1. Introduction

High serum cholesterol levels have been associated with cardiovascular diseases (CVD), a leading cause of death in the world.\(^1\)\(^2\) Atherosclerosis is a disease of medium and large arteries, characterized by progressive thickening of arterial intima. Occlusion of these vessels may ultimately lead to myocardial infarction.\(^3\) This is a leading cause of morbidity and mortality in the US and developed as well as developing countries.

Cholesterol levels are affected by various factors such as rate of endogenous cholesterol synthesis, biliary cholesterol excretion and dietary cholesterol absorption.\(^3\) Several lipid lowering strategies, especially HMG-CoA reductase inhibitors (‘Statins’) or cholesterol synthesis inhibitors have been developed\(^1\)\(^2\) and are currently in therapeutic use. Nevertheless, a substantial number of patients who receive a statin monotherapy, do not achieve the ultimate treatment goals.\(^1\)\(^6\) Moreover, augmenting the dose of statins may also increase adverse side effects.\(^1\)\(^7\) Given the limitations of the statins and other lipid lowering agents such as fibrates and bile acid sequestrants, the research for discovering novel lipid lowering agents continues and studies are currently targeting the process of inhibition of absorption of intestinal cholesterol. The plant sterols and stanols\(^1\)\(^8\), ACAT inhibitors\(^1\)\(^9\), microsomal triglyceride transfer protein (MTP) inhibitors\(^1\)\(^10\), and Niemann-pick C1-L1 ligand inhibitors (NPC1-L1)\(^1\)\(^11\) are candidate compounds that lower intestinal cholesterol absorption.

For absorption of cholesterol in the intestine, esterification of cholesterol was found to be a rate limiting step in intestine. The seminal work of Norum\(^1\)\(^2\), Heiders\(^1\)\(^3\), Field\(^1\)\(^4\) and others\(^1\)\(^5\) ultimately led to the conclusion that Acyl CoA: cholesterol O-acyltransferase (ACAT; EC 2.3.1.26)\(^1\)\(^6\) was one of the primary enzymes responsible for the esterification of cholesterol in intestinal mucosal cells and it played vital role for the esterification and absorption of intestinal cholesterol.

1.1 Atherosclerosis

Atherosclerosis (also known as arteriosclerotic vascular disease or ASVD) is a condition in which an artery wall thickens due to the build-up of fatty materials, mainly cholesterol. Atherosclerosis affects mainly the walls of large arterial blood vessels as a result of accumulation of macrophage white blood cells and low-density lipoproteins (plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the
macrophages by the functional high-density lipoproteins (HDL). It is commonly referred to as hardening of the arteries.

1.2 Pathophysiology of atherosclerosis

Atherogenesis is the developmental process of atheromatous plaques (Fig. 1). It is characterized by remodeling of arteries leading to subendothelial accumulation of fatty substances called plaques. The pathogenesis of atherosclerosis involves a complex series of events, similar to a chronic inflammatory process, with the formation of atherosclerotic plaque as the end result. Injury to the endothelial cell of the artery, resulting in endothelial cell dysfunction, is the first step in the process. Activated endothelial cells attract leukocytes and vascular smooth muscle cells (VSMC), which accumulate and proliferate in the arterial wall. These cellular components produce an excessive amount of connective tissue matrix. The ultimate end point is the formation of a mature fibrous plaque. The plaque rupture, hemorrhage of the rupture plaque, and formation of emboli or thrombosis make the lesions more complicated. A thorough understanding of the pathogenesis of atherosclerosis is essential for the development of strategies for the prevention of the disease and for the development of new and effective treatments.⁴

1.3 Acyl Coenzyme A: Cholesterol O-Acyl Transferase (ACAT)

The acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) is a small enzyme family comprising three homologous members, namely acyl-coenzyme A: cholesterol O-acyltransferase 1 and 2 (ACAT-1 and ACAT-2), and acyl-coenzyme A: diacglycerol acyltransferase 1 (DGAT-1). ACAT has generated interest as a potential means of exploring the atherosclerotic disease.

Figure 1: Cut section of atheromatous artery⁷
process by both lipid and non-lipid mechanisms. Earlier biochemical studies using cell fractionation assays revealed that ACAT activity is found only in microsomal fraction, but not in soluble fraction, suggesting that ACAT is an integral membrane enzyme.

ACAT family enzymes perform important biological functions. ACAT-1 and ACAT-2 are critical for *in vivo* cholesterol homeostasis. At the single cell level, they prevent free excess cholesterol from building up in the cell membranes. At physiological level, they contribute toward formation of cholesteryl esters as part of neutral lipid cargo, to be packaged into the cores of very low-density lipoproteins and chylomicrons. Under pathophysiological conditions, these enzymes convert excess cholesterol into cholesteryl esters in cholesterol-loaded macrophages. The macrophages are gradually converted into foam cells, which is a hallmark of early lesions of atherosclerosis.

### 1.3.1 Identification of ACAT isozymes

The ACAT activity was known as early as the 1970s, but purification of the enzyme failed due to its presence in minute quantities in various tissues. Using an ACAT-deficient Chinese hamster ovary (CHO) cell line, Chang’s laboratory at Dartmouth College first cloned the full-length cDNA of human ACAT-1 in 1993. The cloning of human ACAT-1 is a milestone in research on ACAT family.

ACAT-1 knockout in mice results in decreased cholesterol esterification in fibroblasts and adrenal membranes that markedly reduces cholesterol ester levels in adrenal glands and peritoneal macrophages, suggesting that ACAT-1 plays a major role in these tissues. However, the liver of ACAT-1 deficient mice contains substantial amounts of cholesterol esters and exhibits no reduction in cholesterol esterification activity, suggesting other unknown ACAT isozyme(s) is present in the liver. In 1998, these laboratories reported the cloning of ACAT-2 enzyme, an homologous isozyme of ACAT-1.

### 1.3.2 ACAT expression and regulation

The tissue distribution of ACAT-1 and ACAT-2 are quite different. ACAT-1 is more ubiquitous and has been found in macrophages, adrenal glands, hepatocytes, enterocytes, renal tubular cells and neurons, whereas ACAT-2 has been found in the apical region of the intestinal villi and in the hepatocytes. ACAT-1 is expressed in macrophage-derived foam cells present in human atherosclerotic lesions. Adiponectin, an adipocytokine that has been shown to inhibit foam cell formation, can down-regulate ACAT-1 expression in macrophages derived from...
human peripheral mononuclear cells. Parini et al. demonstrated that ACAT-2 is also expressed in human liver. ACAT-2 expression was shown to be up-regulated in a puromycin-induced rat nephritic syndrome model. This study reported a correlation between the ACAT-2 protein expression and the plasma total cholesterol.

1.3.3 Membrane topology of ACAT enzymes

The ACAT family enzymes are integral endoplasmic reticulum (ER) membrane proteins with multiple transmembrane domains (TMDs) as predicted by TMD algorithms. Membrane topology is important for understanding substrate-binding and catalysis of membrane enzymes. Therefore, various experimental methods have also been designed to investigate membrane topology. The membrane topology of ACAT-1 has been experimentally studied using different approaches. In 1999, Lin et al. first proposed a 7-TMD model for ACAT-1 based on the results of HA-tag insertion and subsequent immunofluorescence observation after selective permeabilization of the cell membrane and the ER membrane. In this model, they also proposed that the two long hydrophobic polypeptide stretches and a long hydrophilic polypeptide stretch, rich in conserved residues, were located in the ER lumen. One year later, Joyce et al. proposed a 5-TMD model for ACAT-1 based on their C-terminal truncation method. In this model, they reported the three hydrophobic polypeptide stretches located in the cytosol. In 2005, Guo et al. reported a 9-TMD model for ACAT-1 based on their cysteine-scanning mutagenesis.

Figure 2: A general ER membrane topology model for ACAT family enzymes. The possible cholesterol/diacyglycerol- regions [binding region (TMD7) is shown in red, and other TMDs are shown in blue. The possible acyl-coenzyme A-binding the loop between TMD6 and TMD7 and the loop between TMD4 and TMD5] are shown in green. The position of the active site His in TMD7 is indicated by a white star.
and subsequent cysteine-specific modification approach.\textsuperscript{34} In the 9-TMD topology model (Fig.2), all long hydrophobic polypeptide stretches are imbedded in the membrane bilayer. In this model, a long polypeptide stretch rich in conserved hydrophilic and hydrophobic residues between TMD6 and TMD7 is located in the cytosol. This peptide stretch may form the binding site of acyl-coenzyme A that is synthesized in the cytosol and is impermeable to the ER membrane. In the 9-TMD model, the so-called active site His-460 of ACAT-1 is located in a membrane sealed region at the luminal end of TMD7. This location seems to be responsible for catalysis.

The membrane topology of ACAT-2 has also been experimentally studied using two different approaches.\textsuperscript{35} HA-tag insertion and subsequent immunofluorescence observation led to a 2-TMD model,\textsuperscript{35} while the C-terminal truncation approach led to a 5-TMD model.\textsuperscript{33} However, sequence analysis shows that ACAT-2 contains nine long hydrophobic peptide stretches corresponding to the nine TMDs of ACAT-1 (Fig. 2). So probably, ACAT-2 also contains nine TMDs. In the proposed 9-TMD model, all long hydrophobic peptide stretches of ACAT-2 are imbedded in the membrane bilayer; the probable acyl-coenzyme-A binding site between TMD6 and TMD7 is located in the cytosol; the so-called active site His-434 is located at the luminal end of TMD7 (Fig. 2).

For ACAT family enzymes, a general topology model with nine TMDs (Fig. 2) has been proposed. Among these, TMD7 is crucial because it is probably involved in substrate-binding and catalysis. TMD7 is rich in conserved residues. The absolutely conserved His-460 at the luminal end is proposed to be an active site. Probably other conserved residues are responsible for cholesterol/diacylglycerol-binding. Since cholesterol and diacylglycerol are insoluble in water, ACAT family enzymes may use the membrane-bound cholesterol and diacylglycerol as substrates. Two long cytosolic loops (between TMD6 and TMD7, and between TMD4 and TMD5) are rich in conserved hydrophobic and hydrophilic residues. They are probably involved in binding of acyl-coenzyme A that is synthesized in the cytosol and is impermeable to the ER membrane.\textsuperscript{18}

1.4 Role of ACATs in cholesterol metabolism

During the past twenty years many types of therapeutic agents have been shown to limit the absorption of cholesterol into the mucosal cell of the intestinal lumen. The mechanism by which most of these agents act is still unknown. Such diverse structural types as neomycin, guar
gums, surfactants, sucrose polyesters and plant sterols have all been reported to act as cholesterol absorption inhibitors.  

1.4.1 Role of ACATs in intestinal wall

Even though cholesterol is absorbed exclusively in the unesterified form, most cholesterol appearing in the lymph is esterified by various long-chain unsaturated fatty acids. Moreover, the mass of cholesterol esters in lymph increases in proportion to the amount of cholesterol absorbed. Thus, re-esterification of cholesterol occurs during the absorptive process. The bulk of available data suggests that the enzyme responsible for this action is ACAT, an enzyme that utilizes long-chain fatty acyl coenzyme A and cholesterol as substrates. Theoretically, this enzyme would allow the continued passive uptake of dietary cholesterol into the mucosal cells. Indeed, it has been shown in cholesterol-fed rats that inhibition of ACAT appears to have beneficial effects on plasma cholesterol via the prevention of the absorption of dietary cholesterol. In recent years the implications of inhibiting this enzyme in other tissues (e.g. liver, artery wall) for the treatment of hypercholesterolemia and atherosclerosis, have become more clear. As illustrated in Fig.3, dietary cholesterol enters the small intestine from the stomach in the form of a crude emulsion. Endogenous cholesterol also enters from the bile. Together with pancreatic digestive enzymes, the bile converts this emulsion into mixed micelles which can incorporate the insoluble cholesterol into their hydrophobic cores. There is general agreement that cholesterol esters must be hydrolyzed to free cholesterol before absorption. This is achieved through the action of pancreatic cholesterol ester hydrolase. The micelles cross the unstirred water layer, a negatively charged hydrophilic region that is separate from the bulk aqueous phase of the lumen, and transfer free cholesterol to the brush border of the mucosal cells, where it is esterified by ACAT to cholesterol esters. These esters are then incorporated into chylomicrons, which are secreted into the lymph. Earlier controversy over whether cholesterol ester hydrolase or ACAT was the rate-limiting enzyme for cholesterol absorptions has been resolved by experiments with inhibitors that have shown that in vitro and ex vivo inhibition of ACAT activity can be correlated with the inhibition of cholesterol absorption, at least in rats and rabbits.
1.4.2 Role of ACATs in hepatocytes

The importance of ACAT in the liver, especially in humans, is less clear compared to its documented importance in the intestine and artery. The liver receives its cholesterol from a number of sources, including endogenous biosynthesis, removal of the lipolytic remnants of chylomicrons via the chylomicrons/remnant receptor and catabolism of LDL via the LDL receptor pathway. This cholesterol is then esterified and stored within the hepatocytes. At least a portion of the resulting ester is available for packaging into very low density lipoproteins (VLDL) and subsequent secretion back into the plasma compartment. A number of studies imply that ACAT inhibition in the liver may result in lowering of plasma lipids. For example, in perfused rat liver the concentration of cholesterol esters is correlated positively with VLDL secretion, suggesting that esterification is required for VLDL formation. More recently it has been shown that ACAT may be required for the secretion of apoB-containing lipoproteins, both in cultured human liver (HepG2) cells and perfused primate liver. However, the possibility remains that the cholesterol substrate of the enzyme may accumulate and causes down regulation of the LDL receptor. This has been observed in HepG2 cells (but not in fibroblasts) with the Sandoz
ACAT inhibitor SaH58035.\textsuperscript{46} Also, an inverse relationship between biliary cholesterol output and ACAT activity has been described in rodents, and patients with gallstones have a reduced ability to esterify free cholesterol because of decreased hepatic ACAT activity.\textsuperscript{47} It is therefore possible that liver ACAT inhibition may result in a lithogenic bile (high in free cholesterol) and subsequent gallstone formation.

1.4.3 Role of ACATs in arterial walls

The accumulation of lipid-laden foam cells of monocyte origin in the aortic intima is an early event in the development of atherosclerosis.\textsuperscript{48} Monocyte-macrophages take up and degrade native LDL at a slow rate via the LDL receptor. However, certain chemically modified forms of LDL are taken up very rapidly by a distinct receptor, designated as scavenger receptor. Recent \textit{in vitro} and \textit{in vivo} studies\textsuperscript{49} support the hypothesis that there is an oxidative modification of LDL that targets it for uptake by the macrophages through the scavenger receptor. This uptake is not regulated; there is no feedback control comparable to the LDL receptor, and macrophages continue to take up these particles as long as they are available. Once inside the cell, the cholesterol esters are hydrolysed and immediately re-esterified by ACAT. This results in a massive accumulation of intracellular esters, resulting in the appearance of characteristic foam cells\textsuperscript{50}. Administration of an ACAT inhibitor with systemic bioavailability would be expected to decrease the accumulation of cholesterol esters and prevent foam cell formation within the artery wall.\textsuperscript{51} The free cholesterol thus generated may then be removed by HDL or other acceptors and targeted back to the liver. This mechanism provides direct anti-atherosclerotic activity for an ACAT inhibitor, and thus targets the site of the disease process.