ABCA1, LXRα, and LXRβ and increased expression of SREBP2 in chondrocytes from cartilage samples, which leads to further cholesterol accumulation (Tsezou et al., 2010; Kostopoulou et al., 2012).

Current information suggests that mitochondria dysfunction and oxidative stress may be the major mechanism of cholesterol-induced chondrocyte disorder. Mitochondria dysfunction and elevated ROS production were found in primary culture of human articular chondrocytes treated with high cholesterol, which was associated with increased MMP13 and RUNX2 and decreased SOX9 (Farnaghi et al., 2017). In addition, a recent study found that cholesterol and its metabolites directly activate retinoic acid-related orphan receptor alpha in chondrocytes, which upregulates matrix-degrading enzymes and increases the risk of osteoarthritis (Choi et al., 2019).

A protective role for statin use against osteoarthritis has been demonstrated in animal studies and clinical trials (Gierman et al., 2014; Haj-Mirzaian et al., 2019). However, other cholesterol-lowering drugs, such as ezetimibe and a PCSK9 inhibitor, have no such effect (van Gemert et al., 2021; Gierman et al., 2014). Thus, the role of statins in the treatment of osteoarthritis is more likely due to their anti-inflammatory effects. Further studies are needed to better determine the effect of cholesterol-lowering strategies on osteoarthritis.

Excess cholesterol accumulation in the brain may induce Alzheimer's disease

The brain is highly enriched in cholesterol compared with other tissues (Chan et al., 2012). In the past two decades, the relationship between cholesterol and Alzheimer's disease has been intensively investigated. Zambón et al. reported a higher incidence of cognitive impairment in familial hypercholesterolemia (Zambón et al., 2010). Accordingly, high prevalence of hypercholesterolemia is found in individuals with cognitive decline, and no ethnic differences are found among Whites, African Americans, and Hispanics (Gupta, 2021). A Mendelian randomization study using 380 genetic variants associated with low LDL-cholesterol levels as instrumental variables also suggested that low plasma LDL-cholesterol levels have a causal effect in reducing the risk of Alzheimer's disease (Benn et al., 2017). The hydroxylation mediated by CYP46A1 is the major elimination pathway for cholesterol in brain. An animal study found that knockdown of the *Cyp46a1* gene in hippocampal neurons induced increased amounts of neuronal cholesterol, followed by enhanced production of β-amyloid and cognitive deficits (Djelti et al., 2015).

Because lipoproteins cannot cross the intact blood-brain barrier, cholesterol in the brain is mainly synthesized *in situ* by astrocytes (Dietschy and Turley, 2001). Although plasma cholesterol cannot directly affect neurons, animal studies have found that hypercholesterolemia may damage the integrity of the blood-brain barrier (de Oliveira et al., 2020; Rapp et al., 2008). Considering the transport and clearance of β-amyloid by the blood-brain barrier is an important factor in the pathogenesis of Alzheimer's disease, plasma cholesterol may be involved in the progression of Alzheimer's disease by altering the clearance of β-amyloid in the brain (Sweeney et al., 2018).

The accumulation of cholesterol in neurons may increase the sensitivity of NMDA-mediated excitotoxicity (del Toro et al., 2010), which is an important mechanism for many neurodegenerative processes (Armada-Moreira et al., 2020). Another role of excess cholesterol in Alzheimer's disease is to enhance the production of β-amyloid, as cholesterol in lipid rafts promotes the activity of γ-secretase (the enzyme responsible for β-amyloid production) (Di Paolo and Kim, 2011; Gamba et al., 2012; Xiong et al., 2008). Furthermore, cholesterol accumulation-induced mitochondria dysfunction and ROS generation also contribute to the accumulation of neurotoxic β-amyloid (Fernández et al., 2009). In addition to neurons, microglia are also important targets of cholesterol, as cholesterol- and/or 25-hydroxycholesterol (25-OHC)-induced microglia activation and neuroinflammation contribute to the onset of Alzheimer's disease (Thirumangalakudi et al., 2008; Wong et al., 2020).

Because lipophilic statins easily cross the blood-brain barrier, these cholesterol-lowering drugs have been proposed as potential neuroprotectors (Wood et al., 2010). Animal studies have demonstrated that statins may ameliorate cognitive disorders in amyloid precursor protein transgenic mice (Kurata et al., 2012; Zhou et al., 2016). Also, reduced membrane cholesterol levels in nerve terminals induced by cyclodextrin may protect neurons under stress conditions through decreasing glutamate release (Krisanova et al., 2012; Pastukhov et al., 2020). However, the clinical use of statins and other cholesterol-lowering drugs in Alzheimer's disease is still controversial because they have shown no benefit on the primary outcome of Alzheimer's disease in large randomized controlled trials and in a Mendelian randomization study (McGuinness et al., 2014, 2016; Benn et al., 2017).

It should be noted that in the brain, almost all of cholesterol is unesterified under normal conditions, while in the affected regions with amyloid plaques, the cholesterol ester contents are

significantly increased (Chan et al., 2012; Tajima et al., 2013). Thus, ACAT1 inhibitors, which inhibit the formation of cholesterol ester, have been proposed in this field. Avasimibe, an ACAT1 inhibitor that has been tested in phase III clinical trials, specifically reduces β-amyloid levels in a mouse model (Huttunen et al., 2010; Shibuya et al., 2015). These observations suggest that the balance between the esters and free cholesterol is also a key point in the control of amyloidogenesis (Alavez-Rubio and Juarez-Cedillo, 2019). The role of cholesterol in Alzheimer's diseases still needs further investigation.

Excess cholesterol accumulation may induce immune dysfunction

High levels of cholesterol also affect the immune system. *Abca1/g1*-deficient mice develop systemic lupus erythematosus symptoms. In the same study, the researchers found that *Abca1/g1*-deficient dendritic cells displayed an increased accumulation of intracellular cholesterol, which was associated with activation of the inflammasome and enhanced pro-inflammatory cytokine secretion (Westerterp et al., 2017). Augmentation of free cholesterol levels in the plasma membrane affects lipid raft dynamics, which leads to increased TCR clustering and immunological synapse formation and facilitates TCR-dependent signaling in *Acat1*-deficient $CD8^+$ T cells (Yang et al., 2016b). Cholesterol accumulation in the plasma membrane also facilities the activation of macrophages by affecting membrane TLRs in *Abca1/g1*-deficient macrophages (Yvan-Charvet et al., 2008).

In addition, animal experiments show that some oxidized cholesterol species, such as 25-OHC, 27-OHC, and 7α,25-OHC, also play an important regulatory role in the immune response. For example, 25-OHC enhances the inflammatory response of macrophages by promoting the expression of inflammatory mediators (Gold et al., 2014). 27-OHC upregulates the levels of inflammatory cytokines such as IL-8, IL-1β, and TNF-α in premonocytes through the TLR4/NF-κB pathway (Gargiulo et al., 2015). Another oxysterol, 7α,25-OHC, mainly plays a chemotactic role. The infiltration of $CD4^+$ T cells into the central nervous system induced by 7α,25-OHC is reduced after knocking out cholesterol 25 hydroxylase (Chalmin et al., 2015).

The use of statins in autoimmune diseases has been proposed for decades, and studies have reported beneficial effects on inflammatory and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (Gurevich et al., 2005). However, this may be due to a direct inhibitory effect of statins on immune signaling pathways via blockade of GTPase isoprenylation (Zeiser, 2018), as mentioned above for pancreatic β cells. Nevertheless, non-statin cholesterol-lowering drugs, such as sevelamer (McGettigan et al., 2016) and ezetimibe (Cho et al., 2020), also present anti-inflammatory and immunomodulatory effects. Collectively, due to the lack of epidemiological evidence on the association between hypercholesterolemia and immune diseases, further studies are needed to evaluate the potential toxic effect of excess cholesterol on the immune system.

Excess cholesterol accumulation may increase the incidence of severe COVID-19

A number of studies have shown associations between elevated cholesterol levels and bacterial or viral infections, but the role of cholesterol in infection is still a matter of controversy. Membrane fusion is a key step for the entry of the SARS-CoV-2 virus. Considering the fundamental role cholesterol plays in cell membrane architecture, it may also be involved in regulating the entry of viruses into host cells. By summarizing the general characteristics of the SARS virus, Radenkovic et al. proposed that high cholesterol levels may increase the density of lipid rafts on the plasma membrane that harbor angiotensin-converting enzyme 2 (ACE2) receptors, thus promoting the process of viral endocytosis (Radenkovic et al., 2020). This hypothesis was supported by SARS-CoV-2 pseudo-virus entry assay (Li et al., 2021; Sanders et al., 2021). In addition to lipid rafts and the ACE2 protein, the SARS-CoV-2 S protein was also found to bind directly to cholesterol to facilitate SARS-CoV-2 entry (Wei et al., 2020a).

COVID-19 severity is linked to many chronic diseases, such as cardiovascular disease, hypertension, obesity, and diabetes, in which cholesterol plays a crucial role. Thereby, lowering cholesterol levels, more specifically LDL-cholesterol levels, may reduce the risk of complications caused by COVID-19 (Cao et al., 2020). In contrast, decreased LDL-cholesterol levels are found in individuals with severe COVID-19 (Fan et al., 2020). The authors considered this observation to be the result of liver injury as pro-inflammatory cytokines are found to be dramatically elevated in individuals with COVID-19, and these cytokines, such as IL-1β and IL-6, strongly alter liver function and inhibit cholesterol efflux (Wei et al., 2020b). On the other hand, hypocholesterolemia-induced SREBP2 activation leads to a cytokine storm as SREBP2-induced cholesterol biosynthesis was suppressed by Sestrin-1 and PCSK9 expression in individuals with COVID-19, while SREBP2-induced IL-1β and TNF-α expression is upregulated (Lee et al., 2020).

Despite the controversial role of cholesterol in COVID-19, in a large retrospective cohort study in China, statins have been reported to reduce the mortality of COVID-19 in the matched statin and non-statin groups (Zhang et al., 2020b). Statins also present potentially protective effects against severe COVID-19 infection (Proto et al., 2021), especially for individuals with familial hypercholesterolemia (Vuorio et al., 2021). Furthermore, another study found that statin-based therapies slowed the progression of COVID-19, while steroid-based anti-inflammation therapies have no such effect (Rodriguez-Nava et al., 2020). However, whether the role of statins in this context may be explained either by their direct cholesterol-lowering effect or by their anti-inflammatory effects remains for further study. Collectively, the role of cholesterol in COVID-19 is still unclear and further research is necessary to provide insights into the molecular mechanism of cholesterol in COVID-19.

CONCLUSION

The adverse effects of excess cholesterol accumulation in multiple tissues and organs were noticed by Ho and Taylor in high-cholesterol-diet-fed rabbits a half a century ago (Ho and Taylor, 1971). However, this phenomenon has not been given much attention until recently. As described above, epidemiological data and evidence from clinical trials, animal studies, and *in vitro* experiments suggest that cholesterol not only leads to macrophage foaming in atherosclerosis, but can also

Table 1. Cholesterol and diseases

Diseases	Cholesterol-induced dysfunctions	Studies on cholesterol-lowering therapies and outcome
Atherosclerosis	induces foaming of macrophages	statins and ezetimibe are recommended in guidelines
NAFLD/NASH	induces inflammation, foaming of Kupffer cells, formation of "crown-like structures," endoplasmic reticulum stress, oxidative stress, and mitochondrial dysfunction	statins and ezetimibe alone or in combination improve liver functions and dissolution of cholesterol crystals and prevent liver fibrosis by inhibiting JNK activation
Islet β cell dysfunction	disturbs glucose metabolism	cyclodextrin ameliorates glucose-induced insulin secretion and reduces β cell apoptosis
	induces ER stress and oxidative stress	ezetimibe protects the function of β cells
	alternates of insulin granules	statins decrease insulin secretion
Renal dysfunction	induces ER stress, lipid deposition, and inflammation	statins slow the decline in the glomerular filtration rate
	perturbs protein reabsorption in renal proximal tubules	cyclodextrin protects BTBR *ob*/*ob* mice from the development of diabetic kidney disease
		LDL apheresis reduces albuminuria and protects podocytes
Pituitary-thyroid axis dysfunction	increases the secretion and expression of TSH in pituitary	statin therapy decreases the risk of hypothyroidism
	induces mitochondrial dysfunction and ER stress in thyroid follicular cells	
Testosterone deficiency	induces ER stress and mitochondrial dysfunction	–
	decreases the expression of enzymes involved in testosterone synthesis	
Osteoporosis	increases the activity of osteoclasts	statins increase bone mineral density and osteoblast differentiation
	decreases the activity of osteoblasts	cholestyramine and colestipol are not associated with a significant protective effect on osteoporosis risk
Osteoarthritis	induces chondrocyte dysfunctions and apoptosis	statins slow the osteoarthritis progression
		ezetimibe and PCSK9 inhibitor have no such effect
Cognitive disorder	increases the sensitivity of NMDA-mediated excitotoxicity	statins ameliorate cognitive disorders in animal model
	increases the production of β-amyloid	cyclodextrin protects neurons under stress conditions
	induces mitochondria dysfunction and ROS generation	
	disturbs the β-amyloid clearance via blood-brain barrier	
Immune dysfunction	activates inflammasomes	statins have beneficial effects on systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis
	disturbs immune signalization and oxidative stress	
COVID-19	increases the density of lipid rafts and promotes viral endocytosis	statins slow the progression of and reduce the mortality from COVID-19
	increases the risk of complications caused by COVID-19	

accumulate in multiple tissues and organs. This excess cholesterol accumulation plays an important role in the pathogenesis, development, and prognosis of multiple diseases (Table 1).

In this article, the term excess cholesterol accumulation refers to an accumulation of excess cholesterol in corresponding tissues or organs. The purpose of our article is to highlight the toxicity of excess cholesterol in multiple diseases and to remind clinicians and researchers to be more involved in this topic. This concept of "cholesterol toxicity" may help us better understand the pathophysiology of cholesterol-related diseases and suggests that targeting excess cholesterol may be an effective treatment strategy for these disease conditions. So far, most of the evidence on this topic has come from epidemiological data or rodent studies, and the causal relationship between excess cholesterol and the listed diseases still needs further work, especially in the use of cholesterol-lowering drugs. Also, further studies are needed to reveal the underlying mechanisms of cholesterol toxicity and to predict new related diseases of excess cholesterol accumulation.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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