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## Novel targets in the liver to treat cardiometabolic diseases

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# **CHAPTER 1**

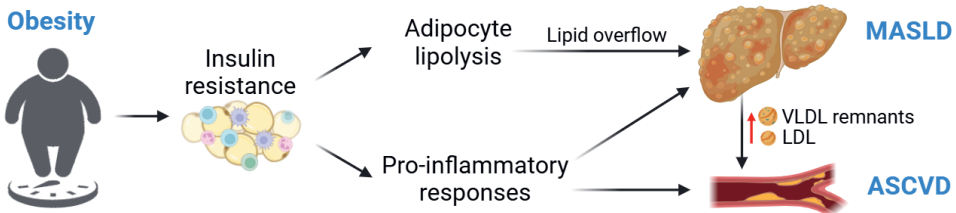
## **General Introduction and Outline**



# General Introduction

## 1. Cardiometabolic diseases

Obesity is a growing worldwide epidemic, globally affecting more than 0.89 billion adults by 2022 <sup>1,2</sup>, which is projected to increase to more than 1.53 billion adults by 2035 <sup>2,3</sup>. Obesity is defined by body mass index (BMI; body weight (kg) divided by height squared (m<sup>2</sup>)) of 30 kg/m<sup>2</sup> or more <sup>2</sup>. Numerous serious health risks have been strongly associated with obesity, including a series of cardiometabolic diseases, e.g., steatotic liver disease, type 2 diabetes, cardiovascular diseases and other metabolic disorder-related diseases. Obesity can trigger insulin resistance in adipose tissues and impair adipocyte function, increasing lipolysis in adipocytes. This causes lipid overflow to organs such as the liver and leads to the development of metabolic dysfunction-associated steatotic liver disease (MASLD). In turn, MASLD contributes to atherosclerosis development due to excessive production by the steatotic and insulin-resistant liver of lipoproteins including very low-density lipoprotein (VLDL) remnants and low-density lipoproteins (LDL) as their lipolytic end products, which are causal in the development of atherosclerotic cardiovascular disease (ASCVD). Meanwhile, the pro-inflammatory responses induced by insulin resistance further aggravate the development of MASLD and ASCVD. These diseases have become the leading causes of increased morbidity and mortality in the world <sup>4,5</sup>. A schematic overview of the contribution of obesity to the development of MASLD and ASCVD is shown in **Figure 1**.



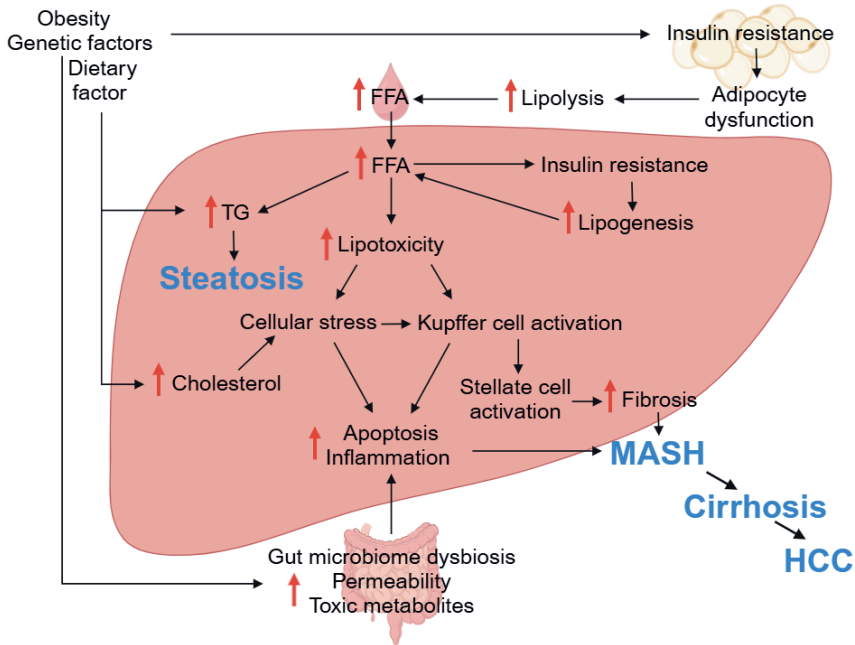
**Figure 1. An overview of the effects of obesity on MASLD and ASCVD.** See the text in Section 1 for the explanation. ASCVD, atherosclerotic cardiovascular disease; MASLD, metabolic dysfunction-associated steatotic liver disease; (V)LDL, (very) low-density lipoprotein.

### 1.1. Metabolic dysfunction-associated steatotic liver disease

MASLD is one of the most widespread chronic liver diseases, affecting over one-third of the global population <sup>6</sup>. 'MASLD' has since 2023 been adopted as the new term for 'non-alcoholic fatty liver disease' (NAFLD). The new nomenclature has been introduced to emphasize the important influence of metabolic risk factors on the pathogenesis of steatotic liver disease, in addition to avoiding the stigmatizing term 'fatty liver', and is assumed to

reshape clinical and preclinical practice and lead to improved patient treatment <sup>7</sup>. MASLD is characterized by excess accumulation of fat, mainly triglycerides (TG), in the liver (steatosis in  $\geq 5\%$  of hepatocytes) <sup>8</sup>. MASLD was initially hypothesized to be progressing according to the ‘two-hit’ theory <sup>9</sup>. Within this theory, the ‘first hit’ is lipid accumulation in the liver, inducing steatosis, which results from an imbalance between hepatic lipid uptake, synthesis, storage, utilization and secretion, mostly related to excess calorie intake in the course of developing obesity. Induction of steatosis would make the liver more susceptible to secondary insults, e.g., oxidative stress, mitochondrial dysfunction and inflammatory cytokine production, together promoting inflammatory cascades through activating the resident macrophages of the liver (i.e., Kupffer cells) as the ‘second hit’, thereby accelerating MASLD development.

However, more recent insights suggested the ‘two-hit’ hypothesis may be too simplistic to explain the mechanism underlying MASLD progression. Thus, the ‘multiple-hit’ theory has been proposed that offers a more accurate explanation by taking into account complex and multiple parallel factors simultaneously contributing to MASLD development <sup>10</sup>. Obesity, genetic factors and/or dietary factors trigger insulin resistance in adipose tissues, causing adipocyte dysfunction and subsequently increased lipolysis that leads to overflow of free fatty acids (FFA) towards the liver. This causes hepatic insulin resistance, resulting in increased lipogenesis. As a result, hepatic TG production increases, which along with excessive intake of dietary TG, leads to hepatic steatosis. Meanwhile, the overload of FFA and intake of dietary cholesterol induces hepatic lipotoxicity, initiating cellular stress and Kupffer cell activation. Additionally, gut microbiome dysbiosis driven by obesity and/or genetic factors can increase the production of toxic metabolites and increase gut permeability. All of these factors can collectively contribute to apoptosis and inflammation in the liver, attracting monocytes from the circulation to become additional hepatic macrophages. Ultimately, Kupffer cell activation stimulates stellate cells to produce collagen, contributing to fibrosis. These consequently drive the development of metabolic dysfunction-associated steatohepatitis (MASH). MASLD and MASH are reversible, while MASH can further develop through advanced fibrosis to severe irreversible liver damage if left untreated, causing cirrhosis and liver cancer (i.e., hepatocellular cancer, HCC). The ‘multiple-hit’ theory of MASLD pathogenesis is graphically illustrated in **Figure 2**.

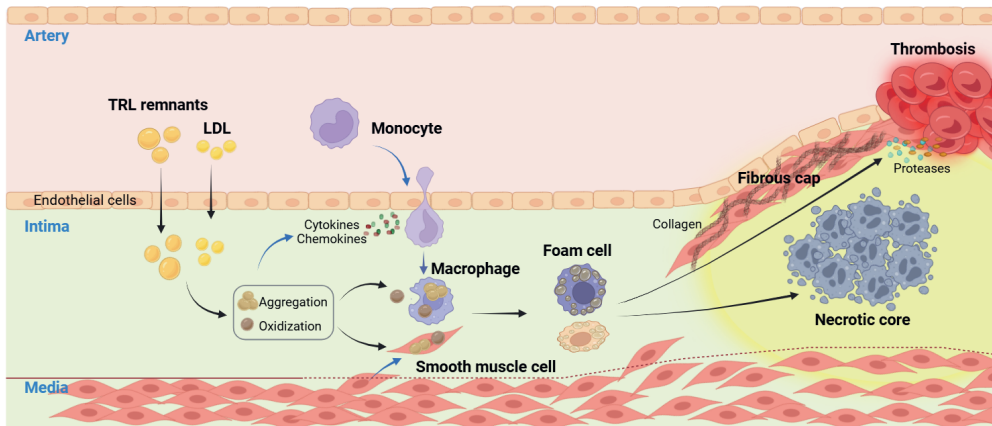


**Figure 2. The 'multiple-hit' theory of MASLD pathogenesis.** See text in Section 1.1 for explanation. FFA, free fatty acids; HCC, hepatocellular cancer; MASH, metabolic dysfunction-associated steatohepatitis; TG, triglycerides.

## 1.2. Atherosclerotic cardiovascular disease

Cardiovascular diseases (CVDs) are the leading cause of global death accounting for 19.8 million global deaths in 2022<sup>11</sup> and are projected to cause 35.6 million deaths in 2050<sup>12</sup>. The dominant CVD is ASCVD, which is caused by atherosclerosis that is characterized by the buildup of lipid plaques within arterial walls and thus leading to narrowing and hardening of arteries. Accumulation of circulating triglyceride-rich lipoprotein (TRL) remnants and LDL in the arterial subendothelial layer is the initial phase of atherosclerosis development<sup>13,14</sup>. Here, TRL remnants and LDL undergo modifications, e.g., aggregation and oxidation, that stimulate endothelial cells to secrete pro-inflammatory cytokines and chemokines, recruiting monocytes into the intima to differentiate into macrophages<sup>13-15</sup>. Macrophages within the intima engulf these modified LDL and TRL remnants, which convert them into 'foam cells' that are laden with mainly cholesterol, resulting in an early stage of an atherosclerotic plaque (i.e., 'lesion'). Foam cells can induce an advanced inflammatory response that promotes the proliferation of smooth muscle cells from media into the intima, where they can be converted into additional foam cells. Smooth muscle cells also produce collagen to form fibrous caps and stabilize the plaques. Through the accumulation of lipids and increased inflammation, foam cells can undergo apoptosis and release lipids and cell

debris, resulting in the formation of a necrotic lesion core and aggravating plaque development <sup>15</sup>. Meanwhile, foam cells secrete proteases to destabilize the fibrous cap, which may lead to plaque rupture, triggering platelet aggregation and thrombosis <sup>16</sup>. Eventually, thrombus formation can lead to a variety of serious cardiovascular events, such as myocardial infarction ('heart attack') and stroke. The development of an atherosclerotic plaque is graphically presented in **Figure 3**.



**Figure 3. Atherosclerotic plaque development.** See text in Section 1.2 for explanation. LDL, low-density lipoprotein; TRL, triglyceride-rich lipoprotein.

Given that MASLD and ASCVD share common risk factors and involve similar pathogenetic features, including lipid accumulation and chronic inflammation [3], some of the therapeutic options that are beneficial for one disease might also be potentially applicable to the other.

## 2. Therapeutic approaches for cardiometabolic diseases

### 2.1. Currently available therapies

#### 2.1.1. Lifestyle interventions

Cardiometabolic disease development is strongly associated with lifestyle factors. Since lifestyle interventions are diverse, effective and less expensive compared to other therapy options, they are highly recommended in cardiometabolic disease management <sup>17,18</sup>.

##### 2.1.1.1. Dietary interventions

Dietary interventions, including calorie restriction, specific dietary-nutrient patterns and meal timing strategies, have all shown beneficial effects in cardiometabolic diseases.

Calorie restriction is one of the most effective dietary interventions to reduce the risk of cardiometabolic diseases<sup>19-23</sup>. Restricting calorie intake (1) markedly reduces body mass and improves the lipid profile in the liver and blood<sup>24,25</sup>, (2) suppresses systemic inflammation by inhibiting pro-inflammatory cytokine secretion<sup>26,27</sup>, (3) and activates various molecular signaling pathways. These include AMP-activated protein kinase-related pathways<sup>28,29</sup>, which are linked with the protection against MASLD<sup>30</sup> and ASCVD<sup>31</sup>.

Improving dietary patterns also have been proven useful for preventing/treating cardiometabolic diseases. For example, the 'Mediterranean diet' entails a high intake of plant-based foods and healthy fats (from olives and nuts), moderate intake of fish and dairy products, and limited intake of red meat and sweets<sup>32</sup>. As such, it has a high ratio of monounsaturated fatty acids to saturated fatty acids and is rich in fiber. This diet reduces BMI and LDL-C<sup>33</sup> and improves human insulin resistance<sup>34</sup>. Another popular dietary pattern is the ketogenic diet, characterized by a rigorous reduction in carbohydrates and high consumption of fats and proteins<sup>35</sup>. This diet induces metabolic adaptations by shifting the body to use fat as the main energy source, and increases insulin sensitivity, reduces body weight and improves lipid profiles<sup>36,37</sup>. However, contradicting studies showed that the ketogenic diet can elevate LDL-C and therefore increase ASCVD risk in humans<sup>38,39</sup>.

Time-restricted eating (TRE) is another intervention that aims to restrict the meal timing without altering diet choices. Secretion of key metabolic hormones and metabolic responses is regulated by internal circadian clocks and TRE can serve as a strong *Zeitgeber* to optimize circadian biology<sup>40</sup> thereby improving cardiometabolic health. In both mouse studies<sup>41-43</sup> and clinical trials<sup>44-48</sup>, TRE showed beneficial effects with respect to attenuating cardiometabolic derangements by reducing body weight, improving insulin sensitivity and lipid profiles, at least partly through modulating the composition of the gut microbiome.

### **2.1.1.2. Physical exercise interventions**

Physical exercise is widely recognized as a crucial element of cardiometabolic disease preventive/therapeutic initiatives and is emphasized in numerous health promotion guidelines. Regular physical exercise training has been shown to reduce insulin resistance<sup>49</sup>, improve lipid profiles<sup>50,51</sup> and suppress inflammation<sup>52,53</sup>, thereby preventing the progression of MASLD<sup>54-56</sup> and ASCVD<sup>57,58</sup>. Of note, the effectiveness of physical exercise in MASLD and ASCVD depends on the modality, intensity and timing of the exercise and differs in different populations. For instance, aerobic exercise elicits a better anti-inflammatory effect than resistance exercise<sup>59</sup> and it is more effective than resistance exercise in decreasing circulating lipids, at least shown in an elderly population<sup>60</sup>. Nevertheless, with comparable duration, frequency and period, resistance exercise training consumes less energy, which is more feasible for the individuals who with low cardiorespiratory endurance or cannot tolerate aerobic exercise<sup>61</sup>. Apart from the modality, exercise intensity also strongly affects the effectiveness of physical exercise. Moderate-intensity exercise training

markedly improves BMI, circulating lipids and inflammation markers compared to low-intensity exercise training <sup>62</sup>. High-intensity interval exercise is superior to moderate-intensity continuous exercise in ameliorating MASLD progression <sup>63</sup>. Recently, the effect of timing of exercise interventions for cardiometabolic health has been evaluated, with seemingly conflicting outcomes. While a recent randomized controlled trial reported that exercise in the morning is more efficient at reducing cardiometabolic risk factors in comparison to exercise in the afternoon <sup>64</sup>, the majority of studies showed that late exercise rather than early exercise improves metabolic profiles <sup>65-68</sup>.

Although lifestyle interventions show pronounced benefits and are considered valuable for cardiometabolic disease treatment, they suffer from limitations: (1) long-term adherence is required to achieve certain improvements and the dropout rate is often high in intervention programs <sup>69,70</sup>; (2) effectiveness highly depends on the intensity/variety of the intervention and individual factors <sup>71,72</sup>; (3) environmental and social factors pose unique implementation challenges <sup>73-75</sup>. Thus, lifestyle interventions should ideally be combined with pharmacological treatments for maximum benefit.

## **2.1.2. Pharmacological therapies**

### **2.1.2.1. Therapy for metabolic dysfunction-associated steatotic liver disease**

Until recently, for MASLD no specific effective drugs were available on the market. Improving lifestyle, e.g., dietary and physical exercise interventions <sup>76</sup>, and managing insulin/lipid levels with other medications, like metformin <sup>77</sup> and statins <sup>78</sup>, indirectly control MASLD development to some extent, and have been the primary options for the patients with MASLD. However, these interventions/treatments have limited efficacy and therapeutic benefits as they do not directly target the underlying pathological mechanisms of MASLD. Therefore, extensive research efforts have been dedicated for searching effective pharmacological therapies.

Only in March 2024, rezdiffra (also known as Resmetirom) has been conditionally approved by the US Food and Drug Administration (FDA) as the first drug for the treatment of adults with noncirrhotic MASH <sup>79</sup>. Rezdiffra is a liver-targeted agonist for thyroid hormone receptor-beta (THR- $\beta$ ), a high-affinity nuclear receptor for thyroid hormone with a crucial role in the regulation of lipid metabolism and energy homeostasis. The THR- $\beta$  pathway is generally impaired in patients with MASLD <sup>80,81</sup>, suggesting that activating this pathway may be beneficial. Indeed, rezdiffra has shown profound efficacy in a phase 2 clinical trial by markedly reducing hepatic fat, markers of inflammation and fibrosis after 12 weeks and 36 weeks of treatment in patients with MASH <sup>82</sup>. Recently, a phase 3 clinical trial in patients with NAFLD and presumed NASH confirmed the efficacy of rezdiffra and in addition established its safety and tolerability <sup>83</sup>.

Although rezdifra may be an important advance for MASLD management, confirmation of its clinical benefit by completing a still ongoing 54-month study was requested by the FDA, which will be required for its continued approval<sup>79</sup>. In addition, its clinical application still faces some challenges, for instance in patient selection, therapy monitoring and further investigation of its potential risks and side effects<sup>84</sup>, warranting the search for additional pharmacological therapies.

### **2.1.2.2. Therapies for atherosclerotic cardiovascular disease**

The development of ASCVD is attributed to many risk factors, including obesity, genetic factors and lifestyle-related risk factors including, but not limited to, physical inactivity, unhealthy diet and smoking. Among these, high circulating cholesterol is the primary causal factor in the initiation and progression of atherosclerosis. Thus, lowering circulating cholesterol, especially within TRL remnants and LDL, is currently the primary goal and the most effective pharmacological management for ASCVD.

#### ***HMGCR inhibition***

Statins are a class of medications used by millions of people worldwide to lower cholesterol and treat ASCVD. The first observation of cholesterol being abundant in atherosclerotic plaques inspired the development of statins<sup>85</sup>. In 1976, the Japanese biochemist Akira Endo was the first to isolate mevastatin from *Penicillium citrinum*<sup>86</sup>, marking the birth of statins. Lovastatin, was approved by the FDA as the first statin in 1987<sup>87</sup> and as of now, 7 additional statins have been discovered and introduced to the market<sup>88</sup>. Statins reduce cholesterol by inhibiting hydroxymethylglutaryl-CoA reductase (HMGCR), the enzyme catalyzing the conversion of hydroxymethylglutaryl-CoA into mevalonate within the cholesterol synthesis pathway, mainly in the liver<sup>89</sup>. Statins compete with binding of hydroxymethylglutaryl-CoA to the active site of HMGCR and thereby interfere with cholesterol synthesis. On the one hand, this decreases the hepatic production of VLDL, which is the primary source of circulating TRL remnants and LDL. On the other hand, the reduction of intracellular cholesterol activates sterol regulatory element-binding proteins (SREBPs) to increase the expression of the low-density lipoprotein receptor (LDLR), accelerating the uptake TRL remnants and LDL from the circulation<sup>90</sup>. Statins have been tested in numerous clinical trials and the results are consistent: statins reduce plasma cholesterol levels (within TRL remnants and LDL) by 25-35%, thereby slowing atherosclerosis progression and improving survival in patients with coronary heart disease<sup>89,91-96</sup>.

Although statins are generally well-tolerated, they have side effects, of which muscle pain has been most frequently reported. Statin treatment impairs the mitochondrial respiratory capacity of skeletal muscle, increasing the risk of muscle injury and causing pain<sup>97-100</sup>. However, it should be noted that a recent meta-analysis revealed that most (>90%) of all reports of muscle complaints by participants on statin treatment were actually not caused by statins<sup>101</sup>. Another reported side effect is liver toxicity, evident from a dose-dependent

increase in circulating transaminases<sup>102,103</sup>, which is considered as an indication of liver damage<sup>104</sup>. Also, atorvastatin treatment also is associated with cholestatic liver injury and simvastatin treatment with hepatocellular damage<sup>105</sup>, albeit these side effects are very rare<sup>106</sup>.

Admittedly, nearly four decades of clinical investigation have proven the efficacy and safety of statins, and have shown in the general population that their benefits far outweigh their risks. Even so, patient adherence to long-term use of statins is poor, frequently caused by safety concerns<sup>107</sup>.

### ***NPC1L1 inhibition***

In addition to inhibiting hepatic cholesterol synthesis, reducing intestinal cholesterol absorption is another strategy to lower LDL-C and the risk of ASCVD. For this purpose, ezetimibe (ezetrol) has been approved by the FDA and European Medicines Agency (EMA) in 2002. Ezetimibe is an antagonist of Niemann Pick C1 like 1 (NPC1L1), which plays a crucial role in cholesterol absorption in the intestine. By inhibiting NPC1L1, ezetimibe reduces the uptake of cholesterol within chylomicrons<sup>108,109</sup>. Many preclinical animal studies have reported that the effects of ezetimibe on reducing LDL-C levels are beneficial for reducing ASCVD risk<sup>109-111</sup>. Likewise, the effectiveness of ezetimibe in lowering circulating cholesterol levels has been shown in clinical trials<sup>112,113</sup>. It should be noted that lowering cholesterol absorption with ezetimibe monotherapy has been demonstrated to upregulate HMGCR activity in humans<sup>114</sup>. Considering such compensatory effects, ezetimibe is often studied/recommended as an add-on to statins rather than as a monotherapy. Ezetimibe plus statin therapy led to a more pronounced reduction in LDL cholesterol levels in clinical trials compared to monotherapy<sup>115-117</sup> and the combination therapy of ezetimibe plus low-dose statin allows those patients who are intolerant to single high-dose statin therapy to achieve the therapeutic LDL-C target<sup>118</sup>.

### ***PCSK9 inhibition***

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an endopeptidase that is secreted from the liver into the circulation where it can bind the LDLR and promote lysosomal LDLR degradation upon cellular internalization, leading to an overall decrease of hepatic LDLR abundance. As a result, LDLR-mediated uptake of TRL remnants and LDL is reduced, resulting in their accumulation in the circulation. Accordingly, inhibiting PCSK9 has been considered an ideal therapeutic target for hypercholesterolemia or hypercholesterolemia-driven ASCVD<sup>119</sup>. In 2015, FDA and EMA approved the PCSK9-inhibiting antibodies alirocumab (prudent) and evolocumab (repatha) for treating hypercholesterolemia, particularly in the individuals who have homozygous familial hypercholesterolemia (HoFH) caused by genetic mutations and characterized by impaired LDLR-mediated lipoprotein clearance pathway. In 2021, the FDA also approved a liver-targeted small interfering RNA that inhibits PCSK9 expression in hepatocytes (inclisiran), which needs to be administered only a few times a year<sup>120</sup>.

1

These PCSK9 inhibitors remarkably reduce plasma levels of cholesterol in TRL remnants and LDL <sup>121</sup> and lower risk of cardiovascular events in clinical trials <sup>122-125</sup>. In addition to its significant cholesterol-lowering effect, studies also demonstrate anti-inflammatory effects of PCSK9 inhibition <sup>126,127</sup>, likely due to stimulating macrophage autophagy and inhibiting oxidative stress <sup>128</sup>. Overall, PCSK9 inhibitors offer additional options to treat hypercholesterolemia in patients who are at risk of cardiovascular events, especially for those patients failing to achieve the target cholesterol levels with traditional therapies, like statins and ezetimibe. However, the implementation of PCSK9 inhibitor therapy is hindered by several challenges, especially for relatively high expense and inconvenient administration approach (requiring injections), which thus limit the accessibility of this therapy to the patients <sup>129</sup>.

Despite quite some pharmacological treatments for MASLD and ASCVD have benefited millions of people, limitations remain. Side effects, drug accessibility, and especially residual cardiometabolic risk <sup>130</sup> warrant the investigation into additional safe, effective and low-cost treatments.

## 2.2. Promising novel therapeutic strategies

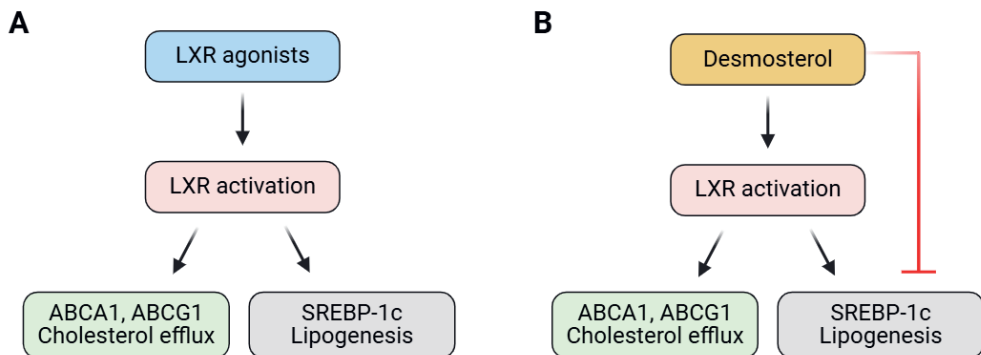
### 2.2.1. Targeting LXR

Since the liver X receptor (LXR) was identified as a nuclear receptor playing a crucial role in the transcriptional control of lipid metabolism in the 1990s <sup>131,132</sup>, interest has emerged to investigate its role in lipid-related diseases. Accumulation of cholesterol in macrophages activates LXR to induce the expression of ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), which facilitate the transfer of excess cholesterol to high-density lipoproteins (HDL), which is thought to transport the cholesterol back to the liver for direct biliary secretion or indirect biliary secretion after conversion into bile acids <sup>133</sup>. Cholesterol export from macrophages suppresses pro-inflammatory responses driven by excessive cellular cholesterol. In addition, LXR activation can prevent inflammation by inhibiting NF- $\kappa$ B signaling which is crucial for inducing immune responses <sup>134</sup>.

Given its favorable effects in the regulation of cholesterol homeostasis and inflammation, activating LXR has been an emerging therapeutic target for diseases related to disorders in cholesterol metabolism. In the past two decades, many mouse studies have shown an atheroprotective effect of LXR activation <sup>135-138</sup>. However, beyond targeting genes involved in cholesterol efflux (ABCA1 and ABCG1) and inducing an anti-inflammatory program in macrophages, LXR activation also stimulates the expression of SREBP-1c in hepatocytes, the key transcription factor inducing lipogenesis, as observed with synthetic LXR agonists such as T0901317 and GW3965 in animal experiments <sup>139-141</sup>. Also in MASLD patients, LXR expression positively correlated with hepatic steatosis <sup>142</sup>, and SREBP-1c and its targeted

lipogenic genes were upregulated<sup>143</sup>. Therefore, although several synthetic LXR agonists have reached phase 1 of clinical trials, none were continued because of lipogenic side effects<sup>144</sup> (**Figure 4A**) including dyslipidemia<sup>145</sup>.

An overload of cholesterol in macrophages results in product inhibition of  $\Delta$ 24-dehydrocholesterol reductase (DHCR24), the terminal enzyme in cholesterol synthesis, resulting in the accumulation of desmosterol, the last intermediate in the Bloch pathway of *de novo* cholesterol synthesis<sup>146</sup>. Desmosterol has been identified as a selective and the most potent endogenous ligand of LXR, reprogramming lipid metabolism and suppressing inflammation via activating LXR target genes and, remarkably, inhibiting SREBP-1c activity<sup>146</sup> (**Figure 4B**). A study found that depleting desmosterol in macrophages by conditional overexpression of DHCR24 in myeloid cells downregulates the expression of ABCA1 and ABCG1 in macrophages, thereby promoting lipid accumulation and inflammation, thereby accelerating atherosclerosis development in mice<sup>147</sup>.



**Figure 4. LXR activation by synthetic LXR agonists or desmosterol.** **A**, LXR activation by LXR agonists not only promotes cholesterol efflux by enhancing the expression of ABCA1 and ABCG1, but also activates the SREBP-1c pathway inducing lipogenesis. **B**, Desmosterol, the precursor of cholesterol, acts as an endogenous LXR ligand and it can promote cholesterol efflux by activating ABCA1 and ABCG1 without causing lipogenesis by inhibiting the SREBP-1c pathway. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; LXR, liver X receptor; SREBP-1c, sterol regulatory element-binding protein-1c.

Interestingly, Müller et al have previously generated SH42 as the first selective DHCR24 inhibitor<sup>148</sup>, and it has been subsequently showed that SH42 treatment by i.p. injection successfully increases systematic desmosterol levels in mice, thereby activating ABCA1 in macrophages and promoting inflammation resolution in peritonitis<sup>149</sup>. Importantly, in contrast to synthetic LXR agonists, SH42 did not increase the lipogenic program in hepatocytes<sup>149</sup>. Given that attenuation of macrophage activation is expected to be

beneficial in the treatment of both MASLD and ASCVD, research into the potential beneficial effects SH42 in these diseases is warranted.

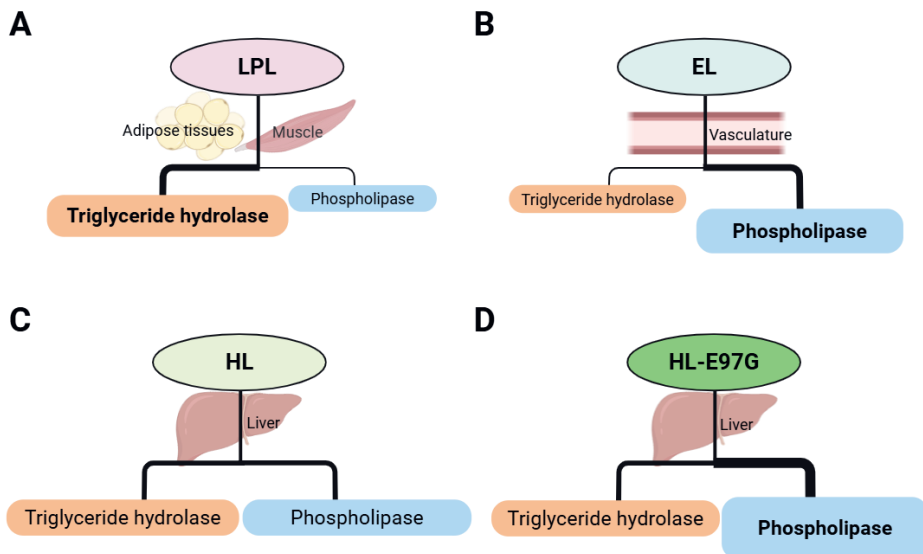
### 2.2.2. Targeting lipases

While lowering circulating cholesterol is undoubtedly a primary strategy in cardiometabolic diseases, regulating other lipoprotein components, including TG and phospholipids, have gained increasing attention. The glycerol-sn-1-fatty acid hydrolases including lipoprotein lipase (LPL), endothelial lipase (EL) and hepatic lipase (HL) are the key enzymes in hydrolyzing TG and/or PL to regulate lipoprotein metabolism<sup>150,151</sup>. These three lipoprotein lipases can be distinguished by their tissue-specific expression and different enzymatic activities (**Figure 5**).

LPL is an enzyme mainly produced in adipocytes within brown and white adipose tissues, cardiomyocytes within the heart and myocytes within skeletal muscle, after which LPL is translocated to the vascular lumen, bound to glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1). As LPL has high TG hydrolase and low phospholipase activity (**Figure 5A**), it is primarily responsible for hydrolyzing TG in calculating TRLs<sup>152</sup>. Humans with loss-of-function mutations in the LPL gene have increased risk of ASCVD<sup>153</sup>. Unlike LPL, EL is mainly synthesized by endothelial cells in the vasculature and has low TG hydrolase but high phospholipase activity (**Figure 5B**). EL can promote the catabolism of  $\beta$ -VLDLs, containing more cholesterol compared to regular VLDLs, suggesting its anti-atherogenic effect<sup>154</sup>. By remodeling lipoproteins through hydrolyzing TG and PL, LPL and EL have been found to promote the clearance of TRLs and LDL<sup>155,156</sup>. LPL and EL activities are regulated by angiopoietin-like protein 3 (ANGPTL3), which is mainly expressed by the liver. Increasing LPL and EL activity by inhibiting ANGPTL3 has been shown to attenuate hyperlipidemia and atherosclerosis development in mice<sup>157-159</sup> with the lipid-lowering effect of ANGPTL3 inhibition being LDLR-independent<sup>160</sup>. As such, the ANGPTL3 inhibitor evinacumab was approved by FDA and EMA in 2021 for the treatment of patients with HoFH and it can largely reduce TRL and LDL-cholesterol levels<sup>161,162</sup>. A recent study showed that evinacumab also reduces LDL-C in pediatric patients with HoFH, but long-term follow-up studies are required to provide definitive evidence of efficiency and safety<sup>163</sup>.

In contrast to the extensive research on LPL and EL, studies on the role of HL in lipoprotein metabolism are limited and current findings are inconsistent. HL is specifically expressed in hepatocytes within the liver, with a balanced hydrolytic activity of TG hydrolase and phospholipase (**Figure 5C**). HL deficiency attenuates atherosclerosis development in mice<sup>164</sup>, and HL has been suggested to contribute to atherogenic lipid profile by forming small-dense LDL in humans<sup>165</sup>. However, low HL activity is associated with increased cardiovascular risk in humans<sup>166</sup>. In addition, HL has been shown to have an important role in promoting the clearance of atherogenic lipoproteins through its noncatalytic ligand activity<sup>167,168</sup>. Although these findings are conflicting, HL may be a target for ASCVD

treatment. Interestingly, a novel rare variant in the hepatic lipase gene (*LIPC*), encoding the gain-of-function variant HL-E97G, was recently identified in a French family with individuals carrying this variant having very low plasma TG and cholesterol levels. This HL gene variant specifically enhances the phospholipase activity of HL without affecting the TG hydrolase activity<sup>169</sup> (**Figure 5D**). Mice expressing HL-E97G in hepatocytes present a similar hypolipidemic phenotype as found in humans<sup>169</sup>, suggesting it may be a promising target to treat hyperlipidemia. Of note, HL-E97G lowers cholesterol levels not only in LDL, but also in HDL that may have elicited effects in atherosclerosis. Therefore, to elucidate whether improving HL function may be worthwhile to pursue as a novel strategy in the prevention and treatment of ASCVD, it is imperative to study the effects of HL-E97G on hypercholesterolemia-driven atherosclerosis development.

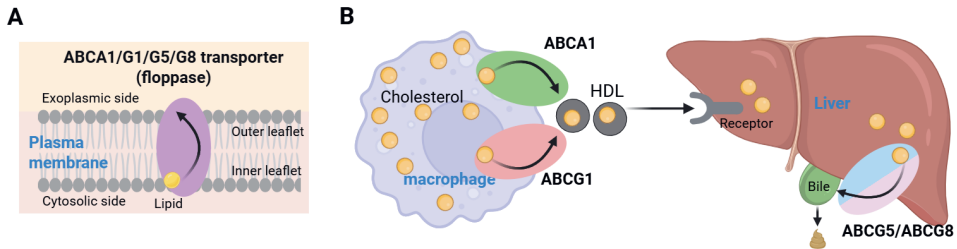


**Figure 5. Lipases responsible for lipoprotein hydrolysis and their tissue-specific expression and enzymatic activities.** See text in Section 2.2.2 for explanation. EL, endothelial lipase; HL, hepatic lipase; HL-E97G, gain-of-function mutant of hepatic lipase; LPL, lipoprotein lipase.

### 2.2.3. Targeting ABC transporters

Lipid transport between cells and organs is vital to maintain cellular/organ function and strongly influences the development of cardiometabolic diseases, with ABC transporters playing key roles. ABC transporters are a superfamily of transmembrane proteins that are responsible for the ATP-powered transportation of various substrates across cellular membranes. The human genome contains 48 ABC genes encoding 44 membrane transporters that belong to 5 subfamilies, A, B, C, D and G<sup>170-172</sup>. These ABC transporters play important roles in various diseases, such as cancer, Alzheimer's disease, MASLD and ASCVD. A few ABC transporters have been linked to lipid transport in cardiometabolic diseases by acting as a floppase to facilitate lipid efflux by transporting lipids from the cytosolic side (inner leaflet) to the exoplasmic side (outer leaflet) of plasma membranes (**Figure 6A**), for example, ABCA1, ABCG1 and ABCG5/ABCG8<sup>173</sup>. As discussed above in section 2.2.1, ABCA1 and ABCG1 promote cholesterol efflux from macrophages to HDL, which can transfer cholesterol to the liver for secretion into VLDL and secretion into bile without and with previous conversion into bile acids. Deficiency of ABCA1 and ABCG1 in mice has been shown to lead cholesterol accumulation in macrophages and accelerate atherosclerosis development<sup>174,175</sup>. Similarly, the heterodimer ABCG5/ABCG8<sup>176</sup> enhances the efflux of cholesterol from the liver into bile, facilitating cholesterol excretion (**Figure 6B**). ABCG5/ABCG8 knockout mice showed increased hepatic lipid accumulation and proinflammatory cytokines, both of which are key indicators of the development of MASLD<sup>177</sup>. On the other side, enhancing ABCG5/ABCG8 expression in mice increases cholesterol excretion, and reduces plasma cholesterol levels, thereby preventing atherosclerosis progression<sup>178</sup>. These transporters have thus been well-studied and shown crucial roles in the treatment of cardiometabolic diseases.

It would be interesting to identify other ABC transporters as targets to beneficially modulate cardiometabolic diseases. An understudied ABC transporter is ABCA6, first cloned in 2001 and given its name based on structural similarities with ABCA1<sup>179</sup>. ABCA6 is mainly expressed in the liver [173]. Cell separation studies showed that within the liver ABCA6 is exclusively expressed by hepatocytes<sup>180</sup>. ABCA6 was later found to be localized in the plasma membrane of hepatocytes at the basolateral surface<sup>181</sup>, suggesting a potential role for ABCA6 in transferring substrates between hepatocytes and the blood. A genome-wide association study specifically in a Dutch population revealed that a human *ABCA6* missense variant (i.e., rs77542162; p.Cys1359Arg) is associated with increased total cholesterol and LDL-C levels<sup>182</sup>. Expression of this variant in wild-type mice did not affect plasma TC levels<sup>181</sup>, likely related to the very low TC levels in wild-type that are hard to modulate, which warrants studying the effect of modulating hepatocytic ABCA6 expression on lipid levels and atherosclerosis in a relevant mouse model for human cardiometabolic diseases.



**Figure 6. ABCA/G-subfamily transporters are involved in lipid/cholesterol transport.** **A**, ABCA1, ABCG1, and the ABCG5/G8 heterodimer act as floppases that can facilitate lipid efflux by transporting cholesterol from the cytosolic side (inner leaflet) to the exoplasmic side (outer leaflet) of plasma membranes. **B**, ABCA1 and ABCG1 are involved in transfer of cholesterol from macrophages to HDL which can bring the cholesterol to the liver for utilization/metabolism. The heterodimer ABCG5/ABCG8 promotes the efflux of cholesterol from the liver to bile, facilitating cholesterol excretion. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; HDL, high-density lipoprotein.

### 3. Outline of this thesis

Cardiometabolic diseases including MASLD and ASCVD have become major global health problems. They are among the most prevalent chronic diseases globally, substantially contributing to morbidity and mortality. As the liver is central in regulation of lipid metabolism and systemic inflammation, strongly influencing the progression of MASLD and ASCVD, this thesis aims to investigate novel therapeutic targets in the liver to treat these diseases through experimental studies in relevant mouse models.

**Chapter 1** provides a general introduction about the pathogenesis of MASLD and ASCVD, summarizes currently available treatments, including lifestyle and pharmacological interventions, and highlights their current limitations. Meanwhile, promising novel therapeutic targets are described, forming the background and motivation for the experimental studies performed in this thesis.

Based on the findings that DHCR24 inhibition increases desmosterol, which activates LXR to lower inflammation and enhance cholesterol efflux from macrophages, in **Chapter 2** we aimed to investigate the effects of selective DHCR24 inhibition on lipid metabolism and inflammation in the context of diet-induced MASLD development. Therefore, we fed APOE\*3-Leiden.CETP mice, a well-established humanized mouse model for the study of (cardio)metabolic diseases, with a MASLD-inducing diet while treating them without and with the specific and selective DHCR24 inhibitor SH42 and evaluated the effects on lipid metabolism, hepatic inflammation and hepatic steatosis. Next, we aimed to evaluate the effects of SH42 on atherosclerosis development. To this end, in **Chapter 3**, we fed APOE\*3-

Leiden.CETP mice and *Ldlr*-deficient (*Ldlr*<sup>-/-</sup>) mice, a more inflammatory mouse model, an atherogenic diet and treated them without and with SH42, to determine the lipoprotein profile, blood immune cell composition and atherosclerotic lesion size, severity and composition in the aortic root.

Based on our previous observation that HL-E97G, a novel rare variant encoded by *LIPC* gene, enhances lipoprotein remodeling and lipid clearance in APOE\*3-Leiden.CETP mice, in **Chapter 4** we further explored the role of this HL variant in lipoprotein metabolism and ASCVD development. To this end, we assessed the effect of adenovirus-associated virus (AAV) vector-mediated expression of HL-E97G in hepatocytes of APOE\*3-Leiden.CETP mice on lipoprotein profile and atherosclerosis development. To evaluate whether the effects induced by HL-E97G are dependent on the LDLR, the same experiment was next carried out in *Ldlr*<sup>-/-</sup> mice.

Since ABCA6 is likely involved in cholesterol metabolism, and mainly expressed by liver hepatocytes, in **Chapter 5** we aimed to elucidate the function of hepatocytic ABCA6 on lipoprotein metabolism and ASCVD development. To do so, *Abca6* was disrupted down in hepatocytes of APOE\*3-Leiden.CETP mice by AAV-CRISPR technology, and effects on plasma lipids, lipoprotein kinetics, and atherosclerosis development were evaluated. To further explain the underlying mechanism, similar measurements were performed disruption of *Abca6* in hepatocytes of *Ldlr*<sup>-/-</sup> mice.

Finally, in **Chapter 6** we discuss the results of these experimental studies and the therapeutic potential of the results for prevention and treatment of cardiometabolic diseases.

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