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Understanding the molecular mechanisms behind lipid homeostasis: the role of apoE and hepatic ACAT 2 in atherogenesis

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INTRODUCTION

ATHEROSCLEROSIS: AN OVERVIEW

Atherosclerosis (athero = soft gruel-like deposit, and sclerosis=hardness) is complex pathology that involve deposition of cholesterol, fats, cellular metabolites, calcium and various other substances on the inner lining of the artery[1]. This disease of the large arteries is the primary cause of heart disease and stroke, and is the underlying cause of about 50% of all deaths in westernized societies[2]. In 2002, the most recent year for which worldwide data from the World Health Organization[3] are available, global mortality due to CVD was estimated at 16.7 million deaths. Of these, CHD was the single most important cause identified, accounting for 7.2 million (43%) deaths. Stroke accounted for an additional 5.5 million (33%) annual deaths. The remaining cardiovascular deaths were due to hypertensive, inflammatory, or rheumatic heart diseases, or other forms, such as tumors of the heart, vascular tumors of the brain, disorders of heart muscle/cardiomyopathy, heart valve disorders, and disorders of the lining of the heart[3]. The incidence of CHD mortality has increased since 1990 as life expectancies have risen. Although CHD death rates have declined in western countries in response to improvements in disease management, rates are expected to increase in developing countries in the future[3].

Under a very precise and literary point of view, arteriosclerosis can be defined as a chronic disease characterized by abnormal thickening and hardening of the arterial walls with resulting loss of elasticity, whereas atherosclerosis is characterized by atheromatous deposits in and fibrosis of the inner layer of arteries. Hence atherosclerosis can be considered as an advanced stage of arteriosclerosis, since plaque formation comes at later stages of the disease[1]. Atherosclerosis initiating event may be different under different condition, but it is suggested that endothelial dysfunction is one of the major event. Such a dysfunction may occur

due to many factors (e.g. vessel injuries and collagen exposure, metabolite deposition in the vessel wall, change in vascular reactivity due to change in the rate or the force of blood flow) [1].

Atherosclerotic lesions begin as fatty streaks underlying the endothelium of large arteries. In humans, fatty streaks can be found in the aorta in the first decade of life, the coronary arteries in the second decade, and the cerebral arteries in the third or fourth decades[2]. They consist of smooth muscle cells (SMCs), lymphocytes and mainly of macrophages[4]. The formation of fatty streaks is initiated by adherence of circulating monocytes to activated endothelial cells at lesion-prone sites within the arteries. Adherent monocytes subsequently migrate into the subendothelial space in response to locally produced chemo-attractant molecules, where they further differentiate into macrophages and start to accumulate lipids (mainly cholesterol esters) by the interaction with modified low density lipoprotein (LDL)[4]. When macrophages take up more lipoprotein cholesterol than they can excrete, the cholesterol is stored in the cytoplasm by means of cholesteryl esters droplets. These droplets give the cytoplasm a foamy appearance at electron microscope observation, thus accounting for the term of foam cells[5]. Then, fatty streaks could progress and develop into advanced atherosclerotic plaques. The American Heart Association (AHA) Committee on Vascular lesion provided a pathobiological nomenclature and classification of human atherosclerotic lesions, dividing them in 6 stage of development (from type I to type VI)[6, 7]. Lesions type I, II and III are the silent precursors of types IV, V and VI, advanced plaques associated with clinical events (see **FIGURE 1** and **TABLE I**).

The development of atherosclerotic plaques occurs after LDL accumulation within the sub-endothelial matrix of artery wall, leukocyte extravasation, foam cell formation, smooth muscle cell proliferation and connective tissue production[8]. With the secretion of fibrous elements by

SMCs, occlusive fibrous plaques develop and increase in size[2]. Low density lipoproteins represent the main source of cholesterol not only for peripheral tissues but also for arterial wall. Moreover it has been demonstrated that accumulation of LDL in blood vessels is greater when level of circulating LDL are raised, and both transport and retention of LDL are increased in the preferred sites for lesion formation[2].

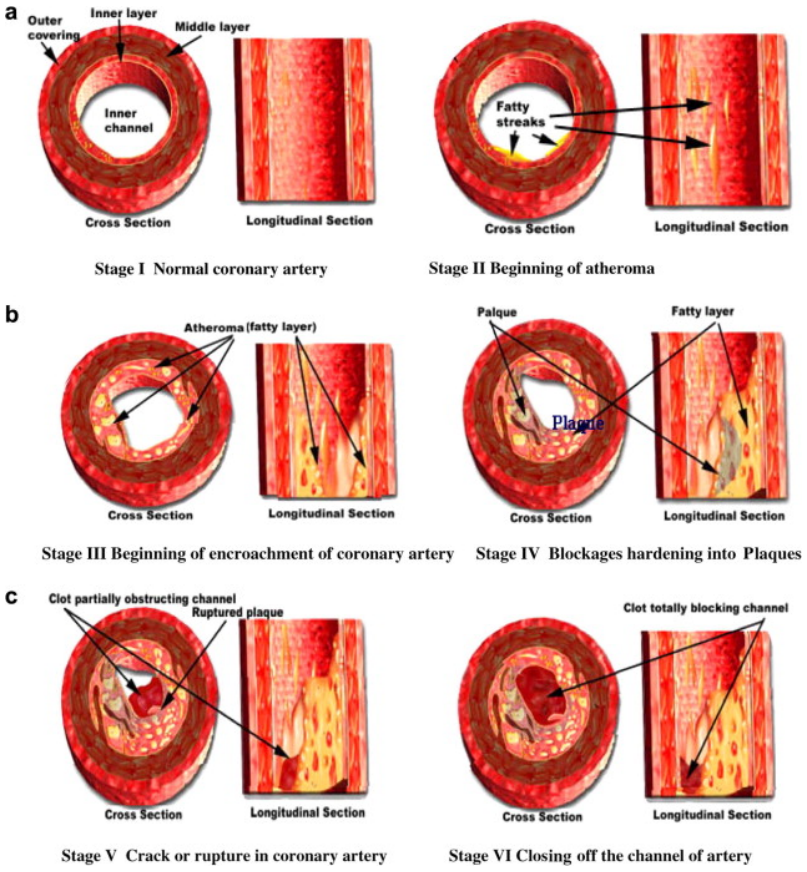

FIGURE 1 - Different stages in progression of atherosclerosis (from[9])

LDL diffuse passively through endothelial cell (EC) junction and their retention in the arterial wall seems to involve interaction between the extracellular matrix and the apolipoprotein B (apoB), which is the major protein component of low density lipoprotein. An ionic interaction of positively charged regions of apolipoprotein (apo) B with matrix proteins,

including proteoglycans, collagen, and fibronectin, is thought to initiate the LDL retention process[10]. In addition, other apoB-containing particle (i.e. lipoprotein (a) and remnants), can accumulate in the intima and promote atherosclerosis[2]. It has been shown that native LDL is not able to load macrophages so rapidly to transform them into foam cells, and so it was proposed that LDL, trapped within vessel wall, does undergo modification, including oxidation, lipolysis, proteolysis and aggregation. Such modifications contribute to inflammation as well as foam cell formation[2]. Macrophages within atheroma show a substantial upregulation of the scavenger receptor that normally function in the recognition and internalization of pathogens and apoptotic cells. Brown, Goldstein and co-workers gave it the name of "scavenger receptor" (SR) to distinguish the uptake of modified LDL by macrophages from LDL uptake via the classical LDL receptor (LDLr) which, in contrast with SR, is feedback inhibited by cellular cholesterol accumulation[5, 11]. The most frequently studied modification of low density lipoprotein is the oxidation, that in-vivo seems to occur by the action of several enzymes, such as NAD(P)H oxidase, xanthine oxidase, mieloperoxidase, nitric oxide synthase (NOS), lipoxygenase and mitochondrial electron transport chain[12]. In vivo it has been shown that products derived from oxygen, chlorine, nitrogen and lipid peroxidation induced free radicals are the major oxidants involved in oxidized LDL (oxLDL) formation. Consistently with the demonstrated role of oxidative stress and oxLDLs in atherosclerosis, it has been shown that variable levels of oxLDL are detectable in human plasma and F_2-isoprostanes (IsoPs) can be measured in human body fluids such as plasma and urine using highly precise methods. The F_2-IsoPs represent, in 2005, the most established index of oxidative stress status in vivo in humans[13]. Both these factors are proposed and considered as strong and independent risk factors for coronary heart disease (CHD) [14, 15]. It has been proposed that OxLDLs

exert their atherogenic activity not only inducing foam cells formation, but also by means of a proinflammatory, immunogenic, cytotoxic and apoptotic activity. They are shown to stimulate adhesion molecule expression in endothelial cells, resulting in increased monocyte recruitment. Moreover OxLDLs seem to promote monocyte differentiation, to stimulate in macrophages the production and secretion of proinflammatory cytokines and chemokines, and to inhibit macrophage migration ability[2, 16]. As mentioned above, low density lipoproteins, trapped inside atheroma, undergo several modification reactions leading to atherogenic compounds: it has been demonstrated that LDL glycation is at least as important as oxidation in atherogenesis (for review see[17]). It has been shown that both glycated LDL (glycLDL) and oxLDL were localized in macrophage-derived foam cells in the atherosclerotic lesions with the native LDL confined to the extracellular matrices. Glycation and oxidation of LDL probably take place simultaneously in vivo, leading to additional modification of LDL (i.e. Glycoxidated LDL)[17]. Post-secretory glycation of proteins is a process of non-enzymatic condensation of glucose with proteins to form stable covalent adducts leading to structural alterations and, consequently, to functional abnormalities.

TABLE I – Classification of human atherosclerotic lesions	
Type I	The inner lining of the normal coronary artery is smooth and free of blockages or obstructions
Type II	With increasing age, lipids or fatty substances (cholesterol and triglycerides) are deposited as fatty streaks which are only minimally raised and do not produce any obstruction or symptoms. This is just the beginning of atheroma.
Type III	Further increase in builtup of fatty layers, atheroma, begins to encroach the inner channel which starts interfering with the free blood flow through coronary artery, thereby exposing the person to more risk of coronary artery disease
Type IV	With fibers beginning to grow in the fatty layers of the atheroma, the blockages harden into plaques, which increase the encroachment in the inner channels of the coronary artery. This encroachment may be up to 50% or more of its diameter and leads to obstruction sufficient to decrease the blood flow of heart muscle, even in the time of its increased need (exercise, emotional stress). This leads to elevation in blood pressure and heart rate.
Type V	In some cases, plaques within the inner lining of the coronary artery may develop a slight crack or rupture, which stimulates the production of blood clots. The clots also get into the crack and cause it to rise and further obstruct the channel of the artery. The supply of the blood flow to the heart muscle is substantially reduced and the patient begins to have severe and prolonged chest pain that occurs at rest. This is known as unstable angina.
Type VI	In case the clot does not fully close the channel of the artery and sufficient blood flow is maintained to the heart muscle, a heart attack may not develop, provided appropriate and prompt treatment is effected. However, the clot may continue to grow in many cases. This can completely fill the open channel of the artery and cutoff blood flow to the part of the heart muscle to which it is supplying.

Created and adapted by using classification from[9]

The non-enzymatic glycation of LDL takes place principally in apoB. Lysine is the major amino acid that undergoes glycation with 2–17% of LDL–lysine residues being glycated. Because of their glycemic status, diabetic patients showed higher serum levels of glycated apoB and glycLDL compared to non diabetic subjects, but high levels of serum glycLDL were also found in hyperlipidemic non diabetic patients[17].

Ischemia, a restriction in blood supply, is one of the most frequent clinical manifestations associated to atherosclerosis, and the loss of luminal diameter secondary to plaque growth seems to not be the major determinant for ischemic event. In fact, it has been known for 20 years that there is a compensatory adaptive enlargement or "positive remodeling" of atherosclerotic human coronary arteries, with preservation of the cross-sectional area of the lumen in the early stages of the disease process. This knowledge is based on the findings of autopsy studies that demonstrated that the coronary lumen did not decrease in size until the atheroma occupied >40% of the area encompassed by the outside wall of the artery[18]. Pathological studies suggest that the development of acute coronary events depends principally on the composition and vulnerability of the plaque rather than severity of stenosis. Hence, the fibrous cap weakening seems to be a key role player in ischemic attack genesis. Several evidences have supported the concept that the protective fibrous cap, far from being fixed and static, actually can undergo continuous and dynamic remodeling and displays considerable metabolic activity. The progressive erosion of this element could cause the partial or the total rupture of atherosclerotic plaque, leading to the activation of coagulation cascade and thrombus formations. Thrombosis represents the final stage of atherosclerosis and leads to clinical manifestations and adverse events in the coronary, cerebro-vascular and vascular disctrict[19].

COMPLEX AETIOLOGY: INFLAMMATION AND DYSLIPIDEMIA

Over the past 50 years, epidemiological studies have shown what kind of complexity is behind atherosclerosis, revealing a very large number of risk factors involved[2]. These can be divided into factors with an important genetic component, and those that are largely environmental. The relative abundance of the different plasma lipoproteins appears to be of primary importance, since raised levels of atherogenic lipoproteins are a prerequisite for most forms of the disease[2]. Each of the genetic risk factors involves multiple genes. Experimental studies with genetic crosses in animals maintained under similar environmental conditions could clearly show this complexity. Such studies in rodents have revealed dozens of genetic loci that contribute to lipoprotein levels, body fat and other risk factors. Moreover, the interactions between risk factors increase the complex aetiology. Frequently, these are not simply additive (e.g. the effects of hypertension on coronary heart disease (CHD) are considerably amplified if cholesterol levels are high).[2] The importance of genetics and environment in human CHD has been examined in many family and twin studies, and it has been shown that the common forms of CHD result from the combination of an unhealthy environment, genetic susceptibility and our increased lifespan[2]. The concept that atherosclerosis is a chronic inflammatory disease, initiated by monocytes adhesion to activated endothelial cells, is widely accepted and has been confirmed by several clinical and experimental observation[2, 4, 20, 21]. "Inflammation is a complex set of interactions among soluble factors and cells that can arise in any tissue in response to traumatic, infectious, post-ischaemic, toxic or autoimmune injury"[22] and seems to be involved in every stage of atherosclerosis. During fatty streaks formation, endothelium activation results in secretion of chemokines (e.g. MPC-1 and IL-8), adhesion molecules (e.g. ICAM-1,

VCAM-1, E-selectin, P-selectin). These molecules enhance monocyte and lymphocyte recruitment and infiltration in the sub-endothelial space[21]. Inflammation seems to play an important role also in the final stage of the pathology, when clinical events occur. Cells inside plaque secrete matrix metal proteases (MMPs), which degrade the cellular matrix protein leading to fibrous cap weakening[23]. Moreover every cell types involved in atherogenesis (i.e. endothelial cells, SMC, monocytes/macrophages, lymphocytes (T, B, NKT), dendritic cells, and mast-cells) secrete or are stimulated by peptides, glycoproteins, protease and cytokines.

Though inflammation allows understanding several mechanisms involved in atherosclerosis, it must be reminded that lipid accumulation is an essential factor for the development of this disease. Moreover, as mention above, raised levels of atherogenic lipoproteins appears to be of primary importance. Dyslipidemia can be defined as the disorder of lipid metabolism leading to an increase (hyperlipoproteinemia) or a decrease (hypolipoproteinemia) of plasma lipoprotein, often associated with their qualitative alteration. The role of plasma lipoproteins in atherosclerosis came under study more than 50 years ago, at the same time as epidemiologic data linking cholesterol and heart disease were becoming available[24]. The lipid hypothesis, which described the role of dietary saturated fat in raising serum cholesterol and the risk of CHD, could not be adequately tested because the treatment options of the time—fibrates, anion-exchange resins, and nicotinic acid—were limited in efficacy and poorly tolerated[24, 25]. Key discoveries in the 1970s, including the identification of the hepatic LDL-C receptor and characterization of hepatic hydroxymethyl glutaryl coenzyme A (HMG-CoA reductase), the enzyme that controls the rate of cholesterol biosynthesis, led to development of HMG-CoA reductase inhibitors, beginning with lovastatin and simvastatin in the 1980s[24, 26-30]. Clinical trials

in the 1990s confirmed the lipid hypothesis and established LDL-C as the primary treatment target[24, 25, 31]. A publication from the Reduction of Atherothrombosis for Continued Health (REACH) Registry of patients (N=67,888) with stable atherosclerotic clinical syndromes highlights that hypercholesterolemia is prevalent, yet undertreated, and correlated generally with cardiovascular event rates around the world[32, 33]. It was shown that substantial proportions of these patients had total cholesterol levels above 5.18 mmol/L (200 mg/dL), with a high of 64.4% in Eastern Europe. Such high rates of hypercholesterolemia are remarkable because more than 75% of these patients were receiving lipid-lowering therapy, predominantly statins. Since 1994, 8 placebo-controlled trials that enrolled 67,462 patients with dyslipidemia have evaluated the efficacy of statin therapy for the primary or secondary prevention of CHD events. In these trials, LDL-C reductions ranging from 25% to 35% corresponded with relative risk reductions of 24% to 40% for CHD death or nonfatal myocardial infarction[25, 34-41], suggesting that, despite the good improvement, a substantial risk of CHD events remains.

Substantial epidemiologic evidence suggests a negative linear correlation between HDL-C levels and the incidence of CHD. An inverse relationship between HDL-C and coronary artery disease (CAD) was established in the Framingham study in the 1970s. This analysis of 2815 men and women aged 49–82 years identified HDL-C as a powerful risk factor inversely associated with the incidence of CAD[42]. Similarly, in the early 1980s, the Prospective Cardiovascular Munster (PROCAM)study[43] evaluated 4559 male participants aged 40–64 years and found a strong negative linear correlation between the incidence of CAD and HDL-C levels (CHD risk ratio of 4.0 for HDL-C <25 mg/dL versus 1.0 for HDL-C >65 mg/dL, $p < 0.001$). Different prospective studies has estimated a 2% (men) and 3 % (women) increase in cardiovascular risk for every 1 mg/dL reduction in serum HDL-C[44]. A post hoc analysis[45] of the Treating

to New Targets (TNT) study[46] was conducted to evaluate the predictive value of HDL-C for the 5-year risk of major CV events. It was showed that HDL-C was a significant predictor of major CV events (*P*=0.05) across the population of statin-treated patients with CHD, and high levels of HDL-C significantly reduced the 5-year risk of major CV events (*P*=0.03), even in patients with low LDL-C (<70 mg/dL) [45]. The finding that HDL-C remained a significant predictor of major CV events in patients with very-low LDL-C supports the clinical relevance of HDL-C as a therapeutic target. Statin-treated patients who achieve a low LDL-C goal still have a substantial residual risk that can perhaps be reduced by increasing the HDL-C concentration[45]. In 2006, the phase III clinical trial ILLUMINATE (Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events) was prematurely terminated because of an increased risk of death and cardiac events in patients receiving torcetrapib[47], a novel drug targeted to cholesterol ester transfer protein (CETP) inhibition to increase HDLc. The CETP inhibition approach is still widely debated, and the particular concern is if the deleterious effect of torcetrapib resides in its intrinsic properties (failure of the molecule) or the whole concept of CETP inhibition is erroneous (failure of the mechanism)[48-51]. One interesting concept that has also been highlighted is the complexity of the relationship between HDL and protection from cardiovascular disease. Functionality has become an important factor for consideration in the development of new agents that raise HDL-cholesterol. The most important function of HDL is its ability to facilitate cholesterol efflux from peripheral cells, particularly macrophages and foam cells, and enhancing reverse cholesterol transport (RCT). Recently it has been demonstrated by in-vitro cellular cholesterol efflux assay that in murine peritoneal macrophages foam cells the ABCA1-mediated efflux to human serum contributes ~80% of the stimulated component of total cholesterol efflux, with ABCG1-mediated efflux responsible for the

remaining 20% of the stimulation of efflux[52]. Very similar results were also obtained using in vivo mouse RCT assays[53]. Thus apolipoprotein A-I (apoA-I), the apolipoprotein of nascent HDL and the preferred acceptor for ABCA1 mediated efflux (see below), seems to play a central role in cholesterol efflux, consistent with their value in predicting cardiovascular risk (for review see[54]).

LIPOPROTEIN METABOLISM

Cholesterol, triglycerides (TG, known also as triacylglycerols) and phospholipids (PL) are the major plasma lipid compounds, whereas fewer amount of other lipophilic compounds, such as lipophilic vitamins and hormones, are detectable. Since their lipophilic nature, they are internalized and carried through blood stream by macromolecular complexes called lipoprotein, resulting from lipid binding to specific protein called apolipoprotein. Liver and intestine are the main organs responsible for lipoprotein synthesis and secretion. Lipoproteins are globular particles characterized by high mass. They consist of a central non-polar nucleus (mainly rich in TG and cholesteryl esters (CE)) which is enveloped by a polar membrane made up of PL, proteins and free cholesterol (FC). Lipoprotein differs one from each other because of density, apolipoprotein and lipid composition. The apolipoprotein content is an extremely important factor for lipoprotein metabolism. Apolipoproteins act indeed not only as structural component, but as enzymatic cofactors and receptor binding element. Lipoproteins can be isolated from plasma by density ultracentrifugation and be classified as follow to:

- Chylomicrons and remnants
- Very Low density lipoprotein (VLDL)
- Intermediate density lipoprotein (IDL)
- Low density lipoprotein (LDL)
- High density lipoprotein (HDL)

The handling of lipoproteins in the body is referred to as lipoprotein metabolism, and in physiological condition it contributes to maintain body lipid homeostasis. It is divided into two pathways, exogenous and endogenous, depending in large part on whether the lipoproteins in

question are composed chiefly of dietary (exogenous) lipids or whether they originated in the liver (endogenous).

ENDOGENOUS PATHWAY

In order for the body to make use of dietary lipids, they must first be absorbed from the small intestine. Since these molecules are oils, they are essentially insoluble in the aqueous environment of the intestine. The solubilization (or emulsification) of dietary lipids is therefore accomplished by means of bile salts, which are synthesized from cholesterol in the liver and then stored in the gallbladder; they are secreted following the ingestion of fat. The emulsification of dietary fats renders them accessible to pancreatic lipases (primarily lipase and phospholipase A_2 (PLA_2). These enzymes, secreted into the intestine from the pancreas, generate free fatty acids and mixtures of mono- and diacylglycerols from dietary triglycerides. Pancreatic lipase degrades triglycerides at the 1 and 3 positions sequentially to generate 1,2-diacylglycerols and 2-acylglycerols. Phospholipids are degraded at the 2 position by pancreatic PLA_2 releasing a free fatty acid and the lysophospholipid. The products of pancreatic lipases then diffuse into the intestinal epithelial cells, where the re-synthesis of triglycerides occurs. Dietary triacylglycerols and cholesterol are conjugated with several apolipoprotein (e.g. apoB-48, apoC-I, apo-CIII) and incorporated into nascent chylomicrons. These are synthesized by intestinal epithelial cells and then secreted into the blood through lymphatic system. In the bloodstream, HDL particles transfer apoC-II and apoE to the nascent chylomicrons. ApoE, interacting with plasma membrane heparan sulfate proteoglycans, binds chylomicron with endothelial cells and by apoC-II, chylomicrons activate lipoprotein lipase (LPL). LPL catalyzes a hydrolysis reaction that ultimately releases glycerol and fatty acids from the chylomicrons. Glycerol and fatty acids can be absorbed in peripheral

tissues, especially adipose and muscle, for energy and storage. After removal TG removal from the non polar nucleus of chylomicrons, by the action of apoC-I and apoC-III particles leave endothelial cells and start to exchange apolipoprotein with the other lipoprotein present in the blood stream. This phase results in the formation of a new lipoprotein called remnant chilomicron enriched in CE, apoB-48 and apoE. Through apoE binding to specific receptors (about 50% LDLr) on hepatocytes, remnants are taken up by the liver by endocytosis and then degradated in lysosomes. Experimental studies by electronic microscopy have shown a new possible pathway (LDLr independent) for hepatic uptake of remnants[55]. Remnants pass through endothelial cell-fenestrae to the space of Disse where they bind initially to proteoglycan-bound apo E and hepatic lipase as well as LDLR, all of which are anchored to the microvillar membrane. Proteoglycan-bound hepatic lipase binds and hydrolyzes remnant-lipids, increasing exposure of the endocytic receptor-binding domain of apo E. Additional proteoglycan-bound apo E on microvilli acquired by the remnants increases the affinity of the remnant particles for LDL receptor related protein (LRP)[55].

ENDOGENOUS PATHWAY

Newly synthesized hepatic triacylglycerols and cholesterol, as well as dietary triacylglycerols and cholesterol, are assembled with apoB in the liver to form VLDL. The synthesis of VLDL has been extensively studied in cultured hepatic cell system[56-59]. By the results of these studies, a two-step models of VLDL assembly has been proposed[60]. 1. In the first step, pre-VLDL is synthesized in the presence of lipids. In this step APOB is cotranslationally lipidated with the assistance of the microsomal triglyceride transfer protein (MTTP) that acts both as a lipid transporter and as a chaperon[61-63]. This process takes place in the endoplasmic reticulum. During the second step, mature VLDL is formed by bulk lipid

addition via fusion of pre-VLDL with large TG droplets. The confinement of the second step is under debate and both Golgi apparatus[64] and smooth ER[64, 65] have been implicated. Finally, in the absence of lipids the nascent APOB is degraded (III). The two-step model is compatible with the idea that the availability of lipids determines the stability of APOB. In agreement with this hypothesis, it was shown that unlipidated or underlipidated APOB is the subject of presecretory proteasomal degradation[66-68]. VLDLs secreted by human hepatocytes ranges from 350 Å to 700 Å depending on the amount of TG in the lipid core. There are two secretion-competent subclasses of VLDL particles: large buoyant TG-rich VLDL 1 and smaller, denser and cholesteryl ester rich VLDL2. The VLDL particles secreted by the liver pass through a number of remodeling steps in the circulation involving lipolysis and protein transfer through LPL and HL, giving rise to IDLs and ultimately LDL. Remaining VLDL can be removed from the circulation by the hepatic uptake mediated by LRP and the VLDL receptor (VLDLr). LDL lipoproteins, mainly cholesterol enriched, interact with LDLr that is expressed in every mammalian cells and bind to apoB-100 and apo-E present in LDL. After binding to LDLr, LDL undergo endocytosis and then are internalized into lysosoms, where the acid lipases hydrolyze CE. Thus, free cholesterol is available for membrane needs, steroid hormone synthesis and storage (after re-esterification by the ACAT enzyme (for details see below)). Before the interaction with LDL receptor, low density lipoproteins can be modified by the action of cholesterol ester transfer protein (CETP) that transfer cholesterol ester (CE)

TABLE II - LIPOPROTEIN CLASSIFICATION AND FEATURES						
CLASS	Density (g/ml)	Diameter (nm)	% protein	% cholesterol	% phospholipid	% triacylglycerol
HDL	>1.063	5-15	33	30	29	8
LDL	1.019-1.063	18-28	25	50	21	4
IDL	1.006-1.019	25-50	18	29	22	31
VLDL	0.95-1.006	30-80	10	22	18	50
CHILOMICRON	<0.95	100-1000	<2	8	7	84

from HDL to LDL (only when hypertriglyceridemia is present, to VLDL[69]) in exchange for triglyceride (TG), and viceversa[70]. LDLr mediated cellular cholesterol influx results in a reduction of cellular cholesterol synthesis, reduction in LDLr expression and increase of CE synthesis. The intracellular and membrane amounts of fatty acids and cholesterol are constantly coordinated with de novo lipid synthesis which is controlled by the transmembrane sterol regulatory element binding proteins (SREBPs) [71]. In the presence of cholesterol or oxysterols, SREBP, SCAP (SREBP cleavage activating protein) and INSIG (insulin inducible gene) form a complex which is retained in the ER. When the concentration of cellular sterols is low, INSIG is released from SREBP-SCAP which is transported to the Golgi apparatus where SREBP undergo two sequential proteolytic cleavages by Site-1 and Site-2 proteases[72]. SREBP-1_a, SREBP-1_c and SREBP-2 proteins are encoded by 2 unique genes (i.e. SREPBF-1 and SREPBF-2) and belong to the SREBP family of basic-helix-loop-helix-leucine zipper transcription factors. The SREBPs differ in their tissue-specific expression, their target gene specificity and the relative potencies of their trans-activation domain. Though SREBP-1_a and -1_c proteins regulate gene involved in the

synthesis of mono- and poly-saturated fatty acids (PUFA) and their incorporation into triglycerides and phospholipids, SREBP-1_a is more potent transcription factor. The expression of SREBP-1_c predominates in mouse liver in the fed state. Conversely SREBP-1_a and SREBP-2 are expressed ubiquitously. SREBP-2 preferentially activates gene involved in the uptake and biosynthesis of cholesterol[71].

REVERSE CHOLESTEROL TRANSPORT

The concept of "reverse cholesterol transport" (RCT) was first introduced in 1968 by Glomset to describe the process by which extrahepatic (peripheral) cholesterol is returned to the liver for excretion in the bile and ultimately the feces[73]. The return of this "peripheral" cholesterol to the liver is necessary to balance cholesterol intake and de novo synthesis and thus to maintain whole-body cholesterol homeostasis[74]. The inverse relationship between RCT and atherosclerosis was suggested by Ross and Glomset[75], who hypothesized that atherosclerotic lesions develop when an imbalance occurs between the deposition and removal of arterial cholesterol after endothelial injury. Some years later it was suggested that on the basis of the inverse relation between HDL cholesterol (HDL-C) and cardiovascular disease, increasing HDL should be considered as a way to increase clearance of cholesterol from the arterial wall to prevent cardiovascular disease[76]. In humans, approximately two-thirds of cholesterol is transported by low-density lipoproteins (LDLs) and ~20% by high-density lipoproteins (HDLs); the remaining cholesterol is carried by very low density lipoprotein (VLDL) particles. The risk of premature cardiovascular disease is positively correlated with LDL levels and negatively correlated with HDL levels. Cholesterol is an essential component of eukaryotic membrane and a fundamental precursor of steroid hormones and bile acids. It has been demonstrated that a physiological free cholesterol (FC)/phospholipid ratio in cellular membranes is necessary to maintain proper membrane fluidity, or more precisely, a proper range of membrane fluidities. Cholesterol is, in fact, able to reduce membrane fluidity, contrasting the effect of unsatured fatty acids contained in membrane phospholipids. It is abundant in the cellular plasma membrane and in the membrane of Golgi apparatus[77], especially in lipid rafts, which are areas of the plasma membrane

enriched in proteins that participate in signal transduction[78]. Non-hepatic cells acquire cholesterol through uptake of lipoproteins and de novo synthesis and yet (apart from steroidogenic tissues that convert cholesterol to steroid hormones) are unable to catabolize it, and this seems to explain the physiological needs for RCT process[74]. Several studies have been demonstrated that excess unesterified cholesterol (FC) is toxic to cells (for review see[79]), and many mechanisms have been proposed to explain this cytotoxicity (i.e. loss of membrane fluidity, disruption of membrane domain, induction of apoptosis, Intracellular cholesterol crystallization, formation of toxic oxysterols, alteration of gene expression)[79]. Therefore, cells have developed several ways to protect themselves against free-cholesterol induced toxicity. One possible proposed mechanism is indentified in the activity of acyl-conzyme A:cholesterol acyltransferase, and specifically ACAT1, that transform free cholesterol in cholesterol ester (CE), subsequently stored in cytoplasmic lipid droplets, to be available for later on cell needs (for more details see review[80] and below).

It has been well established and clearly demonstrated that one key pathway, in preventing cellular cholesterol accumulation, is the efflux of cholesterol to extracellular "acceptors" (physiological represented by high density liprotein (HDL)). Cellular cholesterol efflux is the first step of RCT pathway, and is also considered critical and rate limiting stage for the entire process. Physiologically, RCT clearly occurs from all peripheral tissues, it has often been measured and discussed as a general peripheral process. More recently it has been suggested and strongly supported the concept to the more specific term "macrophage RCT" when discussing this process as it relates to atherosclerosis[74]. As explained, in atherosclerotic lesions, the primary cell type that is overloaded with cholesterol is the macrophage, and therefore, it makes more sense to conceptualize and measure RCT as a macrophage-

specific phenomenon when the aim is to focus on its antiatherosclerotic characteristics[74].

The entire process of RCT can be summarized in 3 fundamentals steps:

- uptake of cholesterol from cells by specific acceptors (cholesterol efflux)
- HDL remodeling
- hepatic cholesterol re-uptake through lipoprotein receptors

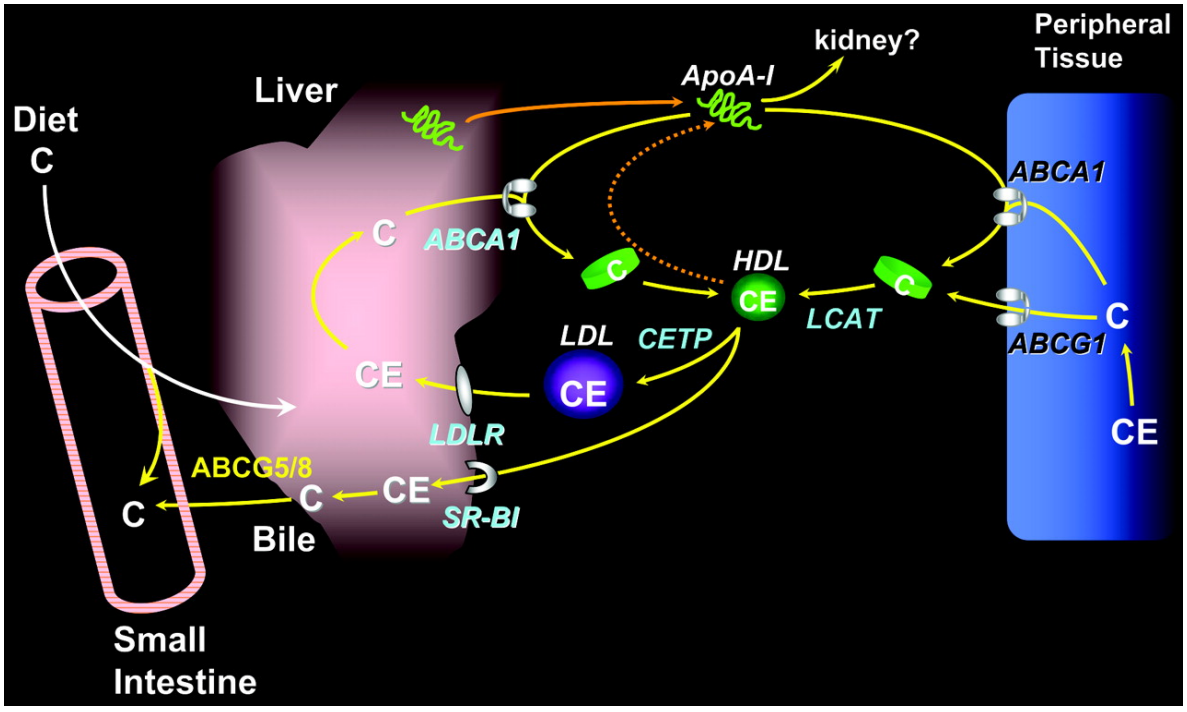

FIGURE 2 – Schematic representation of Reverse Cholesterol Transport (from[81])

CHOLESTEROL EFFLUX PATHWAY: MECHANISMS AND TRANSPORTERS INVOLVED

Cellular cholesterol efflux is the transfer of FC molecule from intracellular compartment towards an extracellular acceptor[82]. There are 3 known mechanisms of FC flux: the aqueous passive diffusion, the accelerated and bidirectional SR-BI-mediated FC flux, and the unidirectional active transport mediated by the ATP binding cassette (ABC) transporters[83].

Aqueous passive diffusion occurs thanks to the sufficient hydrophilic properties of FC molecule. Cholesterol molecules are desorbed at the lipid/water interface, they diffuse through aqueous phase, and subsequently they are absorbed by the acceptor[84]. Water phase around the cell has been shown to represent a barrier to passive diffusion[85], and this kind of mechanism is facilitate when the ratio FC/phospholipids is elevated in the cellular membrane and reduced in the cholesterol acceptor[82]. It has been demonstrated that the rate of aqueous diffusion can be increase by several factors able to reduce the membrane lipid-bilayer density (i.e. high curve radius in cellular plasma membrane, low ratio sphingomyelin/phosphatidyl choline and levels of unsaturated phospholipids)[85]. Cholesterol efflux by aqueous diffusion occurs in every cell type, it is generally slow, not saturable, and inefficient in depleting cells from cholesterol excess. The transfer is bidirectional and regulated by the concentration gradient between cell and acceptor[86]. The rate of this pathway is strongly related to the structure and size of the acceptor. The particle size seems to have a key role, because it affects the collision with cholesterol molecule within aqueous phase (big size acceptor seems to be less efficient)[84].

Cholesterol efflux by passive diffusion can be facilitate by plasma membrane proteins such as the scavenger receptor type BI (SR-BI) [83]. Initially identified as receptor for LDL and modified LDL (i.e. AcLDL and OxLDL) [87, 88], it has been demonstrated that not only SR-BI is able to bind HDL with high affinity leading to the selective uptake of CE contained in these lipoprotein[89], but it can also mediate cellular FC efflux towards phospholipid-rich acceptors like HDL[90]. It is a 82-kDa membrane glycoprotein (509 amino acids) containing a large extracellular domain and two transmembrane domains with short cytoplasmic amino- and carboxy-terminal domains[88]. Major sites of SR-BI expression are the liver and steroidogenic glands. SR-BI is also present in other tissues and cells including the brain, the intestine, macrophages, endothelial cells, and astrocytes and it is clustered in specialized plasma membrane domains including caveolae, microvillar channels, and microvillar extensions[91]. It has been demonstrated that SR-BII (also called SR-BI.2) can be generated by the alternative splicing of SR-BI mRNA: this protein shows a cytoplasmic carboxy-terminal domain completely different. SR-BII has been shown to be strongly less efficient than SR-BI both in CE uptake from HDL and in cholesterol efflux to HDL. This observation suggested the importance of carboxy-domain for SR-BI activity[92]. SR-BI mediated cellular cholesterol efflux occurs preferentially to mature HDL, and is bidirectional and dependent on concentration gradient[83]. Several studies demonstrated that inhibition of HDL binding to SR-BI, by means of specific antibody, leaded to a big reduction of cholesterol efflux, suggesting that HLD-binding could be a key-role player for SR-BI mediated FC efflux[93]. It has been clearly established that SR-BI - mediated efflux occurs only if the phospholipids are present in the structure of cholesterol acceptor. Several data indicated that phospholipids-enrichment of HDL or total serum increased cholesterol efflux by SR-BI. Conversely, efflux decreased when HDL were depleted in

phospholipid by the treatment with phospholipase A2 (PLA2). No cellular FC efflux occurs in presence of lipid-free apolipoproteins[94, 95]. Not only the quantity of phospholipids seems to affect this process, nut also the quality. It has been demonstrated that phosphatidyl choline-enrichment of HDL leaded to an higher extent increased SR-BI efflux than sphingomyelin-enrichment[94]. Nowadays, the real mechanism of action of this protein in mediating cellular FC efflux still remains unclear[83].

The ATP-binding cassette (ABC) protein ABCA1 was first cloned in 1994 and is member of a large family of protein including 49 mammalian transmembrane transporters (of whom 13 are sub-classified as ABCA) that transport a wide diversity of substrates across the lipid bilayers in an energy-dependent manner[96]. The special role of *ABCA1* in cholesterol efflux became apparent when it was identified as the gene that is mutated in Tangier disease (in its homozygous or compound heterozygous form)[97-100]. Previous study showed that cholesterol-enriched fibroblasts and macrophages from patients with Tangier disease lacked the ability to release both phospholipid and FC to lipid-free apolipoproteins but that efflux to mature HDL was normal[101, 102]. Clinical studies demonstrated that individuals with this pathology have almost no HDL cholesterol, and their apo A-I remains poorly lipidated and is rapidly catabolized. Thus it has been suggested and demonstrated that, in mediating the efflux of FC and phospholipid from cells, ABCA1 also mediates the lipidation of apo AI and the formation of nascent HDL (for review see [83, 103, 104]). Pre-ß HDL generally contain 2 copies of apoA-I per particle and 10% by mass of lipid (free cholesterol and phospholipids). In contrast with passive diffusion and SR-BI mediated efflux, ABCA1 mediates unidirectional transport of cellular phospholipid and FC using ATP as an energy source, and the preferred acceptor is lipid poor apolipoproteins such as apolipoprotein A-I (apoA-I)[103]. ABCs transporters

have been structurally classified into 2 groups: *1*) whole, transporters having two similar structural units joined covalently; and *2*) half, transporters of single structural units that form active heterodimers or homodimers[105]. ABCA1 (whole) is a 2,261-amino-acid integral membrane protein with two halves of similar structure. Each half has a transmembrane domain containing six helices and a nucleotide binding domain (NBD) containing two conserved peptide motifs known as Walker A and Walker B, which are present in many proteins that utilize ATP, and a Walker C signature unique to ABC transporters. It has been predicted that ABCA1 shows NH_2 terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds[105]. Several models have been proposed to explain how ABC proteins, and ABCA1 because of structure homology, transport amphipathic lipids out of cells [106]. The flippase model seems to be the most correct. It involves two conformations for the transporter: (a) the transporter is accessible to the cytoplasmic leaflet of the plasma membrane where the substrate binds and (b) the substrate is released into the outer leaflet and thus accessible to fluid-phase acceptors[106]. Another model suggests a hydrated channel for the lipid to travel. However, this seems thermodynamically unlikely in the case of phospholipids. It has been also suggested a variation of the flippase model. This proposes that the substrate is transferred to the outer leaflet but its release is conditional on interaction of the transporter with an acceptor[106]. By means of the crystal structure obtained for the bacterial Sav1866 ABC transporter a (the first of one full ABC transporter)[107] it has been shown an intimate interaction between transmembrane helices and the formation of a cavity, that is shielded from the cytoplasm and inner leaflet of the membrane bilayer, but accessible to the outer leaflet and extracellular environment. Since the cavity is lined with polar amino acid residues, it was suggested that transport could occur through an

'extrusion' mechanism. ATP hydrolysis could enable the transporter to adopt a conformation binding to the substrate on the cytoplasmic side of the membrane. This could occur through structural changes transmitted through intracellular loops that connect the transmembrane helices[107]. It has been demonstrated that many of the human mutations in ABCA1 are in such connecting loops, several of these loops interact closely with the NBD[108]. ABCA1 is localized both in the plasma membrane and intracellular compartments and it appears to target specific membrane domains for lipid secretion. These regions seem to be sensitive to accumulation of cholesterol and other lipophilic compounds that could feed into intracellular compartments and become substrate for ACAT[104]. ABCA1 removes cholesterol that would otherwise accumulate as cytosolic cholesteryl ester lipid droplets. One possibility for the link between ABCA1 and ACAT is that both proteins function to protect cells from incorporating excess free cholesterol into the endoplasmic reticulum where it may disrupt the peptide biosynthetic machinery[104]. It has been demonstrated that when cholesterol esterification is blocked in sterol-loaded macrophages, the potentially cytotoxic free cholesterol that accumulates is a preferred substrate for the ABCA1 pathway[109]. Regarding these observations, two models have been proposed to account for the ability of ABCA1 to target specific lipid domains and mediate cholesterol efflux. The exocytosis model implies that excess intracellular cholesterol is packaged into transport vesicles or rafts, perhaps in the Golgi apparatus, which translocate to domains in the plasma membrane containing ABCA1. The retroendocytosis model suggests that ABCA1- and apolipoprotein-containing vesicles endocytose to intracellular lipid deposits, where ABCA1 pumps lipids into the vesicle lumen for release by exocytosis[104]. Cross-linking experiments has showed that apo-AI can bind to ABCA1[110], and it has been suggested that binding of apo-AI might be necessary but not sufficient

to promote cholesterol efflux[104]. Recent findings have suggested that both plasma membrane and intracellular cholesterol pool are mobilized by ABCA1 during apoA-I lipidation. Though the highest level of cholesterol and ABCA1 in plasma membrane, it has been suggested an important role of intracellular cholesterol pool, especially the late endosomal/lysosomal compartment, in mediating ABCA1 cholesterol efflux. The mobilization of this specific cholesterol pool by internalized ABCA1, and apoA-I co-internalization, suggest the possibility of intracellular nascent HDL formation[111]. ABCA1 is widely expressed throughout animal tissues where it may have multiple and diverse functions. In humans, ABCA1 mRNA was reported to be most abundant in liver, placenta, small intestine, and lung. In mice, ABCA1 mRNA was reported to be most abundant in liver, kidney, adrenal, heart, bladder, testis, and brain[112]. Interestingly, measurements of ABCA1 protein levels in tissues showed discordance with mRNA abundance, in that some tissues with high mRNA levels (kidney, heart, bladder, and brain) had relatively low protein levels[112]. These observations are consistent with the possibility that posttranscriptional regulation plays a major role in tissue expression of ABCA1. ABCA1 expression is regulated by a variety of mechanisms (for review see[104]). Briefly: the transcription of ABCA1 is induced by nuclear orphan receptors, liver X receptors (LXR) α or ß, and retinoid X receptor (RXR), alone or in combination, in which case, the effect is additive. Oxysterols, including 27-, 22(R)-, and 20(S)-hydroxycholesterol, are potent ligands of the LXR, whereas 9-*cis*-retinoic acid is an effective RXR activator. The upregulation seen with cholesterol enrichment of cells is also probably mediated by the LXR/RXR system. ABCA1 transcription is also stimulated in murine macrophages by cAMP. Post transcriptional regulation of ABCA1 is afforded by apoA-I binding, which stabilizes the receptor and prevents its degradation, whereas unsaturated fatty acids seem to promote its turnover.

ATP binding cassette transporter G1 (ABCG1) belongs to the ABC half-type transporters and it is a 678-amino acid, 75.6-kDa integral protein. It has been proposed to act as homodimer in human and murine macrophages[113]. Other evidence has been suggested that ABCG1 may heterodimerise with ABCG4, as the proteins are closely related in amino acid sequence, and have nearly identical intron locations at their structural genes[114]. Like ABCA1, it is expressed in cholesterol loaded macrophages and seems to be active in cholesterol depletion, mediating its cellular efflux[115]. In cells, ABCG1 is expressed both in the plasma membrane and cytoplasmic compartment, and mediates the transport of cholesterol from cells to high density lipoprotein (HDL) but not to lipid-depleted apolipoprotein A-I. Moreover it has been suggested to be only a cholesterol transporter. [115] Conversely another study reported the ability of ABCA1 to mediate phospholipid cellular efflux (especially sphingomyelin) [116]. Studies from the same research group have also suggested that the ABCG1-mediated efflux of cholesterol and SM is dependent on the cellular SM level and distribution of cholesterol in the plasma membrane[117]. It has been proposed that ABCA1 and ABCG1 could efflux cholesterol in peripheral tissues by a synergistic relationship, where ABCA1 lipidates any lipid-poor/free apoA-I to generate nascent or pre-beta-HDL. These particles in turn may serve as substrates for ABCG1-mediated cholesterol export[118]. The transcription of ABCG1 is regulated by LXR/RXR system and the synthesis of oxysterols in macrophages seems to induce ABCG1 expression[119]. Moreoverit has been demonstrated that LXR activation induces redistribution of ABCG1 from macrophage intracellular sites to the plasma membrane and increases cholesterol mass efflux to HDL in an ABCG1-dependent pathway[120]. Northern blot analyses showed abundant expression of ABCG1 protein in the liver, lung and spleen[121], and dot blot analysis

revealed ubiquitous expression of ABCG1[122].Several studies confirm significant ABCG1 mRNA expression in the brain. In situ hybridization studies in mouse brain indicate mRNA mRNA is widely expressed in both the ventricular and mantle zones of embryonic brains and in both gray and white matter of postnatal brains. ABCG1 was also found to be expressed in all cortical layers as well as in the striatum and thalamus, and in many different classes of neurons in the mouse CNS. qRT-PCR analysis and western blot analysis of human fetal brain cells indicated the highest ABCG1 expression in microglia followed by oligodendrocytes, neurons and astrocytes. (for review see[123]).

Human ABCG5 was identified as the human homolog of the rodent isoform that was induced in the liver by treatment with LXR agonist, T0901317. ABCG5 is localized adjacent to ABCG8 on chromosome 2p21 and seems to be coordinately regulated with ABCG8 through common regulatory elements (such as the nuclear receptor LXR). As consequence this 2 transporters have similar tissue- and cell-specific expression patterns[124]. ABCG5 and ABCG8 proteins act as heterodimeric transporter[125], and are expressed at high levels in the canalicular membrane of hepatocytes, where they play an essential role in hepatobiliary cholesterol transport. It has been demonstrated that in Abcg5 Abcg8 (−/−) knockout mice biliary cholesterol concentrations are extremely low, compared with wild-type animals[126]. In addition, ABCG5/ABCG8 are expressed at lower levels in the apical membrane of enterocytes in small intestine and colon, where they limit the absorption of sterols from the lumen[124, 127]. It has been also shown that mutations in either ABCG5 or ABCG8 cause sitosterolemia, a rare autosomal recessive disorder characterised by accumulation of both plant-derived sterols (primarily sitosterol, but also campesterol, stigmasterol, and brassicasterol) and animal-derived sterol (cholesterol) in plasma and

tissues, leading to the development of xanthomas[124, 128]. Recent evidences seem to suggest that expression of the bile salt transporter ABCB4 is required for ABCG5/ABCG8-mediated biliary sterol secretion, suggesting that cholesterol excretion into bile requires bile salt micelles as cholesterol acceptor [129].

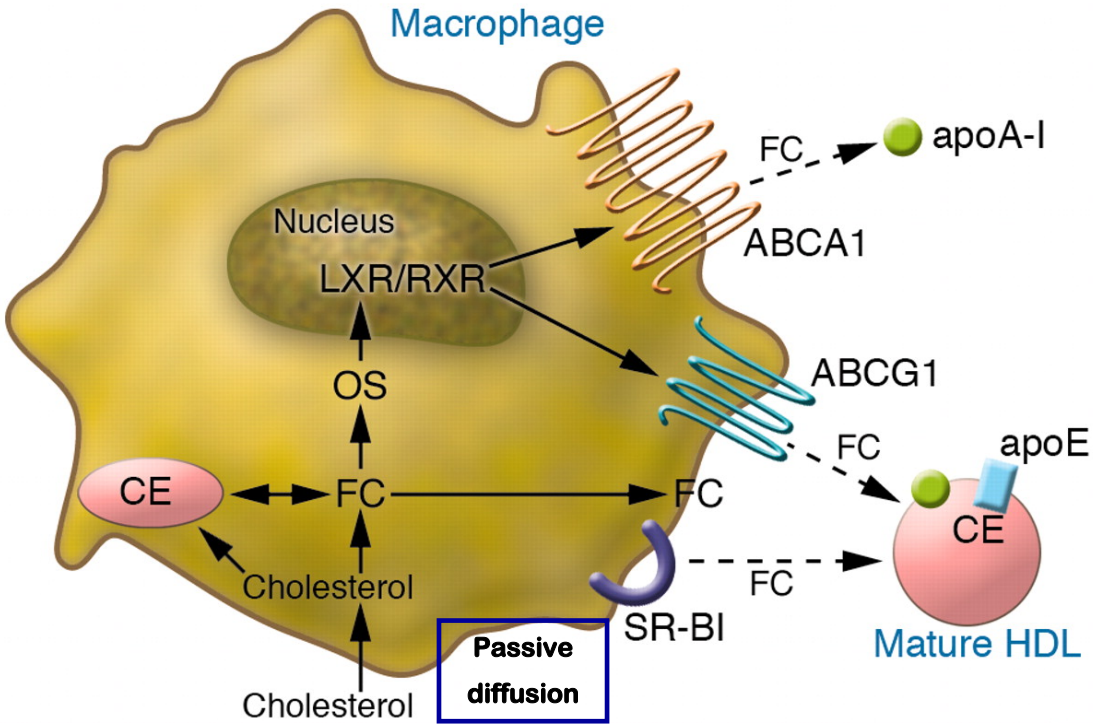

Figure 3 – Schematic representation of cellular cholesterol efflux (modified from[130])

THE SECOND AND THIRD STEP OF REVERSE CHOLESTEROL TRANSPORT

The nascent HDL particles undergo an intravascular process of maturation and remodeling through a several metabolic and enzymatic reactions. Initially, Lecithin cholesterol acyltransferase (LCAT) catalyzes the transfer of 2-acyl groups from lecithin to FC, generating CE (i.e. cholesteryl linoleate) and lysolecithin[70]. CE is more hydrophobic than FC and moves to the core of the lipoprotein particle, resulting in the modification of HDL particle shape. The discoidal preβ-HDL is transformed by LCAT activity in a more mature and spherical HDL (α-HDL)[131]. LCAT has been long proposed to play a critical role in promoting reverse cholesterol transport, by maintaining the correct cholesterol concentration gradient between cells and plasma HDL[73]. A defect in LCAT function would be expected to enhance atherosclerosis by interfering with this process. In a very recent review by Rader and Rothblat' s group[132], it has been suggested that while LCAT is clearly important for HDL metabolism, it could have not such a critical role in macrophages reverse cholesterol transport[132]. Moreover, our group has recently demonstrated that serum from LCAT deficient patients show the same capacity of control serum to decrease the cholesterol content of cholesterol-loaded macrophages due to a higher efflux potential via ABCA1 [133]. HDL particles distribution appear strongly altered in carriers of LCAT gene mutation, being characterized by a reduced content in HDL_2 particles, and by accumulation of preβ-HDL responsible for the greater ABCA1 mediated cholesterol efflux observed. The results from the study suggest that functional LCAT is not required for appropriate macrophage cholesterol depletion and RCT, since cell cholesterol can be taken up by discoidal preβ-HDL, and then delivered to the liver possibly through SR-BI[133].

In this intravascular stage of RCT, a-HDL particles undergo the activity of cholesteryl ester transfer protein (CETP). This enzyme is a hydrophobic glycoprotein, secreted by the liver and the adipose tissue that circulates in plasma bound to lipoprotein. CETP promote the transfer of cholesterol ester (CE) from HDL to LDL (only when hypertriglyceridemia is present, to VLDL[69]) in exchange for triglyceride (TG), and viceversa[70]. Low density lipoprotein return then to the liver and, interacting with LDL receptor release CE to hepatocytes. It has been suggested that the major part of HDL-CE follows this indirect pathway to be uptaken by the liver. However, the final step of RCT is mediated by other mechanisms, as occur in CETP deficient animals (e.g. mice). The most studied pathway of RCT involves the selective uptake of HDL-cholesterol by the hepatic SR-BI. SR-BI knockout mice have increased plasma HDL-cholesterol levels resulting from impaired hepatic uptake[134], while hepatic overexpression of SR-BI reduced plasma HDL-cholesterol levels due to increased hepatic uptake[135, 136]. It has been demonstrated that overexpression of hepatic SR-BI in mice promoted macrophage RCT despite reduced plasma HDL-cholesterol concentrations; conversely, ablation of SR-BI markedly reduced macrophage RCT despite increased plasma HDL-cholesterol concentrations[137]. Another mechanism proposed for the final step of RCT in the absence or disfunction of CETP, is the apolipoprotein E (apoE) enriched HDL pathway (HDL_{apoE}). Since apoE is an effective ligand for LDLr, it has been proposed that $_{HDLapoE}$ could directly deliver CE to the liver interacting with LDLr[138].

HDL PARTICLE CLASSIFICATION

HDL is a class of heterogeneous lipoproteins containing characterized by high density (>1.063 g/mL) and small size (5–17 nm). Human HDLs contain two main apolipoproteins: apoA-I and apoA-II which account, respectively, for 70 and 20% of the HDL protein. There are also several minor apolipoproteins in HDL, including apoA-IV, the C-apolipoproteins, apoD, apoE and apoJ[139], and apoM[140]. As mentioned above, by the action of different plasma and cellular factors HDLs undergo a continuous process of remodeling, leading to several HDL sub-populations and fractions detectable in human blood. Several criteria can be used to classify HDL sub-fractions, depending on the different techniques for separation:

- Electronic microscopy: spherical and discoidal particles
- Density (d) gradient by ultracentrifugation:
  HDL_2(1.063<d<1.125 gr/ml) and HDL_3 (1.125<d<1.121 gr/ml)
- Polyacrylamide gel separation (by size):
  HDL_{3c}< HDL_{3b}< HDL_{3a}< HDL_{2a}< HDL_{2b}
- Apolipoprotein composition:
  A-I HDL and A-I/A-II HDL
- Agarose gel 2D electrophoresis:
  α, preβ and γ-migrating HDL

HDL remodeling and metabolism consists of changement in lipids and apolipoprotein content and involves at least 10 different proteins: ABCA1, LCAT, SR-BI, CETP (see above), apoA-I, PLTP, LPL, HL, EL and cubulin[140, 141].

Phospsholipid transfer protein (PLTP) transfers surface PL from VLDL and chylomicrons to HDL during TG lipolysis and accounts for most of the PL transfer in human plasma. PLTP remodels HDL particles into larger HDL particles by particle fusion, subsequently releasing apoA-I [142]. Targeted disruption of PLTP in mice results in 60% reduction in HDL and apoA-I levels [143]because of enhanced clearance of HDL particles. Human PLTP transgenic mice have increased levels of pre-β1 HDL, apoA-I and PL[144]. The role of PLTP in human pathophysiology has yet to be elucidated.

Lipoprotein lipase (LPL) is secreted by many tissues of the body, mainly by adipose tissue and muscle. It is bound in the luminal surface of endothelial cells as homodimers to heparan sulfate proteoglycans and can be released by administration of heparin[145]. It mediates the hydrolysis of TG, transforming large TG-rich particles (VLDL, chylomicrons) into smaller TG-depleted remnant lipoproteins. Redundant surface lipid (FC, PL) and apolipoproteins are subsequently transferred from chylomicrons to HDL, increasing HDL-C and apoA-I plasmatic levels.[141]
Like LPL, Hepatic lipase (HL) and endothelial lipase (EL) are two member of the TG lipase family[141]. HL is synthesized by hepatocytes, and has greater activity against HDL than VLDL or chylomicrons. It converts large HDL_2 particles into smaller HDL_3 and lipid-poor apoA-I by TG hydrolysis. HL is most effective in hydrolyzing HDL if the HDL is TG enriched; hypertriglyceridemic conditions HL increases apoA-I catabolism and decreases HDL levels[141]. EL appears to have relatively more phospholipase activity than TG lipase activity and appears to have a greater preference for HDL over apoB-containing lipoproteins[146].
Levels of ApoA-I in plasma are strongly correlated with HDL levels. In agreement with the studies on HDL, low apoAI has been shown to be equivalent or better than low HDL cholesterol as a risk marker for atherosclerosis or cardiovascular events[147, 148]. Liver and intestine are the two major organs secreting nascent HDL as lipid-poor apoA-I or small

particles containing apoA-I and phospholipids and with pre-ß mobility on electrophoresis. The newly secreted particles acquire additional cholesterol and phospholipids via ABCA1, forming pre-ß HDL. It is likely that pre-ß HDL particles are formed very rapidly when monomeric apoA-I is exposed to cells.

Although the liver is the central modulator of cholesterol homeostasis in the body and the major site of synthesis of apoA-I, this organ erroneously has not been considered as source of cholesterol for lipidation of circulating apoA-I and HDL biogenesis.

Recent findings have shown that ABCA1 plays a key role in hepatic cholesterol efflux, inducing pathways that modulate cholesterol homeostasis in the liver, and establish the liver as a major source of plasma HDL-C. [149]. By in vitro study it has also been demonstrated that ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes[150]. Moreover, the idea of liver as a major organ for HDL synthesis is strongly supported by the demonstration that in mice with specific hepatic ABCA1 disruption lead to a decrease of 80% in HDL-C, while the decrease in HDL induced in full ABCA1$^{-/-}$ mice was of 95% [151].

APOLIPOPROTEIN E AND APOE KNOCK OUT MOUSE MODEL

The apolipoprotein E (apoE) is a 34kDa glicoprotein contained in all lipoproteins other than LDL, whose physiological role consists in the regulation of lipoprotein metabolism. Plasma apoE is mainly produced by the liver (>75%) and the brain is the second major organ of its synthesis. Macrophages also synthesize apoE [152]. This apolipoprotein accomplishes this function mainly by promoting the hepatic clearance of HDL, VLDL, IDL and chylomicrons through the binding with LDL and chylomicron-remnant receptor[153] and inhibiting trygliceride lipolysis[154]. ApoE deficiency (really rare in human) or mutation causes an impairment of lipoprotein plasma profile, leading to hypercholesterolemia, related to the accumulation of chylomicron remnants in plasma and early development of atherosclerosis[155, 156]. Mice lacking apoE (apoE$^{-/-}$) provided the first practical model of hyperlipidemia and atherosclerosis. ApoE$^{-/-}$ mice exhibit abnormal deposition of lipids in the proximal aorta and liver even at 3 months of age, indicating that the lack of this apolipoprotein is sufficient to initiate the atherogenic process. The lesion development and plaque composition in apoEKO mice is also similar to that in humans, establishing it as an excellent animal model for studying the pathogenesis of atherosclerosis. A small collection of foam cells that are tightly adhered to the aortic valve begin to appear in mice at about two months of age. With time the complexity of the lesion increases to have fibrous caps, a necrotic core of foam cell debris, cholesterol crystals and calcifications. Large advanced plaques are often associated with the thickening of medial and adventitial tissue, accompanied by chronic inflammation[157]. The proatherogenic alteration in lipoprotein composition derived from apoE deficiency in mice was well characterized by Zhang and colleagues[158]: marked increase in total cholesterol, significant reduction in HDL-C, moderate raise of

triglycerides. More specific characterization of lipoprotein structure lead to the discovery that apoE deficient causes not only a quantitative, but also a qualitative modification of lipoproteins: whereas in normal mouse plasma HDL migrates in alpha position and VLDL/LDL in pre-beta and beta position, in apoE deficient mouse plasma the distribution of lipids shifted towards the position where lipoproteins of lower density migrate[158]. Moreover, substantial alterations in apolipoprotein content and distribution in lipoproteins was observed: accumulation of Apolipoprotein A-I (apoA-I), A-IV and B-48 in VLDL. Interestingly, the total amount of apoA-I is not different between normal and apoE deficient mice.

ACAT ENZYMES: STRUCTURE, TISSUE DISTRIBUTION AND FUNCTION

ACAT stands for acyl-conzyme A:cholesterol acyltransferase and is an integral membrane enzyme located in the endoplasmic reticulum (ER) in a variety of cells and tissues. It catalyzes the synthesis of cholesterol esters (CE) conjugating a free cholesterol (FC) molecule and a fatty acyl-CoA[159]. Approximately 15 years ago, two different ACAT genes (Soat1 and Soat2) encoding for the two different ACAT enzymes (ACAT1 and ACAT2) have been identified. Soat1 was first identified in 1993 thanks to its ability to functionally complement the ACAT deficiency of a Chinese hamster ovary (CHO) cell mutant[160]. Some years later the first description of the effect of ACAT1 gene disruption in mice foreshowed the subsequent identification of a second ACAT enzyme[161]: these ACAT1$^{-/-}$ mice showed essentially normal hepatic cholesterol esters concentration, synthesis rate and response to ACAT inhibitors[161]. In 1998 the Soat2 gene was simultaneously identified in monkeys, mice and humans[162-164]. Whereas the two protein isoforms catalyse the same chemical reaction, it has become clear that the apparent physical structure of the protein and the physiological role in cholesterol metabolism are different from one another[165]. Both ACAT1 and ACAT2 are membrane-bound enzymes predicted by computer models to have as many as 8 transmembrane domains for ACAT1 and 7 for ACAT2[162]. Membrane topology studies have been performed by two research groups reporting different models for insertion of ACAT enzymes into and across ER. Joyce et al. showed that ACAT1 and ACAT2 used 5 transmembrane domains (3 of those were the same one for both enzymes) with serine residues essential for the activity (Ser_{269} and Ser_{249} for ACAT1 and ACAT2 respectively). Interestingly the developed topology model placed Ser_{269} on the cytoplasmic side of ER membrane, whereas

the analogous serine in ACAT2 was positioned in the ER lumen[166]. In other studies by Lin et al. 7 transmembrane domains were predicted to be used for ACAT1, while only 2 were predicted to be used in ACAT2[167, 168]. Different methodologies were used by the two groups, and nowadays it still not clear which model for the insertion of these proteins into and across the membrane might be correct, but both studies suggested fundamental structural differences between the 2 enzymes. This is consistent with the understanding that has been progressed from the discovery of the two ACAT isoforms, and that has clearly demonstrated their different tissue distribution and physiological function. ACAT1 mRNA is ubiquitously expressed in mammalian tissues: expression levels are highest in the adrenals glands, macrophages and sebaceous glands[160, 169, 170]. Studies on non human primates indicated that ACAT1 expression was greatest in globet cells and interstitial macrophages of the small intestine, Kupffer cells of the liver, adrenocortical cells, and in cells of Bowman's capsule and distal tubules in kidney[171]. In mice, this isoform is expressed at the highest level in macrophages, adrenocortical cells, dermal sebaceous glands and preputial glands[169, 170]. Conversely, ACAT2 is expressed only in small intestine and liver, as demonstrated by studies in adult nonhuman primates[4] (where the expression of ACAT2 protein has shown to be limited to only 2 cell types, small intestinal enterocytes and hepatocytes[171]) and mice[163]. For several years the only available ACAT1 and ACAT2 localization data in human claimed that ACAT2 was not located in the hepatocytes of the adult liver[164, 172, 173]. As in animals, ACAT1 was demonstrated to be highly expressed in human atherosclerotic lesion[174], in macrophages and hormone producing cell[17], but conversely with animals[171], ACAT1 was suggested to be the major enzyme present both in human hepatocytes and Kupffer cells, [172, 175]. In 2004 Parini at al. clearly demonstrated by that as in animals, also in the adult human liver ACAT2 was expressed in the ER of all hepatocytes,

whereas ACAT1 was only detectable within Kupffer cells[176]. Interestingly, samples analyzed in this study were snap-frozen human liver biopsy taken at the time of the surgery for gallstone removal, and not post mortem tissues as in the previous studies. Studies on the 5'-untranslated region of human ACAT2, from the same research group, have identified the hepatic nuclear factor 1 (HNF1) as one of the main transcription factor of this enzyme in hepatocytes elucidating the mechanisms that control the liver-specific expression of the human ACAT2 gene[177]. In vitro it has been shown the possibility to find both ACAT1 and ACAT2 expressed in HepG2 cells and Caco2 cells and some have claimed that isolated hepatocytes may contain both ACAT enzymes[172, 173] and macrophages may express both[178], but evidence support the fact that in mammals enterocytes and hepatocytes are the only 2 cell types to abundantly express ACAT2 without expressing detectable amount of ACAT1. Both ACAT1 and ACAT2 seem to be not expressed in a physiological settings in the same cell types in any tissue of the body[80]. Hence, the various types of studies performed on acyl-conzyme A:cholesterol acyltransferase (see above) generally support the likehood that ACAT1 and ACAT2 are structurally different enzymes, localized in different cell types, and have separate physiologic functions in cholesterol metabolism pathway[80] (see figure 4): ACAT1, found in highest quantities in tissues that store cholesterol esters in cytoplasmic lipid droplets (i.e. maprophages), is suggested to provides essential "housekeeping" functions to prevent free cholesterol-induced cytotoxicity, in response to excessive cholesterol availability within the cells, and presumably to the needs of membrane function. Conversely ACAT2, confined in hepatocytes and small intestine enterocytes, synthesize cholesterol esters (mainly oleate and palmitate) that may be incorporated into lipid storage droplets (from which FC can be

regenerated) or incorporated into apoB-containing lipoproteins and secreted into plasma[80].

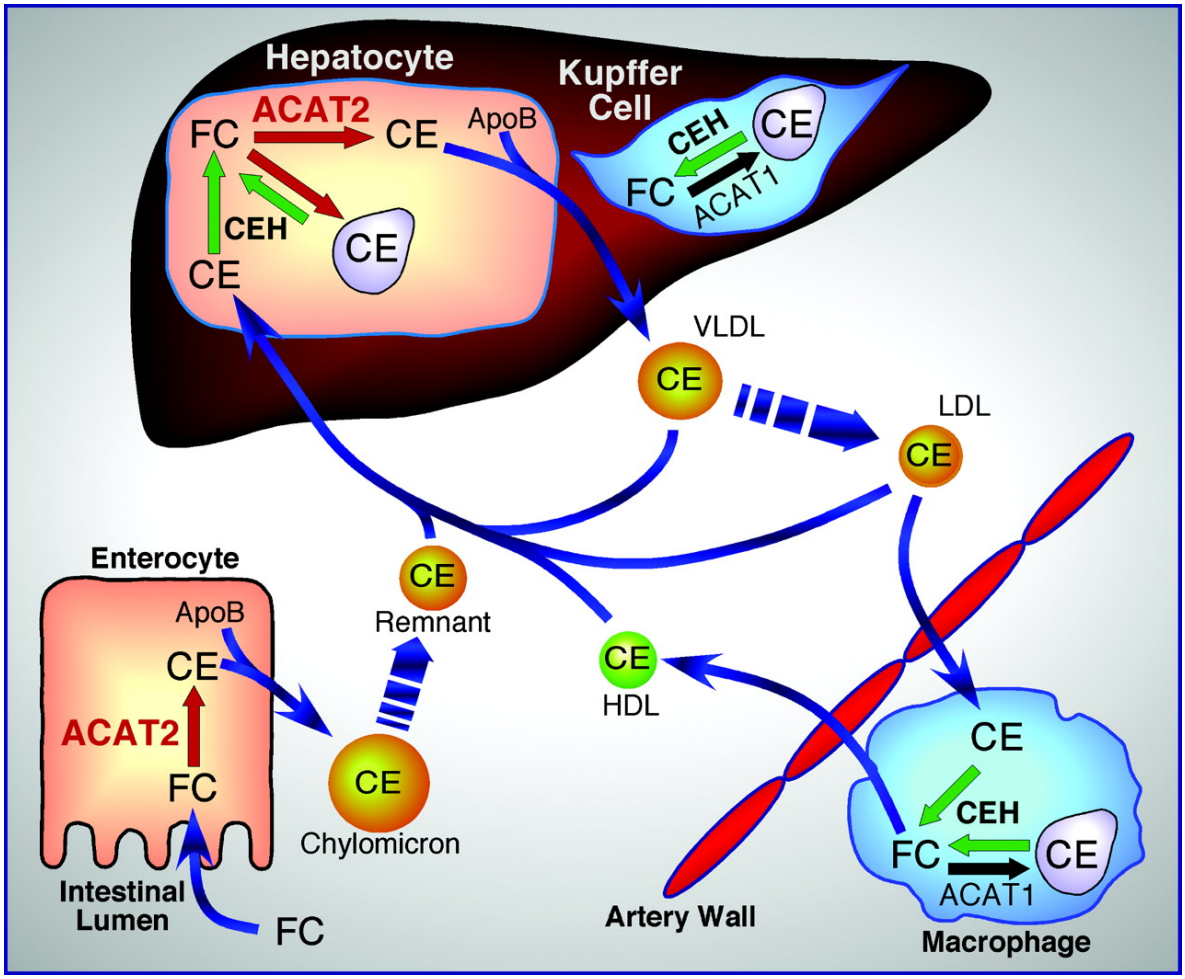

Figure 4 - Diagram of the separate roles of ACAT1 and ACAT2 in cholesterol metabolism in the liver, enterocyte, and macrophages within the artery wall (from[80])

SPECIFIC ACAT2 INHIBITOR TO RETARD ATHEROSCLEROSIS: MAY BE THE FUTURE?

After the discovery of the ACAT reaction, inhibitors of intracellular esterification as a means to prevent the arterial CE accumulation in atherosclerosis have been experimentally investigated for two decades. Because FC can be fluxed out from cells and incorporated in HDL, the rational to inhibit ACAT activity was that altering the intracellular cholesterol balance in favor of FC production would promote more efficient cholesterol efflux, activation of RCT, and deflation of atheroma[179]. A second rational for ACAT inhibition therapy approaches was to decrease hepatic and intestinal CEs formation, resulting in decreases in plasma levels of apoB-containing lipoprotein: thus the incorrect identification of ACAT1 as the exclusive esterifying enzyme in human liver[172, 175] has generated incorrect physiologic models, which in part have contributed to wrong therapeutic approaches aimed to unspecific inhibition of ACAT1 and ACAT2 (see editorial[179]). In the clinical settings, ACAT inhibitors available failed the aim to retard atherosclerosis: this is the result demonstrate by two different clinical trials, the Avasimibe and Progression of Lesion on Ultrasound (A-PLUS) study, and the more recent ACAT Intravascular Atherosclerosis Treatment Evaluation (ACTIVATE) trial[180, 181]. As suggested by Fazio and Linton[179], regarding the ACTIVATE outcomes, "A bold conclusion cab be drawn from the study by Nissen et al.: non selective ACAT inhibition in an ineffective antiatherosclerosis therapy and is probably harmful." As already mentioned it has been demonstrated that, as in animals ACAT2 is the predominant cholesterol esterifying enzyme in the liver and seems to exclusively take part in the synthesis and secretion of CE in apoB-containing lipoprotein, conversely to ACAT1 confined in Kupffer cells[176]. For this reason ACAT2 has been proposed as a new potential

therapeutic target for prevention of CVD[80]. Much key information has been learned from animals models with gene deletion of either ACAT1 or ACAT2. It has been demonstrated that the marked alteration in cholesterol homeostasis caused by ACAT1 disruption led to massive deposition of unesterified cholesterol in mouse skin and brain[182, 183]. Moreover, macrophages lacking ACAT1 increased atherosclerosis in mice with dyslipidemia, probably owing to toxic effect of free cholesterol and to apoptosis of macrophages in the vessel wall[184], and ACAT unspecific pharmacological inhibition has been shown to promote rather than decrease atherosclerosis in mice and rabbits[185]. Thus, it has been suggested that inhibition of ACAT1 enzyme should not be aim at[80]. On the contrary, deletion of ACAT2 has been consistently atheroprotective. Atherosclerosis in ACAT2 deficiency was first studied in apoE/ACAT2 double knockout mice (for review see[80]). ApoE/ACAT2 double knockout mice had greatly reduced levels of aortic atherosclerosis compared to the apoE single knockout controls. The lipid core of plasma apoB-containing lipoproteins of apoE/ACAT2 double knockout mice consisted almost entirely of triglycerides with extremely low levels of CE. The plasma levels of apo-B were unchanged, suggesting that the amount of ACAT2-derived CE present in the lipoprotein core was of vital importance. The protection from atherosclerosis in ACAT2 deficient mice has also been established in animals challenged with diets containing different fatty acids (FA). Regardless of the FA present in the diet (i.e n-trans FA and saturated FA) animals deficient for ACAT2 did not develop atherosclerosis[186]. Thus, ACAT2 plays an essential role in facilitating dietary fat type-specific atherosclerosis. A successful protection from atherosclerosis has also been obtained by decreasing the activity of hepatic ACAT2 in mice injected with anti-sense oligonucleotide therapy, which interferes with and reduces ACAT2 mRNA[187]. Using the same therapeutic approach, depletion of hepatic ACAT2 activity in mice also

revealed a non-biliary route for fecal cholesterol excretion[188]. In non-human primates the atherogenic role for ACAT2 has been established. For many years, it was recognized that plasma low density lipoproteins (LDL) enriched in ACAT2-derived CE (cholesteryl oleate and cholesteryl palmitate) accumulate in the plasma of monkeys that are more susceptible to the development of the coronary artery atherosclerosis (for review see[80]). Subsequently, observations were made that during liver perfusion, the secretion rate of CE was highly correlated to the extent of coronary artery atherosclerosis and to the CE enrichment of plasma LDL, indicating a direct correlation between hepatic ACAT2 activity and atherogenesis. In humans, several publications showed that higher proportions of CE derived from ACAT2 are associated with a increased incidence of complications from CVD[80]. Moreover in the Atherosclerosis Risk in Communities (ARIC) study, Ma et al. [189] found in both men and women (2872 participants) that the average carotid intima-media thickness (IMT) was associated significantly and positively with the proportion of ACAT2 derived cholesterol. Furthermore also in the ULSAM study, a positive association between ACAT2 derived CE (cholesteryl palmitate and oleate) and CVD death has been identified[190].

AIM

Apolipoprotein E is a plasma protein that serves as a ligand for low density lipoprotein reptors. Through its interaction with these receptors, apoE participates actively in the transport of cholesterol and other lipids among various cells of the body[191]. Hence, apoE has been suggested to act as an important key-player in several critical step of the lipoprotein metabolism pathway. Mice lacking this apolipoprotein develop early and spontaneously atherosclerotic plaques. Thus, apoE$^{-/-}$ mice provide the first and one of the most useful models to study hyperlipidemia and atherosclerosis[157].

Acyl-conzyme A:cholesterol acyltransferase 2, confined in hepatocytes and small intestine enterocytes, synthesize cholesterol esters (mainly oleate and palmitate) that are incorporated into apoB-containing lipoproteins and secreted into plasma[80]. In human there are several indication that ACAT2-derived cholesteryl esters accumulate in plasma in association with coronary heart disease. In monkeys and mice, this accumulation has also been highly correlated with increased atherosclerosis[80].

The general aim of this work was to investigate the molecular mechanisms involved in lipoprotein metabolism and lipid homeostasis trying to further clarify the role played by these two proteins.

Specific aim 1:

Beyond the influence on plasma lipoprotein profile, it has been demonstrated that promotion of cholesterol release from foam cells is clearly secondary to apoE expression and secretion in macrophages, the most important cholesterol accumulating cells in atherosclerosis[192]. Thus, the deletion of apoE in mice may produce an atherogenic phenotype also because of an impaired mobilization of cholesterol from macrophages. Hence, we could expect a possible negative effect on

the process of reverse cholesterol transport in apoE$^{-/-}$. To verify this hypothesis we measured the macrophagic RCT in this mouse model of atherogenesis.

Specific aim 2:

Disruption ACAT2 in mice has lead to an increased triglyceride content in VLDL particle and to a diminished trigliceride content in the liver[80]. These findings may involve a LXRs stimulation resulting from the increased free cholesterol levels inside hepatocyte, as consequence of ACAT2 deficiency. It has been extensively demonstrated that the liver x receptors, for which different oxysterols serve as ligand, are key players in the regulation of lipoprotein ad lipid metabolism. By regulating the expression of SREBP-1$_c$, ACC, FAS and SCD-1, LXRs influence metabolism of fatty acids, triglycerides, and triglycerides rich lipoprotein. Moreover, similarly to what can be conceived for triglycerides secretion into VLDL, disruption of ACAT2 could lead to an increase in ABCA1 expression in hepatocytes, resulting in greater cholesterol efflux into nascent HDL.

Thus, the aim of this part of the work was to further investigate the effect of specific hepatic ACAT2 knock down in mice by means of anti-sense oligonucleotide therapy (ASO6), trying to unmask a possible effect mediated by LXR stimulation and to unmask a possible link between ACAT2 and HDL synthesis in mice.

MATERIALS AND METHODS

Animals and experimental design

IN VIVO REVERSE CHOLESTEROL TRANSPORT STUDY

10 apoE$^{-/-}$ (B6.129P2-*Apoe*^{tm1Unc}/J) mice and 10 C57Bl/6 (WT) mice (about10 week older) were used as MPM donors. Macrophagic RCT was quantified in apoE$^{-/-}$ (B6.129P2-*Apoe*^{tm1Unc}/J) (n=5) mice and C57Bl/6 (WT) mice (n=5) as described below.

ASO6 STUDY

Male C57Bl/6 (WT) mice and male LXRa/b double knock-out (LXR doko) mice (on a pure C57Bl/6 genetic background) were treated for 4 weeks with twice a week injection (7 animals/group) of:

- anti-sense oligonucleotide (ASO) specifically targeted to the hepatic ACAT2 gene (ASO6) – 25 mg/kg
- ASO bearing a non-sense sequence (Ctrl) – 25 mg/kg

4 weeks prior start and for all the duration of the experiment, animals were fed with 10% SAT (cal) and 0.2% cholesterol (w/w) diet. Animals were fasted for 4 hrs prior sacrifice.

Quantification of Macrophagic RCT in-vivo

Macrophagic RCT in vivo was evaluated as previously described[193], but using murine peritoneal macrophages (MPM) instead of J774 macrophages. After MPM harvesting, cells were incubated for 48 hours with [3H]-cholesterol (5 µCi) and AcLDL (100 µg/ml). Subsequently, MPM from wild type mice were intraperitoneally injected into wild type mice (WT/WT), and macrophages from apoE-/- mice were injected into apoE-/- mice (apoE/apoE). Mice were successively separated into individual cages, and after 48 hours from the injection mice were sacrificed and blood and liver were collected. Plasma was separated and counted in a β-counter. Livers were extracted by Bligh and Dyer method and counted by liquid scintillation addition. Feces were collected at 24 h and 48 h

after the injection of radiolabeled cells extracted by the Bligh and Dyer method, and counted by liquid scintillation addition.

Evaluation of plasma efflux potential

Mice were sacrificed and blood was collected from the heart, recovered in plastic tubes and anticoagulated with sodium citrate 3.8% (1 part citrate:9 part blood). Plasma was isolated by low speed centrifugation and stored at -80°C until use. Aliquots of plasma were used as acceptors in cholesterol efflux experiments.

Measurement of passive diffusion and ABCA1-mediated cell cholesterol efflux

Efflux by passive diffusion was measured using control J774 macrophages. ABCA1-mediated cholesterol efflux was measured using control J774 macrophages and J774 macrophages treated with cpt-cAMP to upregulate ABCA1[194]. Cells were grown in RPMI with 10% FCS, incubated at 37°C, 5% CO2, seeded in 12-well plates and utilized at 80-90% of confluence. Monolayers were washed with PBS and incubated for 24 h in medium containing [^{3}H]cholesterol (4 μCi/ml). The labeling medium contained 1% FCS and 2 μg/ml of an ACAT inhibitor. Following 24 h labeling period, cells were washed and incubated with 0.2% BSA, with or without 0.3 mM cpt-cAMP for 18 h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol; these cells provide baseline (time 0) values for total [^{3}H]cholesterol content. Stimulated and unstimulated monolayers containing [^{3}H]cholesterol were washed with PBS and incubated for efflux time (4 h) in the presence of 2% (v/v) serum Cell media were centrifuged to remove floating cells, and radioactivity in the supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as: (cpm in medium at 4h / cpm at time 0) x 100. The ABCA1-mediated cholesterol efflux was

calculated as the percentage efflux from stimulated J774 macrophages minus the percentage efflux from unstimulated J774 cells.

Measurement of ABCG1-mediated cell cholesterol efflux

CHO-K1 cells stably expressing hABCG1 were generated as previously described[118]. Parent and hABCG1-expressing cells were labeled for 24 h with [^{3}H]-cholesterol, washed, and equilibrated for 90 minutes in serum-free medium, then incubated in efflux medium containing BSA (1 mg/mL) in the presence of 2% (v/v) serum for efflux time (6 h). Cell media were centrifuged to remove floating cells and cells were lysed in 0.1% (v/v) Triton X-100. Cells and media were assayed for radioactivity and cholesterol efflux in medium samples calculated as a percentage of total cholesterol (cells plus medium) in the culture. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells minus the percentage efflux from CHO-K1 parent cells.

Measurement of SR-BI-mediated cell cholesterol efflux

SR-BI-mediated cholesterol efflux was measured using Fu5AH rat hepatoma cells, a stable highly SR-BI-expressing cell line[195]. Cells were seeded in 12-well plates and grown in DMEM medium with 5% FCS for 2 days, and labeled with 4 μCi/ml [3H]cholesterol for 24 h in medium with 1% FCS and 2 μg/ml of an ACAT inhibitor, to ensure that all labeled cholesterol was present as free cholesterol. Cells were then incubated for 18 h with 0.2% BSA. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol; these cells provide baseline (time 0) values for total [3H]cholesterol content. Cells were then washed with PBS and incubated in the presence of 2% (v/v) serum for efflux time (4 h). Cell media were centrifuged to remove floating cells, and radioactivity in the supernatant was determined by liquid scintillation

counting. Cholesterol efflux was calculated as: (cpm in medium at 4h / cpm at time 0) x 100.

Antisense Oligonucleotides

The 20-mer phosphorothioate oligonucleotides containing 2'-0-methoxyethyl groups at positions 1 to 5 and 15 to 20 with sequences targeted to mouse ACAT2. The sequences of these ASOs are as follows: ASO 6: 5'-TTCGGAAATGTTGCACCTCC-3'; ASO control: 5'-GTCGCTCAACATCTGAATCC-3'. The ASO control is not complementary to the ACAT2 sequence and does not hybridize with any specific gene target.

ASOs were obtained from ISIS Pharmaceuticals, Carlsbad, CA, USA, by means of a collaboration.

Plasma lipoprotein profile

Plasma lipoproteins were separated by size exclusion chromatography, and the total cholesterol, free cholesterol, triglyceride content were determined by a system for on-line detection [196]

Hepatic lipid analysis

Liver and extracted in chloroform-methanol (2:1, v/v). 7a-hydroxy-4-cholesten-3-one (C4) levels were determined in liver lipid extract by the LC-MS/MS method with $^{2}H_{6}$-labelled C4 as internal standard as previously described[197]. Hepatic total cholesterol , cholesterol ester , free cholesterol , triglyceride mass was measured in liver lipid extracts using enzymatic assays as described previously[188, 198]. Protein content was determined according to Lowry's method.

Relative RNA expression level measurements

Hepatic total RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA (High Capacity cDNA Reverse

Transcription kit, Applied Biosystem). Real-time quantitative PCR assays were performed in triplicate using SYBR-Green (Power SYBR Green Master PCR master mix, Applied Biosystem). Primers (primer sequences are available on request) were designed using Primer Express 2.0, all with sequences crossing exon-exon boundaries. Data were calculated by the delta-C_t method, expressed in arbitrary units, and were normalized by the signals obtained from the same cDNA for endogenous gene mRNA expression.

Western Blot analysis

3 increasing amounts of liver membranes from each mouse sample were separated on a NuPage 3-8% Tris-Acetate gel (Invitrogen) and then transferred onto nitrocellulose membranes (Invitrogen). After blocking in 5% nonfat dry milk in PBS-T (PBS with 0.1% Tween-20), the nitrocellulose membranes were incubated overnight at 4°C with rabbit primary antibody in 5% nonfat milk powder in PBS-T. After washing with PBS-T, secondary antibody HPR-conjugated was added for 2 hours. The signals were detected using the SuperSignal chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL) and a Fuji BAS 1800 analyzer (Fuji Photo Film Co.) and quantified by Image Gauge software (Science Lab 98, version 3.12; Fuji Photo Film Co.).

Liver membrane preparation

Liver samples (200mg) were homogenized in 3-mL ice-cold buffer containing 20mM Tris-HCl, 0.25 M sucrose, and 2mM $MgCl_2$. A protease inhibitor cocktail (complete MINI Roche diagnostic) was added to the buffer before homogenization. The homogenate was then centrifuged for 10 minutes at 2000x*g* (4°C) to remove fats. The resulting intermediate phase was centrifuged for 45 minutes at 32000rpm by Beckman

Ultracentrifuge XL-70. The pellet from this spin was resuspended in 100 µl of Lysis Buffer containing 80 mM NaCl, 50 mM Tris-HCl, 2 mM $CaCl_2$, 1% TritonX-100 and protease inhibitor cocktail, and immediately frozen at –80°C.

Isolation of microsomes and enzymatic analysis

Liver samples (50 to 150 mg) were homogenized in 3-mL ice-cold buffer containing 0.1 mol/L K_2HPO_4, 0.25 mol/L sucrose, and 1 mmol/L EDTA, pH 7.4. A protease inhibitor cocktail (Sigma) was added to the buffer before homogenization. The homogenate was then centrifuged for 15 minutes at 12 000*g* (4°C) to remove cell debris. The resulting supernatant was centrifuged for 60 minutes at 100 000*g*. The microsomal pellet from this spin was resuspended in 0.1 mol/L K_2HPO_4 at pH 7.4 and immediately frozen at –80°C.

For Western blot analyses, 100 µg of microsomal protein was analyzed after suspension in protein solubilization buffer (120 mmol/L Tris, pH 6.8; 20% glycerol, 4% sodium dodecyl sulfate, and bromophenol blue). Dithiothreitol was added to a final concentration of 100 mmol/L, and samples were incubated at 37°C for 30 minutes. Separation and blotting were performed as described earlier with the antibodies made against monkey enzyme *N*-terminal sequences[199]. Detection was accomplished by chemiluminescence, with exposure times being adjusted to maximize band intensity. Total ACAT enzymatic activity was determined with hepatic microsomes as previously described[200], except preincubation included a cholesterol-saturated solution of ß-hydroxypropyl cyclodextrin for 30 minutes before addition of ^{14}[C]oleoyl Co-A. In separate tubes, pyripyropene A, a specific ACAT2 inhibitor[201], was included in the preincubation and reaction mixture at a concentration of 5 µmol/L to separately identify ACAT1 (uninhibited) and ACAT2 (total–ACAT1) activities.

2D gel electrophoresis

Plasma HDL subclasses were separated by 2D electrophoresis, in which agarose gel electrophoresis was followed by non denaturing polyacrylamide gradient gel electrophoresis and subsequent immunoblotting[202]. In the first dimension, plasma (5 μl) was run on a 0.5% agarose gel; agarose gel strips containing the separated lipoproteins were then transferred to a 3-20% polyacrylamide gradient gel. Separation in the second dimension was performed at 30mA for 4 hours. Fractionated HDL were then electroblotted onto a nitrocellulose membrane and detected with an anti apoA-I antibody.

Statistical analysis

RCT and efflux results were analyzed by Student's t test with the use of GraphPad Prism Software, San Diego, CA. Efflux data are reported as means of triplicates ± S.D. Significance of differences in ASO6 study was determined for each group of values by 2-way ANOVA using Statistica 7.0 software, StatSoft Inc., Tulsa, USA. A P value less than 0.05 was considered significant.

RESULTS

REVERSE CHOLESTEROL TRANSPORT STUDY

By means of a method that has been widely applied to physiological and pharmacological studies[193, 203], we tested the mobilization of radiolabeled cholesterol from macrophages, injected into the peritoneum of recipient mice, to plasma, liver and feces to evaluate the role of apoE in the antiatherosclerotic process of RCT in vivo. In the first experiment, macrophages from wild type mice were injected into wild type mice (WT/WT), and apoE deficient macrophages were injected into apoE$^{-/-}$ mice (apoE/apoE). 48 hours after injection of MPM apoE/apoE showed an higher content of [^{3}H]-cholesterol in plasma compared to WT/WT (**FIGURE 5 (A)**). Plasma total cholesterol measured by colorimetric method was 5 fold increased in apoE deficient mice compared to wild type (35.44±8.71mg/dl vs 175.43±27.39 mg/dl in WT/WT and apoE/apoE mice respectively). An opposite trend was observed for [^{3}H]-cholesterol measured in liver and faeces, since samples from apoE$^{-/-}$ mice showed a dramatic reduction of tracer compared to wild type (**FIGURE 5 (B)/(C)**). Total RCT, calculated as the sum of cholesterol mobilized from macrophages and thus detected in plasma, liver and feces, was higher in WT/WT mice (5.38% ±0.74vs 3.97% ±1.11Student's t test p=0.05). These data indicate that reverse cholesterol transport is impaired in absence of apoE.

To further investigate the role of apoE in the RCT and particularly its involvement in the first step, plasma from WT/WT or apoE/apoE mice was tested as cholesterol acceptor. This assay was performed in different cell models, in order to investigate the specific mechanism of efflux involved.

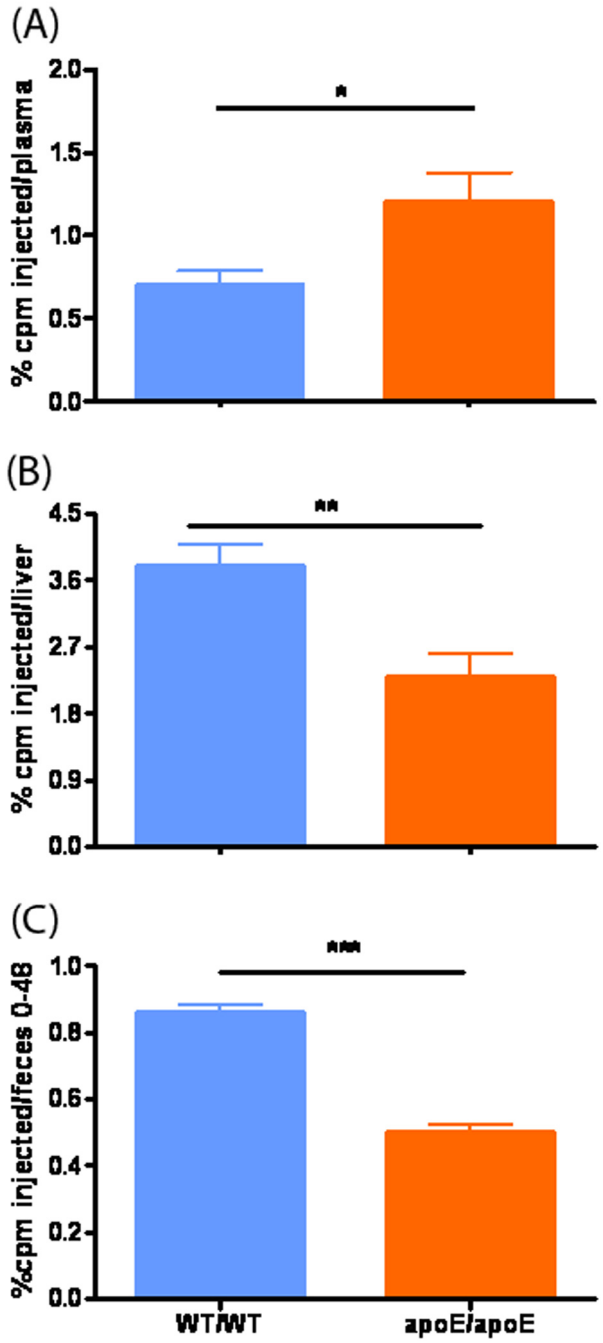

FIGURE 5 - Macrophage-specific RCT in vivo: Macrophage-derived [3H]-cholesterol in plasma (A), liver (B) and feces (C). Aliquots of plasma were counted in a β-counter. Samples of hepatic tissue and feces were extracted by Bligh and Dyer method in order to isolate the sterol fraction. Quantification of radioactivity content was performed by liquid scintillation counting. Results were expressed as amount of radioactivity in whole plasma, liver or total feces. Student's t test *p<0.05, **p<0.01, ***p<0.001.

The evaluation of SR-BI-mediated efflux was assessed in rat hepatoma Fu5AH cells, where high amounts of this transporter are naturally present[195]. The incubation of these cells for 4 hours with 2% plasma from apoE/apoE promoted more efficient release of cholesterol than plasma from WT/WT (**FIGURE 6**).

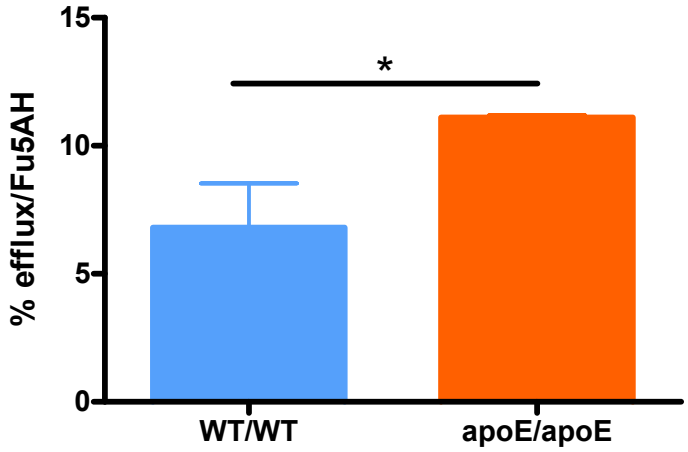

FIGURE 6 – Plasma efflux potential by SR-BI mediated mechanism: Fu5AH were radiolabeled with [^{3}H]-cholesterol, equilibrated in an albumin-containing medium and exposed to 2% plasma for 4h. Efflux was expressed as cpm in medium/cpm To*100. Student's t test *p<0.05

A similar trend was observed when passive diffusion- and ABCG1-mediated cholesterol efflux were evaluated, respectively in J774 macrophages and CHO cells (**FIGURE 7** and **FIGURE 8**). Conversely, no difference in the capacity of plasma from WT/WT and apoE/apoE mice to drive lipid release by ABCA1 was observed (**FIGURE 9**).Taken together these results suggest that the deletion of apoE in mice, impairs the RCT in vivo in spite of an increase in plasma efflux potential.

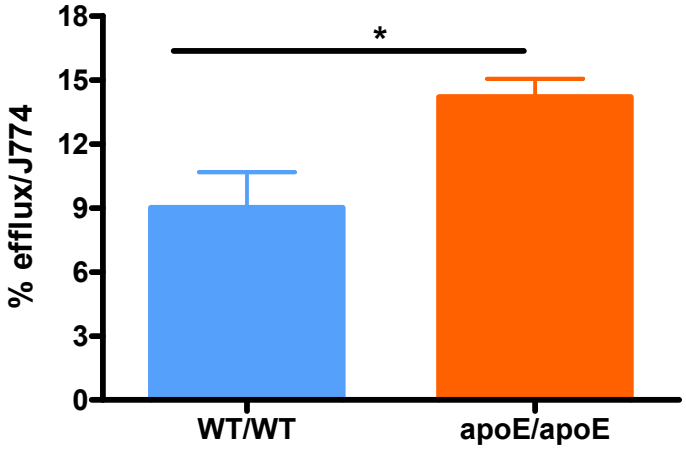

FIGURE 7 - Plasma efflux potential by acqueous passive diffusion: J774 macrophages were radiolabeled with [^{3}H]-cholesterol, equilibrated in an albumin-containing medium and exposed to 2% plasma for 4h. Efflux was expressed as cpm in medium/cpm To*100. Student's t test *p<0.05

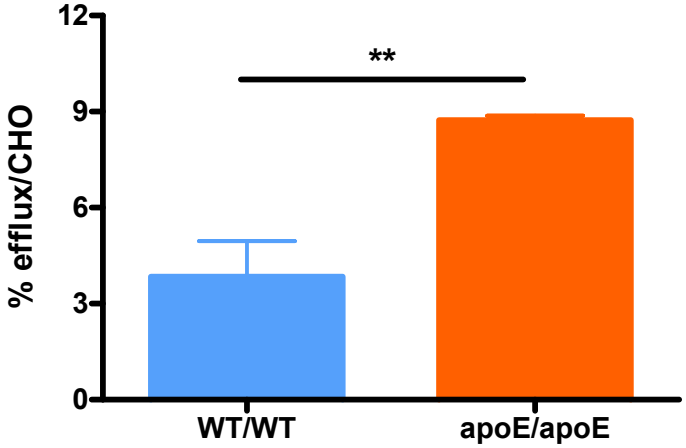

FIGURE 7 - Plasma efflux potential by ABCG1 mediated mechanism: Parent and hABCG1-expressing CHO cells were radiolabeled with [^{3}H]-cholesterol, equilibrated in an albumin-containing medium and exposed to 2% plasma for 4h. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells minus the percentage efflux from CHO parent cells Efflux was expressed as cpm in medium/cpm To*100. Student's t test *p<0.005

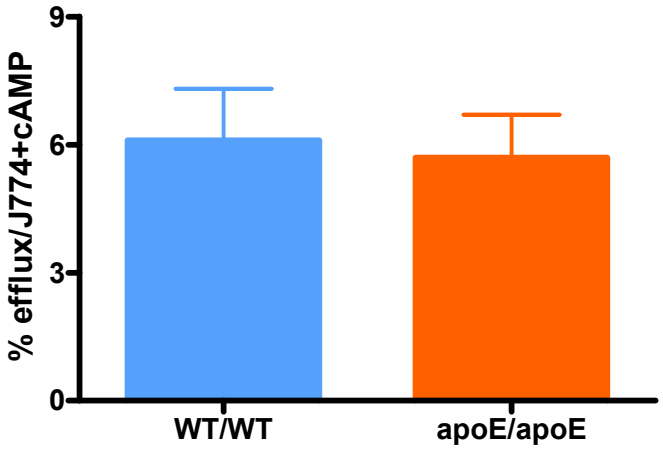

FIGURE 9 - Plasma efflux potential by ABCA1 mediated mechanism: J774 macrophages were radiolabeled with [^{3}H]-cholesterol, equilibrated in an albumin-containing medium in presence of cpt-cAMP 0.3mM and exposed to 2% plasma for 4h. Efflux was expressed as cpm in medium/cpm To*100.

ASO6 STUDY

We initially measured the SOAT2 mRNA levels in the liver by RT-PCR, and the expression of ACAT2 protein in liver microsomes by western blot analysis. As expected, ASO6 led to a reduction of ACAT2 both at mRNA and protein levels, in both WT and LXR double knockout mice (**FIGURE 10**). Analysis of ACAT2 activity in liver microsomes showed a significant reduction in both animal strains treated with ASO6 (Student's t test $p<0.001$), while ACAT1 activity was not affected by the anti-sense oligonucleotide treatment (**FIGURE 11**). It should be pointed out that the ACAT1 activity observed in the hepatic microsomal fraction is the contribution of

Kupffer cells present in the liver: ACAT1 is confined only to these cells whereas ACAT2 only to hepatocytes (for review see[80]). LXRa mRNA expression in the liver was slightly decreased in WT mice treated with ASO6 ($p<0.05$), while LXRβ expression was not affected by ACAT2 disruption. As expected both LXR genes were absent in LXR DOKO mice. (**FIGURE 12**)

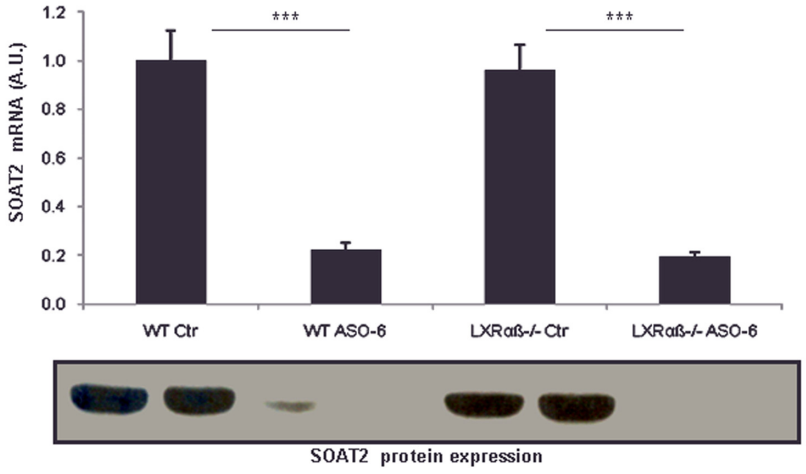

FIGURE 10 – Effect of ASO6 treatment on ACAT2 mRNA and protein expression in the liver: mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl. 2-way ANOVA, ***p<0,001. Western blot analysis was performed on liver microsomes as described in methods.

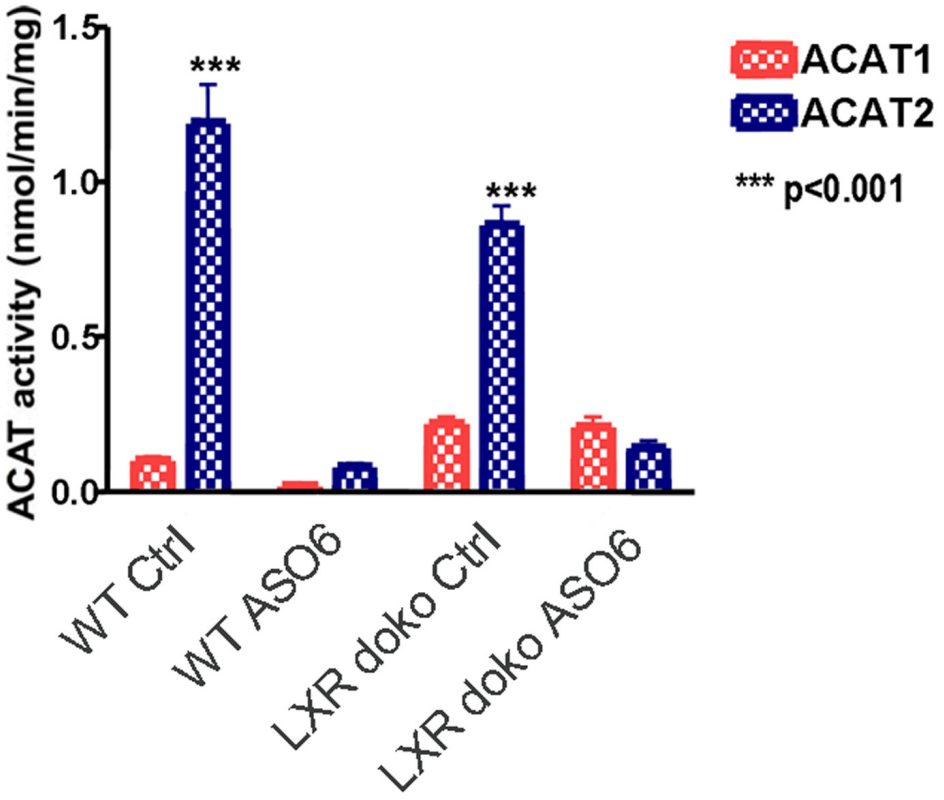

FIGURE 11 – Effect of ASO6 treatment on ACAT2 and ACAT1 activity in liver microsomes (for details see methods). Student's t test ***p<0.001

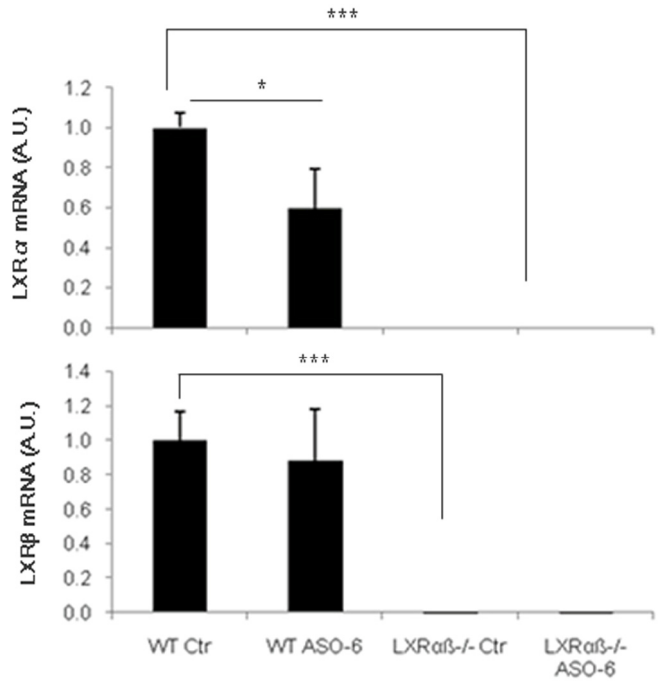

FIGURE 12 – Effect of ASO6 treatment on LXRa and LXRβ gene expression in the liver: mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl. 2-way ANOVA, *p<0.05 ***p<0.001.

PLASMA LIPOPROTEIN PROFILE

The analysis of plasma lipoprotein fractions revealed that ASO6 led to a slight increase in HDL-total cholesterol and to a clear increase in HDL-free cholesterol content. The latter was more pronounced in LXR DOKO mice (**FIGURE 13 (A)/(B)**). Serum triglycerides were increased in apoB-containing lipoprotein fraction by ACAT2 down-regulation, and this effect was clearly not LXR-dependent (**FIGURE 13 (c)**)

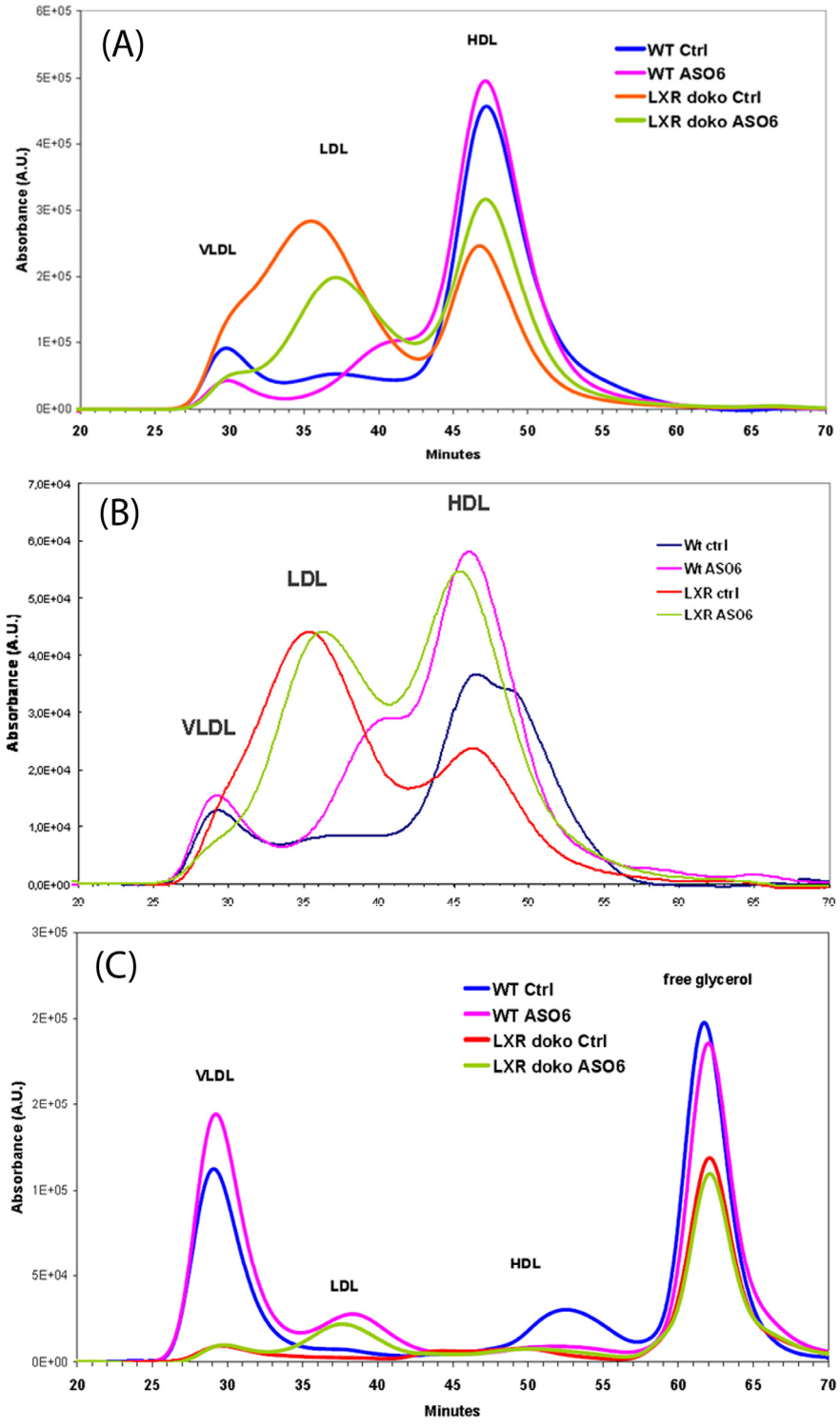

FIGURE 13 – Plasma lipoprotein profile: (A) Total cholesteol, (B) Free cholesterol, (C) Triglycerides. Plasma lipoproteins were separated by size exclusion chromatography, and the TC, FC, TG content were determined by a system for on-line detection [196]

LIVER LIPID COMPOSITION

Hepatic total cholesterol content was about 4 fold higher in LXR DOKO mice than in WT (390.04 ± 38.8 mg /gr Protein vs. 92.21 ± 8.44 mg/gr Protein, n=6, 2-way ANOVA $p<0.001$). ASO6 treatment lead to a 71% and 24% decrease of hepatic TC in WT and LXR DOKO mice, respectively (**FIGURE 14 (A)**) ACAT2 down-regulation did not affect the hepatic free cholesterol content both in WT and LXR DOKO mice. The latter showed a significantly increased FC amount compared to WT (**FIGURE 14 (B)**). Also, cholesteryl ester levels were 4.6-fold higher in the liver of LXR DOKO mice than in WT. In WT mice, ASO6 treatment reduced hepatic CE levels by 90%, while only a 29% reduction was observed in LXR DOKO mice receiving ASO6 (**FIGURE-14 (C)**). In WT mice hepatic TG were about 7.5 fold higher than in LXR DOKO. ACAT2 down-regulation lead to a modest reduction in hepatic TG content (22% and 38% reduction in WT and LXR DOKO mice respectively). (**FIGURE-14 (D)**).

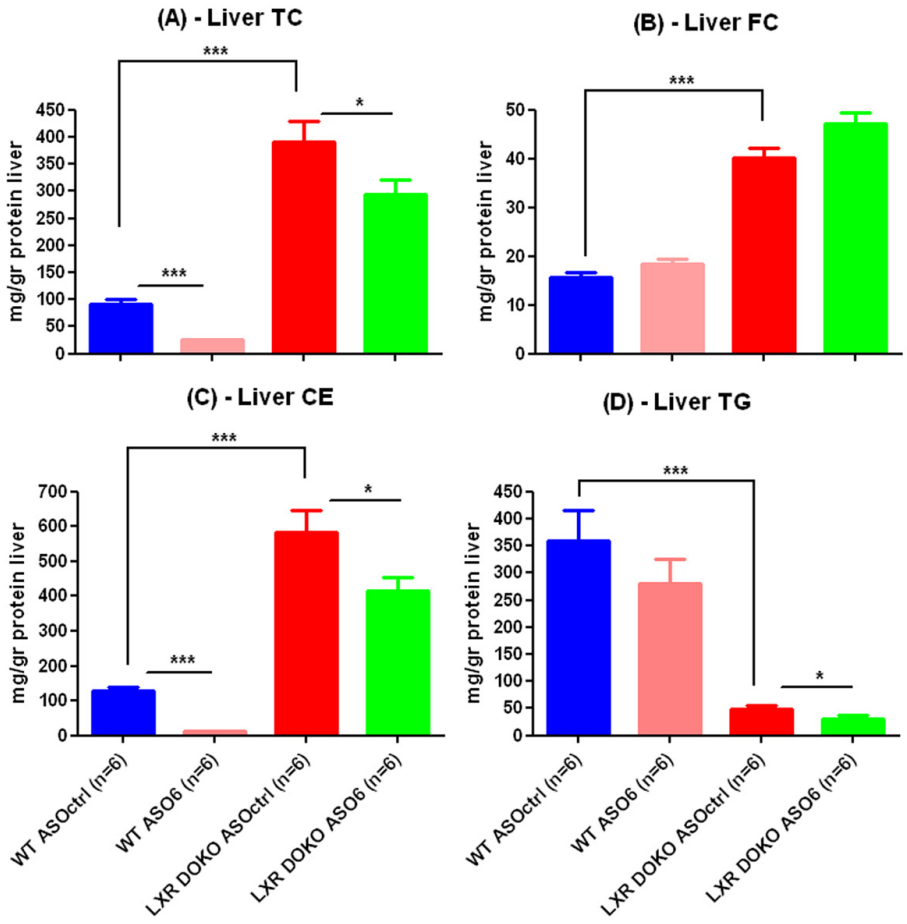

FIGURE 14 – Effect of ASO6 treatment on liver lipid composition. Hepatic total cholesterol , cholesterol ester , free cholesterol , triglyceride mass was measured in liver lipid extracts by enzymatic assays. 2-way ANOVA, *p<005, ***p<0.001

ANALYSIS OF HEPATIC GENE AND PROTEIN INVOLVED IN LIPID HOMEOSTASIS

Down-regulation of hepatic ACAT2 activity by ASO treatment should affect to the pool of free cholesterol that can signal to and determine a down-regulation of the SREBP-system. This indeed led to down regulation of the expression of the gene limiting cholesterol synthesis, but only in WT mice. If no differences in hepatic expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase and synthase between the two different genotypes, ASO6 treatment lead to reduction of mRNA expression of

these two genes only in WT mice (**FIGURE 15 (A)/(B)**). A similar trend was observed also for LDLr and PCSK9 mRNA: significant reduction induced by ACAT2 down-regulation was shown in WT group, while in LXR DOKO mice no statistically significant effects were seen (**FIGURE 15 (C)/(D)**). Despite the higher decrease in PCSK9 expression, compared to the decrease in LDLr expression, the net effects of ASO6-tretamnet was a down regulation of LDLr protein expression in liver membranes of WT mice (46% decrease)(**FIGURE 16**). Interestingly, we found that LXR DOKO mice showed undetectable levels of LDLr protein in liver membranes by western blot analysis (**FIGURE 16**).

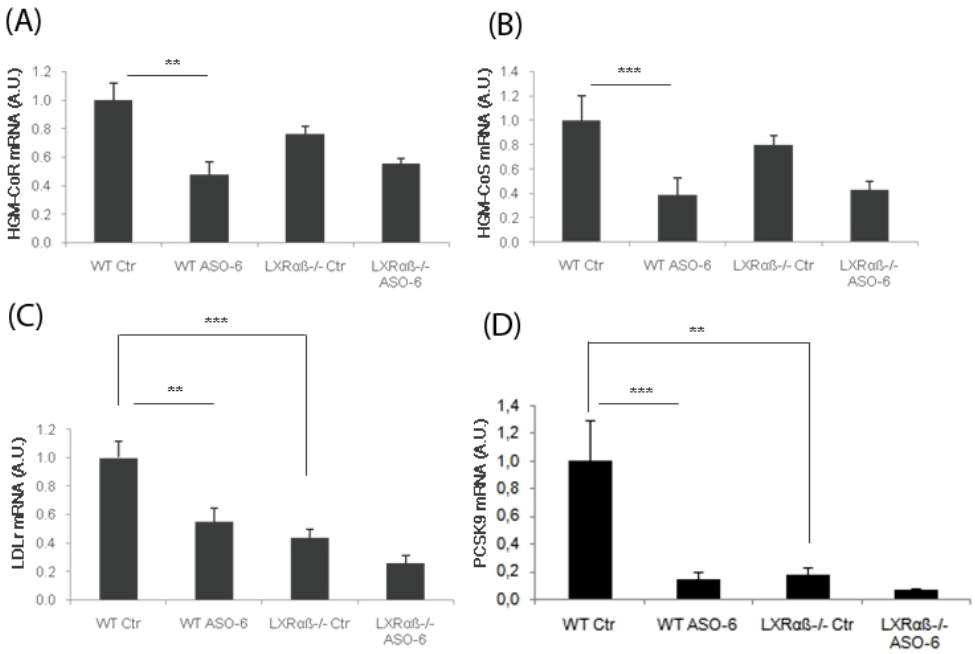

FIGURE 15 – Effect of ASO6 treatment on hepatic gene esxpression: (A) HMG-CoA reductase, (B) HMG-CoA Synthase, (C) LDL receptor, (D) PCSK9. mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl. 2-way ANOVA, **p<0.005 ***p<0.001.

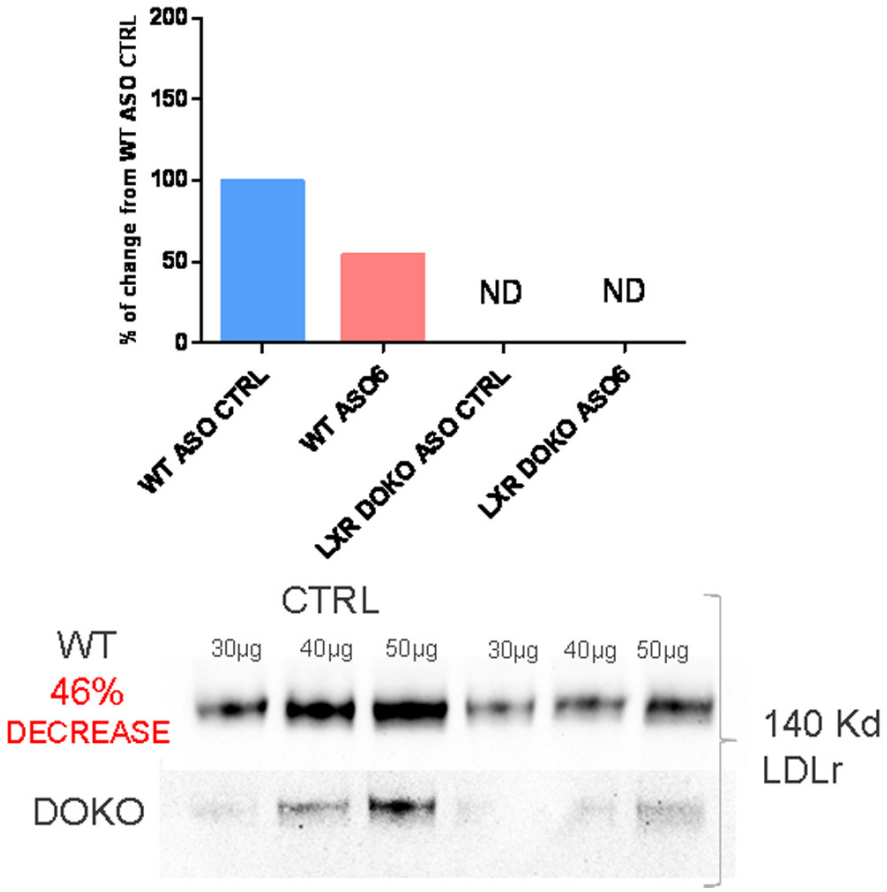

FIGURE 16 – Effect of ASO6 treatment on LDLr protein expression in liver membrane.

Quantification of mRNA by RT-PCR of the genes involved in bile acids synthesis and cholesterol transport revealed that ASO6 had no effect on the hepatic mRNA levels of CYP7A1, ABCG5 and ABCG8, both in WT and LXR DOKO mice. As expected, all these gene were down regulated in LXR DOKO mice compared to WT (**FIGURE 17 (A)/(C)/(D)**) Small heterodimer partner (SHP) mRNA expression was similar in both genotypes, and ASO6-treatment resulted in a down-regulation only in LXR DOKO mice (**FIGURE 17 (B)**). Quantification of 7a-hydroxy-4-cholesten-3-one (C4), a surrogate marker for bile acid synthesis, in hepatic lipid extract showed an increase following ASO6-treatment both

in WT and LXR DOKO mice (**FIGURE 18**). As expected and consequence of CYP7A1 down-regulation, C4 levels were significantly lower and the increase by ASO6-treatment smaller in LXR DOKO mice when comparet to WT animals.

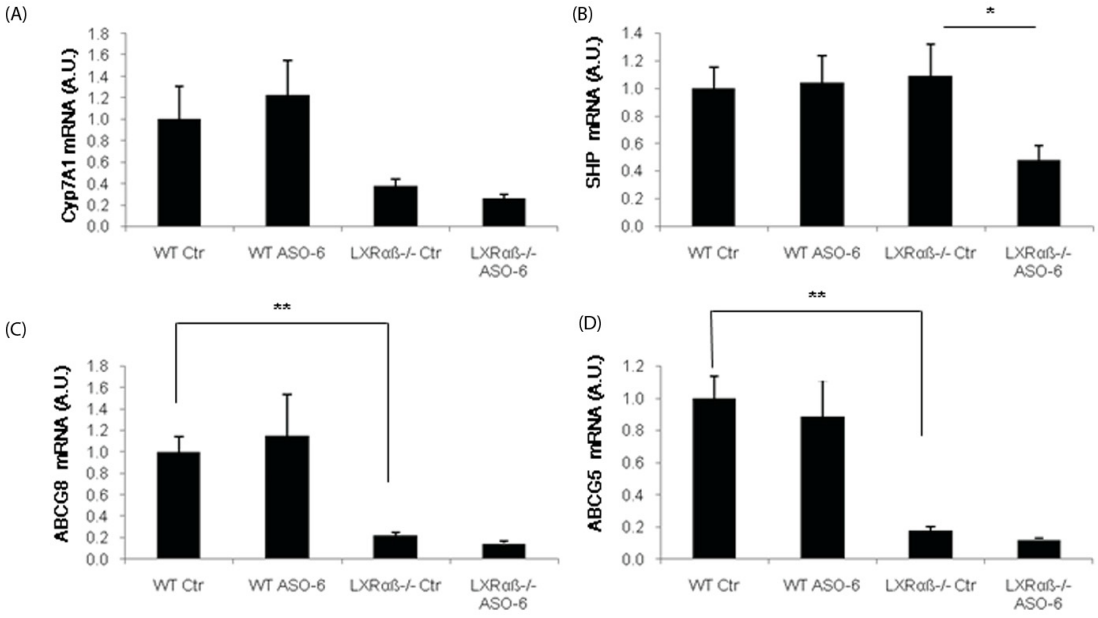

FIGURE 17 – Effect of ASO6 treatment on hepatic mRNA expression of gene involved in bile acids synthesis and cholesterol transport: (A) Cyp7A1, (B) SHP, (C) ABCG8, (D) ABCG5. mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl. 2-way ANOVA, *p<0.05, **p<0.005.

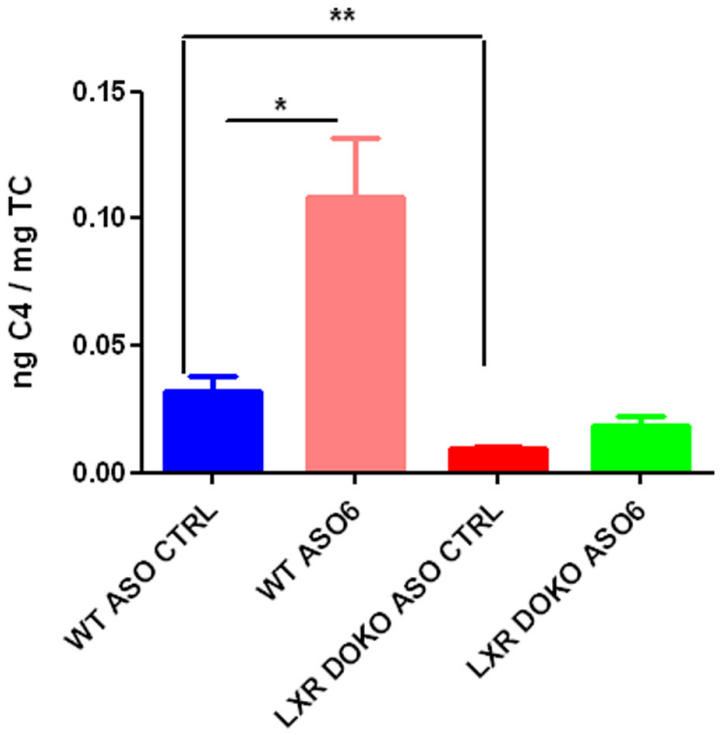

FIGURE 18 – Quantification of 7a-hydroxy-4-cholesten-3-one (C4) in the liver. Data are corrected for hepatic total cholesterol content, and expressed as Mean value for each group (n=6) ± SEM, 2-way ANOVA *p<0.01, **p<0.005.

Hepatic mRNA levels of the gene involved in cholesterol efflux from hepatocytes (i.e. ABCA1, and SR-BI) were not affected by oligonucleotide therapy (**FIGURE 19**), but ASO6 lead to an increase of ABCA1 and SR-BI protein in the liver membrane from both animal genotypes. SR-BI protein was increased by treatment of 28% and 27% for WT and LXR DOKO respectively (**FIGURE 20**).

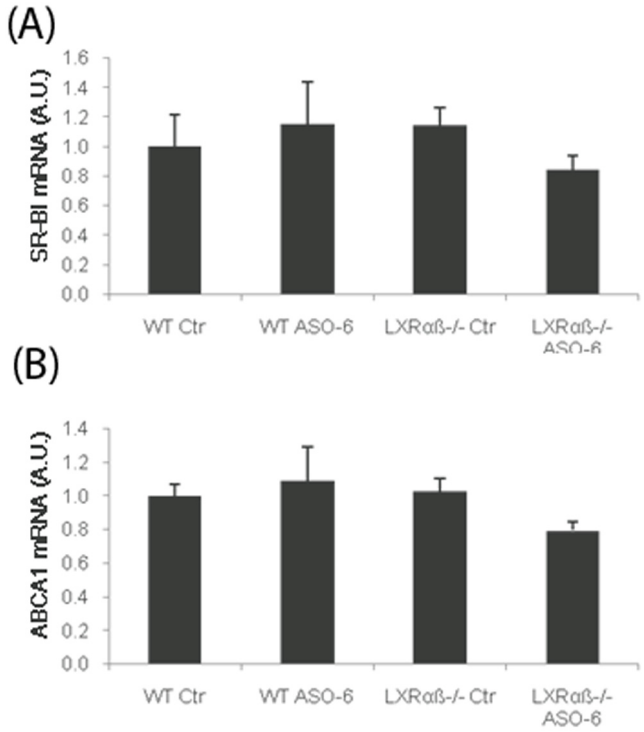

FIGURE 19 – Effect of ASO6 treatment on hepatic mRNA expression of cholesterol transport er gene(A) SR-BI, (B) ABCA1. mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl.

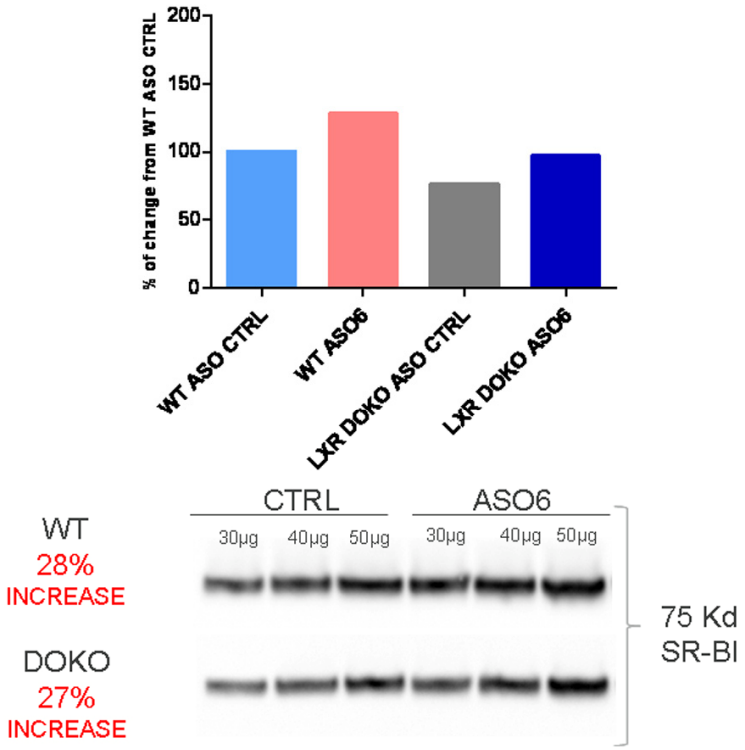

FIGURE 20 – Effect of ASO6 treatment on SR-BI protein expression in liver membrane.

Interestingly, ASO6 lead to a 134% increase of ABCA1 protein in liver membrane from WT mice and to a higher increase (188%) in LXR DOKO liver membranes (**FIGURE 21**). Although the minor effects seen for triglyceride content in the liver (see above), ASO treatment led to down-regulation of all the gene involved in fatty acid synthesis, likely mediated by a SREBP1-c down-regulation (i.e. Fatty acid synthase, hepatic stearoyl-CoA deaturase 1 and acetyl-CoA carboxylase 1)(**FIGURE 22**). Triacylglycerol hydrolase 1 and 2 mRNA expression resulted significantly reduced in LXR DOKO KO mice compared to WT, suggesting TGH-1 and TGH-2 as new LXR gene target. ASO6 did not seem to affect the expression of this gene in WT mice, while in LXR DOKO mice ACAT2 down-regulation lead to a slight increase of TGH-1 mRNA and a decrease in TGH-2 mRNA levels (**FIGURE 23**).

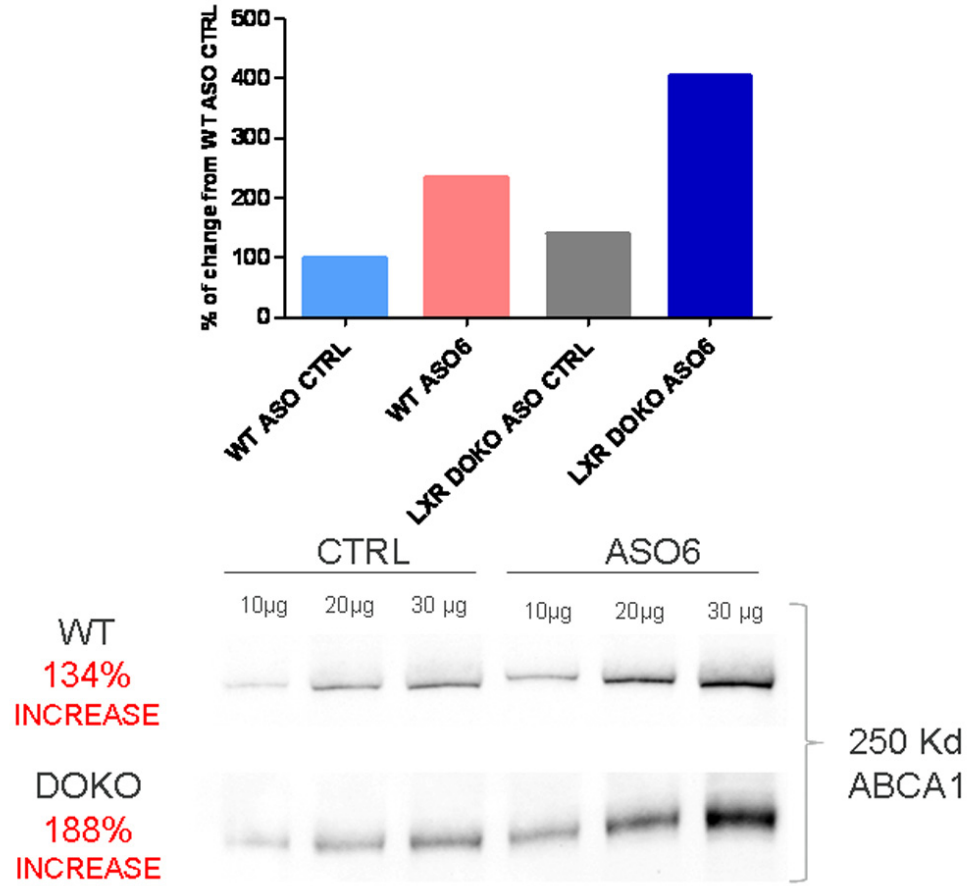

FIGURE 21 – Effect of ASO6 treatment on ABCA1 protein expression in liver membrane.

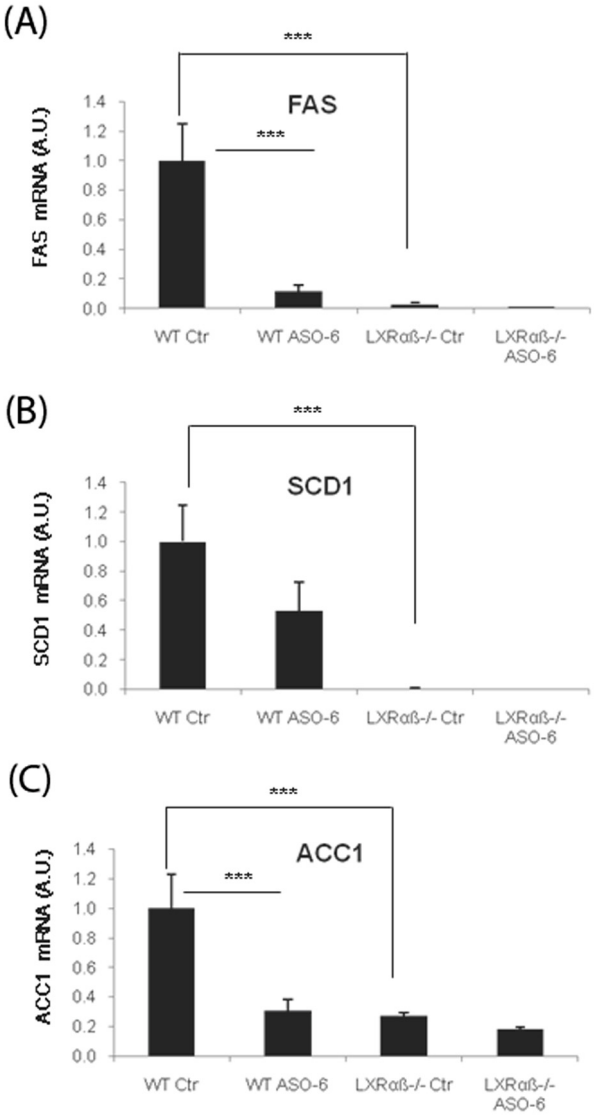

FIGURE 22 – Effect of ASO6 treatment on hepatic mRNA expression of genes involved in fatty acid synthesis: (A) fatty acid synthase, (B) stearoyl-CoA deaturase 1, (C) acetyl-CoA carboxylase 1. mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for TFIIB mRNA expression and normalized to WT Ctrl.

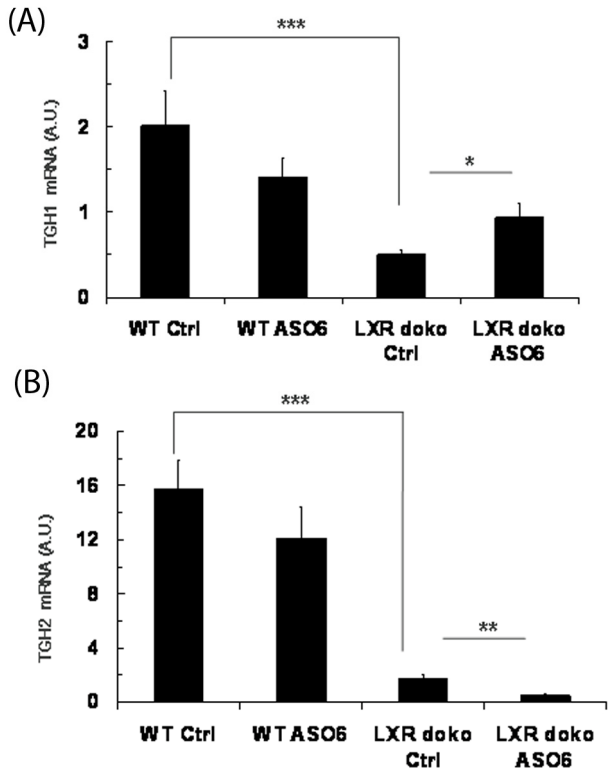

FIGURE 23 – Effect of ASO6 treatment on hepatic mRNA expression of Triacylglycerol hydrolase 1 (A) and 2 (B) mRNA expressions were analyzed by real-time RT-PCR. Data are standardized for 18S mRNA expression and normalized to WT Ctrl.

HDL subpopulation distribution

Since the observed increase in plasma HDL-C and ABCA1 protein in the liver secondary to ACAT2 down-regulation, we decided to analyze mouse sera by 2D-gel electrophoresis to identify possible changes in HDL sub-fractions. In WT mice ASO6 treatment did not affect small pre-beta HDL percentage (**FIGURE 24**), but lead to clear changes in HDL subpopulation distribution: HDL becomes more heterogeneous, with the appearance in the alpha region of particles of lager size (**FIGURE 25**). A different trend was observed in LXR DOKO mice, in which ASO6

treatment induced a significant increase in pre-beta HDL percentage (**FIGURE 24**) and to a shift of the alpha HDL towards smaller size particles (**FIGURE 25**).

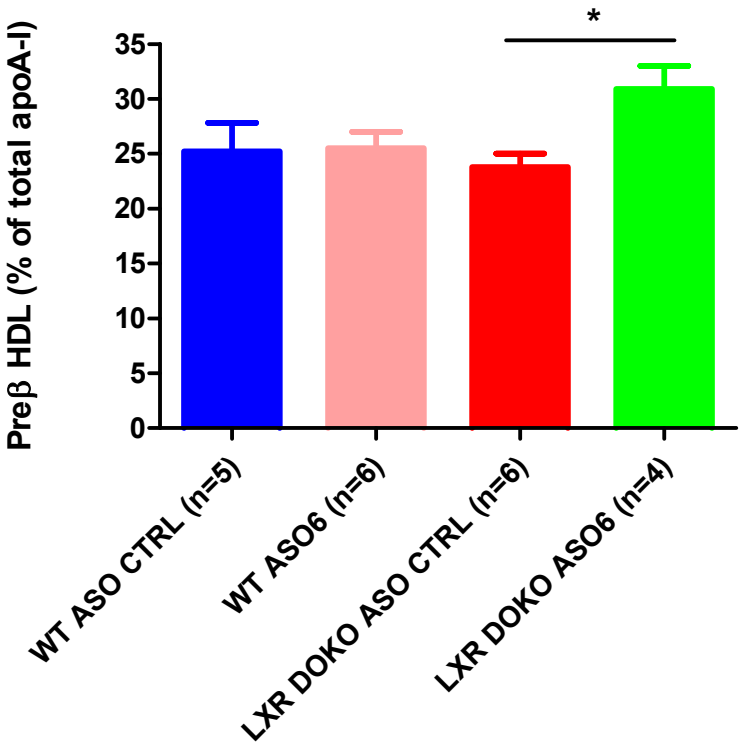

FIGURE 24 – % of total apoA-I in preβ HDL subfraction. Student's t test $p<0.05$

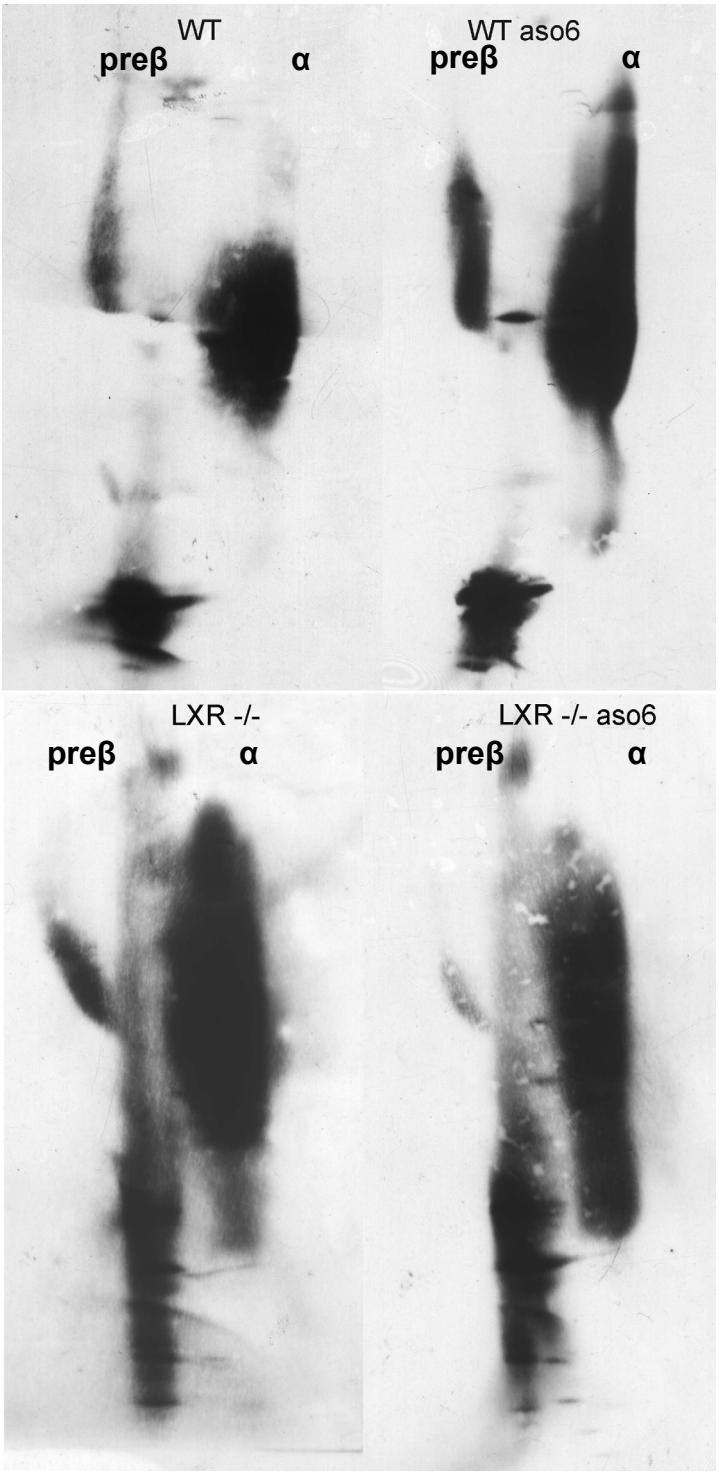

FIGURE 25 – HDL subclasses. HDL subclasses were separated by 2D electrophoresis and transferred onto a nitrocellulose membrane, on which lipoproteins were detected with an anti apoA-I antibody.

DISCUSSION

IN-VIVO REVERSE CHOLESTEROL TRANSPORT STUDY

The atheroprotective role of apoE is well documented by several works, showing the impact of its deletion on atherosclerosis development. This activity is at least in part related to the modulation of lipoprotein profile, but it has been shown that independently of this, apoE selective expression in macrophages protects towards the development of atherosclerosis in hypercholesterolemic mice. Since RCT is an antiatherosclerotic process possibly influenced by circulating lipoprotein (particularly HDL) we aimed to evaluate apoE role in this physiological pathway. We manage a method that allows the determination of RCT that occurs specifically from macrophages. Here we used peritoneal macrophages from WT or apoE-/- mice enriched with 3H-cholesterol and injected into WT or apoE deficient mice respectively. Cholesterol is delivered from macrophages to the plasma compartment and successively returns to the liver and feces. The quantification of radioactivity in plasma, liver and faeces allows to measure every single step of RCT. The amount of radioactivity in plasma is significantly higher in apoE/apoE mice, consistent with increased level of circulating lipoproteins. In contrast, reduced amount of cholesterol in liver and feces was detected, consistent with previously published data showing that apoE KO mice present a reduced uptake of CE-HDL[204] and that apoE promotes biliar excretion[205]. The quantification of total RCT, estimated as the sum of radioactive cholesterol in plasma, liver and feces, revealed that more radioactivity is mobilized within WT/WT mice, indicating that the whole process is impaired in absence of apoE. To investigate whether increase of lipid efflux may account for the increased RCT in WT/WT mice, we tested plasma capacity to drive cholesterol release from cells. However, cholesterol efflux to apoE KO mice plasma by passive diffusion, SR-BI and ABCG1 was improved. This probably reflects

the higher content of apoB-containing lipoproteins, that are known to be good and efficient acceptors of cholesterol through these mechanisms. However, it should be reminded that passive diffusion and SR-BI-mediated efflux are bidirectional processes, whose global effect depends on lipid concentration gradient between cells and acceptors. It is therefore conceivable that apoE/apoE mouse plasma is also able to promote lipid influx, thus enriching cell cholesterol content. Conversely, plasma capacity to drive ABCA1-mediated efflux was similar in both animal groups.

Taken together these results suggest that apoE essential for a functional RCT.

ASO6 STUDY

Inhibition of intracellular esterification of cholesterol as a means to prevent the arterial CE accumulation in atherosclerosis has for many years been considered as potential strategy. Much key information has been learned from mice with gene deletion either of ACAT1 or ACAT2. In hypercholesterolemic ACAT1-/- animals, the ACAT1 deficiency led to a marked alteration in cholesterol metabolism leading to massive accumulation of unesterified cholesterol causing numerous skin and brain lesions. The atherosclerotic lesions in the apoE-/- and LDLr-/- mice lacking ACAT1 had reduced levels of neutral lipids and a paucity of macrophages which may reflect cell death via apoptotic mechanisms [182]. On the contrary, deletion of ACAT2 has been consistently atheroprotective (for review see[80]). A successful protection from atherosclerosis has been obtained by decreasing the activity of hepatic ACAT2 in mice injected with anti-sense oligonucleotide therapy, which

interferes with and reduces ACAT2 mRNA[187]. Using the same therapeutic approach in the present work we investigated the role of hepatic ACAT2 disruption in triglyceride and cholesterol metabolism in LXRa/β double knock-out mice and in wild type mice fed with 10% SAT (cal) and 0.2% cholesterol (w/w) diet. The use of these two animal models was aimed to find a possible LXR activation secondary to ACAT2 inhibition. No evident LXR activation followed down-regulation of hepatic ACAT2 activity by ASO treatment in male mice. In WT mice ASO6 seemed to affect the pool of free cholesterol that could signal to and determine a down-regulation of the SREBP-system. Indeed, the effect observed in this animal group was a decreased mRNA expression of HMG-CoR, HMG-CoS. ASO6 led also to a down-regulation LDLr mRNA and to a higher extent decrease in PCSK9 expression. Nevertheless, the net effect was a down regulation of LDL-receptor protein expression in liver membranes from WT mice. Interestingly, we found that LXR DOKO mice showed undetectable levels of LDLr protein in liver membranes by western blot analysis, suggesting a defect in LDLr recycling (more work will have to be performed to confirm this hypothesis). Furthermore in wild-type mice, the lack of LXR activation and the down-regulation of the SREBP-system after ASO treatment suggest that LXRs are not sensitive to phisologic variation of cholesterol levels within the cell, conversely to the SEBRP-system.

It has been demonstrated that disruption of ACAT2 in mice leads to increased plasma triglyceride to reduced hepatic triglyceride (for review see[80]). In the present study serum triglycerides were increased in apoB-containing lipoprotein fraction by ACAT2 down-regulation, and this effect was clearly not LXR-dependent. Curiously, hepatic ACAT2 knock down led only to a modest reduction in hepatic TG content both in WT and LXR DOKO mice. Never the less ASO treatment led to down-regulation of all genes involved in fatty acid synthesis, likely mediated by the SREBP1-c down-regulation.

As described above, animals were on a 10% SAT (cal) and 0.2% cholesterol (w/w) diet four weeks prior start and for all the duration of ASO6 treatment. This condition probably caused a strong hepatic fat load, leading to the observed partial effect of ASO treatment in reducing liver TG content. We demonstrated that cholesteryl ester levels were 4.6-fold higher in the liver of LXR DOKO mice than in WT, and ASO6 treatment reduced hepatic CE levels by 90% in WT animals, while only a 29% reduction was observed in LXR DOKO mice receiving ASO6. For the first time we identified TGH1 and TGH2 as new LXR-target gene. Since it has been shown that triacylglycerol hydrolase catalyses not only the hydrolysis of TG but also of CE, TGH1 and TGH2 reduced expression observed in LXR DOKO may explain the elevated hepatic CE-content in the livers of LXR DOKO mice. ACAT2 down-regulation did not affect the hepatic free cholesterol content both in WT and LXR DOKO mice. Similar results were shown in a recent mouse study using the antisense oligonucleotide approach for hepatic ACAT2 activity down-regulation[206]. In this elegant study, depletion of hepatic ACAT2 resulted in reduced packaging of cholesterol into apoB-containing lipoprotein, whereas HDL levels remained unchanged. Despite the reduced CE accumulation in the liver of ASO treated mice, no hepatic free cholesterol accumulation occurred and neutral fecal sterol excretion was promoted[206]. Brown et al. supported the concept that when hepatic cholesterol esterification is limited, the liver doesn't deal with FC burden by a new mechanism where none of the traditional pathway (i.e. repression of the novo synthesis, conversion to bile acid, efflux into nascent HDL) are involved[206]. They proposed a non-biliary route for fecal cholesterol excretion[206]. Our work was also aimed to unmask a possible link between ACAT2 and HDL synthesis in mice. In a recent study on gallstone and gallstone-free patients we demonstrated a significant negative correlation between the hepatic ACAT2 activity and the

plasma levels of HDL cholesterol and apoA-I (manuscript in preparation). This important finding is in line with the hypothesis that when hepatic ACAT2 is low, free cholesterol may be fluxed through ABCA1 to apoA-I and thus increasing nascent HDL particles. It should be also reminded that the study by Brown et al. [206] was performed on apoB100only/LDLr-/- mice: being apoB-100 highly expressed and LDLr lacking, the resulting elevated secretion of VLDL and the dramatically decreased LDL-clearance could mask the positive effect of ACAT2 inhibition on HDL-C mediated by the ABCA1 pathway: FC could be forced into the larger amount of apoB-100-containig particles or utilized as substrate for bile acid synthesis rather than be fluxed into nascent HDL.

In the present work we demonstrated that down-regulation of hepatic ACAT2 activity is coupled to an increase in protein expression of ABCA1 in the liver, providing us with the mechanism by which ACAT2 may regulate HDL levels in plasma. Indeed the analysis of plasma lipoprotein profile revealed that ASO6 treatment led to a slight increase in HDL-total cholesterol and to a clear increase in HDL-free cholesterol content. Moreover in WT mice ASO6 treatment did not affect small pre-beta HDL percentage, but lead to clear changes in HDL subpopulation distribution: HDL becomes more heterogeneous, with the appearance in the alpha region of particles of lager size. A different trend was observed in LXR DOKO mice, in which ACAT2 down-regulation induced a significant increase in pre-beta HDL percentage and to a shift of the alpha HDL towards smaller size particles.

In conclusion, the use of anti-sense oligonuclotide targeted to hepatic ACAT2 revealed a new pathway by which the liver contributes to HDL metabolism and identify ACAT2 as an important intracellular hepatocyte player in the determination of cholesterol fluxes either into apoB-containing lipoprotein secretion or into apoAI lipoprotein synthesis.

ABBREVIATIONS

ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
ABCG5	ATP-binding cassette G5
ABCG8	ATP-binding cassette G8
ACAT	acyl-CoA:cholesterol acyltransferase
ACC1	acetyl-CoA carboxylase 1
AcLDL	acetylated LDL
apoA-I	apolipoprotein A-1
apoB	apolipoproteinB
apoE	apolipoprotein E
ASO	antisense olionucleotide
C4	7a-hydroxy-4-cholesten-3-one
Ca	calcium
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
Cyp7a	cholesterol 7 alpha hydroxylase
EC	endothelial cell
EL	endothelial lipase
ER	endoplasmic reticulum

FA	fatty acid
FAS	fatty acid synthase
FC	free cholesterol
glycLDL	glycosilated LDL
HDL	high-density lipoprotein
HDL-C	HDL cholesterol
HL	hepatic lipase
HMG-CoR	3- hydroxymethyl glutaryl coenzyme A reductase
HMG-CoS	3- hydroxymethyl glutaryl coenzyme A synthase
ICAM	intracellular adhesion molecule
IDL	intermediate density lipoprotein
IL	interleukins
INSIG	insulin inducible gene
IR	insulin receptor
LCAT	lecithin:cholesterol acyl transferase
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LOX-1	lectin-like oxidized low-density lipoprotein receptor-1
Lp (a)	lipoprotein (a)
LPL	lipoprotein lipase
LPS	lipopolysacchride

LXR	liver X receptor
LXRE	LXR response elements
MCP-1	macrophage chemotactic protein
MCSF	macrophage colony-stimulating factor
MMPs	matrix metalloproteinases
MPM	murine pedritonel macrophages
MTTP	microsomal triglyceride transfer protein
NBD	binding domain
NFkB	nuclear factor-kappa B
NO	nitric oxide
NPC	Niemann-Pick type C
OxLDL	oxidized LDL
PLA2	phospholipase A2
PLC	Phospholipase C
PLTP	phospholipid transfer protein PLTP
PPAR	peroxisome proliferator-activated receptors
PUFA	poly-unsaturated fatty acids
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SCAP	SREBP cleavage activating protein
SCD-1	stearoyl CoA desaturase

SHP	Small heterodimer partner
SMC	smooth muscle cells
SR-B1	scavenger receptor B-1
SREBP	sterol response element-binding protein
TC	total cholesterol
TG	triglyceride
TGH1	Triacylglycerol hydrolase 1
TGH2	Triacylglycerol hydrolase 2
TLR	Toll-like receptor
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein
VLDLr	VLDL receptor

REFERENCES

1. Tiwari RL, Singh V, Barthwal MK. Macrophages: an elusive yet emerging therapeutic target of atherosclerosis. *Medicinal research reviews.* 2008;28(4):483-544.

2. Lusis AJ. Atherosclerosis. *Nature.* 2000;407(6801):233-241.

3. Organization WH. The atlas of heart disease and stroke. Available at: http:www.who.int/cardiovascular_diseases/resources/atlas/en.

4. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell.* 2001;104(4):503-516.

5. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annual review of biochemistry.* 1983;52:223-261.

6. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis, thrombosis, and vascular biology.* 1995;15(9):1512-1531.

7. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* 1995;92(5):1355-1374.

8. Khalil MF, Wagner WD, Goldberg IJ. Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology.* 2004;24(12):2211-2218.

9. Jain KS, Kathiravan MK, Somani RS, Shishoo CJ. The biology and chemistry of hyperlipidemia. *Bioorganic & medicinal chemistry.* 2007;15(14):4674-4699.

10. Boren J, Olin K, Lee I, Chait A, Wight TN, Innerarity TL. Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *The Journal of clinical investigation.* 1998;101(12):2658-2664.

11. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proceedings of the National Academy of Sciences of the United States of America.* 1979;76(1):333-337.

12. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arteriosclerosis, thrombosis, and vascular biology.* 2005;25(1):29-38.

13. Morrow JD. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. *Arteriosclerosis, thrombosis, and vascular biology.* 2005;25(2):279-286.

14. Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brummer J, Gutzki FM, Berger J, Frolich JC, Boger RH. Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study. *Circulation.* 2004;109(7):843-848.

15. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation.* 2005;112(5):651-657.

16. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. *Free radical biology & medicine.* 2000;28(12):1815-1826.

17. Younis N, Sharma R, Soran H, Charlton-Menys V, Elseweidy M, Durrington PN. Glycation as an atherogenic modification of LDL. *Current opinion in lipidology.* 2008;19(4):378-384.

18. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. *The New England journal of medicine.* 1987;316(22):1371-1375.

19. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation.* 2001;104(3):365-372.

20. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. *Nature medicine.* 2002;8(11):1218-1226.

21. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine.* 2005;352(16):1685-1695.

22. Nathan C. Points of control in inflammation. *Nature.* 2002;420(6917):846-852.

23. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation.* 2005;111(25):3481-3488.

24. Thompson GR, Barter PJ. Clinical lipidology at the end of the millennium. *Current opinion in lipidology.* 1999;10(6):521-526.

25. Mahley RW, Bersot TP. Drug therapy for hypercholesterolemia and dyslipidemia. In: Hardman JG LL, ed. *Goodman & Gilman's The Pharmacological Basis of Therapeutics. 11th ed.* New York: McGraw-Hill Medical Publishing Division; 2006:933–966.

26. Shapiro DJ, Rodwell VW. Regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol synthesis. *The Journal of biological chemistry.* 1971;246(10):3210-3216.

27. Goldstein JL, Brown MS. Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. *Proceedings of the National Academy of Sciences of the United States of America.* 1973;70(10):2804-2808.

28. Brown MS, Goldstein JL. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proceedings of the National Academy of Sciences of the United States of America.* 1974;71(3):788-792.

29. Endo A, Kuroda M, Tanzawa K. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal

metabolites, having hypocholesterolemic activity. *FEBS letters.* 1976;72(2):323-326.

30. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hensens O, Hirshfield J, Hoogsteen K, Liesch J, Springer J. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proceedings of the National Academy of Sciences of the United States of America.* 1980;77(7):3957-3961.

31. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama.* 2001;285(19):2486-2497.

32. Bhatt DL, Steg PG, Ohman EM, Hirsch AT, Ikeda Y, Mas JL, Goto S, Liau CS, Richard AJ, Rother J, Wilson PW. International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *Jama.* 2006;295(2):180-189.

33. Steg PG, Bhatt DL, Wilson PW, D'Agostino R, Sr., Ohman EM, Rother J, Liau CS, Hirsch AT, Mas JL, Ikeda Y, Pencina MJ, Goto S. One-year cardiovascular event rates in outpatients with atherothrombosis. *Jama.* 2007;297(11):1197-1206.

34. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet.* 1994;344(8934):1383-1389.

35. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *The New England journal of medicine.* 1995;333(20):1301-1307.

36. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *The New England journal of medicine.* 1996;335(14):1001-1009.

37. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *The New England journal of medicine.* 1998;339(19):1349-1357.

38. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, Langendorfer A, Stein EA, Kruyer W, Gotto AM, Jr. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *Jama.* 1998;279(20):1615-1622.

39. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet.* 2002;360(9326):7-22.

40. Sever PS, Dahlof B, Poulter NR, Wedel H, Beevers G, Caulfield M, Collins R, Kjeldsen SE, Kristinsson A, McInnes GT, Mehlsen J, Nieminen M, O'Brien E, Ostergren J. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet.* 2003;361(9364):1149-1158.

41. Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, Ford I, Gaw A, Hyland M, Jukema JW, Kamper AM, Macfarlane PW, Meinders AE, Norrie J, Packard CJ, Perry IJ, Stott DJ, Sweeney BJ, Twomey C, Westendorp RG. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. *Lancet.* 2002;360(9346):1623-1630.

42. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *Jama.* 1986;256(20):2835-2838.

43. Kannel WB. High-density lipoproteins: epidemiologic profile and risks of coronary artery disease. *The American journal of cardiology.* 1983;52(4):9B-12B.

44. Wilson PW. High-density lipoprotein, low-density lipoprotein and coronary artery disease. *The American journal of cardiology.* 1990;66(6):7A-10A.

45. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM, Kastelein JJ, Bittner V, Fruchart JC. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *The New England journal of medicine.* 2007;357(13):1301-1310.

46. LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *The New England journal of medicine.* 2005;352(14):1425-1435.

47. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine.* 2007;357(21):2109-2122.

48. Tall AR. CETP inhibitors to increase HDL cholesterol levels. *The New England journal of medicine.* 2007;356(13):1364-1366.

49. Tall AR, Yvan-Charvet L, Wang N. The failure of torcetrapib: was it the molecule or the mechanism? *Arteriosclerosis, thrombosis, and vascular biology.* 2007;27(2):257-260.

50. Schaefer EJ, Asztalos BF. Increasing high-density lipoprotein cholesterol, inhibition of cholesteryl ester transfer protein, and heart disease risk reduction. *The American journal of cardiology.* 2007;100(11 A):n25-31.

51. El Harchaoui K, van der Steeg WA, Stroes ES, Kastelein JJ. The role of CETP inhibition in dyslipidemia. *Current atherosclerosis reports.* 2007;9(2):125-133.

52. Adorni MP, Zimetti F, Billheimer JT, Wang N, Rader DJ, Phillips MC, Rothblat GH. The roles of different pathways in the release of cholesterol from macrophages. *Journal of lipid research.* 2007;48(11):2453-2462.

53. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Tall AR, Rader DJ. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *The Journal of clinical investigation.* 2007;117(8):2216-2224.

54. Khuseyinova N, Koenig W. Apolipoprotein A-I and risk for cardiovascular diseases. *Current atherosclerosis reports.* 2006;8(5):365-373.

55. Havel RJ, Hamilton RL. Hepatic catabolism of remnant lipoproteins: where the action is. *Arteriosclerosis, thrombosis, and vascular biology.* 2004;24(2):213-215.

56. Boren J, Graham L, Wettesten M, Scott J, White A, Olofsson SO. The assembly and secretion of ApoB 100-containing lipoproteins in Hep G2 cells. ApoB 100 is cotranslationally integrated into lipoproteins. *The Journal of biological chemistry.* 1992;267(14):9858-9867.

57. Boren J, Rustaeus S, Olofsson SO. Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. *The Journal of biological chemistry.* 1994;269(41):25879-25888.

58. Rustaeus S, Lindberg K, Stillemark P, Claesson C, Asp L, Larsson T, Boren J, Olofsson SO. Assembly of very low density lipoprotein: a two-step process of apolipoprotein B core lipidation. *The Journal of nutrition.* 1999;129(2S Suppl):463S-466S.

59. Stillemark P, Boren J, Andersson M, Larsson T, Rustaeus S, Karlsson KA, Olofsson SO. The assembly and secretion of apolipoprotein B-48-containing very low density lipoproteins in McA-RH7777 cells. *The Journal of biological chemistry.* 2000;275(14):10506-10513.

60. Hooper AJ, van Bockxmeer FM, Burnett JR. Monogenic hypocholesterolaemic lipid disorders and apolipoprotein B metabolism. *Critical reviews in clinical laboratory sciences.* 2005;42(5-6):515-545.

61. Rustaeus S, Stillemark P, Lindberg K, Gordon D, Olofsson SO. The microsomal triglyceride transfer protein catalyzes the post-translational assembly of apolipoprotein B-100 very low density lipoprotein in McA-RH7777 cells. *The Journal of biological chemistry.* 1998;273(9):5196-5203.

62. Wang S, McLeod RS, Gordon DA, Yao Z. The microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. *The Journal of biological chemistry.* 1996;271(24):14124-14133.

63. Wu X, Zhou M, Huang LS, Wetterau J, Ginsberg HN. Demonstration of a physical interaction between microsomal triglyceride transfer protein and apolipoprotein B during the assembly of ApoB-containing lipoproteins. *The Journal of biological chemistry.* 1996;271(17):10277-10281.

64. Tran K, Thorne-Tjomsland G, DeLong CJ, Cui Z, Shan J, Burton L, Jamieson JC, Yao Z. Intracellular assembly of very low density lipoproteins containing apolipoprotein B100 in rat hepatoma McA-RH7777 cells. *The Journal of biological chemistry.* 2002;277(34):31187-31200.

65. Rusinol A, Verkade H, Vance JE. Assembly of rat hepatic very low density lipoproteins in the endoplasmic reticulum. *The Journal of biological chemistry.* 1993;268(5):3555-3562.

66. Borchardt RA, Davis RA. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *The Journal of biological chemistry.* 1987;262(34):16394-16402.
67. Dixon JL, Furukawa S, Ginsberg HN. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *The Journal of biological chemistry.* 1991;266(8):5080-5086.
68. White AL, Graham DL, LeGros J, Pease RJ, Scott J. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. A function of the ability of apolipoprotein B to direct lipoprotein assembly and escape presecretory degradation. *The Journal of biological chemistry.* 1992;267(22):15657-15664.
69. Guerin M, Le Goff W, Lassel TS, Van Tol A, Steiner G, Chapman MJ. Atherogenic role of elevated CE transfer from HDL to VLDL(1) and dense LDL in type 2 diabetes : impact of the degree of triglyceridemia. *Arteriosclerosis, thrombosis, and vascular biology.* 2001;21(2):282-288.
70. Franceschini G, Maderna P, Sirtori CR. Reverse cholesterol transport: physiology and pharmacology. *Atherosclerosis.* 1991;88(2-3):99-107.
71. Raghow R, Yellaturu C, Deng X, Park EA, Elam MB. SREBPs: the crossroads of physiological and pathological lipid homeostasis. *Trends in endocrinology and metabolism: TEM.* 2008;19(2):65-73.
72. Espenshade PJ, Hughes AL. Regulation of sterol synthesis in eukaryotes. *Annual review of genetics.* 2007;41:401-427.
73. Glomset JA, Norum KR. The metabolic role of lecithin: cholesterol acyltransferase: perspectives form pathology. *Advances in lipid research.* 1973;11:1-65.
74. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation.* 2006;113(21):2548-2555.
75. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science (New York, N.Y.* 1973;180(93):1332-1339.
76. Miller GJ, Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet.* 1975;1(7897):16-19.
77. Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. *The Journal of clinical investigation.* 2002;110(5):597-603.
78. Lange Y. Disposition of intracellular cholesterol in human fibroblasts. *Journal of lipid research.* 1991;32(2):329-339.
79. Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *The Journal of clinical investigation.* 2002;110(7):905-911.
80. Rudel LL, Lee RG, Parini P. ACAT2 is a target for treatment of coronary heart disease associated with hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology.* 2005;25(6):1112-1118.
81. Oram JF, Vaughan AM. ATP-Binding cassette cholesterol transporters and cardiovascular disease. *Circ Res.* 2006;99(10):1031-1043.
82. Johnson WJ, Mahlberg FH, Rothblat GH, Phillips MC. Cholesterol transport between cells and high-density lipoproteins. *Biochimica et biophysica acta.* 1991;1085(3):273-298.

83. Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH. Importance of different pathways of cellular cholesterol efflux. *Arteriosclerosis, thrombosis, and vascular biology*. 2003;23(5):712-719.

84. Phillips MC, Gillotte KL, Haynes MP, Johnson WJ, Lund-Katz S, Rothblat GH. Mechanisms of high density lipoprotein-mediated efflux of cholesterol from cell plasma membranes. *Atherosclerosis.* 1998;137 Suppl:S13-17.

85. Phillips MC, Johnson WJ, Rothblat GH. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochimica et biophysica acta.* 1987;906(2):223-276.

86. Yokoyama S. Release of cellular cholesterol: molecular mechanism for cholesterol homeostasis in cells and in the body. *Biochimica et biophysica acta.* 2000;1529(1-3):231-244.

87. Krieger M, Herz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annual review of biochemistry.* 1994;63:601-637.

88. Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *The Journal of biological chemistry.* 1994;269(33):21003-21009.

89. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science (New York, N.Y.* 1996;271(5248):518-520.

90. Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *The Journal of biological chemistry.* 1997;272(34):20982-20985.

91. Owen JS, Mulcahy JV. ATP-binding cassette A1 protein and HDL homeostasis. *Atheroscler Suppl.* 2002;3(4):13-22.

92. Webb NR, Connell PM, Graf GA, Smart EJ, de Villiers WJ, de Beer FC, van der Westhuyzen DR. SR-BII, an isoform of the scavenger receptor BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. *The Journal of biological chemistry.* 1998;273(24):15241-15248.

93. Gu X, Lawrence R, Krieger M. Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. *The Journal of biological chemistry.* 2000;275(13):9120-9130.

94. Yancey PG, de la Llera-Moya M, Swarnakar S, Monzo P, Klein SM, Connelly MA, Johnson WJ, Williams DL, Rothblat GH. High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *The Journal of biological chemistry.* 2000;275(47):36596-36604.

95. Jian B, de la Llera-Moya M, Ji Y, Wang N, Phillips MC, Swaney JB, Tall AR, Rothblat GH. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *The Journal of biological chemistry.* 1998;273(10):5599-5606.

96. Annilo T, Chen ZQ, Shulenin S, Costantino J, Thomas L, Lou H, Stefanov S, Dean M. Evolution of the vertebrate ABC gene family: analysis of gene birth and death. *Genomics.* 2006;88(1):1-11.

97. Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM, Oram JF. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *The Journal of clinical investigation.* 1999;104(8):R25-31.

98. Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nature genetics.* 1999;22(4):347-351.

99. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nature genetics.* 1999;22(4):352-355.

100. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest J, Jr., Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nature genetics.* 1999;22(4):336-345.

101. Francis GA, Knopp RH, Oram JF. Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease. *The Journal of clinical investigation.* 1995;96(1):78-87.

102. Remaley AT, Schumacher UK, Stonik JA, Farsi BD, Nazih H, Brewer HB, Jr. Decreased reverse cholesterol transport from Tangier disease fibroblasts. Acceptor specificity and effect of brefeldin on lipid efflux. *Arteriosclerosis, thrombosis, and vascular biology.* 1997;17(9):1813-1821.

103. Tall AR. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *Journal of internal medicine.* 2008;263(3):256-273.

104. Oram JF, Heinecke JW. ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. *Physiological reviews.* 2005;85(4):1343-1372.

105. Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. *Journal of lipid research.* 2001;42(7):1007-1017.

106. van Meer G, Halter D, Sprong H, Somerharju P, Egmond MR. ABC lipid transporters: extruders, flippases, or flopless activators? *FEBS letters.* 2006;580(4):1171-1177.

107. Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC transporter. *Nature.* 2006;443(7108):180-185.

108. Brunham R, Yang C, Maclean I, Kimani J, Maitha G, Plummer F. Chlamydia trachomatis from individuals in a sexually transmitted disease core group exhibit frequent sequence variation in the major outer membrane protein (omp1) gene. *The Journal of clinical investigation.* 1994;94(1):458-463.

109. Thorngate FE, Yancey PG, Kellner-Weibel G, Rudel LL, Rothblat GH, Williams DL. Testing the role of apoA-I, HDL, and cholesterol efflux in the atheroprotective action of low-level apoE expression. *Journal of lipid research.* 2003;44(12):2331-2338.

110. Chroni A, Liu T, Fitzgerald ML, Freeman MW, Zannis VI. Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry.* 2004;43(7):2126-2139.

111. Boadu E, Bilbey NJ, Francis GA. Cellular cholesterol substrate pools for adenosine-triphosphate cassette transporter A1-dependent high-density lipoprotein formation. *Current opinion in lipidology.* 2008;19(3):270-276.

112. Wellington CL, Walker EK, Suarez A, Kwok A, Bissada N, Singaraja R, Yang YZ, Zhang LH, James E, Wilson JE, Francone O, McManus BM, Hayden MR. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. *Laboratory investigation; a journal of technical methods and pathology.* 2002;82(3):273-283.

113. Vaughan AM, Oram JF. ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *Journal of lipid research.* 2006;47(11):2433-2443.

114. Cserepes J, Szentpetery Z, Seres L, Ozvegy-Laczka C, Langmann T, Schmitz G, Glavinas H, Klein I, Homolya L, Varadi A, Sarkadi B, Elkind NB. Functional expression and characterization of the human ABCG1 and ABCG4 proteins: indications for heterodimerization. *Biochemical and biophysical research communications.* 2004;320(3):860-867.

115. Vaughan AM, Oram JF. ABCG1 redistributes cell cholesterol to domains removable by high density lipoprotein but not by lipid-depleted apolipoproteins. *The Journal of biological chemistry.* 2005;280(34):30150-30157.

116. Kobayashi A, Takanezawa Y, Hirata T, Shimizu Y, Misasa K, Kioka N, Arai H, Ueda K, Matsuo M. Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1. *Journal of lipid research.* 2006;47(8):1791-1802.

117. Sano O, Kobayashi A, Nagao K, Kumagai K, Kioka N, Hanada K, Ueda K, Matsuo M. Sphingomyelin-dependence of cholesterol efflux mediated by ABCG1. *Journal of lipid research.* 2007;48(11):2377-2384.

118. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(3):534-540.

119. Baldan A, Tarr P, Lee R, Edwards PA. ATP-binding cassette transporter G1 and lipid homeostasis. *Current opinion in lipidology.* 2006;17(3):227-232.

120. Wang N, Ranalletta M, Matsuura F, Peng F, Tall AR. LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. *Arteriosclerosis, thrombosis, and vascular biology.* 2006;26(6):1310-1316.

121. Croop JM, Tiller GE, Fletcher JA, Lux ML, Raab E, Goldenson D, Son D, Arciniegas S, Wu RL. Isolation and characterization of a mammalian homolog of the Drosophila white gene. *Gene.* 1997;185(1):77-85.

122. Klucken J, Buchler C, Orso E, Kaminski WE, Porsch-Ozcurumez M, Liebisch G, Kapinsky M, Diederich W, Drobnik W, Dean M, Allikmets R, Schmitz G.

ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proceedings of the National Academy of Sciences of the United States of America.* 2000;97(2):817-822.

123. Kim WS, Weickert CS, Garner B. Role of ATP-binding cassette transporters in brain lipid transport and neurological disease. *Journal of neurochemistry.* 2008;104(5):1145-1166.

124. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science (New York, N.Y.* 2000;290(5497):1771-1775.

125. Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC, Hobbs HH. ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. *The Journal of biological chemistry.* 2003;278(48):48275-48282.

126. Yu L, Gupta S, Xu F, Liverman AD, Moschetta A, Mangelsdorf DJ, Repa JJ, Hobbs HH, Cohen JC. Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. *The Journal of biological chemistry.* 2005;280(10):8742-8747.

127. Repa JJ, Dietschy JM, Turley SD. Inhibition of cholesterol absorption by SCH 58053 in the mouse is not mediated via changes in the expression of mRNA for ABCA1, ABCG5, or ABCG8 in the enterocyte. *Journal of lipid research.* 2002;43(11):1864-1874.

128. Lee MH, Lu K, Patel SB. Genetic basis of sitosterolemia. *Current opinion in lipidology.* 2001;12(2):141-149.

129. Vrins C, Vink E, Vandenberghe KE, Frijters R, Seppen J, Groen AK. The sterol transporting heterodimer ABCG5/ABCG8 requires bile salts to mediate cholesterol efflux. *FEBS letters.* 2007;581(24):4616-4620.

130. Rader DJ. Molecular regulation of HDL metabolism and function: implications for novel therapies. *J Clin Invest.* 2006;116(12):3090-3100.

131. Attie AD, Kastelein JP, Hayden MR. Pivotal role of ABCA1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis. *Journal of lipid research.* 2001;42(11):1717-1726.

132. Rader DJ, Alexander ET, Weibel GL, Billheimer J, Rothblat GH. Role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *Journal of lipid research.* 2008.

133. Calabresi L, Favari E, Moleri E, Adorni MP, Pedrelli M, Costa S, Jessup W, Gelissen IC, Kovanen PT, Bernini F, Franceschini G. Functional LCAT is not required for macrophage cholesterol efflux to human serum. *Atherosclerosis.* 2008.

134. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(23):12610-12615.

135. Wang N, Arai T, Ji Y, Rinninger F, Tall AR. Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in

transgenic mice. *The Journal of biological chemistry.* 1998;273(49):32920-32926.

136. Kozarsky KF, Donahee MH, Rigotti A, Iqbal SN, Edelman ER, Krieger M. Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature.* 1997;387(6631):414-417.

137. Zhang Y, Da Silva JR, Reilly M, Billheimer JT, Rothblat GH, Rader DJ. Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport in vivo. *The Journal of clinical investigation.* 2005;115(10):2870-2874.

138. Mahley RW, Huang Y, Weisgraber KH. Putting cholesterol in its place: apoE and reverse cholesterol transport. *The Journal of clinical investigation.* 2006;116(5):1226-1229.

139. Barter PJ. Hugh sinclair lecture: the regulation and remodelling of HDL by plasma factors. *Atherosclerosis.* 2002;3(4):39-47.

140. Nielsen LB, Dahlback B. Lipid metabolism: why is apoM an apolipoprotein? *Current opinion in lipidology.* 2008;19(6):622-623.

141. Wang M, Briggs MR. HDL: the metabolism, function, and therapeutic importance. *Chemical reviews.* 2004;104(1):119-137.

142. Qin S, Kawano K, Bruce C, Lin M, Bisgaier C, Tall AR, Jiang X. Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoA-IV-rich lamellar lipoproteins. *Journal of lipid research.* 2000;41(2):269-276.

143. Jiang XC, Bruce C, Mar J, Lin M, Ji Y, Francone OL, Tall AR. Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *The Journal of clinical investigation.* 1999;103(6):907-914.

144. Jiang X, Francone OL, Bruce C, Milne R, Mar J, Walsh A, Breslow JL, Tall AR. Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. *The Journal of clinical investigation.* 1996;98(10):2373-2380.

145. Camps L, Reina M, Llobera M, Vilaro S, Olivecrona T. Lipoprotein lipase: cellular origin and functional distribution. *The American journal of physiology.* 1990;258(4 Pt 1):C673-681.

146. Rader DJ, Jaye M. Endothelial lipase: a new member of the triglyceride lipase gene family. *Current opinion in lipidology.* 2000;11(2):141-147.

147. Francis MC, Frohlich JJ. Coronary artery disease in patients at low risk--apolipoprotein AI as an independent risk factor. *Atherosclerosis.* 2001;155(1):165-170.

148. Walldius G, Jungner I. The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. *Journal of internal medicine.* 2006;259(5):493-519.

149. Basso F, Freeman L, Knapper CL, Remaley A, Stonik J, Neufeld EB, Tansey T, Amar MJ, Fruchart-Najib J, Duverger N, Santamarina-Fojo S, Brewer HB, Jr. Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *Journal of lipid research.* 2003;44(2):296-302.

150. Sahoo D, Trischuk TC, Chan T, Drover VA, Ho S, Chimini G, Agellon LB, Agnihotri R, Francis GA, Lehner R. ABCA1-dependent lipid efflux to

apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *Journal of lipid research.* 2004;45(6):1122-1131.

151. Timmins JM, Lee JY, Boudyguina E, Kluckman KD, Brunham LR, Mulya A, Gebre AK, Coutinho JM, Colvin PL, Smith TL, Hayden MR, Maeda N, Parks JS. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *The Journal of clinical investigation.* 2005;115(5):1333-1342.

152. Elshourbagy NA, Liao WS, Mahley RW, Taylor JM. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. *Proc Natl Acad Sci U S A.* 1985;82(1):203-207.

153. Mahley RW, Ji ZS. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J Lipid Res.* 1999;40(1):1-16.

154. Mahley RW, Rall SC, Jr. Apolipoprotein E: far more than a lipid transport protein. *Annu Rev Genomics Hum Genet.* 2000;1:507-537.

155. Ghiselli G, Schaefer EJ, Gascon P, Breser HB, Jr. Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. *Science.* 1981;214(4526):1239-1241.

156. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* 1992;71(2):343-353.

157. Pendse AA, Arbones-Mainar JM, Johnson LA, Altenburg M, Maeda N. ApoE knock-out and knock-in mice: atherosclerosis, metabolic syndrome and beyond. *J Lipid Res.* 2008.

158. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* 1992;258(5081):468-471.

159. Buhman KF, Accad M, Farese RV. Mammalian acyl-CoA:cholesterol acyltransferases. *Biochimica et biophysica acta.* 2000;1529(1-3):142-154.

160. Chang CC, Huh HY, Cadigan KM, Chang TY. Molecular cloning and functional expression of human acyl-coenzyme A:cholesterol acyltransferase cDNA in mutant Chinese hamster ovary cells. *The Journal of biological chemistry.* 1993;268(28):20747-20755.

161. Meiner VL, Cases S, Myers HM, Sande ER, Bellosta S, Schambelan M, Pitas RE, McGuire J, Herz J, Farese RV, Jr. Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proceedings of the National Academy of Sciences of the United States of America.* 1996;93(24):14041-14046.

162. Anderson RA, Joyce C, Davis M, Reagan JW, Clark M, Shelness GS, Rudel LL. Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *The Journal of biological chemistry.* 1998;273(41):26747-26754.

163. Cases S, Novak S, Zheng YW, Myers HM, Lear SR, Sande E, Welch CB, Lusis AJ, Spencer TA, Krause BR, Erickson SK, Farese RV, Jr. ACAT-2, a second mammalian acyl-CoA:cholesterol acyltransferase. Its cloning, expression,

and characterization. *The Journal of biological chemistry*. 1998;273(41):26755-26764.

164. Oelkers P, Behari A, Cromley D, Billheimer JT, Sturley SL. Characterization of two human genes encoding acyl coenzyme A:cholesterol acyltransferase-related enzymes. *The Journal of biological chemistry*. 1998;273(41):26765-26771.

165. Rudel LL, Lee RG, Cockman TL. Acyl coenzyme A: cholesterol acyltransferase types 1 and 2: structure and function in atherosclerosis. *Current opinion in lipidology*. 2001;12(2):121-127.

166. Joyce CW, Shelness GS, Davis MA, Lee RG, Skinner K, Anderson RA, Rudel LL. ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane. *Molecular biology of the cell.* 2000;11(11):3675-3687.

167. Lin S, Cheng D, Liu MS, Chen J, Chang TY. Human acyl-CoA:cholesterol acyltransferase-1 in the endoplasmic reticulum contains seven transmembrane domains. *The Journal of biological chemistry*. 1999;274(33):23276-23285.

168. Lin S, Lu X, Chang CC, Chang TY. Human acyl-coenzyme A:cholesterol acyltransferase expressed in chinese hamster ovary cells: membrane topology and active site location. *Molecular biology of the cell.* 2003;14(6):2447-2460.

169. Uelmen PJ, Oka K, Sullivan M, Chang CC, Chang TY, Chan L. Tissue-specific expression and cholesterol regulation of acylcoenzyme A:cholesterol acyltransferase (ACAT) in mice. Molecular cloning of mouse ACAT cDNA, chromosomal localization, and regulation of ACAT in vivo and in vitro. *The Journal of biological chemistry*. 1995;270(44):26192-26201.

170. Meiner V, Tam C, Gunn MD, Dong LM, Weisgraber KH, Novak S, Myers HM, Erickson SK, Farese RV, Jr. Tissue expression studies on the mouse acyl-CoA: cholesterol acyltransferase gene (Acact): findings supporting the existence of multiple cholesterol esterification enzymes in mice. *Journal of lipid research*. 1997;38(9):1928-1933.

171. Lee RG, Willingham MC, Davis MA, Skinner KA, Rudel LL. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *Journal of lipid research*. 2000;41(12):1991-2001.

172. Chang CC, Sakashita N, Ornvold K, Lee O, Chang ET, Dong R, Lin S, Lee CY, Strom SC, Kashyap R, Fung JJ, Farese RV, Jr., Patoiseau JF, Delhon A, Chang TY. Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *The Journal of biological chemistry*. 2000;275(36):28083-28092.

173. Chang TY, Chang CC, Lin S, Yu C, Li BL, Miyazaki A. Roles of acyl-coenzyme A:cholesterol acyltransferase-1 and -2. *Current opinion in lipidology*. 2001;12(3):289-296.

174. Miyazaki A, Sakashita N, Lee O, Takahashi K, Horiuchi S, Hakamata H, Morganelli PM, Chang CC, Chang TY. Expression of ACAT-1 protein in human atherosclerotic lesions and cultured human monocytes-macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 1998;18(10):1568-1574.

175. Sakashita N, Miyazaki A, Takeya M, Horiuchi S, Chang CC, Chang TY, Takahashi K. Localization of human acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) in macrophages and in various tissues. *The American journal of pathology*. 2000;156(1):227-236.
176. Parini P, Davis M, Lada AT, Erickson SK, Wright TL, Gustafsson U, Sahlin S, Einarsson C, Eriksson M, Angelin B, Tomoda H, Omura S, Willingham MC, Rudel LL. ACAT2 is localized to hepatocytes and is the major cholesterol-esterifying enzyme in human liver. *Circulation*. 2004;110(14):2017-2023.
177. Pramfalk C, Davis MA, Eriksson M, Rudel LL, Parini P. Control of ACAT2 liver expression by HNF1. *Journal of lipid research*. 2005;46(9):1868-1876.
178. Sakashita N, Miyazaki A, Chang CC, Chang TY, Kiyota E, Satoh M, Komohara Y, Morganelli PM, Horiuchi S, Takeya M. Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) is induced in monocyte-derived macrophages: in vivo and in vitro studies. *Laboratory investigation; a journal of technical methods and pathology*. 2003;83(11):1569-1581.
179. Fazio S, Linton M. Failure of ACAT inhibition to retard atherosclerosis. *The New England journal of medicine*. 2006;354(12):1307-1309.
180. Tardif JC, Gregoire J, L'Allier PL, Anderson TJ, Bertrand O, Reeves F, Title LM, Alfonso F, Schampaert E, Hassan A, McLain R, Pressler ML, Ibrahim R, Lesperance J, Blue J, Heinonen T, Rodes-Cabau J. Effects of the acyl coenzyme A:cholesterol acyltransferase inhibitor avasimibe on human atherosclerotic lesions. *Circulation*. 2004;110(21):3372-3377.
181. Nissen SE, Tuzcu EM, Brewer HB, Sipahi I, Nicholls SJ, Ganz P, Schoenhagen P, Waters DD, Pepine CJ, Crowe TD, Davidson MH, Deanfield JE, Wisniewski LM, Hanyok JJ, Kassalow LM. Effect of ACAT inhibition on the progression of coronary atherosclerosis. *The New England journal of medicine*. 2006;354(12):1253-1263.
182. Accad M, Smith SJ, Newland DL, Sanan DA, King LE, Jr., Linton MF, Fazio S, Farese RV, Jr. Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest*. 2000;105(6):711-719.
183. Yagyu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perrey S, Tamura Y, Ohashi K, Okazaki H, Yahagi N, Shionoiri F, Iizuka Y, Harada K, Shimano H, Yamashita H, Gotoda T, Yamada N, Ishibashi S. Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *The Journal of biological chemistry*. 2000;275(28):21324-21330.
184. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RV, Jr. Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *The Journal of clinical investigation*. 2001;107(2):163-171.
185. Perrey S, Legendre C, Matsuura A, Guffroy C, Binet J, Ohbayashi S, Tanaka T, Ortuno JC, Matsukura T, Laugel T, Padovani P, Bellamy F, Edgar AD. Preferential pharmacological inhibition of macrophage ACAT increases plaque formation in mouse and rabbit models of atherogenesis. *Atherosclerosis*. 2001;155(2):359-370.
186. Bell TA, 3rd, Kelley K, Wilson MD, Sawyer JK, Rudel LL. Dietary fat-induced alterations in atherosclerosis are abolished by ACAT2-deficiency in ApoB100 only, LDLr-/- mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27(6):1396-1402.

187. Bell TA, 3rd, Brown JM, Graham MJ, Lemonidis KM, Crooke RM, Rudel LL. Liver-specific inhibition of acyl-coenzyme a:cholesterol acyltransferase 2 with antisense oligonucleotides limits atherosclerosis development in apolipoprotein B100-only low-density lipoprotein receptor-/- mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26(8):1814-1820.

188. Brown JM, Bell TA, 3rd, Alger HM, Sawyer JK, Smith TL, Kelley K, Shah R, Wilson MD, Davis MA, Lee RG, Graham MJ, Crooke RM, Rudel LL. Targeted depletion of hepatic ACAT2-driven cholesterol esterification reveals a non-biliary route for fecal neutral sterol loss. *J Biol Chem*. 2008;283(16):10522-10534.

189. Ma J, Folsom AR, Lewis L, Eckfeldt JH. Relation of plasma phospholipid and cholesterol ester fatty acid composition to carotid artery intima-media thickness: the Atherosclerosis Risk in Communities (ARIC) Study. *The American journal of clinical nutrition*. 1997;65(2):551-559.

190. Warensjo E, Riserus U, Gustafsson IB, Mohsen R, Cederholm T, Vessby B. Effects of saturated and unsaturated fatty acids on estimated desaturase activities during a controlled dietary intervention. *Nutr Metab Cardiovasc Dis*. 2008.

191. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science (New York, N.Y.* 1988;240(4852):622-630.

192. Bellosta S, Mahley RW, Sanan DA, Murata J, Newland DL, Taylor JM, Pitas RE. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *The Journal of clinical investigation*. 1995;96(5):2170-2179.

193. Zhang Y, Zanotti I, Reilly MP, Glick JM, Rothblat GH, Rader DJ. Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. *Circulation*. 2003;108(6):661-663.

194. Bortnick AE, Rothblat GH, Stoudt G, Hoppe KL, Royer LJ, McNeish J, Francone OL. The correlation of ATP-binding cassette 1 mRNA levels with cholesterol efflux from various cell lines. *The Journal of biological chemistry*. 2000;275(37):28634-28640.

195. de la Llera-Moya M, Rothblat GH, Connelly MA, Kellner-Weibel G, Sakr SW, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. *Journal of lipid research*. 1999;40(3):575-580.

196. Parini P, Johansson L, Bröijersén A, Angelin B, Rudling M. Lipoprotein profiles in plasma and interstitial fluid analyzed with an automated gel-filtration system. *Eur J Clin Invest*. 2006;36:98-104.

197. Lovgren-Sandblom A, Heverin M, Larsson H, Lundstrom E, Wahren J, Diczfalusy U, Bjorkhem I. Novel LC-MS/MS method for assay of 7alpha-hydroxy-4-cholesten-3-one in human plasma. Evidence for a significant extrahepatic metabolism. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;856(1-2):15-19.

198. Temel RE, Tang W, Ma Y, Rudel LL, Willingham MC, Ioannou YA, Davies JP, Nilsson LM, Yu L. Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *J Clin Invest*. 2007;117(7):1968-1978.

199. Rudel LL, Davis M, Sawyer J, Shah R, Wallace J. Primates highly responsive to dietary cholesterol up-regulate hepatic ACAT2, and less responsive primates do not. *The Journal of biological chemistry*. 2002;277(35):31401-31406.

200. Carr TP, Parks JS, Rudel LL. Hepatic ACAT activity in African green monkeys is highly correlated to plasma LDL cholesteryl ester enrichment and coronary artery atherosclerosis. *Arterioscler Thromb*. 1992;12(11):1274-1283.

201. Lada AT, Davis M, Kent C, Chapman J, Tomoda H, Omura S, Rudel LL. Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness. *Journal of lipid research*. 2004;45(2):378-386.

202. Favari E, Lee M, Calabresi L, Franceschini G, Zimetti F, Bernini F, Kovanen PT. Depletion of pre-beta-high density lipoprotein by human chymase impairs ATP-binding cassette transporter A1- but not scavenger receptor class B type I-mediated lipid efflux to high density lipoprotein. *The Journal of biological chemistry*. 2004;279(11):9930-9936.

203. Zanotti I, Poti F, Pedrelli M, Favari E, Moleri E, Franceschini G, Calabresi L, Bernini F. The LXR agonist T0901317 promotes the reverse cholesterol transport from macrophages by increasing plasma efflux potential. *J Lipid Res*. 2008;49(5):954-960.

204. Arai T, Rinninger F, Varban L, Fairchild-Huntress V, Liang CP, Chen W, Seo T, Deckelbaum R, Huszar D, Tall AR. Decreased selective uptake of high density lipoprotein cholesteryl esters in apolipoprotein E knock-out mice. *Proc Natl Acad Sci U S A*. 1999;96(21):12050-12055.

205. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arterioscler Thromb Vasc Biol*. 2004;24(6):1006-1014.

206. Brown JM, Bell TA, 3rd, Alger HM, Sawyer JK, Smith TL, Kelley KD, Shah RA, Wilson MD, Davis MA, Lee RG, Graham MJ, Crooke RM, Rudel LL. Targeted depletion of hepatic ACAT2-driven cholesterol esterification reveals a non-biliary route for fecal sterol loss. *J Biol Chem*. 2008.