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**VALIDATION OF A NOVEL HYPOTHESIS OF GENERATING  
FOAM CELLS BY ITS USE TO STUDY REVERSE CHOLESTEROL  
TRANSPORT**

by

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A dissertation submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy  
in the Department of Biomedical Sciences  
in the College of Medicine  
at the University of Central Florida  
Orlando, Florida

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## ABSTRACT

Generation of foam cells, an essential step for reverse cholesterol transport (RCT) studies, uses the technique of receptor dependent macrophage loading with radiolabeled acetylated Low Density Lipoprotein (Ac-LDL). In this study, we used the ability of a biologically relevant detergent molecule, Lysophosphatidylcholine (Lyso PtdCho), to form mixed micelles with cholesterol or cholesteryl ester (CE) to generate macrophage foam cells. Fluorescent or radiolabelled cholesterol / Lyso PtdCho mixed micelles were prepared and incubated with RAW 264.7 or mouse peritoneal macrophages. Results showed that such micelles were quite stable at 4°C and retained the solubilized cholesterol during one month storage. Macrophages incubated with cholesterol or CE (unlabeled, fluorescently labeled or radiolabeled) / Lyso PtdCho mixed micelles accumulated CE as documented by microscopy, lipid staining, labeled oleate incorporation, and by thin layer chromatography (TLC). Such foam cells unloaded cholesterol when incubated with high density lipoprotein (HDL) and not with oxidized HDL (Ox-HDL). We propose that stable cholesterol or CE / Lyso PtdCho micelles would offer advantages over existing methods.

Oxidative stress is associated with heart failure (HF). Previously our research group observed that the patients with low left-ventricular ejection fraction showed accumulation of high level of oxidized LDL (Ox-LDL) when compared with the heart failure patients with normal range of ejection fraction (EF). HDL is known to be atheroprotective and one of its important antioxidative functions is to protect LDL from oxidative modifications. However, HDL itself undergoes oxidation and Ox-HDL becomes functionally poor. It is expected to have a diminished ability to promote reverse cholesterol

transport. Therefore, it was hypothesized that the quality of HDL present in the patients with EF would more compromised than those present in the patients with normal EF. Functionality of HDL was evaluated by measuring its cholesterol efflux capacity from foam cells generated *in vitro*. Functionality of HDL, which is strongly related to the oxidative modifications of HDL was further estimated by measuring paraoxonase 1 (PON1) enzyme activity associated with HDL. Higher the PON1 activity and RCT ability, better is the functionality of HDL.

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## LIST OF ABBREVIATIONS

|                  |   |
|------------------|---|
| <b>ABCA1</b>     | ATP- binding cassette A1                |
| <b>ABCG1</b>     | ATP- binding cassette G1                |
| <b>ACAT</b>      | Acyl-CoA: cholesterol O-acyltransferase |
| <b>Ac-LDL</b>    | Acetylated low density lipoprotein      |
| <b>ADMEM</b>     | Advanced DMEM                           |
| <b>Apo B-100</b> | Apolipoprotein B-100                    |
| <b>Apo-A1</b>    | Apolipoprotein A1                       |
| <b>ApoA-II</b>   | Apolipoprotein A II                     |
| <b>ApoA-IV</b>   | Apolipoprotein A I                      |
| <b>ApoC</b>      | Apolipoprotein C                        |
| <b>ApoE</b>      | Apolipoprotein E                        |
| <b>ApoJ</b>      | Apolipoprotein J                        |
| <b>CABG</b>      | Coronary artery bypass grafting         |
| <b>CAD</b>       | Coronary artery disease                 |
| <b>CD36</b>      | Cluster of Differentiation 36           |
| <b>CE</b>        | Cholesteryl ester                       |
| <b>CETP</b>      | Cholesterol ester transfer protein      |
| <b>CPM</b>       | Count per minute                        |
| <b>DMEM</b>      | Dulbecco's Modified Eagle Medium        |
| <b>DPM</b>       | Disintegrations per minute              |
| <b>EDTA</b>      | Ethylenediaminetetraacetic acid         |
| <b>EF</b>        | Ejection fraction                       |
| <b>FBS</b>       | Fetal bovine serum                      |
| <b>GSPx</b>      | Glutathione selenoperoxidase            |
| <b>HBSS</b>      | Hank's balanced salt solution           |
| <b>HDL</b>       | High density lipoprotein                |
| <b>HF</b>        | Heart failure                           |
| <b>ICAM-1</b>    | Intercellular adhesion molecule 1       |
| <b>LCAT</b>      | Lecithin: cholesterol acyltransferase   |



|                                 |   |
|---------------------------------|---|
| <b>LDH</b>                      | Lactate dehydrogenase                               |
| <b>LDL</b>                      | Low density lipoprotein                             |
| <b>LPDS</b>                     | Lipoprotein deficient serum                         |
| <b>LVB</b>                      | Left ventricular blood                              |
| <b>LXR</b>                      | Liver X receptors                                   |
| <b>Lyso PtdCho</b>              | Lysophosphatidylcholine                             |
| <b>MCP-1</b>                    | Monocyte chemotactic factor                         |
| <b>MCSF</b>                     | macrophage colony stimulating factor                |
| <b>mm-LDL</b>                   | Minimally modified LDL                              |
| <b>MPO</b>                      | Myeloperoxidase                                     |
| <b>Ox-HDL</b>                   | Oxidized high density lipoprotein                   |
| <b>Ox-LDL</b>                   | Oxidized low density lipoprotein                    |
| <b>PAF-AH</b>                   | Platelet-activating factor-acetyl hydrolase         |
| <b>PB</b>                       | Peripheral blood                                    |
| <b>PBS</b>                      | Phosphate buffer saline                             |
| <b>p-NPA</b>                    | p-Nitrophenyl acetate                               |
| <b>PON1</b>                     | Paraoxonase   |
| <b>PPAR <math>\alpha</math></b> | Peroxisome proliferator activated receptor $\alpha$ |
| <b>PtdCho</b>                   | Phosphatidylcholine                                 |
| <b>RBC</b>                      | Red blood cells                                     |
| <b>RCT</b>                      | Reverse cholesterol transport                       |
| <b>rHDL</b>                     | Reconstituted HDL                                   |
| <b>SAA</b>                      | Serum amyloid A                                     |
| <b>SR</b>                       | Scavenger receptor                                  |
| <b>SR-A1</b>                    | Scavenger receptor class A type 1                   |
| <b>SR-B1</b>                    | Scavenger receptor class B type 1                   |
| <b>TLC</b>                      | Thin layer chromatography                           |
| <b>VCAM-1</b>                   | Vascular adhesion molecule 1                        |
| <b>VLDL</b>                     | Very low density lipoproteins                       |

## **CHAPTER 1: GENERAL INTRODUCTION**

### **Coronary Artery Diseases**

Coronary artery diseases (CAD) are the most common pathobiological conditions associated with atherosclerosis (1). CAD is the main cause of death in Western societies (2). In United States alone one third of all deaths above the age of 35 are caused by CAD. Out of 15 million CAD cases in United States approximately 8 million people are known to suffer heart attacks and around half a million people die from it every year. Moreover, millions of people suffer from severe atherosclerosis without showing any symptoms. The cost to manage CAD which includes health care services and loss of productivity of the sufferers could build up to more than \$100 billion per year (3, 4).

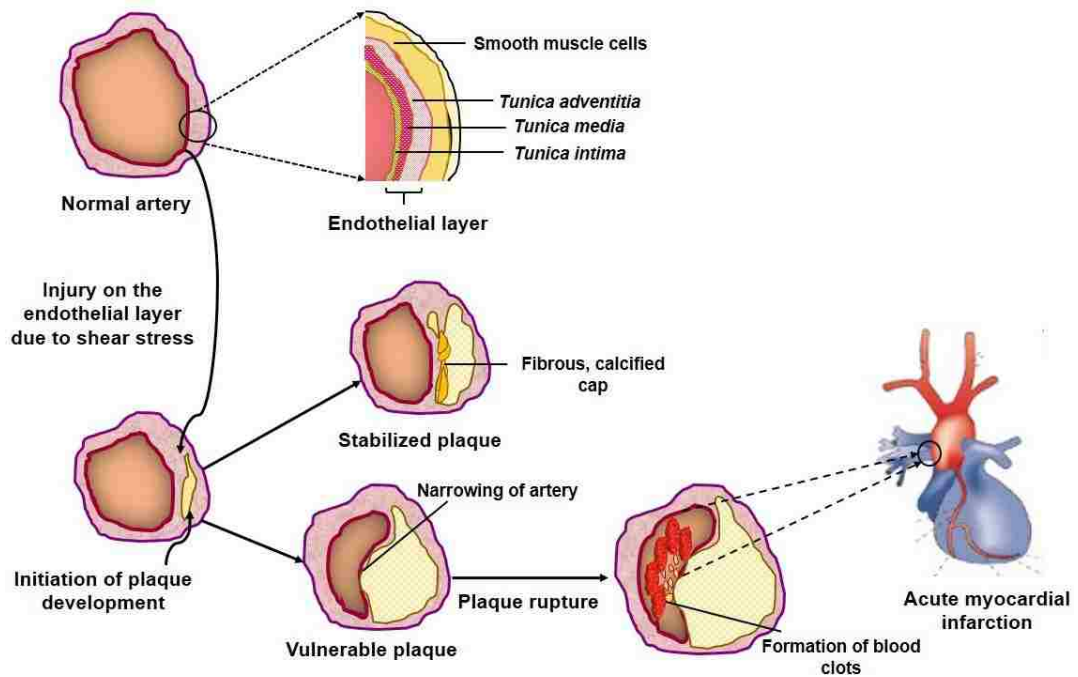
### **Atherosclerosis**

The term atherosclerosis (synonymously used with arteriosclerosis) was first introduced in 1829 by Jean Lobstein (1). Atherosclerosis is often studied as a type of chronic systemic inflammation and is characterized by progressive lesion formation and narrowing of arteries. The risk factors associated with atherosclerosis are often heritable, known as 'run in the family' (5). However, in the modern industrialized society atherosclerosis and CAD are more often initiated by environmental factors, such as, smoking, obesity, diabetes and lack of physical activity. Atherosclerosis is a dynamic process and initiates with the formation of fatty streaks on the innermost layer of arterial wall, known as *tunica intima*. Fatty

streaks contain mainly lipid loaded macrophages known as foam cells and other immune cells such as T-cells, dendritic cells and mast cells (6). Autopsies of young individuals revealed the presence of fatty streaks on the arterial walls which are generally asymptomatic (7). These fatty streaks can develop into atheromas over the time or can regress with the change in life style, diet and exercise (8).

Atheroma or atherosclerotic plaques are complex in structure and contain lipid laden foam cells, extracellular lipid droplets, mainly cholesterol crystals, apoptotic cells and cellular debris in the necrotic core. Plaques are covered by smooth muscle cells and collagen-rich matrix to form a fibrous cap (Figure 1) (6). Advanced lesions are frequently associated with calcium deposition. Calcification of lesions is positively correlated with plaque burden and believed to have a protective action against plaque rupture which is often fatal (9).

Growing plaques can prevent regular blood flow in the coronary artery by progressively narrowing the arterial lumen and cause flow-limiting stenosis and ischemic conditions. However, the most severe clinical implications are generally associated with atherosclerotic plaque rupture. When the fibrous cap is weak or damaged, the plaque becomes vulnerable by the action of activated immune cells and inflammatory cytokine. Disruption of plaque leads to exposure of thrombogenic material located in the core of the plaque, to arterial circulation, resulting in platelet aggregation and the formation of thrombus, which eventually can lead to myocardial infarction, heart failure and ischemic stroke (Fig. 1) (10).



**Figure 1. Atherosclerotic Plaque Development and Rupture.**

Injury to the *tunica intima* of endothelial layer of arterial lining initiates plaque formation. Accumulation of modified LDL set off a chain reaction that leads to generation of fatty streaks. Fatty streaks contain large amount of free and esterified cholesterol, inflammatory immune cells, necrotic cells and foam cells. Calcification can stabilize the atheroma, while rupture of plaque leads to thrombosis which can result in acute myocardial infarction, stroke and heart attack.

## **Low Density Lipoproteins**

Low density lipoproteins (LDLs), the large spherical particles containing cholesteryl ester (CE) core, are the major carrier of cholesterol in plasma. Besides being the largest pool of CE in the human plasma LDL also contains small amount of triglycerides in the neutral lipid core. The highly hydrophobic lipid core is surrounded by amphiphilic phospholipids and apolipoprotein B-100 (apo B-100). LDLs are derived from triglyceride-rich very-low-density lipoproteins (VLDLs) by the action of hepatic lipase. Normally LDL remains in the blood circulation for 2-3 days (11). About 75% of the LDL particles are absorbed by liver *via* LDL receptor for which apolipoprotein B-100 on the LDL serves as the ligand. Binding of LDL to the receptor initiates endocytosis of the particle and fusion of LDL containing vesicle to lysosomes, followed by LDL receptor recycling. Lysosomal degradation of LDL generates fatty acids, cholesterol, glycerol and amino acids as the primary components of LDL. Free cholesterol incorporated in the cytoplasmic cholesterol pool is utilized for membrane and steroid synthesis and remaining cholesterol gets esterified into highly hydrophobic CE (12).

## **Oxidized LDL**

Atherosclerosis is closely associated with the lipid accumulation in the *tunica intima* of the artery. Lipid-laden foam cells, which are found in the atherosclerotic plaque, are known to derive its cholesterol burden from LDL (13). However, macrophages express limited number of LDL receptors and the accumulation of cholesterol in macrophages do not add up to the amount of

cholesterol stored *via* LDL receptor mediated uptake (14), which is also regulated by a feedback mechanism (15). LDL are considered to be risk factors for CAD because oxidative modification of LDL makes them highly atherogenic. Increased plasma level of LDL make them prone to a number of modifications by several ways which in turn induce a series of events leading to fatty streak formation. Increased plasma level of LDL not only increases intimal LDL level, but also causes enhanced adherence of circulating monocytes to the arterial endothelial cell lining. Cells found in the atherosclerotic plaque, such as endothelial cells, smooth muscle cells, fibroblasts, neutrophils, monocytes and macrophages can catalyze the oxidation of LDL. Pro-atherogenic events induced by modified LDL are complex. Oxidized LDL (Ox-LDL) recruits monocytes to adhere and penetrate the arterial intima by stimulating monocyte chemotactic factor (MCP-1) from the endothelial cells. Adherence of monocytes to endothelial cells is associated with Ox-LDL stimulated endothelial secretion of intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1). Ox-LDL can induce the release of macrophage colony stimulating factor (MCSF) which prompts differentiation of monocytes into adherent macrophages. Ox-LDL itself can act as a chemotactic factor to attract patrolling monocytes. 'Trapping' of macrophages in the intimal region initiates the fatty streak formation as the scavenger receptors (SR) present on the macrophages play a crucial role in cholesterol accumulation. Ox-LDL is also believed to cause injury in the endothelial lining of the intima (Fig. 2) (14, 16).

The characteristic feature of modified LDL is the gain of a net negative charge which makes them unrecognizable by classical LDL receptors, but converts them into ligands for SR present on macrophages. Oxidation of lipid component can generate peroxidized lipids which in turn can give rise to aldehydes and leads to the alteration of the structure of the protein moiety. Apolipoproteins themselves can undergo oxidative modification resulting in cross-linking, proteolysis, structural modification and alteration in amino acid compositions of the protein moiety. *In vitro* studies of the oxidative modifications of LDL revealed metal ion such as copper, enzyme such as lipoxygenase (Fig. 2), oxygen radicals, reactive nitrogen species generated by hydrogen peroxide or myeloperoxidases (17), peroxides and superoxides play vital role in the oxidation of LDL and make Ox-LDL recognizable by SR (Fig. 2) (18, 19).

### **Macrophages and Scavenger Receptors**

Macrophages are commonly associated with innate immune system and phagocytosis. In case of atherosclerosis, macrophages play a very critical role in the development of plaque mostly due to its unique features associated with immune surveillance. During the initiation stage of fatty streak formation, once monocytes are recruited and differentiated into macrophages in the intimal region, macrophages express a number of SR. SR are cell-membrane proteins and bind to a wide variety of ligands including chemically or biologically modified lipoproteins (20). In 1979 Goldstein et al. discovered that macrophages utilize their scavenging mechanism to uptake modified LDL from plasma. It was also discovered that the

macrophage mediated endocytosis of modified LDL takes place *via* a different receptor than the classical LDL receptor (21, 22). Expression of SR are closely associated with adherence of cells as fully differentiated macrophages only and not the freshly isolated monocytes express high level of SR (23).

#### *Scavenger Receptor Class A:*

There are two major classes of SR: scavenger receptor class A (SR-A) and scavenger receptor class B (SR-B). SR-AI/II (type I and II) are generally present on the cell-surface of tissue macrophages such as Kupffer cells and can be induced in other cell types (24). Two types of SR-A are derived by alternative mRNA splicing and SR-AI is different from SR-AII by having a cysteine-linked C-terminal extension. SR-A are expressed as transmembrane glycoproteins and the collagen like domain form a positively charged groove due to the presence of lysine and is involved in the binding of negatively charged ligands (25). They play an important role in the phagocytosis of apoptotic cells, in cell adhesion, antigen recognition and processing (20). SR-A bind to several polyanionic macromolecules such as bacterial surface components- lipopolysaccharides and lipoteichoic acid, polyribonucleotides (polyguanosinic acid and polyinosinic acid), dextran sulfate and anionic phospholipids such as phosphatidylserine (25). SR-A are also known as pattern recognition receptors as they bind to microbial surface proteins of the same molecular pattern expressed by a wide range of bacteria (26). SR-A expression are regulated by cytokines and atherosclerotic lesions are known to have abundant expression of SR-A (25). Initially SR-A were thought to be



associated with the binding of acetylated LDL (Ac-LDL). However, as Ac-LDL is absent *in vivo*, the role of SR-A in the endocytosis of Ox-LDL was studied more thoroughly (24). It has been suggested that while SR-A are responsible for the uptake of 80% and 50% of Ac-LDL and Ox-LDL respectively. Even though its efficiency to uptake Ox-LDL is much lesser than that of Ac-LDL, SR-A knockout mice showed significant reduction in atherosclerotic lesions in otherwise atherosclerotic mice model (20).

#### *Scavenger Receptor Class B:*

CD36, a member of SR-B family is associated with Ox-LDL uptake. CD36 along with SR-A are known to be responsible for 75-90% of the uptake and degradation of Ac-LDL and Ox-LDL (27). CD36 is an 88-KDa membrane glycoprotein and unlike SR-A, it is more ubiquitous in expression. CD36 is expressed by adipocytes, skeletal muscle cells, microvascular endothelial and smooth muscle cells along with a number of hematocytes such as platelets, dendritic cells, monocytes and macrophages (20, 24). Previous studies have shown that CD36 can bind to minimally modified LDL (mm-LDL), a form that is capable of initiating monocyte migration and “trapping”. In CD36 knockout mice, Ox-LDL binding and endocytosis by peritoneal macrophages is known to be decreased by 60-80% and the atherosclerotic lesion burden is reduced by 70% when CD36- apoE double knockout mice are fed a high-fat diet (20). In human subjects lacking CD36, monocyte derived macrophages showed 40% reduction in Ox-LDL endocytosis (24). Besides Ox-LDL, the other biologically relevant ligands

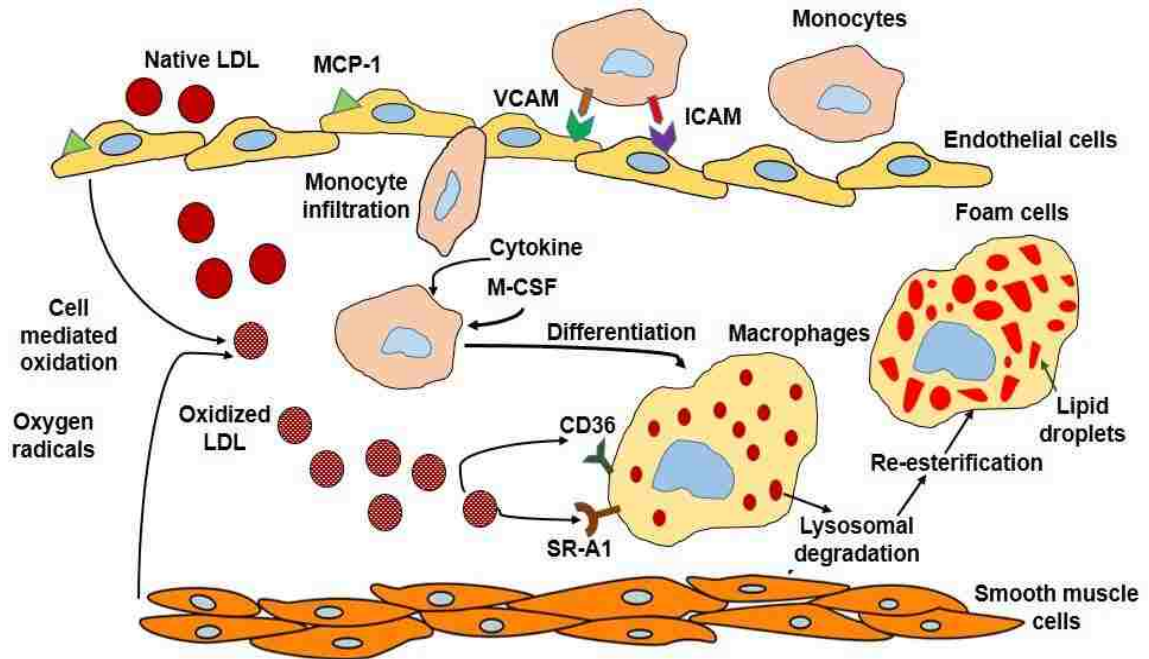
for CD36 are apoptotic cells, cell debris, *Plasmodium falciparum* parasitized erythrocytes, sickle cell erythrocytes, collagens type I and IV, anionic phospholipids and long-chain fatty acids. However, unlike SR-A, CD36 does not bind to a host of polyanionic ligands such as fucoidan, polyribonucleic acids and carrageenan (20, 24).

### **Cholesterol Accumulation and Foam Cells**

Even though the majority of the cholesterol accumulation mediated by modified LDL takes place *via* SR, a number of studies documented different sources and pathways of cholesterol accumulation. Blocking of SR-A and SR-B together prevented the modified LDL uptake by 75-90%, but could not inhibit the cholesterol accumulation completely (28). This observation directed the study to understand the unique and not-so-common pathways of foam cell development. Kruth et al (29, 30) reported the mechanism for receptor-independent uptake of native LDL by macrophages and subsequent development of foam cells. According to this model monocyte-derived macrophages upon activation take up native and modified LDL by fluid-phase endocytosis, otherwise known as macropinocytosis. In this actin-dependent endocytic pathway activated macrophages enclose the surrounding fluid within a vacuole by plasma membrane fusion and engulf the vacuole to internalize the surrounding fluid. This observation was highly valuable as it explains why infectious agents, and inflammatory cytokines released from the atherosclerotic plaque induce LDL uptake in its various forms (29, 30). Furthermore, macrophages can internalize aggregated LDL, which

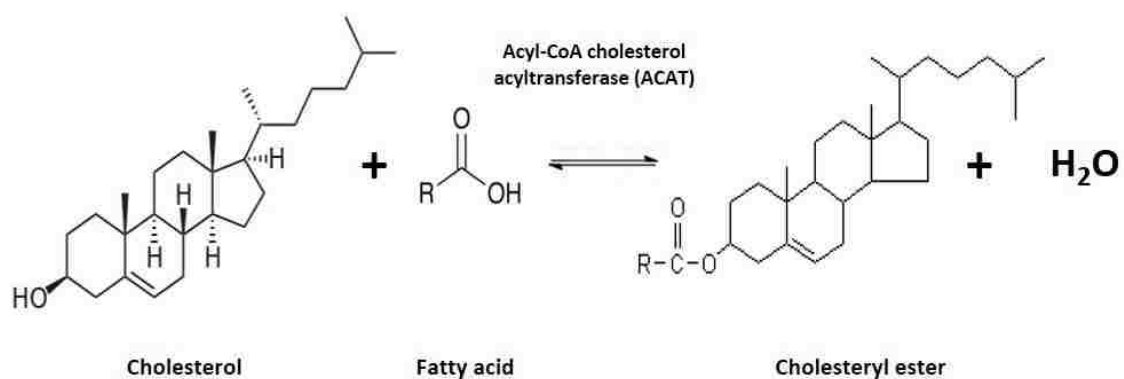
are too large to fit in the pinocytic vesicles, by phagocytosis (31). Yet another model of foam cell development suggests that the presence of sphingomyelinase, an enzyme found in arterial wall and in the atherosclerotic lesions, promotes LDL receptor mediated native LDL and SR mediated Ac-LDL uptake (32).

The fundamental mechanisms associated with the foam cell development are acyl-CoA: cholesterol O-acyltransferase (ACAT) activation and CE accumulation (Fig. 3). Excess of free cholesterol in cells can be highly cytotoxic and generally it is converted into CE for storage. Accumulation of cholesterol by foam cells is a two-step process: i) lysosome mediated hydrolysis of CE present in modified LDL to produce free cholesterol, ii) re-esterification of free cholesterol in presence of ACAT to CE as the lipid droplets in the cytoplasm (33). However, the studies by Tangirala et al (34, 35) suggested that in the advanced stage of atherosclerotic lesion formation lysosome itself can serve as the pool of free cholesterol and CE crystals. In older plaques macrophages and smooth muscle cells exhibited such cytoplasmic and lysosomal lipid droplet accumulation and appeared to be independent of ACAT activation (34, 35). Cholesterol accumulation and retention in both free and esterified form initiate the process of fatty streak formation.



**Figure 2. Role of Modified LDL in the Initiation of Fatty Streak Formation.**

Increased plasma concentration of LDL leads to accumulation of LDL in the subendothelial space and oxidative modification by 15 lipoxygenase. Ox-LDL acts as a chemoattractant and recruits monocytes to differentiate into tissue macrophages. Monocyte adherence to the endothelial layer is facilitated by VCAM and ICAM; migration of monocytes to the subendothelial space is achieved with the help of MCP-1. Differentiation of monocytes to macrophages is associated with M-CSF. Macrophages express scavenger receptors and facilitate the uptake of modified LDL. Cholesterol present in the LDL is quickly esterified into CE by the enzyme ACAT and stored as lipid droplets. Lipid-enriched macrophages or foam cells are the core structure of fatty streaks and eventual atherosclerotic plaques.



**Figure 3. Esterification of Free Cholesterol for storage.**

Free cholesterol which is highly cytotoxic gets converted into highly hydrophobic CE with the help of the enzyme ACAT. CE crystals are stored in the cytoplasm and lysosomes of the cells. Macrophages are highly efficient in esterification and storage of cholesterol. This reaction is reversible and CE is hydrolyzed to produce cholesterol which gets utilized for cell membrane repair or generation and hormone synthesis.

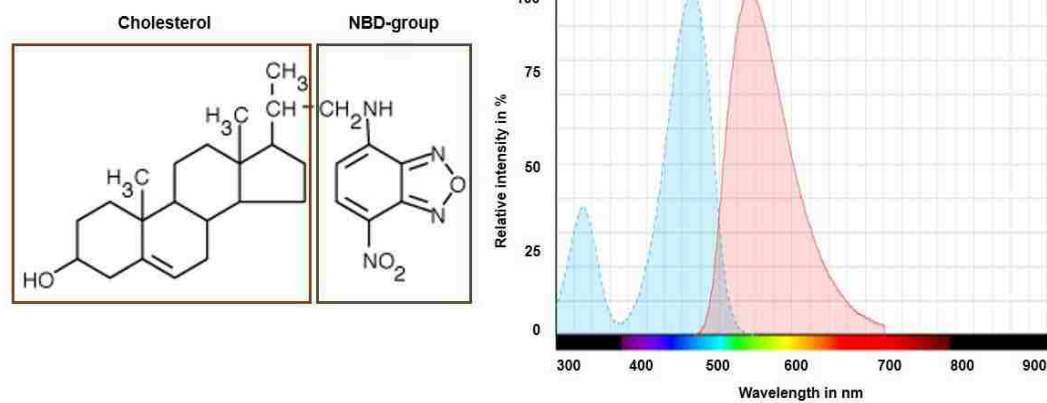
### ***In Vitro* Generation of Foam Cells with Cholesterol Analogue**

Foam cells generated *in vitro* have been a very important tool to study atherosclerotic plaque formation as well as regression. As mentioned earlier, generally modified LDL (Ac-LDL and Ox-LDL) are used to generate foam cells. Isolated LDL undergoes acetylation or oxidation in presence of acetic anhydride or copper ion respectively. To follow the distribution and the fate of the incorporated cholesterol, radiolabeled cholesterol ( $^3\text{H}$  or  $^{14}\text{C}$ -cholesterol) is often used. In recent years fluorescently labeled cholesterol has been used for various studies such as to investigate the distribution of cholesterol in cell membrane (36), to understand the effects of sterol carrier protein and apolipoprotein E (apoE) on high density lipoprotein (HDL) mediated cholesterol efflux (37) and HDL recycling (38), to develop high-throughput cholesterol efflux assays (39) and to explore the mechanism of cholesterol absorption and storage in the *in vivo* animal model (40).

NBD- cholesterol is a commercially available, frequently used fluorescently labeled cholesterol analog. NBD group present in the 22-NBD-cholesterol or 22-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-23, 24-bisnor-5-cholen-3b-ol, is responsible for the fluorescent nature and the excitation wavelength for NBD is 485 nm. The emission spectra range for NBD varies between 535- 545 nm depending on ethanol or aqueous solution respectively (Fig. 4) (41).

Macrophages, hepatocytes, fibroblasts and lymphocytes have been loaded with NBD-cholesterol. Previous studies suggest that NBD-cholesterol can be very effectively used to study the cellular uptake and trafficking of the cholesterol. However, use of NBD-cholesterol to investigate the distribution of cholesterol in

the plasma membrane produced ambiguous results (41). NBD-cholesterol is potentially a reliable, easy-to-use cholesterol analogue and can be substituted for radiolabeled cholesterol.



**Figure 4. Fluorescent analogue of Cholesterol.**

22-NBD-cholesterol has 22-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-23, 24-bisnor-5-cholen-3b-ol, group attached to cholesterol. It is visualized as green fluorescent probe. The excitation wavelength for NBD is 485 nm and the emission spectra range for NBD varies between 535- 545 nm.



## Reverse Cholesterol Transport

In 1968 Glomset (42) proposed the existence of a mechanism which is involved in the transport of cholesterol from peripheral tissues to the liver and is believed to maintain the cholesterol homeostasis (42). Studies from the last four decades have established that Reverse Cholesterol Transport (RCT) is one of the most important defense mechanisms of our body to prevent initial plaque formation. RCT is a mechanism through which free and esterified cholesterol from extrahepatic (peripheral) tissue are effluxed to HDL, travel to liver, and a) get secreted as a component of hepatobiliary salt, b) get reabsorbed in small intestine as a secretory component in bile or c) get excreted via feces (43, 44). RCT from lipid-laden foam cell macrophages are of particular interest because of its atheroprotective role. Free cholesterol is utilized by cells for the generation or repairment of plasma membrane and for the synthesis of hormones by steroidogenic organs. Excess of cholesterol is highly toxic to cells as they cannot catabolize cholesterol. However, in macrophages excess of cholesterol can be esterified into CE and deposited (45). Macrophages and other peripheral tissues efflux excess cholesterol to extracellular acceptors *via* a number of mechanisms. Neutral sterol that is excreted *via* feces is derived mostly as a by-product of cholesterol and bile salt synthesis in liver. Cholesterol effluxed from macrophages contributes relatively small amount to this free cholesterol pool. From liver cholesterol is delivered to intestine as a component of bile and bile salts and in the intestine cholesterol is reabsorbed. Unabsorbed cholesterol along with other neutral sterols gets excreted in feces (46).

### *HDL and Apolipoprotein A-I:*

The heterogeneous lipoprotein HDL contains almost equal amounts of lipid and protein. Functional HDL is characterized by high hydrated density (1.063-1.21 g/ml), elevated protein content and small size (5-17 nm) (47). In the total pool of HDL, the content of lipid, apolipoproteins and enzymes vary significantly resulting in a great diversity in HDL population with different shape, size, density, electrophoretic mobility, charge and antigenicity (48). Heterogeneity of HDL is determined by its apolipoprotein contents, especially by Apolipoprotein A-I (apoA-I) conformation. ApoA-I is the major structural apolipoprotein in HDL, constituting up to 70% of the total protein content. The amphipathic helical structure of apoA-I allows apoA-I associated HDL to switch between discoidal and spherical shape under different stages of cholesterol uptake. Ultracentrifugation of HDL gives rise to two major fraction-i) less dense, larger HDL<sub>2</sub> (1.063-1.125 g/ml) population and ii) more dense, smaller HDL<sub>3</sub> (1.125-1.21 g/ml) population (49). Generally smaller HDL<sub>3</sub> is considered to be more effective in inducing cholesterol efflux, shows better anti-oxidative and anti-inflammatory properties than larger HDL<sub>2</sub>. However, a very elevated level of HDL<sub>3</sub>, a condition which can be associated with impaired HDL maturation and hepatic delivery of cholesterol, can potentially increase the risk of cardiovascular diseases (50).

The extracellular cholesterol acceptor involved in RCT is essentially HDL in different forms. Interaction between lipid-free or lipid-poor apoA-I along with ATP-binding cassette transporter A1 (ABCA1) initiates the efflux of phospholipids and unesterified cholesterol from the cells. Monomolecular apoA-I, which is

synthesized by intestine and liver becomes lipidated during this process to form pre- $\beta$  migrating discoidal complex. Pre- $\beta$  migrating nascent HDL contains at least two molecules of apoA-I, bilayer of phospholipids and little unesterified cholesterol (Fig. 5). Nascent HDL delivers a portion of its unesterified cholesterol content to liver and rest of the free cholesterol is esterified by the action of lecithin: cholesterol acyltransferase (LCAT), which is acquired by nascent HDL from blood plasma. During the process of highly hydrophobic CE accumulation nascent HDL gets converted into spherical  $\alpha$ -migrating mature HDL. CE along with small amount of triglyceride forms the hydrophobic core of the mature HDL which is surrounded by monolayer of phospholipids, unesterified cholesterol and apolipoproteins. A portion of CE gets recycled back as the component of newly formed apoB containing plasma lipoproteins (LDL and VLDL) with the help of cholesterol ester transfer protein (CETP) (51, 52) while majority of CE is delivered to liver (Fig. 6) (53). In liver the majority of the cholesterol gets incorporated as a component of bile salt and gets secreted in the duodenum to emulsify dietary lipid. A small amount of cholesterol is secreted into the bile directly and is re-absorbed by small intestine (54). The non-biliary pathway of RCT involves fecal excretion of neutral sterols (55).

#### *Mechanisms Involved in RCT:*

Cholesterol efflux only from macrophages and not the other peripheral tissues, are studied as classic RCT model and has significant importance in atherosclerotic plaque development. Macrophages present on the arterial wall

have the potential to become foam cells as they take up modified lipoproteins and apoptotic cells by phagocytosis. Previous studies have indicated that macrophages take up more free cholesterol than possibly by any other cells and free cholesterol can induce apoptosis in macrophages (56). As mentioned earlier, macrophages have two lines of defense against toxicity by excess cholesterol loading- i) by esterification of free cholesterol, ii) by cholesterol efflux. Cholesterol efflux from macrophages involves various mechanisms, such as transporter or receptor associated cholesterol efflux and by passive diffusion.

*ATP-Binding Cassette Transporters:*

ABCA1 play a major role is efflux of free cholesterol from macrophages to lipid-poor apoA-I or nascent HDL. ABCA1 is a large integral membrane protein and like other members of ABC superfamily utilizes ATP as energy source to transport metabolites including lipids across the membrane. Tall et al (57) suggest that the membrane domain of ABCA1 binds to apoA-I (57, 58). Close interaction between ABCA1 and apoA-I causes transfer of cholesterol and phospholipids from the inner plasma membrane leaflet to the outer one by a flopping mechanism (59). ApoA-I takes up this cholesterol and phospholipid by absorption and forms nascent HDL (57). Several other models have been proposed to explain the high affinity of ABCA1 towards nascent HDL. Nonetheless, the importance of ABCA1 in RCT mechanism is unquestionable. Tangier disease which is characterized by loss-of-function mutation in both the ABCA1 alleles, shows severe cholesterol accumulation in peripheral macrophages as well as HDL deficiency. *In vivo* mice

models have further established the critical role of ABCA1 in RCT and therefore in prevention of atherosclerosis (45, 60).

ATP-binding cassette transporter G1 (ABCG1) is the other transporter from ABC family involved in RCT. However, ABCG1 shows preference for mature HDL and not for lipid-poor apoA-I. Disruption of ABCG1 expression results into massive cholesterol deposition in peripheral tissues including macrophages (61). Many studies suggest that ABCA1 and ABCG1 function cooperatively and the synergistic action of these two transporters is responsible for the majority of cholesterol efflux from macrophages (62, 63). As expected, when ABCA1 and ABCG1 were knocked down simultaneously in the *in vitro* or *in vivo* models, cholesterol deposition increased severely as the amount of cholesterol efflux was decreased. Increase of atherosclerotic plaque formation and decreased rate of RCT were far more evident in the cases of dual knock down than the observed in the absence of either of the transporters. ABCA1 and ABCG1 are regulated by liver X receptors (LXR $\alpha$  and LXR $\beta$ ) which in turn are strongly regulated by cholesterol levels in plasma (64, 65).

#### *Role of SR-B1 in RCT:*

Scavenger receptor class B type 1 (SR-B1) is a cell-surface HDL receptor which is known to promote cholesterol efflux to mature, spherical  $\alpha$ -HDL rather than to nascent HDL. Previous studies suggest that the conformation of apoA-I in HDL is a strong determinant of SR-B1/ HDL interaction (66). Other studies showed SR-B1 mediated free cholesterol efflux is also dependent on the phospholipid content of the HDL. SR-B1 mediated cholesterol efflux is markedly increased to

the HDL enriched with phosphatidylcholine while HDL enriched with sphingomyelin facilitates SR-B1 mediated free cholesterol influx. SR-B1 shows this unique feature of facilitating the bidirectional movement of free cholesterol between HDL and cells. The bidirectional movement of cholesterol associated with SR-B1 depends on the cholesterol concentration gradient between HDL and the plasma membrane (66, 67). However, SR-B1 might not play a very crucial role in cholesterol efflux from macrophages like ABCA1 and ABCG1 (68) and more investigation is needed to fully understand the importance and therapeutic value of SR-B1 in cholesterol efflux from atherosclerotic foam cells.

On the other hand, expression of SR-B1 in liver is extremely crucial for HDL metabolism and the maintenance of cholesterol homeostasis. As mentioned earlier, SR-B1 facilitates selective uptake of cholesterol from HDL (and other lipoproteins) without the degradation of the HDL particle. SR-B1 enables the delivery of free, unesterified cholesterol from plasma HDL to liver and steroidogenic tissues by a two-step selective uptake mechanism which also facilitates the recycling of HDL particles (66). In liver and other tissues HDL binds to SR-B1 and cholesterol is efficiently transferred to the cells by diffusion into the plasma membrane (69). As expected, overexpression of hepatic SR-B1 is associated with lower plasma HDL-cholesterol level and increased content and transport of cholesterol into the bile from liver (66).

The diffusion model of RCT proposed by Johnson et al (70), postulates that cholesterol is desorbed from the cell membrane by a spontaneous process and is

taken up by HDL molecules. This pathway between the cell membrane and acceptor molecule involves diffusion of cholesterol in the aqueous phase (70, 71).

*Current Approaches to Study and Improve RCT:*

RCT is known to be inversely related with the risk of atherosclerotic plaque development and cardiovascular diseases. For over a decade efforts have been made to quantify RCT and optimize it as a parameter to understand, predict and treat the cardiovascular diseases better. Initially *in vivo* model for RCT was developed using mice model. Modified LDL labeled with <sup>3</sup>H-cholesterol was utilized to generate macrophage foam cells and these foam cells were injected intraperitoneally in the recipient mice. Plasma and feces samples were collected from these mice at various time points during the experimental duration and the fraction of injected <sup>3</sup>H-cholesterol recovered in the feces was considered as the amount of cholesterol undergoing RCT in a given time starting from macrophages (39, 72). For several years this technique has been used to investigate the effect of different inhibitors or enhancers on various transporter mediated pathways of RCT (73). Radioactive (<sup>14</sup>C) and fluorescently labeled (40) cholesterol have been used for these studies. Attempts have been made to insert macrophage seeded polyethersufone hollow fibers intraperitoneally in animals, to mimic the artery, and to study peripheral cholesterol efflux *in vivo* (74). However, these methods cannot be applied to investigate RCT in human subjects. Until recently studying different components of RCT in human subjects has been invasive, time-consuming

procedures in which hospitalization of patients over a period of time is required to administer labeled cholesterol and to collect blood and fecal samples (75, 76).

Recently attempts have been made to develop the *in vitro* RCT assay which can be used to measure cholesterol efflux from macrophages in presence of patients' serum. Studies by de la Llera-Moya et al (77) and Khera et al (78) used macrophages loaded with <sup>3</sup>H-cholesterol to measure cholesterol efflux capacity in a large patient population. ApoB depleted serum from healthy volunteers or patients with different stages of confirmed CAD were used to measure the ability of serum HDL to induce cholesterol efflux from the macrophages. The study by de la Llera-Moya et al (77) strongly emphasizes the importance of the functionality of HDL over the total count of serum HDL. The study by Khera et al (78) which integrated all the known transporter, receptor and diffusion mediated cholesterol efflux from macrophages, demonstrated that the ability of HDL to induce cholesterol efflux was inversely associated with atherosclerosis and CAD. While this study reinstated the importance of HDL functionality, it also challenges our understanding of RCT and its relationship with HDL in many ways and makes us realize that understanding the complexity of RCT is far from over (77, 78).

Recognizing the key components of RCT and the effort to improve them have been important focus for therapeutic approaches to control atherosclerosis and CAD. The *ex vivo* cholesterol efflux assays can be immensely informative regarding the roles of ABC transporters in RCT or their regulation by LXR. These data can be used to manipulate and develop target-oriented therapeutic approach to improve RCT and therefore, reduce the risk of atherosclerosis. Use of LXR

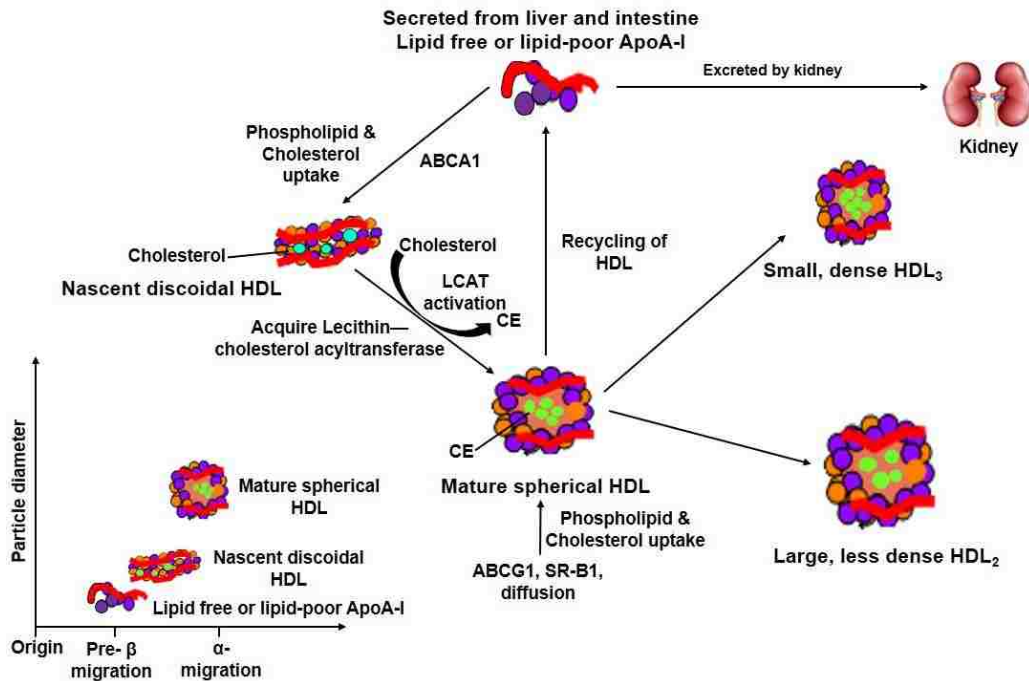


agonists to enhance ABC transporters can be potentially beneficial if the undesirable hepatic steatosis induced by these agonists can be minimized as a side effect. Other approaches explored the possibility to enhance hepatic apoA-I expression by using small molecules like RVX-208 or *via* peroxisome proliferator activated receptor  $\alpha$  (PPAR  $\alpha$ ) agonists. Initial human trial studies involving the introduction of apoA-I or reconstituted HDL (rHDL) directly in the circulatory system showed beneficial effects on RCT and the regression of plaques. Some other therapeutic tactics to improve RCT include oral delivery of apoA-I mimetic peptide, inhibition of CETP to improve HDL concentration in serum, strategies to increase SR-B1 mediated hepatic uptake of cholesterol and direct intestinal secretion of cholesterol (79).

#### *Clinical Relevance of RCT:*

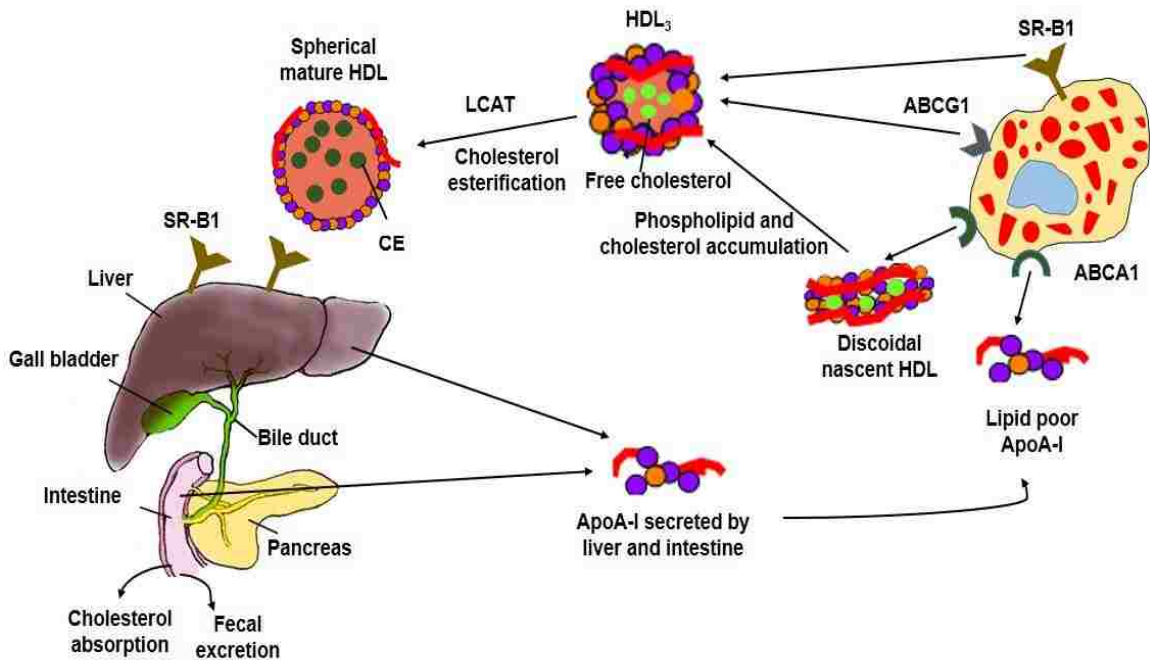
The initial hypothesis regarding linear correlation between HDL concentration and risk of developing CAD has been questioned for a long time. The importance of cholesterol efflux is undeniable as several preclinical studies demonstrated that increased plasma concentration of lipid poor apoA-I not only improves RCT, but also attenuates or regresses atherosclerotic plaque formation (80). Based on these observations, novel therapeutic models have been proposed that will improve the cholesterol efflux capacity of HDL and therefore, HDL functionality. However, some recent studies have questioned the relevance of RCT itself. A study by Li et al (81), demonstrated that the cholesterol efflux capacity is inversely associated with CAD. During this study, initial RCT assays demonstrated

that the cholesterol efflux capacity associated with apoB-depleted serum was inversely associated with the risk of CAD in the study group. Surprisingly, when the same subjects were monitored for the occurrence of CAD for next 3 years, a clear inverse relationship between the cholesterol efflux capacity and the prevalence of CAD emerged (81). This unique and highly important study once again challenges our understanding regarding HDL and its association with CAD. Nonetheless, cholesterol efflux from peripheral tissues and atherosclerotic plaques is a highly important target to control CAD and more emphasis should be given to understand the mechanisms and clinical relevance of RCT.



**Figure 5. Formation of HDL through Various Steps.**

ApoA-I is synthesized and secreted by liver and intestine. ApoA-I in the lipid poor form are the best acceptors of cholesterol and accept cholesterol *via* ABCA1. Upon accepting cholesterol, it forms discoidal, pre  $\beta$ -migrating nascent HDL which can further accept cholesterol and phospholipid *via* ABCG1, SR-B1 and by diffusion. Esterification of free cholesterol by HDL associated LCAT generates spherical,  $\alpha$ - migrating, mature HDL. Depending on size, HDL can be divided into small, spherical HDL<sub>3</sub> and large spherical HDL<sub>2</sub>. During RCT, ApoA-I is recycled and excess of ApoA-I is secreted through the kidney.



**Figure 6. Reverse Cholesterol Transport.**

Free cholesterol and phospholipids are effluxed from the peripheral tissues or macrophage foam cells to HDL *via* ABCA1, ABCG1, SR-B1 or by diffusion. In HDL, cholesterol is esterified into CE by LCAT and transported to the liver by selective uptake through SR-B1. From liver cholesterol gets secreted into the bile as the biliary salt component and reaches the intestine. Here, cholesterol can get recycled or excreted in the feces. Before reaching the liver, HDL can trade CE for triglycerides from VLDL or LDL with the help of CETP.

## **HDL and Anti-atherogenicity**

Anti-atherogenic properties of HDL are mainly attributed, but not restricted to its capacity to induce cholesterol efflux and reverse cholesterol transport. Several studies strongly indicated that HDL prevents oxidation of LDL, enhance endothelial function and repairment (53) and thus prevent the initial steps of fatty streak formation. HDL is also associated with anti-inflammatory and anti-thrombotic properties. All properties taken together indicate HDL can prevent atherosclerotic plaque progression while promoting lesion regression (53). The diverse functionality of HDL is due to the presence of various apolipoproteins, and enzymes involved in lipid metabolism, anti-oxidative mechanisms etc.

### *Antioxidative Effects of HDL:*

In 1990 Parthasarathy et al described oxidative modification of LDL can be reduced or prevented by its co-incubation with HDL (82). Oxidative modification of LDL involves oxidation of phospholipids, generation of toxic short-chain aldehydes and oxidation of protein moieties resulting in the generation of mm-LDL. Functional HDL prevents these modifications by various means.

ApoA-I prevents or delays the formation of mm-LDL by removal of peroxidized fatty acids and phospholipids. Previous studies further indicate that HDL can act as a reservoir for peroxidized lipids and deliver them to the liver for efficient elimination from the body (83). Other apolipoproteins such as apoE, apoA-II and apoA-IV are also associated with the anti-oxidant properties of HDL (47).

Paraoxonase (PON1), an HDL associated enzyme, plays a crucial role as an anti-oxidant and catalyzes hydrolysis of lipid peroxides, fatty acid peroxides and hydrogen peroxides to prevent LDL modification (83). PON1, a 45-kDa glycoprotein hydrolyzes lactones, organophosphate insecticides and nerve gas agents (84). HDL provides a hydrophobic harbor and stabilizes PON1 after its secretion from the liver. Association with HDL and interaction with apoA-I is an essential step for the proper functioning of PON1 (47). It is considered to be a primary contributory factor to the anti-oxidative property of HDL and protects HDL from oxidative modification as well. Patients with cardiovascular disorders are known to have decreased levels and functionally less active PON1 (85). Oxidized phospholipid mediated monocyte chemotaxis and macrophage activation are prevented by PON1 (86). Homocysteinylation, a post-translational modification that can impair normal protein function, is believed to be neutralized by PON1. Homocysteine thiolactone which causes endothelial injury and initiates fatty streak formation, is hydrolyzed and detoxified by PON1. Moreover, PON1 is believed to enhance the production of Lyso PtdCho and polyunsaturated fatty acids by acting like phospholipase A<sub>2</sub>. It has been hypothesized that Lyso PtdCho improves the binding between ABCA1 and HDL and therefore, increases cholesterol efflux (47, 86).

Recent investigations suggest accumulation of oxidized lipids is also prevented by another HDL associated enzyme LCAT (83). Platelet-activating factor-acetyl hydrolase (PAF-AH), glutathione selenoperoxidase (GSPx) are the

other HDL-associated enzymes known to have anti-oxidative properties and detoxify lipid hydroperoxides to less toxic products (47).

*Anti-inflammatory and Other Anti-atherogenic Function of HDL:*

As mentioned earlier, HDL inhibits monocyte infiltration in the endothelial lining induced by Ox-LDL. PON1 and PAF-AH are responsible for this anti-inflammatory property of HDL. Additionally, HDL prevents the expression of cytokine induced cell surface molecules such as VCAM-1, ICAM-1 and E-selectin (47, 87), which are essential for the initiation of monocyte transmigration and macrophage differentiation. Therefore, HDL reduce inflammatory response induced tissue damage. HDL modulates the proper functioning of endothelial cells and prevents Ox-LDL induced endothelial cell death.

Other anti-atherogenic functions attributed to HDL are anti-apoptotic, anti-thrombotic, vasodilatory, anti-infectious activities (47).

**Dysfunctional HDL and Pro-atherogenicity**

Dysfunctionality of HDL has received tremendous attention in the last two decades. A vast amount of researches have been done to identify the factors responsible for drastic modifications of structure and composition of HDL and to address the outcomes associated with HDL dysfunctionality. Acute-phase response, inflammation and oxidative stress render HDL dysfunctional with highly adverse consequences.

Acute and chronic inflammation is known to reduce the concentration of apoA-I in plasma due to decrease synthesis of apoA-I by the liver. Additionally, apoA-I is replaced by serum amyloid A (SAA), an acute phase protein, in HDL (47). As expected, SAA containing small, dense HDL is far less effective to induce cholesterol efflux. Another highly effective way of impairing apoA-I is known to be oxidative modifications of certain apoA-I amino acids. Amino acids of apoA-I undergo chlorination, nitration modifications by myeloperoxidase, an enzyme secreted by arterial wall macrophages. Oxidized apoA-I fails to induce ABCA1 mediated cholesterol efflux and inhibits LCAT activation (88).

Under inflammatory conditions or oxidative stress, HDL-associated enzymes often become dysfunctional or inactive. During acute phase response, PON1 level in plasma is often reduced dramatically or PON1 in HDL is replaced by SAA (89). Oxidative stress induced PON1 inactivation is known to be associated with elevated hepatic expression of SR (86). Decreased level of PON1 is closely associated with aging, diabetes and hypercholesterolemia (47).

Even though modifications of apoA-I and PON1 are mostly responsible for the inactivation of HDL, change in the lipid contents of HDL often leads to HDL dysfunctionality, other mechanisms contribute significantly to this phenomenon. During acute phase response and inflammation, CE present in the core of HDL is often replaced by the triglycerides, resulting in decreased plasma HDL levels. This characteristic is frequently associated with elevated levels of CETP, which is responsible for CE transfer from HDL to triglyceride-rich lipoproteins, in the system (47).



Dysfunctionality of HDL results in reduced cholesterol efflux, decreased oxidative protection of LDL by HDL and increased cholesterol influx by macrophages. In summary, dysfunctional HDL increases the risk of atherosclerosis and cardiovascular diseases by several folds, as opposed to the atheroprotective functions of native HDL.

### **Heart Failure and Left-ventricular Ejection Fraction**

Heart failure (HF) is one of the costliest and common health problem that is complex in nature. The morbidity and mortality rate associated with HF is very high. Population studies show that 30-40% of the patients diagnosed with HF, die within a year while 60-70% of the mortality happens within 5 years. HF happens following the gradual and severe muscle weakness/ stiffening in the heart and inefficient pumping of the blood. As the muscle weakness progresses, the organs and peripheral tissues receive less amount of oxygenated blood and the pressure in the heart gradually increases.

Ejection fraction (EF) refers to the percent of the total amount of blood in the left ventricle, which is pumped out with each heartbeat. A normal heart's EF varies between 55-70% and EF below 40% is a strong indicator of HF. EF 60 indicates that 60% of the total amount of blood from the left ventricle is pumped out with each contraction. Patients with EF below 35% are at a very high risk of life- threatening irregular heartbeats which can lead to sudden cardiac arrest and death. There are 2 types of HF associated with EF: i) Systolic HF or systolic left ventricular dysfunction: it is characterized by low left-ventricular EF and less

efficient left ventricular contraction resulting in reduced circulation of oxygenated blood throughout the body, ii) diastolic HF or HF with preserved left ventricular (LV) function: it is characterized by normal left-ventricular EF and normal contraction, but irregular ventricle relaxation resulting in reduced inflow of blood in the heart.

## **CHAPTER 2: NOVEL TECHNIQUE FOR GENERATING MACROPHAGE FOAM CELLS FOR *IN VITRO* REVERSE CHOLESTEROL TRANSPORT STUDIES**

### **Introduction:**

Increased plasma LDL, an important risk factor for atherosclerosis, leads to its sub-endothelial accumulation. In the intima LDL undergoes several structural and physiological modifications, mediated by oxidation, denaturation, by enzyme actions or by self-aggregation (32, 90-93). Enhanced uptake of modified lipoproteins by macrophages involves at least two crucial scavenger receptors, namely SR-A1 and CD36 (27, 94). In addition, fluid phase pinocytosis of LDL has been suggested to result in foam cell formation (95). Eventually, lesion progression and plaque rupture result in platelet aggregation and the formation of thrombus, which can lead to heart attack or stroke (96). Changes in lifestyle, diet and physical exercise are known to cause regression of atherosclerosis (8).

RCT is a mechanism through which cholesterol from peripheral tissues, especially from macrophage-rich plaques, are effluxed to apoA-I/ HDL, and then transferred to liver for disposal (51, 73, 97). It is currently believed that cholesterol efflux capacity of HDL, and not the HDL cholesterol level, has positive correlation with anti-atherogenicity (98). However, recent studies by Hazen and associates showed a remarkable association of RCT with increased risk for myocardial infarction, stroke, and death. Although RCT assay at the time of sample collection indicated cholesterol efflux activity from macrophages to apoB-depleted serum, as cholesterol acceptor, was inversely associated with risk of prevalent CAD (81),

when subjects were followed for three years a positive correlation between increased cholesterol efflux and increased CAD was observed. Regardless of the implications of RCT, it is obvious that RCT is an important phenomenon that requires not only further investigations but also more efficient methods. Cholesterol efflux from peripheral tissues is promoted by transporters, ABCA1 and ABCG1 (98). RCT also involves uptake of free and esterified cholesterol by hepatic HDL receptor SR-B1 (99).

HDL not only acts as an acceptor for plaque cholesterol (100) but also has been suggested to have several anti-atherogenic functionalities, such as anti-oxidative, anti-inflammatory and anti-thrombotic (53, 87, 101) properties. Oxidation of HDL renders it “dysfunctional” (47, 88) and this dysfunctionality has been suggested to be an important factor in the control of atherosclerosis. Previous studies showed that oxidation of apoA-I impairs the efflux of cholesterol, and thereby RCT (102).

The currently available technique to study RCT *in vitro* involves the use of macrophage foam cells generated by incubation with cholesterol labeled Ac-LDL. Macrophages incubated with radiolabeled Ac-LDL are used as foam cells to study RCT with respect to HDL heterogeneity (103) and to investigate the role of ABCA1 in RCT (39, 77). In recent times, a novel approach has been taken for high-throughput cholesterol efflux studies. Macrophages incubated with radiolabeled free cholesterol were utilized to study cholesterol efflux in presence of serum from human subjects (78, 81). Fluorescently labeled cholesterol (NBD-cholesterol), which has the NBD probe attached to alkyl chain of cholesterol (104), has been

used for several *in vitro* RCT studies. This method has been extended to study RCT in an *in vivo* model (74). Foam cells loaded with radiolabeled Ac-LDL are injected intraperitoneally in animal models, then distribution of labeled cholesterol in plasma, liver and feces were followed and measured (40, 73). To understand and evaluate the functionality of HDL, a speedier and more reliable assay needs to be developed.

The current methods of labeling Ac-LDL with radioactive or fluorescent tags suffer from major deficiencies. First LDL has to be isolated from suitable donors and Ac-LDL needs to be prepared. These lipoproteins are inherently unstable and their shelf life is limited. Our previous studies have demonstrated that LDL undergoes oxidation even during the centrifugation involved in its preparation (90). Longer dialysis and other steps also promote oxidation and such particles have to be stored with Ethylenediaminetetraacetic acid (EDTA) or other additives. Acetylation involves toxic chemicals, such as acetic anhydride, and additional dialysis steps are involved in its isolation. The incorporation of radioactive/fluorescent tags involves physical adsorption methods and yet another purification step to remove unincorporated tracers. In addition, there is variability in the extent of cholesterol incorporation into different preparations of Ac-LDL that might offer inconsistent information regarding RCT when cholesterol efflux data from different patients are compared.

Thus, there is a need for an efficient method for incorporation of cholesterol into macrophages. Past studies from our laboratory demonstrated that a highly non-polar molecule,  $\beta$ -carotene, could be very efficiently solubilized using Lyso

PtdCho as a natural “detergent”. The resulting solution was optically clear, stable and provided an opportunity to enrich cellular  $\beta$ -carotene in a concentration-dependent manner (105). In this study, we use a similar technique to incorporate cholesterol or CE into macrophages to generate foam cells. Lyso PtdCho is very efficiently utilized by macrophages by different ways: a) an acyltransferase reaction yields Phosphatidylcholine (PtdCho) from lyso, b) lyso phospholipase cleaves the acyl ester bond and removes the detergent nature of Lyso PtdCho, and c) inter molecular trans-esterification reaction creates a molecule of PtdCho and glycerophosphorylcholine (106, 107). In contrast, macrophages esterify cholesterol and accumulate CE efficiently (108). Thus, while Lyso PtdCho would be “de-toxified”, CE will accumulate. This process also could be considered biological as Ox-LDL has been suggested to be involved in foam cell formation, and contains substantial levels of Lyso PtdCho (109, 110).

NBD-cholesterol has been previously used in several studies related to uptake, metabolism and HDL-mediated efflux in primary hepatocytes and fibroblasts (38, 111). In recent years NBD-cholesterol has become a significant tool to study cholesterol metabolism because it closely mimics the behavior of cholesterol regarding metabolism and intracellular trafficking (37). In the present study NBD-cholesterol along with a  $^3\text{H}$ -cholesterol have been used as the analogs for cholesterol to demonstrate Lyso PtdCho mediated cholesterol loading, foam cell development and HDL-induced cholesterol efflux.

## **Materials and Methods:**

### *Reagents:*

RPMI 1640, Advanced DMEM (ADMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, penicillin-streptomycin (PS), 1X phosphate buffer saline (PBS), NBD-cholesterol were bought from Invitrogen Life Technologies (Carlsbad, CA). Cholesterol, Lyso PtdCho from egg yolk, oleic acid, 0.9% saline, fucoidan, polyinosinic acid, 4-dimethylaminopyridine and silica gel matrix for thin layer chromatography (TLC) separation were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform, methyl alcohol, hexane, diethyl ether, acetic acid isopropyl alcohol and ethyl alcohol were purchased from VWR international (Randor, PA). [1, 2-  $^3\text{H}$  (N)] cholesterol, [1- $^{14}\text{C}$ ] Oleic acid, [methyl- $^{14}\text{C}$ ] Choline chloride were purchased from American Radiolabeled Chemicals (St. Louis, MO). Oil red O was purchased from Fisher Scientific (Hampton, NH) and oleic anhydride from Santa Cruz biotechnology (Dallas, TX).

### *Cell Culture:*

RAW 264.7 macrophages were cultured in RPMI 1640 medium containing 10% FBS, 1% PS, 1% sodium pyruvate and 1% L-glutamine. Cells were seeded at  $1.2 \times 10^6$ / ml density and incubated at 37° C in a 5 % CO<sub>2</sub> incubator to reach 65-70% confluence overnight. For the experiments involving foam cell development RPMI 1640 with 0.1% Lipoprotein deficient serum (LPDS) was used as incubation medium. Swiss Webster mice were purchased from Charles River (Wilmington, MA) and used to collect peritoneal macrophages. Macrophages from

peritoneal cavity of 10-week old Swiss Webster mice were isolated by peritoneal lavage using 5 ml cold 0.9% saline, followed by centrifugation. Cells were cultured under the same conditions as used for RAW macrophages. HepG2 cells were cultured in ADMEM containing 10% FBS, 1% PS and 1% L-glutamine.

#### *Solubilization of Cholesterol:*

Stock solutions of 5 mM cholesterol and 2 mM NBD-cholesterol were prepared in chloroform. 20 mM Lyso PtdCho stock solution was prepared in chloroform: methanol (1:1). For a typical reaction 3  $\mu$ moles of cholesterol (600  $\mu$ l from the stock), 10 nmoles of NBD-cholesterol (5  $\mu$ l from the stock) and 3  $\mu$ moles of Lyso PtdCho (150  $\mu$ l from the stock) solutions were mixed together in a test tube, followed by drying under nitrogen. 1 ml of sterile PBS was added to the dried content and the mixture was vigorously vortexed for 1-2 minutes. The aqueous solution was filtered through 0.22  $\mu$ m filter. The entire procedure was done under semi-darkness.

In studies with radioactive cholesterol, NBD-cholesterol was substituted with  $^3\text{H}$ -cholesterol to give a final specific radioactivity of 5000 disintegration per minute (DPM)/ nmole of cholesterol.

#### *Synthesis of Fluorescent CE:*

2 mg cholesterol, 0.2 mg NBD-cholesterol and 25 mg oleic anhydride were mixed and dried in a glass tube. 4-dimethylaminopyridine was added to the reaction mixture as a catalyst for esterification (112) and the whole reaction mixture



was heated at 80°C for 30 minutes, until the liquid became bright yellow. The presence of NBD-CE was verified by TLC and later NBD-CE was separated from the reaction mixture by column chromatography. Purity of NBD-CE was checked again by TLC.

*Isolation and Modifications of Lipoproteins:*

Following Institutional Review Board approval, blood was collected in heparinized tubes from consented healthy donors and stored on ice. Blood was centrifuged at 3000 rpm for 20 minutes and plasma was separated. Lipoproteins were isolated from normal plasma by sequential ultracentrifugation using a Beckman TL-100 tabletop ultracentrifuge (Beckman, Palo Alto, CA) (113). The isolated lipoproteins were dialyzed against 0.3 mM EDTA in 1x PBS (pH 7.4) overnight and subsequently filter sterilized. The amount of protein was estimated using the Folin Lowry method. Lipoprotein sample was subjected to oxidation immediately after dialysis. Oxidation of lipoproteins was performed using either 5µM copper or myeloperoxidase (MPO) as described previously (114, 115). LDL was acetylated using acetic anhydride (92). Ac-LDL was dialyzed overnight and filter sterilized using 0.2 µm syringe filter. Isolated and modified lipoproteins were stored at 4°C and used within 1 week of preparation.

Tagging Ac-LDL with <sup>3</sup>H-cholesterol was done as described previously by Kritharides et al (116). Ac-LDL and <sup>3</sup>H-cholesterol in ethanol were co-incubated with RPMI containing 1% LPDS for 24 hours at 37°C. This stock reaction mixture

was diluted to obtain a working solution which contained 10 µg protein/ ml of Ac-LDL and 0.25 µCi/ ml <sup>3</sup>H-cholesterol in RPMI with 0.1% LPDS (116, 117).

#### *Cellular Uptake of Cholesterol:*

RAW 264.7 macrophages were seeded in 12- well plate at 1.2X10<sup>6</sup> cells/ml concentrations to reach 65-70% confluence overnight. Cells were washed with warm sterile PBS once followed by 4 hour of incubation with 0.1% LPDS containing RPMI 1640. After 4 hours these cells were incubated with 40 µl of cholesterol or CE/ Lyso PtdCho mixed micelle filtrate per ml of medium (equivalent of 120 µM concentrations of Lyso PtdCho and cholesterol (or CE) each). For each experiment involving cholesterol (unlabeled and NBD or <sup>3</sup>H-cholesterol) / Lyso PtdCho mixed micelles 40 µM oleic acid was added to the cells during the incubation. Freshly isolated peritoneal macrophages were seeded in 24 - well plate at 2.5X10<sup>6</sup> cells/ml concentrations and used in the same way to demonstrate cholesterol uptake via cholesterol / Lyso PtdCho mixed micelles. After overnight incubation, cells were washed with warm PBS twice and images of live cells were taken under fluorescence microscope (Axio imager, Carl Zeiss AG, Oberkochen, Germany) or cells were fixed with 10% formalin and stained with Oil red O.

#### *Detection of Foam Cells:*

To detect the presence of fluorescent NBD-cholesterol within the cells, images of live cells were taken with AxioCam MRm (Axio imager, Carl Zeiss AG, Oberkochen, Germany). RAW 264.7 macrophages were then lysed by incubating

with 1 ml of methanol at 37°C for 15 minutes. Cell lysates were centrifuged at 3000 rpm for 10 minutes. 100 µl of supernatant was used to measure fluorescence intensity present within the cells. Fluorescence plate reader (Envision 2014 Multilabel Plate Reader, PerkinElmer, Waltham, MA) was used to measure fluorescence intensity at emission spectra of 535 nm upon excitation at 475 nm.

Macrophages were also fixed to stain for CE droplets. After washing with PBS cells were fixed with 10% formalin, made permeable with 60% isopropanol and stained with Oil red O. Images of foam cells were taken under 10X and 40X objectives in the light microscope with the Leica DFC295 camera (Leica camera AG, Solms, Germany). Oil red O stained lipid droplets were further quantified by elution of the stain followed by absorption measurement with a spectrophotometer. Stained wells were rinsed with 60% isopropanol very briefly to remove any residual Oil red O and then dried completely. Stains were eluted with 1 ml isopropanol for 10 minutes at room temperature. After thorough pipetting to ensure complete elution, 100 µl aliquots were taken in triplicates in a 96-well plate and absorbance was measured at 500 nm (118).

*Quantification of Cholesterol Incorporated in Total Extracted Lipid and in CE Fraction:*

Macrophages were incubated with <sup>3</sup>H-cholesterol labeled Ac-LDL or cholesterol (unlabeled and <sup>3</sup>H-cholesterol) / Lyso PtdCho mixed micelles for 18 hours at 37°C. Lipid extraction was done using the method of Bligh and Dyer (119). Lipids were dissolved in 50 µl of chloroform and 5 µl of this was utilized directly to

measure  $^3\text{H}$ -cholesterol incorporation. This procedure was done in triplicates and rest of the reconstituted lipid (~ 35  $\mu\text{l}$ ) was used for TLC analysis. Lipids were separated by TLC on silica gel matrix using hexane/ diethyl ether/ acetic acid (30: 6: 0.5; V/V/V) solvent system (120) and visualized with iodine vapor. Spots of CE from samples incubated with  $^3\text{H}$ -cholesterol labeled Ac-LDL or cholesterol (unlabeled and  $^3\text{H}$ -cholesterol) / Lyso PtdCho mixed micelles were compared with CE standard and analyzed for  $^3\text{H}$ -cholesterol incorporation by liquid scintillation and luminescence Counters (MicroBeta2 Plate Counter, PerkinElmer, Waltham, MA). Count per minute (CPM) values obtained for the incorporation of  $^3\text{H}$ -cholesterol in total extracted lipid or CE fraction of lipid was converted into nmoles of cholesterol and represented subsequently.

*Incorporation of  $^{14}\text{C}$ - Oleic acid in CE within Foam Cells:*

To demonstrate the conversion of cholesterol into CE while using cholesterol/ Lyso PtdCho mixed micelles,  $^{14}\text{C}$ - Oleic acid was introduced during incubation of cells with cholesterol/ Lyso PtdCho mixed micelles. A stock solution of 100 mM oleic acid containing 1  $\mu\text{Ci}$   $^{14}\text{C}$ - Oleic acid was prepared. RAW 264.7 macrophages were incubated with either cholesterol/ Lyso PtdCho or CE/ Lyso PtdCho mixed micelles in presence of 40  $\mu\text{M}$  oleic acid/ ml of medium for 18 hours. After overnight incubation, cells were washed with warm PBS twice and lipid extraction was done with methanol / chloroform (2:1) (119). Chloroform-lipid phase was separated, the solvent was evaporated under nitrogen and the lipids were reconstituted with chloroform. Lipids were separated and CE was identified as

described previously. Spots of CE from samples incubated with cholesterol/ Lyso PtdCho or CE / Lyso PtdCho mixed micelles were compared with CE standard and analyzed for  $^{14}\text{C}$ - Oleic acid incorporation by autoradiography (Cyclone Plus Phosphor Imager- PerkinElmer, Waltham, MA) followed by liquid scintillation and luminescence counting.

*Measurement of Lactate Dehydrogenase Activity:*

Lactate dehydrogenase (LDH) cytotoxicity assay kit (Catalog number- 10008882) was purchased from Cayman Chemical (Ann Arbor, MI) and the assay was performed following the protocol supplied with the kit. In brief, RAW 264.7 macrophages were seeded at  $10^5$  - $10^6$  density in 96-well plate. After treating the cells for 18 hours, 100  $\mu\text{l}$  of medium was collected from each of the well and  $\text{NAD}^+$  (provided with the kit) was added to the medium. Enzymatic activity of LDH oxidized lactate into pyruvate and converted  $\text{NAD}^+$  into  $\text{NADPH}$  and  $\text{H}^+$ . Finally, a colorimetric reaction between  $\text{NADPH}$  and  $\text{H}^+$  and diaphorase (provided with the kit) produced dark-pink formazan. Formation of formazan was measured at 490-520 nm wavelength using a plate-reader (Benchmark Plus Microplate Spectrophotometer System, Bio-Rad laboratories, Hercules, CA) and LDH activity per ml of medium was calculated using standard curve. All the treatments were performed at least in triplicates and repeated four times.

### *Hemolysis of Human RBC:*

Human blood collected in a heparinized tube was centrifuged at 3000 rpm for 20 minutes and red blood cells (RBC) was collected from the pellet and washed. RBC was diluted with 0.9% saline (1:20; vol: vol) and hemolysis in presence of water was performed to determine the optical density value equivalent to 1 and to determine the optimum volume of diluted RBC required for the rest of the experiment. 75  $\mu$ l of diluted RBC was taken in eight glass tubes and 0, 10, 25, 50, 100, 150, 200 and 250  $\mu$ M of Lyso PtdCho micelles, cholesterol/ Lyso PtdCho and CE/ Lyso PtdCho mixed micelles were added to each tube. The volume was made up to 1 ml by adding PBS and the reaction mixtures were incubated at room temperature for 15 minutes. Then the reaction mixtures were centrifuged at 2000 rpm for 15 minutes. Without disturbing the cell pellet, 100  $\mu$ l of supernatant from each glass tube were taken in triplicates in a 96-well plate and absorbance was measured at 540 nm.

### *Synthesis of $^{14}$ C- Lyso PtdCho and Quantification of Lyso PtdCho Metabolism by Macrophages:*

Synthesis of  $^{14}$ C- Lyso PtdCho was done from  $^{14}$ C- Choline chloride. HepG2 cells were incubated with 5  $\mu$ Ci of  $^{14}$ C- Choline chloride in presence of total growth medium, ADMEM that contains 4  $\mu$ g/ ml choline chloride. After 72 hours of incubation lipids were extracted and were separated by thin layer chromatography using chloroform/ methanol/ water (65: 25: 3.5; V/V/V) as the solvent system. PtdCho band was extracted with chloroform: methanol (1: 1) followed by

reconstitution of dried lipid content with 0.8 ml of PBS. This reconstituted PtdCho was subjected to enzymatic conversion into Lyso PtdCho by adding 25  $\mu$ l snake venom phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) (0.5 units) and 80  $\mu$ l of 10 mM CaCl<sub>2</sub>. The reaction was set at 37°C for 4 hours and the reaction mixture was used to run TLC using the previous solvent system with Lyso PtdCho and PtdCho as standards. Lyso PtdCho band from the TLC plate was extracted and was used for further experiments.

<sup>14</sup>C-Lyso PtdCho was mixed with 3  $\mu$ moles unlabeled Lyso PtdCho and 3  $\mu$ moles cholesterol from the previously described stock solutions. The reaction mixture was dried under nitrogen and reconstituted with 1ml PBS by vigorous vortexing. After filtering, 5  $\mu$ l the micelles were used to measure radioactivity count. Cholesterol/ Lyso PtdCho (unlabeled and <sup>14</sup>C labeled) mixed micelles containing at least 5000 CPM/ ml activity (~ 25  $\mu$ l/ ml of medium) were used to incubate RAW 264.7 macrophages with for 18 hours at 37°C. After overnight incubation lipid extraction was done by Bligh and Dyer method and reconstituted lipid was used to run TLC with PtdCho and Lyso PtdCho standards using chloroform/ methanol/ water (65: 25: 3.5; vol: vol: vol) solvent system. Spots corresponding to PtdCho and Lyso PtdCho standards were isolated from the TLC plate and metabolic fate of <sup>14</sup>C-Lyso PtdCho was determined by liquid scintillation and luminescence counters.

### *Quantification of Cellular Cholesterol Efflux:*

RAW 264.7 macrophages and mouse peritoneal macrophages incubated overnight with cholesterol/ Lyso PtdCho mixed micelles were washed with PBS twice and fresh medium was added. Cells were then incubated with 0- 200 µg/ml of native HDL or 25 and 50 µg/ml of Ox-HDL (MPO or Cu mediated) for 4 hours and medium was collected to measure fluorescence intensity. After removal of the medium, RAW 264.7 macrophages were washed with PBS and images under fluorescence microscope were taken. To measure fluorescence intensity within the cells, cell lysates were prepared as described before or cells were fixed and stained with Oil red O to observe CE accumulation.

To study cholesterol efflux, foam cells were also developed using <sup>3</sup>H-cholesterol (and unlabeled cholesterol) / Lyso PtdCho mixed micelles. Efflux of <sup>3</sup>H-cholesterol in medium upon incubation with HDL was measured by liquid scintillation and luminescence counters.

### *Statistical Analysis:*

Each experiment was performed more than three times and each experimental condition was set up in triplicates. All values were presented as mean ± standard deviation. To determine the difference between two groups, unpaired 2-tailed Student's t-test was applied. Analysis of variance (ANOVA) was applied with Dunnett's or Bonferroni's correction for multiple comparisons using GraphPad Prism 5.0 software (San Diego, CA). The minimum level of significance in all tests was  $p < 0.05$ .



## Results:

### *Solubilization of Cholesterol in Aqueous Solution in Presence of Lyso PtdCho:*

Cholesterol was solubilized in PBS by mixing cholesterol and Lyso PtdCho together. To determine if Lyso PtdCho could increase the solubility of cholesterol in PBS, constant amounts of cholesterol, NBD- cholesterol and an increasing amount of Lyso PtdCho were taken in five glass tubes. After drying, vigorous mixing and filtering, aqueous solution was made with 1 ml PBS as described in the methodology. Prepared mixed micelles contained 1 mM cholesterol (200  $\mu$ l from 5 mM stock) and 5  $\mu$ M NBD-cholesterol (2.5  $\mu$ l from 2 mM stock) and Lyso PtdCho ranging from 0 to 500  $\mu$ M (0, 2.5, 5, 12.5 and 25  $\mu$ l from 20 mM stock). Fluorescence intensity was determined in the clear filtrate. Fluorescence intensity of the aqueous solution containing only cholesterol and fluorescent cholesterol, and no Lyso PtdCho was at the background level and increased in the filtrates containing increasing amounts of Lyso PtdCho (Fig. 7a).

To determine if cholesterol could reduce the solubility of NBD-cholesterol in PBS in a concentration-dependent manner, increasing concentrations of cholesterol ranging from 3- 24  $\mu$ M (0.6, 1.2, 3.6 and 4.8  $\mu$ l from 5 mM stock) were taken in four glass tubes. Equimolar concentration of Lyso PtdCho ranging from 3- 24  $\mu$ M (0.15, 0.3, 0.9 and 1.2  $\mu$ l from 20 mM stock) was added to the cholesterol and a constant concentration of 5  $\mu$ M NBD-cholesterol was taken. Mixed micelles in 1 ml of PBS were made as described above. The fluorescence intensity of the

aqueous solution gradually decreased as the concentration of unlabeled cholesterol used to make mixed micelles increased (Fig. 7b).

*Accumulation of Fluorescent Cholesterol in Macrophages:*

RAW 264.7 macrophages were incubated with mixed micelles containing increasing concentrations (0-10  $\mu\text{M}$ ) of NBD-cholesterol for 18 hours. Then the cells were washed with PBS twice and visualized under fluorescence microscope (Fig. 7c). Macrophages showed concentration- dependent increased accumulation of NBD-cholesterol in the cytoplasm, which was further confirmed by measuring the fluorescence intensity in the corresponding cell lysates (Fig. 7d). Fluorescence intensity in cells incubated with 0.25, 0.5, 1, 5 and 10  $\mu\text{M}$  of NBD-cholesterol were observed to be several folds higher than the background level.

*Mixed Micelles Mediated Cholesterol Uptake is a Quick Process:*

RAW 264.7 macrophages were incubated with cholesterol (unlabeled and NBD-cholesterol) / Lyso PtdCho mixed micelles for 0, 2, 4, 6, 12, 18, 24 and 48 hours. Cells for each treatment condition were incubated with 90  $\mu\text{M}$  cholesterol, 300 nM NBD-cholesterol and 90  $\mu\text{M}$  Lyso PtdCho (15  $\mu\text{l}$  of mixed micelles/ 500  $\mu\text{l}$  of medium). At the end of each time point cells were observed under microscope (data not shown) and were lysed to quantify fluorescence intensity in the cell lysates. It was observed that within two hours the fluorescence intensity of the cells treated with mixed micelles increased significantly from the background level. Furthermore, fluorescence intensity in cell lysates after treatment for 4 hours

increased slightly from 2 hours and almost reached a plateau between 4 to 18 hours. However, after a steady increase in fluorescence intensity at 24 hours, there was a sharp decrease in fluorescence intensity level at the 48 hour time point even though it was still several fold higher than the background level (Fig. 7e).

*Mixed Micelles of Cholesterol in Aqueous Solution are Stable:*

Mixed micelles of cholesterol, NBD-cholesterol and Lyso PtdCho in PBS was prepared as described before and aliquots of the filtrate were stored at room temperature, at 4°C and -20°C. Fluorescence intensity of the filtrate from each storing condition was determined at week 0, week 1, week 2 and week 4 from the storage time. Mixed micelle solution from each temperature was filtered again right before their use to measure fluorescence intensity or to treat the cells. We observed that the fluorescence intensity of the solution stored at room temperature started deteriorating rapidly, while the fluorescence intensity of the solutions stored at 4°C and -20°C were fairly stable up to four weeks (Fig 8a). Furthermore, the mixed micelles stored at -20°C for four weeks were used to develop macrophage foam cells (Fig. 8b). Fluorescent cholesterol accumulation in the macrophages was documented by taking images under fluorescence microscope and droplets of CE were identified by Oil Red O staining.

Fluorescent cholesterol accumulation was further quantified by measuring fluorescence intensity in the cell lysates (Fig. 8c) and the accumulated CE droplets were quantified by eluting the Oil red O stain (Fig. 8d). Measurement of fluorescence intensity and elution of Oil red O stain both confirmed the previous

observations regarding the stability of mixed micelles at different temperatures. Cells treated with micelles stored at 4°C and -20°C showed almost no change in NBD-cholesterol or CE droplet accumulation from the starting time point (week 0). However, there was a sharp decrease in the accumulation of NBD-cholesterol and CE droplets in cells treated with micelles stored at room temperature.

In order to substantiate the observations regarding the stability of mixed micelles additional experiments were performed. Mixed micelles were prepared using cholesterol (unlabeled and <sup>3</sup>H-cholesterol) and Lyso PtdCho and filtered micelles were stored as described in the previous section. Measurements of radioactivity in the micelles stored at room temperature, 4°C and -20°C were done at week 0, week 1, week 2 and week 4. Micelles stored under these conditions were further used to incubate RAW 264.7 macrophages for 18 hours. Each time before using, the micelles stored at different temperatures were filtered through 0.22 µm filter. Stability of the <sup>3</sup>H-cholesterol containing micelles (Fig. 8e) mimicked the results of micelles containing NBD-cholesterol. More interestingly, cells treated with 40 µl (per ml of medium) of <sup>3</sup>H-cholesterol containing micelles stored at room temperature showed steadily decreasing incorporation of cholesterol in total extracted lipid (Fig. 8g) as well as in CE fraction (Fig. 8f). Incorporation of cholesterol in total extracted lipid (Fig. 8g) and in CE fraction (Fig. 8f) remained almost the same as that of the freshly prepared micelles when cells were treated with micelles stored at 4°C and -20°C even after four weeks (TLC plate- Fig. 15).

*Mixed Micelles Mediated Cholesterol Uptake does not Cause Cell Cytotoxicity:*

Amount of lactate dehydrogenase secreted into the medium during the incubation of cells with cholesterol (or CE) / Lyso PtdCho mixed micelles was measured to rule out the cytotoxic effect of the mixed micelles (Fig. 9a). Mixed micelles contained 3 mM Lyso PtdCho alone, 3 mM cholesterol or CE with equimolar Lyso PtdCho. Macrophages were seeded in 96-well plate in  $1 \times 10^5$ /well concentration and after 24 hours were washed with PBS twice and incubated with 120  $\mu$ l of RPMI containing 0.1% LPDS for 4 hours. Cells were then incubated with 1  $\mu$ g Ac-LDL per well (bar 2), 50 (2  $\mu$ l from 3 mM Lyso PtdCho containing mixed micelles) and 75  $\mu$ M (3  $\mu$ l) Lyso PtdCho micelles per well (bar 3-4), 50 (2  $\mu$ l from 3 mM cholesterol/ Lyso PtdCho containing mixed micelles) and 75  $\mu$ M (3  $\mu$ l) cholesterol/ Lyso PtdCho mixed micelles per well (bar 5-6) and 25 (1  $\mu$ l from 3 mM CE/ Lyso PtdCho containing mixed micelles) and 50  $\mu$ M (2  $\mu$ l) CE/ Lyso PtdCho mixed micelles per well (bar 7- 8) for 18 hours. All the treatments were done in triplicates and at the end of 18 hours 100  $\mu$ l medium was collected from each well to measure LDH activity in the sample. In this colorimetric assay pink colored formazan was measured. Cells incubated with Lyso PtdCho micelles produced very dark-pink color indicating very high cell lysis; whereas cells treated with cholesterol or CE/ Lyso PtdCho mixed micelles produced very pale pink color as was observed in the cells without any treatment. Thus, cells treated with cholesterol or CE / Lyso PtdCho mixed micelles were devoid of toxicity.

*Cytotoxic Effects of Lyso PtdCho are Greatly Reduced by the Presence of Cholesterol in Mixed Micelles:*

RBC diluted in saline were mixed with Lyso PtdCho micelles or cholesterol/ Lyso PtdCho or CE/ Lyso PtdCho mixed micelles containing 0, 10, 25, 50, 100, 150, 200 and 250  $\mu\text{M}$  of Lyso PtdCho or cholesterol or CE. Cholesterol and CE contain mixed micelles also had equimolar concentrations of Lyso PtdCho. The reaction mixture containing Lyso PtdCho micelles showed almost immediate hemolysis and optical density value of the supernatant of the reaction mixtures were several folds higher when compared with background. Conversely, reaction mixture containing cholesterol/ Lyso PtdCho mixed micelles showed almost no hemolysis even at the end of 15 minutes incubation and optical density value of the supernatant of the reaction mixtures was barely above the background level. Nevertheless, the hemolytic properties of CE/ Lyso PtdCho mixed micelles were in between the other two micelles. At lower concentrations, between 10- 100  $\mu\text{M}$  CE/ Lyso PtdCho did not cause very significant amount of hemolysis, even though the amount of hemolysis was higher than that induced by cholesterol/ Lyso PtdCho mixed micelles. There was a sharp increase in the level of hemolysis in the reaction mixtures containing more than 100  $\mu\text{M}$  CE/ Lyso PtdCho mixed micelles. Between 150- 250  $\mu\text{M}$  concentrations, levels of hemolysis caused by CE/ Lyso PtdCho mixed micelles were almost the same as those of Lyso PtdCho micelles (Fig. 9b).

*Lyso PtdCho Component of Mixed Micelles was metabolized into PtdCho:*

Cells treated with cholesterol/ Lyso PtdCho (unlabeled and <sup>14</sup>C-Lyso PtdCho) mixed micelles for 18 hours demonstrated that more than 75% of Lyso PtdCho, provided as the component of mixed micelles was metabolized into PtdCho (Fig. 9c). The metabolization of Lyso PtdCho to PtdCho was a very quick process, which took place within 2 hours (data not shown) as demonstrated previously (121, 122).

*Foam Cells are developed by Incubating Macrophages with Cholesterol Containing Mixed Micelles:*

RAW 264.7 macrophages and mouse peritoneal macrophages were incubated with mixed micelles for 18h. 40 µl of mixed micelles which was used per ml of medium, contained 120 µM cholesterol (unlabeled), 400 nM NBD-cholesterol and 120 µM Lyso PtdCho (panel iii and vi), while mixed micelles containing CE (24 µl of CE mixed micelles/ ml of medium) had 72 µM unlabeled CE, 240 nM fluorescently labeled CE and 72 µM Lyso PtdCho (panel iv and vii) (Fig. 10a). Ac-LDL (10 µg/ml, panel ii) treated macrophages were used as positive control for all the foam cell development studies. Macrophages incubated with cholesterol / Lyso PtdCho micelles were provided with 40 µM oleic acid to aid CE conversion and accumulation. When observed under the fluorescence microscope, cells treated with NBD-cholesterol containing mixed micelles showed fluorescent cholesterol accumulation within the cells. As expected negative control cells (panel i and v) and cells treated with Ac-LDL (panel ii) did not yield any fluorescent images. To

ensure the accumulation of fluorescent cholesterol was only within the cells, we lysed RAW 264.7 macrophages and measured the fluorescence intensity in the cell lysate. Fluorescence intensity in the cell lysates of negative control was comparable to that of the background level whereas fluorescence intensity was much higher in the lysate of cells incubated with mixed micelles (data not shown).

CE droplet accumulation in RAW 264.7 macrophages was demonstrated by Oil red O staining following the treatment with Ac-LDL, cholesterol/ Lyso PtdCho and CE/ Lyso PtdCho mixed micelles. Oil red O stain was eluted after the images were taken. Eluted Oil red O stain which indirectly quantifies the amount of CE droplets present in the cells was taken in 100  $\mu$ l aliquots and absorption was measured at 500 nm. Optical density value of all the three samples matched very closely and ranged between 0.3-0.4 (Fig. 10b).

*Quantity of CE Accumulated in Foam Cells Developed by Ac-LDL Mediated Uptake or Mixed Micelles Mediated Uptake are Similar:*

RAW 264.7 macrophages were treated with 10  $\mu$ g/ml of Ac-LDL labeled with  $^3$ H-cholesterol or with cholesterol (unlabeled and  $^3$ H-cholesterol) / Lyso PtdCho mixed micelles for 18 hours. Quantification of cholesterol incorporation in total extracted lipid showed, foam cells developed by Ac-LDL mediated and mixed micelles mediated uptake contained 22 nmoles and 26 nmoles of cholesterol (volume of total extracted lipid was 50  $\mu$ l) respectively (Fig. 10c). Cholesterol incorporation in the CE fraction of extracted lipid was 1.5 nmoles for Ac-LDL



mediated foam cell development and 1.8 nmoles for that of mixed micelles (Fig. 10d).

*Cholesterol is esterified into CE during Micelle Mediated Foam Cell Formation:*

RAW 264.7 macrophages incubated with cholesterol / Lyso PtdCho mixed micelles were simultaneously treated with <sup>14</sup>C- Oleic acid to study the incorporation of radioactive oleate during the esterification of cholesterol into CE. 40 μM oleic acid (with 200 pCi radioactivity) / ml of medium was supplied during the incubation of cells with cholesterol or CE / Lyso PtdCho mixed micelles and after overnight incubation lipids were extracted using methanol / chloroform (2:1). TLC separation of the samples from both types of micelle treatments showed distinct spots of CE when compared with the standard CE spot and these spots were further analyzed for radioactivity. Before exposing the TLC plate to iodine vapor, radioautograph image of the TLC plate was taken and image clearly demonstrated much higher oleate incorporation in cells incubated with cholesterol / Lyso PtdCho micelles (Fig. 10e-insert). This observation was further confirmed when analysis for oleate incorporation in CE showed that cells incubated with CE / Lyso PtdCho micelles had a 10-fold percentage decrease in <sup>14</sup>C- Oleic acid incorporation in CE compared to cells treated with cholesterol / Lyso PtdCho micelles (Fig. 10e).

*The Uptake of Cholesterol Micelles is Distinct from that of Ac- LDL:*

Fucoidan and Polyinosinic acid are ligands for SR-A1 and they reduce or inhibit the uptake of Ac-LDL by macrophages (123, 124). However, fucoidan or

polyinosinic acid did not inhibit the accumulation of fluorescent cholesterol or CE in RAW 264.7 macrophages (Fig. 11a- b). 50 µg/ml of fucoidan and 100 µg/ml of polyinosinic acid were used separately to block Ac-LDL mediated foam cell formation. Oil Red O staining demonstrated that macrophages pre-incubated with fucoidan or polyinosinic acid accumulated very little to no CE droplets when treated with Ac-LDL. But under the same conditions, macrophages incubated with cholesterol (or CE) / Lyso PtdCho mixed micelles accumulated plenty of CE droplets (Fig. 11c).

Quantification of CE droplet accumulation by Oil red O stain elution further established this observation. While the optical density of stains eluted from cells treated with Ac-LDL following the pre-incubation with fucoidan or polyinosinic acid decreased by 2-folds from the positive control, the same pre-incubation had little or no effects on the CE droplet accumulation in cells treated with cholesterol or CE/ Lyso PtdCho mixed micelles (Fig. 11d).

The unique nature of foam cell development by mixed micelles was further established by using <sup>3</sup>H-cholesterol labeled Ac-LDL and mixed micelles. Pre-treatment of fucoidan and polyinosinic acid decreased the incorporation of cholesterol both in total extracted lipid and in a CE fraction by 4-folds and 2-folds respectively in case of Ac-LDL mediated foam cell development. Fucoidan and polyinosinic acid did not have any effect on the incorporation of cholesterol in total extracted lipid and in CE fraction (Fig. 17) when foam cell development was mediated by cholesterol (unlabeled and <sup>3</sup>H-cholesterol)/ Lyso PtdCho mixed micelles (Fig. 11a- b).

### *HDL Causes Cholesterol Efflux from Foam Cells:*

Foam cells were developed by incubating RAW 264.7 macrophages and mouse peritoneal macrophages with cholesterol (unlabeled and NBD-cholesterol or  $^3\text{H}$  cholesterol)/ Lyso PtdCho mixed micelles and used for the efflux study in presence of HDL. Foam cells were incubated with 0, 25, 50, 100 and 200  $\mu\text{g}/\text{ml}$  of freshly prepared HDL. After 4 hours medium was collected from each well and RAW 264.7 macrophages were lysed to measure the presence of NBD-cholesterol in the medium and in the cell lysates respectively. Fluorescence intensity in the medium collected from foam cells alone (no HDL) was in the background level and increased in a HDL-concentration dependent manner (Fig. 13a). A similar observation was made with respect to cholesterol efflux when mouse peritoneal macrophages were used (Fig 13a- insert). Inversely the fluorescence intensity in the lysate of foam cells without HDL incubation was the maximum. Fluorescence intensity in the lysate of foam cells decreased gradually as the concentration of HDL in the medium increased (Fig. 13b). Images were taken under fluorescence microscope after the incubation of foam cells with or without HDL. Reduction in fluorescence intensity indicated less amount of NBD-cholesterol remained inside the foam cells when incubated with increasing concentrations of HDL. Study of CE droplet accumulation by Oil red O staining further confirmed our observations (Fig. 13c).

These results were further validated in the efflux study using  $^3\text{H}$ - cholesterol. CPM value for  $^3\text{H}$ - cholesterol efflux was converted into nmoles and presented (Fig. 14a). Further analysis showed that 25- 200  $\mu\text{g}$  of native HDL caused

cholesterol efflux ranging between 10- 25% of cholesterol present in total extracted lipid (Fig. 14b). Preliminary results indicated that most of the cholesterol in the medium was associated with intact, reisolated HDL (data not shown).

*HDL Mediated Cholesterol Efflux is Quantitatively Similar from Foam Cells Developed by Ac-LDL and Mixed Micelles:*

Foam cells developed by <sup>3</sup>H-cholesterol labeled Ac-LDL and cholesterol (unlabeled and <sup>3</sup>H- cholesterol) / Lyso PtdCho mixed micelles were incubated with 0, 25 and 50 µg of native HDL for 4 hours. Quantitatively the cholesterol efflux by 25 and 50 µg of HDL from foam cells developed by mixed micelles were a little bit higher than that from Ac-LDL derived foam cells (Fig. 14c). However, when the percentage of cholesterol efflux was calculated with respect to cholesterol present in total extracted lipid, the efflux rate was very similar. Incubation with 25 and 50 µg of HDL caused 2- fold and 3- fold induction in cholesterol efflux from foam cells developed from either method of cholesterol loading (Fig. 14d).

*Ox-HDL Fails to Cause Cholesterol Efflux:*

Cholesterol efflux from foam cells were compared in presence of 25 µg and 50 µg native HDL and MPO and Cu mediated Ox-HDL. Cholesterol efflux was documented by measuring the fluorescence intensity in medium after incubation of foam cells with 25 µg and 50 µg native HDL and Ox-HDL. Even though Cu Ox-HDL caused some cholesterol efflux, 25 µg and 50 µg native HDL induced much higher cholesterol efflux when compared to that of Ox-HDL counterparts (Fig. 13d). As seen in the figure MPO mediated oxidation of HDL was more effective at

inhibiting cholesterol efflux than copper mediated oxidation. Cholesterol efflux in presence of Ox-HDL was further confirmed by using NBD-cholesterol loaded mouse peritoneal macrophages (Fig. 13e) and <sup>3</sup>H- cholesterol loaded foam cells (data not shown).

### **Discussion:**

In this study, we demonstrated for the first time that macrophage foam cells can be developed using micellized cholesterol or CE. The foremost challenge to incorporate cholesterol in cells is obviously its high hydrophobicity. The amphiphilic property of Lyso PtdCho, which is also a natural detergent, was utilized to form mixed micelles and to solubilize cholesterol and CE (unlabeled and fluorescently labeled) in PBS. A previous study has demonstrated that Lyso PtdCho could form stable, soluble complex with equimolar cholesterol (125) and this technique has been modified successfully to solubilize  $\beta$ -carotene in aqueous solution (105). Augé et al (105) further demonstrated that solubilized  $\beta$ -carotene could be utilized for cellular enrichment of  $\beta$ -carotene in vitro. In the present study, increasing amounts of cholesterol (unlabeled and NBD-cholesterol) were successfully solubilized in PBS by gradually raising the concentration of Lyso PtdCho in the mixed micelles. Solubility of NBD-cholesterol was demonstrated directly by measuring the fluorescence intensity of the aqueous solution. We validated the solubility of cholesterol in aqueous solution indirectly by reducing the solubility of NBD-cholesterol in a cholesterol concentration-dependent manner. As the concentration of cholesterol provided to make mixed micelles was gradually

increased, while keeping the concentration of NBD-cholesterol constant, the fluorescence intensity of the aqueous solution was decreased in a concentration dependent manner. RAW 264.7 macrophages were loaded with NBD-cholesterol and unlabeled cholesterol in a concentration-dependent manner and fluorescently labeled cholesterol enriched foam cells were developed. Foam cells were successfully generated by using mouse peritoneal macrophages as well. We also observed that mixed micelle mediated NBD-cholesterol uptake is a very rapid process and cells were enriched with NBD-cholesterol as quickly as within 2 hours continuing up to 24 hours. Nonetheless, there was a sharp decline in the fluorescence intensity level at 48 hours which was due to detachment of many cells as a result of over-confluence.

It would be worth noting that previous studies demonstrated that NBD-cholesterol has a higher aqueous solubility than cholesterol and might not truly represent the uptake and efflux of cholesterol (37). Higher solubility of NBD-cholesterol has been utilized for uptake studies by directly incubating cells with NBD-cholesterol in ethanol solution (37, 104). The influence of higher solubility of NBD-cholesterol in the results of cholesterol uptake and efflux studies was ruled out by using  $^3\text{H}$ -cholesterol, a more widely used cholesterol analog to repeat the key experiments. Recently, Khera et al (78) and Li et al (81) utilized macrophages labeled with free radioactive cholesterol for high-throughput cholesterol efflux study. The enrichment of macrophages with free  $^3\text{H}$ -cholesterol or  $^{14}\text{C}$ -cholesterol was done by incubating cells with free radioactive cholesterol for 24 hours. However, the goal of our present study was to develop foam cells enriched with

cholesterol and CE, not just with fluorescent or radiolabeled cholesterol, by a novel, reproducible and quick technique. Therefore, Lyso PtdCho micelle mediated cholesterol delivery was a more suitable approach to solubilize and to ensure the delivery of highly hydrophobic cholesterol and CE to the cells. Besides, we documented that the free cholesterol delivered by micelles gets esterified to CE and produces CE droplet enriched foam cells. It is also to be noted that cellular cholesterol efflux is also accompanied by cellular phospholipid efflux. As noted in our studies, Lyso PtdCho is efficiently converted to PtdCho and thus would provide the PtdCho needed for efficient efflux. It remains to be established whether the fluorescence/ radioactivity of both cholesterol and PtdCho are associated with HDL fraction in the medium. Our preliminary results (data not shown) suggests that most of the fluorescence in the medium is associated with isolated HDL after incubation with foam cells containing NBD-cholesterol.

Incorporation of  $^{14}\text{C}$  oleate was studied to ascertain that esterification of cholesterol into CE is indeed the source of lipid droplets seen inside the foam cells upon incubation with cholesterol / Lyso PtdCho micelles. Cells incubated with cholesterol / Lyso PtdCho micelles yielded the CE band comparable to one from CE / Lyso PtdCho micelle treated cells, upon TLC separation (Fig. 16). Nonetheless, cholesterol / Lyso PtdCho micelle treated cells showed very high  $^{14}\text{C}$  oleic acid incorporation, while CE / Lyso PtdCho micelle treated cells had a 10-fold reduction in oleate incorporation. This demonstrated that cholesterol / Lyso PtdCho mixed micelles generated foam cells, which would be physiologically relevant as cholesterol was esterified into CE and appeared as lipid droplets during Oil red O

staining. For all the experiments involving cholesterol (unlabeled and NBD or  $^3\text{H}$ -cholesterol) / Lyso PtdCho micelles, cells were incubated with 40  $\mu\text{M}$  oleic acid along with the micelles. Oleic acid was supplied to facilitate the esterification of cholesterol into CE and has been used in many studies to follow Ac- LDL uptake.

Lyso PtdCho is known to induce cell lysis due to its detergent like properties and causes cell lysis by breaking down the intact cell membrane (125-127). It was observed in the current study that Lyso PtdCho alone is highly soluble in PBS and caused massive cell death. Cell lysis was confirmed by measuring secretion of LDH in medium upon incubation of cells with Lyso PtdCho micelles. However, cell morphology was not affected by the incubation with cholesterol/ Lyso PtdCho mixed micelles, as clearly demonstrated in Fig. 10a (panel iii). We also demonstrated that red blood cells were resistant to lysis when increasing concentrations of cholesterol were present along with Lyso PtdCho. This observation was not surprising as the work of Rand et al (125) explained that stable complex between equimolar cholesterol and Lyso PtdCho causes a conformational change in micelle formation, leading to reduction in the destabilizing and lytic effects of Lyso PtdCho. The reduced lytic effects of cholesterol/ Lyso PtdCho mixed micelles were clearly established by its reaction with RBC. Incubation of RBC with Lyso PtdCho micelles for 15 minutes, lysed RBC releases hemoglobin in the aqueous solution, while the same reaction with an equivalent amount of cholesterol/ Lyso PtdCho mixed micelles had very little to no cell lysis. Nonetheless, CE/ Lyso PtdCho mixed micelles induced more cell lysis causing increased secretion of LDH (data not shown) in the medium and



hemoglobin in the aqueous solution when compared with that of cholesterol/ Lyso PtdCho. One possible explanation could be that CE does not cause conformational changes as cholesterol does during mixed micelle formation and thus CE is less effective in reducing the lytic effects of Lyso PtdCho. After this initial observation, reduced volume of CE/ Lyso PtdCho mixed micelles was used to enrich macrophages with CE. Lytic effects of Lyso PtdCho micelles were clearly in effect even with the concentration as low as 10  $\mu$ M of Lyso PtdCho, while presence of CE up to a concentration of 100  $\mu$ M CE has greatly reduced this lytic effect.

The advantage of using Lyso PtdCho as a membrane fusion agent to deliver cholesterol to cells is two-fold as Lyso PtdCho is metabolized by cells into PtdCho (an acylation reaction) and glycerophosphorylcholine (a transesterification reaction) (121) as previous studies demonstrated this conversion using various cell lines including macrophages (122). The quick metabolism of Lyso PtdCho by cells ensures successful cargo delivery to the cells without influencing RCT. We observed that more than 75% of Lyso PtdCho, delivered as mixed micelles, was metabolized into PtdCho within 2 hours of incubation of cells with cholesterol/ Lyso PtdCho (unlabeled and  $^{14}\text{C}$ - tagged) mixed micelles.

We have further established that micellized CE uptake is uniquely distinguished from Ac-LDL uptake. SR-A1 is actively involved in Ac-LDL binding and Ac-LDL mediated uptake of cholesterol (123). We have used fucoidan and polyinosinic acid, known ligands for SR-A1 (124) to inhibit the binding of Ac-LDL to SR-A1. As expected fucoidan and polyinosinic acid prevented the Ac-LDL mediated foam cell formation, greatly inhibited the incorporation of  $^3\text{H}$ -cholesterol

in total extracted lipid and in the CE fraction of lipids in Ac-LDL treated cells. However, SR-A1 ligands had no effects on cholesterol or CE/ Lyso PtdCho mixed micelle mediated cholesterol delivery and the accumulation of NBD-cholesterol remained unaffected. Additionally, fucoidan and polyinosinic acid had no inhibitory effects in the incorporation of  $^3\text{H}$ -cholesterol in total extracted lipid and in the CE fraction of lipids when macrophages were incubated with cholesterol/ Lyso PtdCho mixed micelles. Thus the micelle mediated cholesterol or CE delivery utilizes the membrane fusion property of Lyso PtdCho, making this foam cell development technique novel and unique. Furthermore, the universality of micelle mediated delivery was established by developing cholesterol enriched hepatocyte, HepG2 cells (data not shown).

Currently the development of foam cells, used to study RCT, typically involves isolation and acetylation of LDL, followed by incorporation of radiolabeled cholesterol. Isolation of LDL is a tedious process and LDL is prone to be oxidized automatically during the isolation process (90). Isolation and acetylation of LDL can vary qualitatively from one preparation to another, resulting in the induction of inflammatory responses in animal models. Most importantly, Ac-LDL preparations are not stable and cannot be stored for a long time. In our present study we have established that cholesterol or CE/ Lyso PtdCho mixed micelle preparation is functionally stable at 4°C and -20°C up to one month while mixed micelle preparation stored at -20°C is stable for even longer time (data not shown). Furthermore, mixed micelle preparation stored at -20°C for one month was used to develop macrophage foam cells, which were qualitatively similar to those

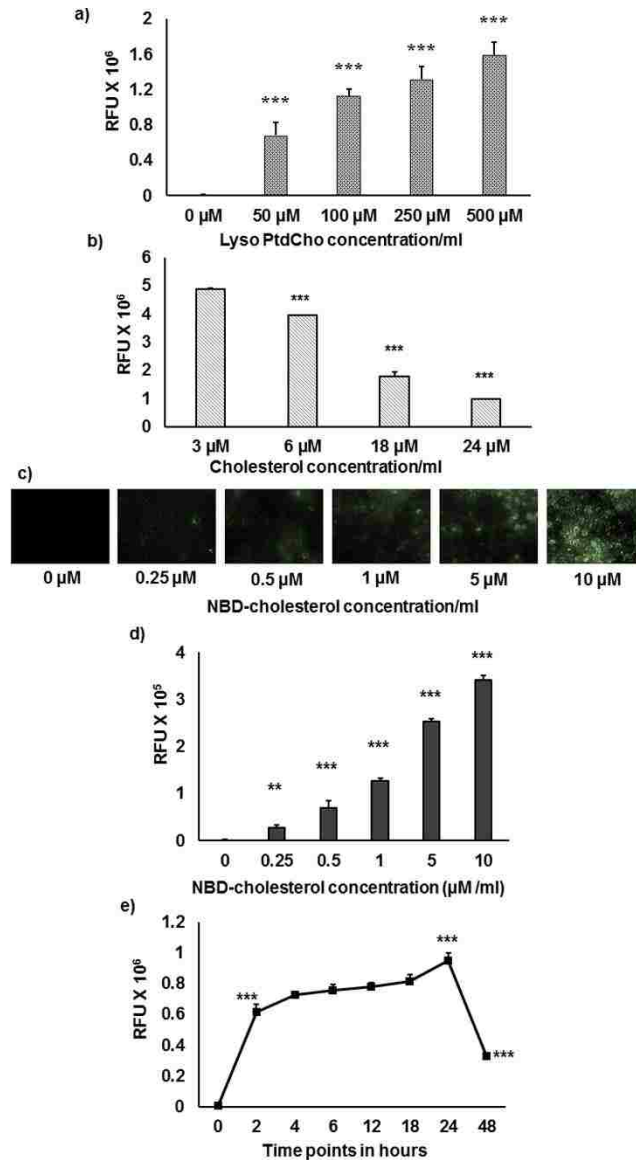
developed with freshly prepared mixed micelles. Quantitative analysis of foam cells developed with mixed micelles stored at 4°C and -20°C up to one month revealed storage time did not affect the accumulation of NBD-cholesterol, CE droplets or incorporation of <sup>3</sup>H-cholesterol in total extracted lipid and in the CE fraction of the lipid. Extraction of lipids from the mixed micelle preparation stored at 4°C and -20°C for several weeks followed by analysis from TLC separation showed no decrease in Lyso PtdCho and cholesterol content (data not shown).

We also observed that mixed micelle mediated cholesterol loading was highly reproducible and the amount of cholesterol incorporated in the total extracted lipid always ranged between 25-27 nmoles when cells were incubated with cholesterol (unlabeled and <sup>3</sup>H-cholesterol) / Lyso PtdCho mixed micelles (data not shown). The same treatment resulted in the incorporation of 1.5- 1.8 nmoles of cholesterol in CE fraction of the lipid when the experiment was repeated five times or more (data not shown).

Foam cells developed by using cholesterol/ Lyso PtdCho mixed micelles were efficiently used to study cholesterol efflux. Increased concentration of HDL caused a concentration-dependent cholesterol efflux from the foam cells and was quantified by measuring the amount of NBD-cholesterol in the medium. For the efflux study, foam cells were also developed using cholesterol (unlabeled and <sup>3</sup>H-cholesterol) / Lyso PtdCho mixed micelles and concentration-dependent efflux of <sup>3</sup>H-cholesterol confirmed our observation to that of NBD-cholesterol efflux. It should also be noted that HDL- mediated cholesterol efflux from foam cells developed by mixed micelles were quantitatively very similar from foam cells

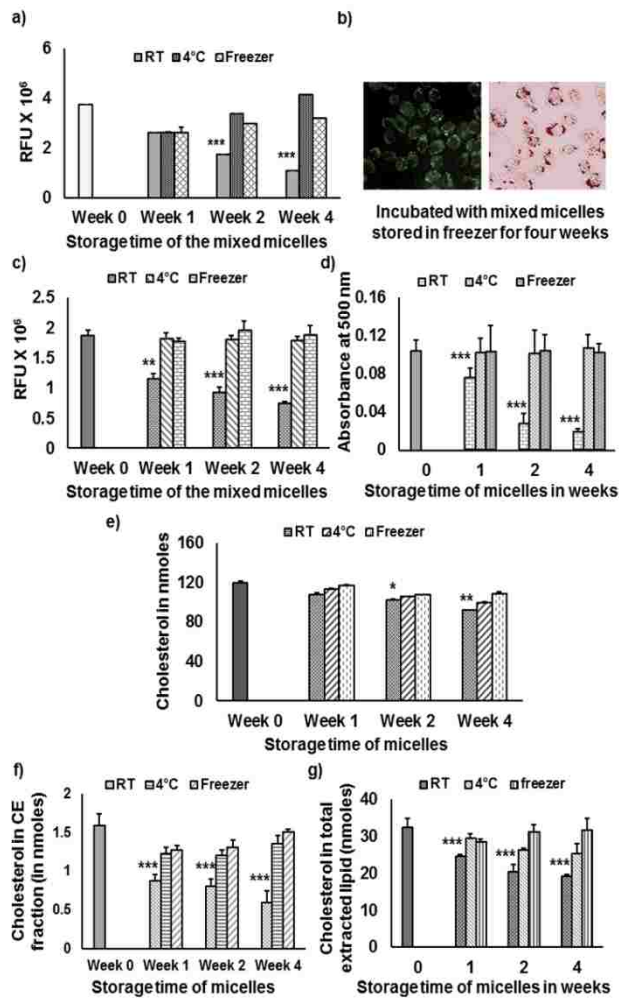
developed by Ac-LDL. Various studies have suggested that oxidation of HDL leads to the loss of its anti-atherogenic property. To determine if the micelle derived foam cells could be utilized to differentiate between native, functional HDL and oxidized, dysfunctional HDL, we used equal concentrations of native and Ox-HDL separately. As we expected Ox-HDL failed to induce any significant cholesterol efflux from the foam cells. Therefore, we conclude that the foam cells developed by using cholesterol/ Lyso PtdCho mixed micelles can be potentially used to study RCT in subjects with or without the background of cardiovascular diseases.

Development of foam cells loaded with fluorescently labeled cholesterol via mixed micelles mediated technique would be less time consuming and highly reproducible. Furthermore, these foam cells could be utilized to screen a large number of human plasma/HDL samples to determine the efficacy of cholesterol efflux from foam cells or RCT.



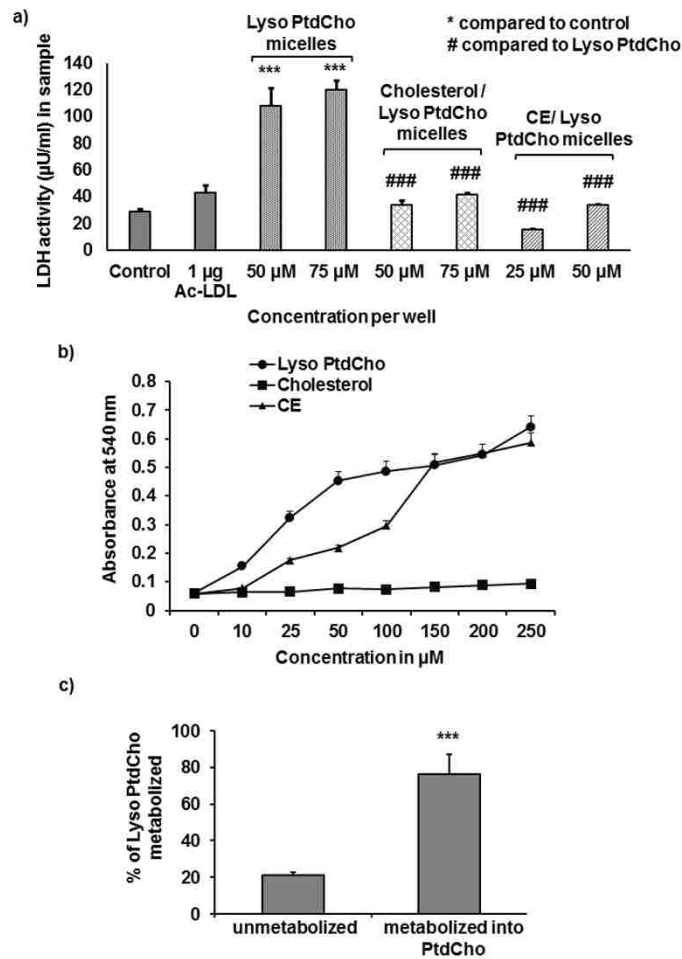
**Figure 7. Solubilization of NBD-cholesterol/ Lyso PtdCho Micelles.**

Cholesterol (unlabeled) and NBD-cholesterol were mixed together and solubilized in presence of Lyso PtdCho to form mixed micelles, a) solubilization of cholesterol and NBD-cholesterol in presence of increasing concentrations of Lyso PtdCho; b) solubilization of NBD-cholesterol is inhibited by cholesterol in a concentration-dependent manner; c) concentration-dependent increased uptake of fluorescent cholesterol by RAW 264.7 macrophages, cells were treated with mixed micelles containing 0- 10 μM NBD-cholesterol, after 18 hrs cells were visualized under fluorescence microscope (40X objectives); d) cells treated as above were lysed using methanol and fluorescence intensity was measured using fluorescence plate reader; e) cells were treated with cholesterol, NBD-cholesterol/ Lyso PtdCho mixed micelles for 0- 48 hrs and fluorescence intensity in the cell lysate were measured. Values are expressed as mean ± SD (n ≥ 3). \*\* P < 0.01, \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test).



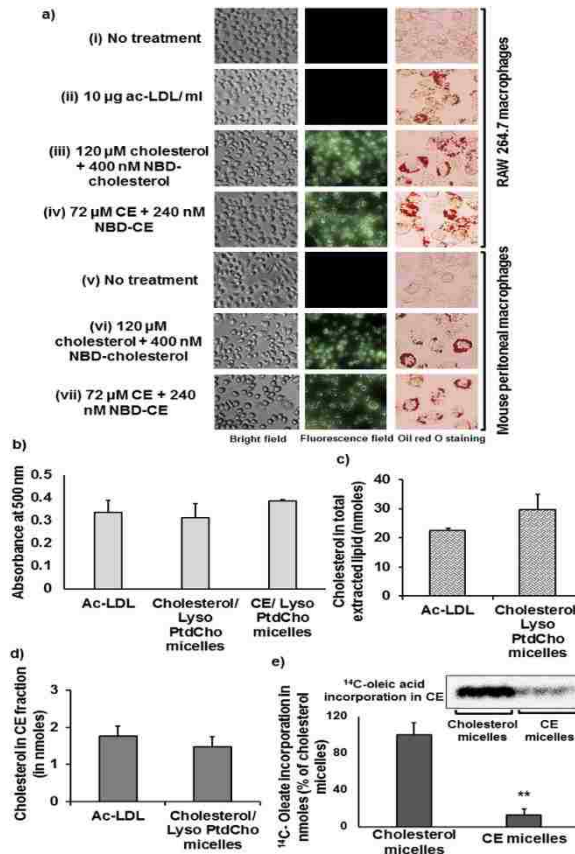
**Figure 8. Stability of NBD-cholesterol/ Lyso PtdCho Micelles.**

a) Cholesterol, NBD-cholesterol/ Lyso PtdCho mixed micelles were stored at room temperature, 4°C and -20°C for week 0- week 4 and fluorescence intensity of the micellized NBD-cholesterol solution stored at different temperatures up to 4 weeks was measured; b) foam cells containing NBD-cholesterol was developed by incubating macrophages with mixed micelles stored at -20°C for four weeks; c) cells were treated with fluorescently labeled mixed micelles stored at room temperature, 4°C and -20°C for week 0- week 4 and fluorescence intensity in the cell lysates were measured; d) absorbance value of Oil red O stain eluted after staining the cells as treated above; e) cholesterol, <sup>3</sup>H-cholesterol/ Lyso PtdCho mixed micelles were stored at room temperature, 4°C and -20°C for week 0- week 4 and radioactivity of the micellized cholesterol solution stored at different temperature up to 4 weeks was measured (count per minute values were converted into nmoles of cholesterol and represented); f) cells were treated with <sup>3</sup>H-cholesterol labeled mixed micelles stored at room temperature, 4°C and -20°C for week 0- week 4 and incorporation of cholesterol in CE fraction were measured; g) incorporation of cholesterol in the total extracted lipid after the treatment of cells was measured as mentioned above. Values are expressed as mean ± SD (n ≥ 3). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test). Significant differences in values for different storage temperatures and time-points are always compared with week 0 value.



**Figure 9. Foam Cells Were Developed Using Cholesterol or CE (Unlabeled) /Lyso PtdCho Mixed Micelles Without Inducing Cytotoxic Effects.**

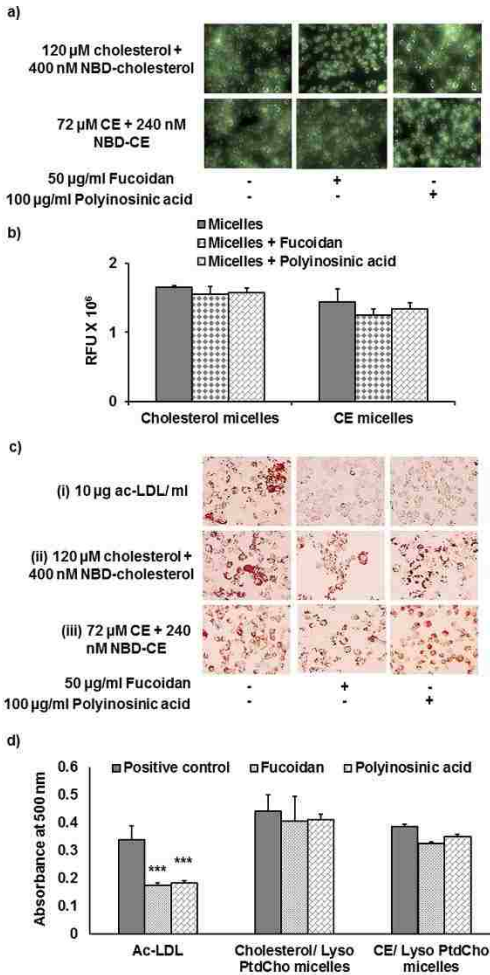
a) Lactate dehydrogenase secreted in the medium was used to quantify LDH activity ( $\mu\text{U}/\text{ml}$ ) in samples treated under different conditions- no treatment, 1  $\mu\text{g}$  Ac-LDL, 50 and 75  $\mu\text{M}$  Lyso PtdCho micelles or cholesterol/ Lyso PtdCho micelles, 25 and 50  $\mu\text{M}$  CE/ Lyso PtdCho micelles per well. P values for samples treated with Lyso PtdCho alone were calculated against control (no treatment), for samples treated with cholesterol or CE /Lyso PtdCho mixed micelles were calculated against the samples treated with Lyso PtdCho alone. Values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*\* or ### P < 0.005 (one-way ANOVA with Bonferroni's multiple comparison test). b) Hemolysis of human RBC was demonstrated by adding increasing concentrations of Lyso PtdCho micelles, cholesterol/ Lyso PtdCho or CE/ Lyso PtdCho mixed micelles to constant concentration of RBC and optical density of the reaction mixture was measured; c) metabolization of  $^{14}\text{C}$ - Lyso PtdCho used as the component of cholesterol/ Lyso PtdCho micelles was demonstrated. Values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*\* P < 0.005 (2-tailed student's t-test).



**Figure 10. Foam Cells Developed Using Mixed Micelles Show Cholesterol Esterification.**

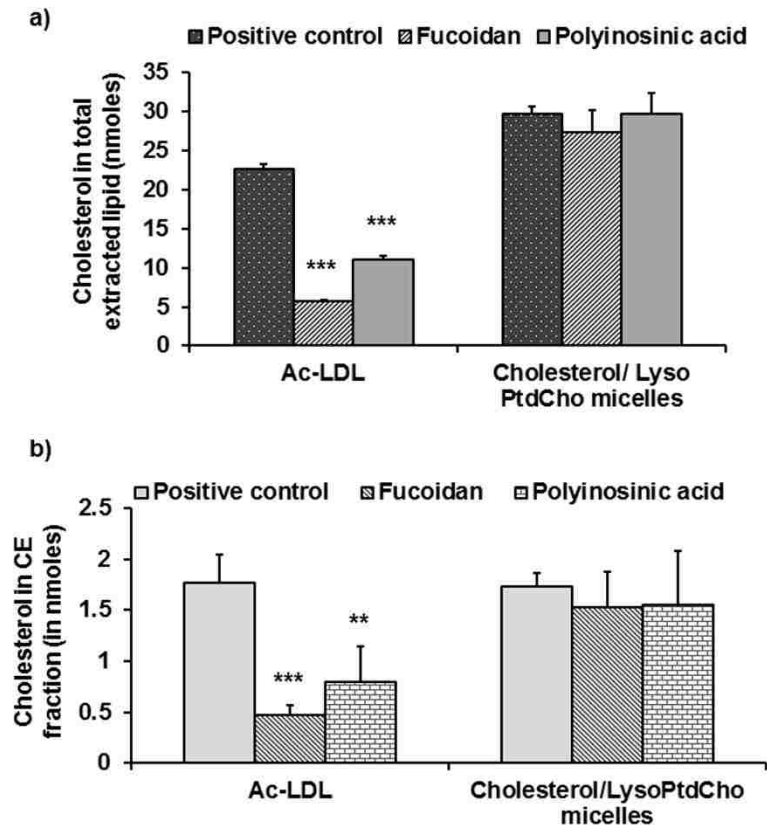
a) RAW 264.7 (panel i- iv) and mouse peritoneal macrophages (panel v-vii) incubated with mixed micelles for 18 hours showed accumulation of NBD-cholesterol (iii- RAW 264.7 macrophages and vi- mouse peritoneal macrophages) and fluorescent CE (iv- RAW 264.7 macrophages and vii- mouse peritoneal macrophages) droplet under fluorescence microscope. CE droplet accumulation demonstrated by Oil Red O staining. CE droplet accumulation was observed in cells treated with Ac-LDL (ii- RAW 264.7 macrophages), cholesterol (iii- RAW 264.7 macrophages and vi- mouse peritoneal macrophages) or CE (iv- RAW 264.7 macrophages and vii- mouse peritoneal macrophages)/ Lyso PtdCho mixed micelles; b) Oil red O stain from RAW 264.7 macrophages treated with Ac-LDL and cholesterol/ Lyso PtdCho or CE/ Lyso PtdCho mixed micelles were eluted and absorbance was measured; c) cells were treated with  $^3\text{H}$ -cholesterol labeled Ac-LDL or cholesterol,  $^3\text{H}$ -cholesterol/ Lyso PtdCho mixed micelles and incorporation of cholesterol in total extracted lipid was measured; d) incorporation of cholesterol in CE fraction was measured following the above mentioned treatment; e) cells incubated with cholesterol or CE / Lyso PtdCho mixed micelles were supplied with 40  $\mu\text{M}$   $^{14}\text{C}$ -oleic acid / ml. Cells incubated with CE / Lyso PtdCho mixed micelles showed almost 10-fold less  $^{14}\text{C}$ -oleic acid incorporation than those treated with cholesterol / Lyso PtdCho mixed micelles. Insert shows radioautography image of  $^{14}\text{C}$ -oleic acid incorporation in CE fraction of cells as treated above. Values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*  $P < 0.01$  (2-tailed student's t-test).





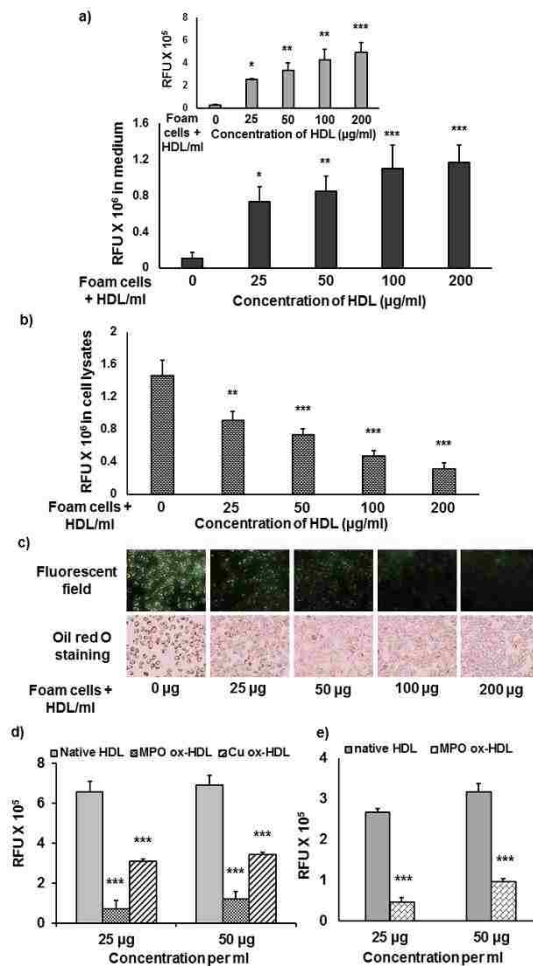
**Figure 11. Mixed Micelle Mediated Foam Cell Formation is not affected by Scavenger Receptor Binding Ligands.**

a) Macrophages were pre-incubated with either 50  $\mu$ g/ ml fucoidan or 100  $\mu$ g/ ml polyinosinic acid, followed by incubation with cholesterol or CE (unlabeled and fluorescently labeled)/ Lyso PtdCho mixed micelles for 18 hours. NBD-cholesterol or fluorescent CE accumulation within macrophages was observed in the cells pre-incubated with fucoidan and polyinosinic acid; b) measurement of fluorescence intensity in cell lysates from cells treated with fluorescently labeled cholesterol or CE with or without fucoidan or polyinosinic acid treatment; c) Macrophages pre-incubated with fucoidan and polyinosinic acid showed very little to no CE droplet accumulation after treatment with Ac-LDL (i). CE droplet accumulation in macrophages pre-incubated with fucoidan and polyinosinic acid was observed after incubation with cholesterol (ii) or CE (iii)/ Lyso PtdCho mixed micelles; d) Oil red O stain was eluted from the cells treated with Ac-LDL and cholesterol or CE/ Lyso PtdCho mixed micelles with or without the pre-incubation of fucoidan or polyinosinic acid and absorption of Oil red O stain at 500 nm was measured. Values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*\*  $P < 0.005$  (one-way ANOVA with Dunnett's multiple comparison test). Significant differences for fucoidan and polyinosinic treatment are always compared with the respective positive control.



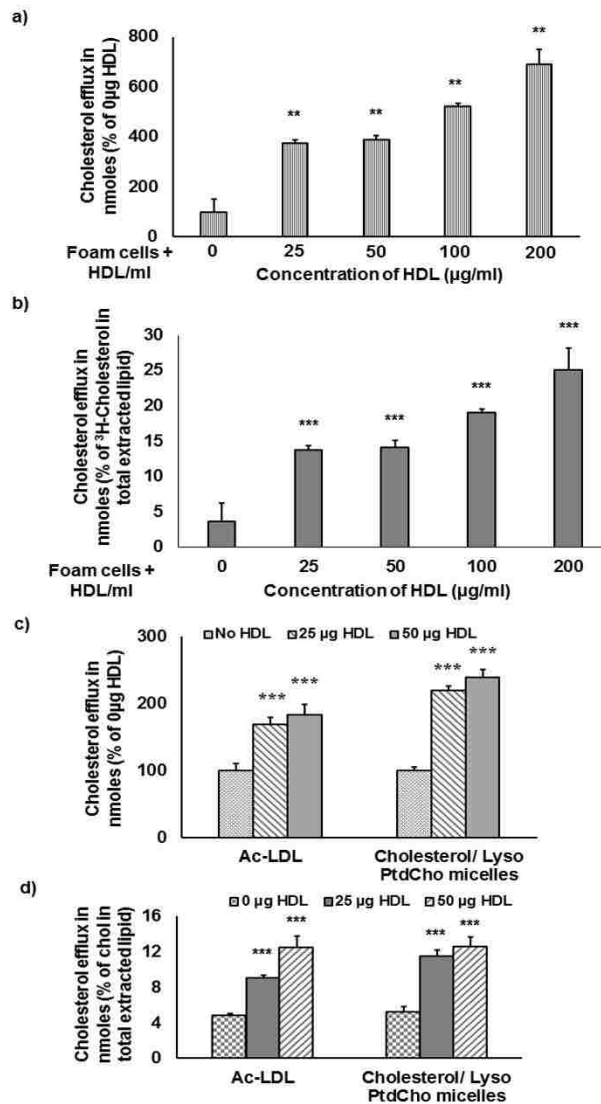
**Figure 12. Incorporation of Cholesterol is not inhibited by Pre- incubation with Fucoidan or Polyinosinic Acid.**

a) RAW 264.7 macrophages were treated with <sup>3</sup>H-cholesterol labeled Ac-LDL or cholesterol, <sup>3</sup>H-cholesterol/ Lyso PtdCho mixed micelles with or without fucoidan and polyinosinic acid pre-incubation and incorporation of cholesterol in total extracted lipid was measured; b) incorporation of cholesterol in CE fraction was measured from cells as treated above. Values are expressed as mean  $\pm$  SD (n  $\geq$  3). \*\* P < 0.01, \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test). Significant differences for fucoidan and polyinosinic treatment are always compared with the respective positive control.



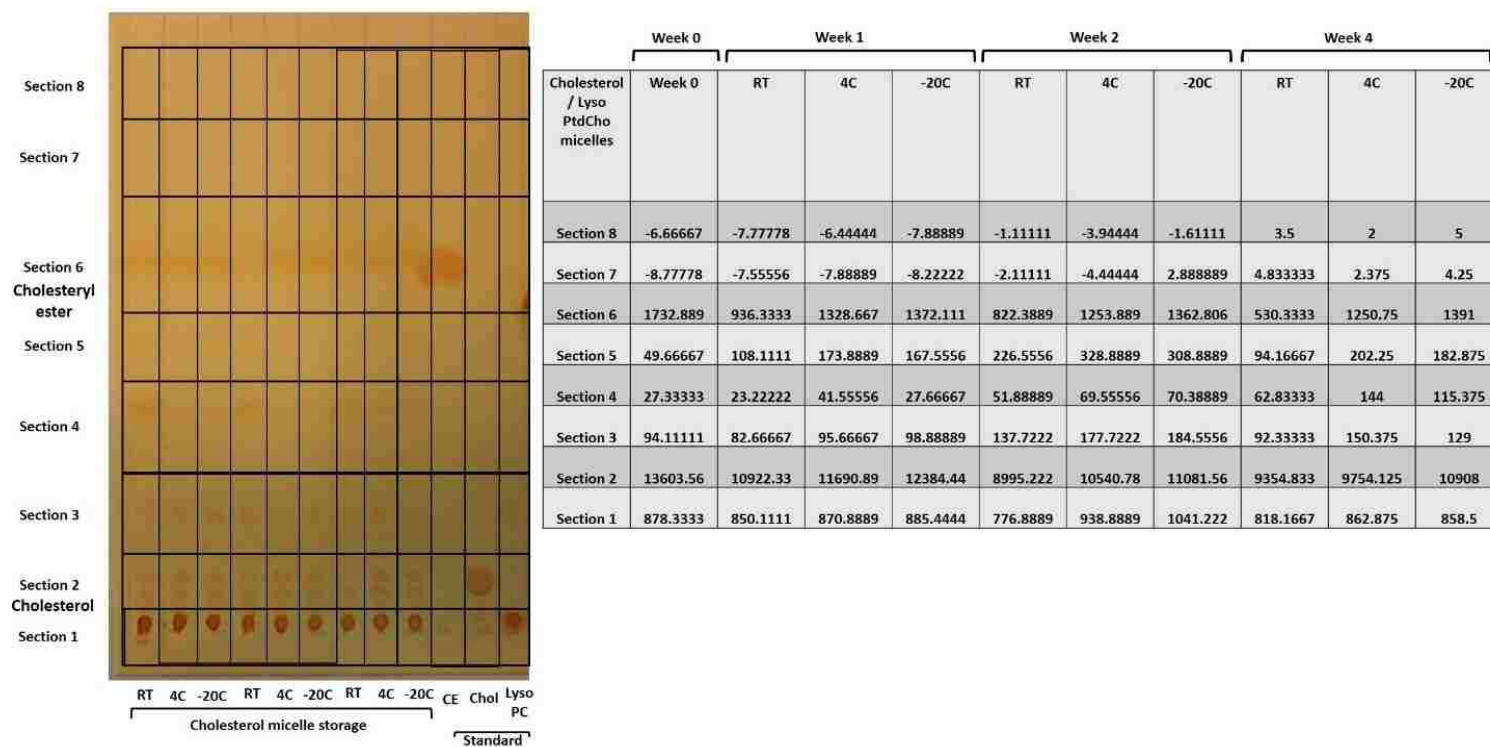
**Figure 13. Cholesterol Efflux From Foam Cells in Presence of HDL.**

Foam cells were developed using cholesterol (unlabeled and NBD-cholesterol or <sup>3</sup>H-cholesterol)/ Lyso PtdCho mixed micelles. Foam cells were incubated with 0- 200 µg/ml of HDL for 4 hours. a) Medium from NBD-cholesterol containing foam cells (RAW 264.7 macrophages) incubated with increasing concentrations of HDL showed concentration-dependent increase in fluorescence intensity. Insert shows mouse peritoneal macrophage derived foam cells incubated with HDL demonstrating concentration-dependent NBD-cholesterol efflux in the medium; b) fluorescence intensity in the corresponding cell lysates decreased as the concentration of HDL increased. \* P < 0.05; \*\* P < 0.01, \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test). c) Fluorescence images and Oil Red O staining showed reduction in CE droplet accumulations in cells after incubation with HDL; d) foam cells were incubated with 25 and 50 µg/ml of native HDL and MPO or Cu mediated Ox-HDL. Cholesterol efflux caused by native HDL was much higher when compared with the same concentration of Ox-HDL. Values are expressed as mean ± SD (n ≥ 3). \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test). Significant differences for Ox-HDL treatments are always compared with the corresponding concentration of native HDL. e) Cholesterol efflux from peritoneal macrophage derived foam cells incubated with 25 and 50 µg/ml of native HDL and MPO mediated Ox-HDL. Values are expressed as mean ± SD (n ≥ 3). \*\*\* P < 0.005 (2-tailed student's t-test).



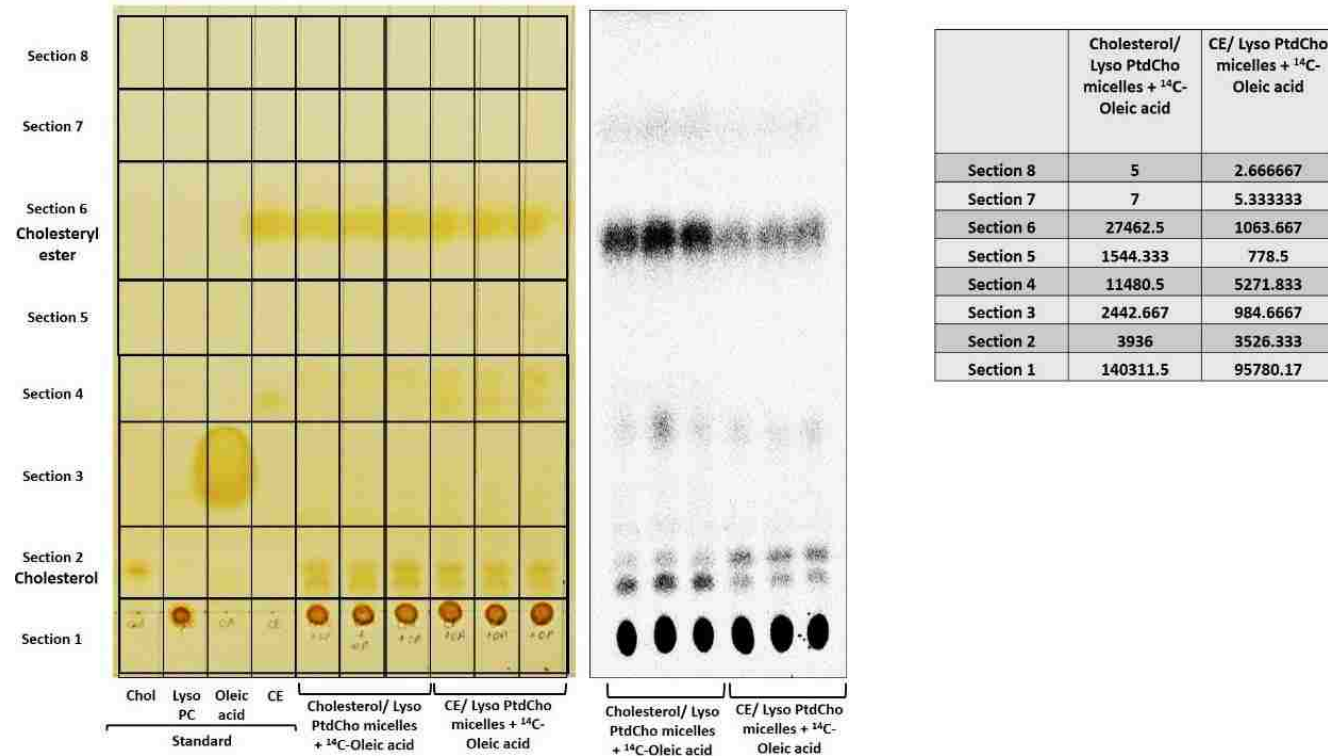
**Figure 14. Ac-LDL and Mixed Micelles Mediated Foam Cells Demonstrated Similar HDL-dependent Cholesterol Efflux.**

a) Foam cells developed by incubation with <sup>3</sup>H-cholesterol containing mixed micelles showed concentration-dependent increase in cholesterol efflux when treated with increasing concentrations of HDL; b) HDL-mediated cholesterol efflux was represented as % of cholesterol incorporated in total extracted lipid; c) foam cells were developed by incubating macrophages with <sup>3</sup>H-cholesterol labeled Ac-LDL or <sup>3</sup>H-cholesterol containing mixed micelles and HDL-mediated cholesterol efflux was studied using 25 and 50 µg/ml of native HDL; d) HDL-mediated cholesterol efflux represented as % of cholesterol incorporated in total extracted lipid. Values are expressed as mean ± SD (n ≥ 3). \*\* P < 0.01, \*\*\* P < 0.005 (one-way ANOVA with Dunnett's multiple comparison test).



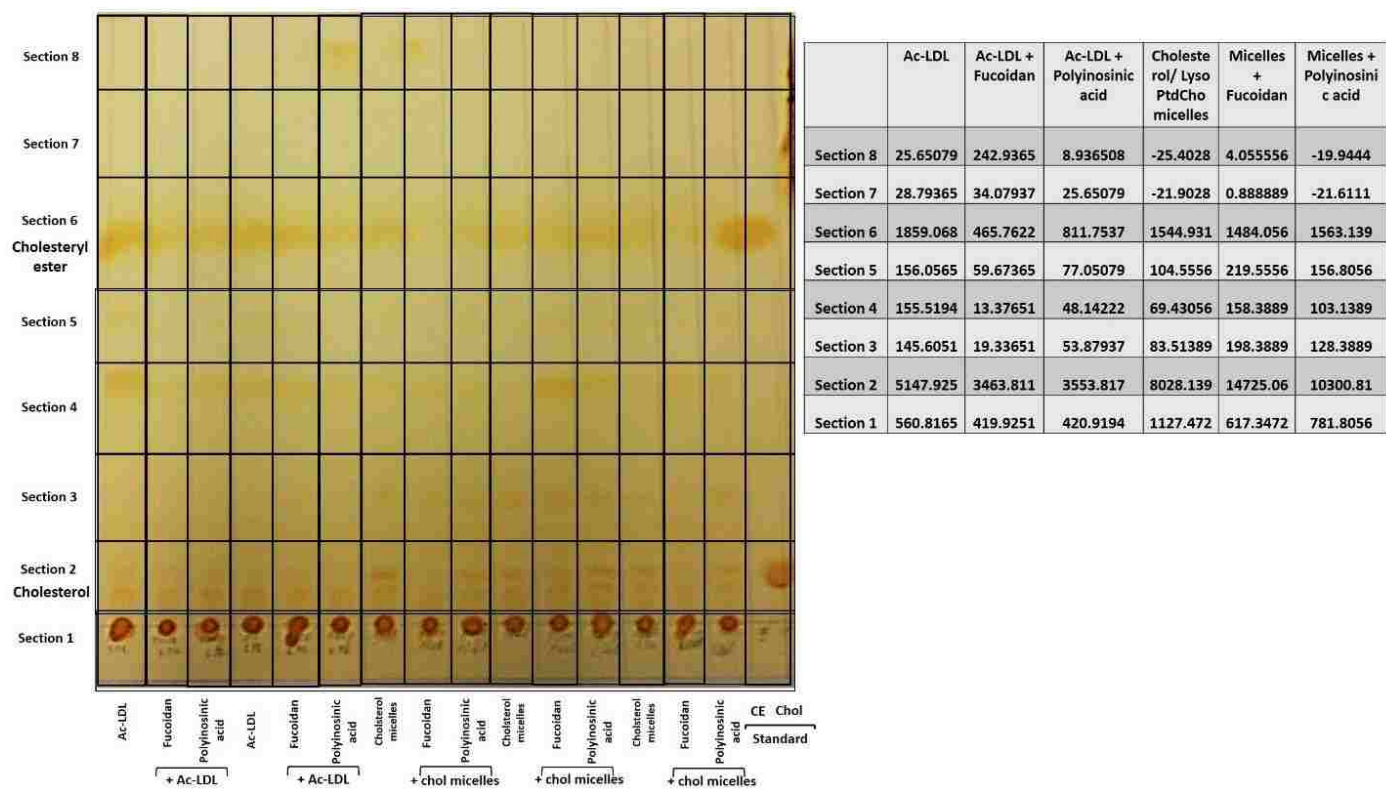
**Figure 15. Extracted Lipid was separated on TLC Plate and TLC Plate Exposed to Iodine Vapor.**

Cholesterol (unlabeled and  $^3\text{H}$ -cholesterol)/ Lyso PtdCho mixed micelles stored at room temperature,  $4^\circ\text{C}$  and  $-20^\circ\text{C}$  for Week 0, Week 1, Week 2 and Week 4 were used to incubate RAW 264.7 macrophages for 18 hours. Extracted lipid was used to run TLC with cholesterol, cholesteryl ester and Lyso PtdCho standards and one of the representative TLC plate was shown here. TLC plate was cut into eight sections for each sample and radioactivity measurement was taken as shown in the table.



**Figure 16. Extracted Lipid was separated on TLC Plate and TLC Plate Exposed to Autoradiography Imager Followed by Exposure to Iodine Vapor.**

Cholesterol/ Lyso PtdCho and CE/ Lyso PtdCho mixed micelles along with <sup>14</sup>C-Oleic acid were used to incubate RAW 264.7 macrophages for 18 hours. Extracted lipid was used to run TLC with cholesterol, cholesteryl ester, oleic acid and Lyso PtdCho standards. TLC plate was cut into eight sections for each sample and radioactivity measurement was taken as shown in the table.



**Figure 17. Extracted Lipid was separated on TLC Plate and TLC Plate Exposed to Iodine Vapor.**

<sup>3</sup>H-cholesterol tagged ac-LDL and cholesterol (unlabeled and <sup>3</sup>H-cholesterol)/ Lyso PtdCho mixed were used to incubate RAW 264.7 macrophages with or without fucoïdan or polyinosinic acid for 18 hours. Extracted lipid was used to run TLC with cholesterol and cholesteryl ester standards. TLC plate was cut into eight sections for each sample and radioactivity measurement was taken as shown in the table.

## **CHAPTER 3: PLASMA FROM HEART FAILURE PATIENTS WITH NORMAL EJECTION FRACTION HAVE BETTER HDL FUNCTIONALITY AS COMPARED TO SUBJECTS WITH POOR EJECTION FRACTION**

### **Introduction**

Population studies from the past few decades have clearly demonstrated that plasma HDL has an inverse relationship with the progression of cardiovascular disease. Some of the atheroprotective functions of HDL are associated with, but not limited to, its role in RCT and the prevention of LDL oxidation (101).

RCT can be considered as the main defense mechanism against atherosclerotic plaque development. The mechanism of RCT involves efflux of cholesterol from the peripheral tissue and lipid-laden macrophages to extracellular cholesterol acceptor HDL, and the transport of cholesterol to liver for recycling or disposal *via* excretion. ABCA1 and ABCG1 are the major transporters involved in the efflux of cholesterol from macrophages. ABCA1 mediated lipid transport involves lipid-free or lipid-poor form of HDL, which contains mostly apolipoproteins. The models for ABCA1 associated lipid transport suggest that binding of ABCA1 with apoA-I induces efflux of cholesterol from the plasma membrane. However, ABCA1 can bind to a wider range of apolipoproteins such as apoA-II, apoA-IV, apoE and apoC. Lipidation of apolipoproteins with cholesterol and phospholipids produces pre- $\beta$  migrating HDL particles which continue to be acceptors of cholesterol effluxed via ABCA1 (59, 73, 98, 128). ABCG1 mediated cholesterol efflux mostly involves mature  $\alpha$ -migrating HDL and ABCG1 associated pathway accounts for 6-12% of the free cholesterol efflux from the cell membranes (62).



Plasma HDL density varies between 1.06- 1.21 g/ml and the population of human plasma HDL constitutes a heterogeneous group of particles which can be distinguished based on their size, density, electrophoretic mobility, lipid and apolipoprotein composition. Mature HDL can be categorized into 2 major populations based on the separation done by ultracentrifugation, i) large, spherical HDL<sub>2</sub> and ii) small, dense, protein-rich HDL<sub>3</sub>. Several studies investigating the characteristics of HDL<sub>3</sub> revealed that majority of the apoA-I in the plasma associated with HDL<sub>3</sub> and this sub-fraction of HDL is also enriched with several anti-oxidant enzymes such as PON1, PAF-AH or lipoprotein associated phospholipase A2 and LCAT. Thus, HDL<sub>3</sub> is believed to play a critical role not only in RCT but also in the anti-oxidative functions associated with HDL (49, 129). Additionally, HDL, especially HDL<sub>3</sub> is also known to act as an anti-apoptotic factor and protects macrophages from Ox-LDL induced apoptosis by stimulating cholesterol efflux (130).

Studies from the last two decades strongly suggested that HDL prevents the oxidative modification of LDL and Ox-LDL induced cytotoxicity which are the crucial events to initiate atherosclerotic plaque development (101). Co-incubation with HDL not only decreased the metal ion or cell induced oxidation of LDL, but also reduced the uptake of modified LDL by macrophages. HDL acts as a scavenger for lipid peroxides along with preventing the formation of lipid peroxides (131, 132). Association of HDL with various enzymes is believed to be responsible for its anti-oxidative properties. One of the most potent enzymes involved in the anti-oxidative activities of HDL is PON1. Serum PON1 is a calcium dependent

esterase, synthesized and secreted by the liver. PON1 catalyzes the hydrolysis of organophosphates, insecticides, aromatic carboxylic acid esters, carbamates and nerve gas and produces less toxic products (133). HDL provides stability to this 43-KDa enzyme after its secretion from liver. The affinity of apoA-I to PON1 for binding is very high and apoA-I is believed to stimulate the lactonase activity of PON1. Previous studies indicated that PON1 protects LDL from oxidation and stimulates cholesterol efflux from macrophages (134, 135). Protection of LDL from oxidation is believed to be mediated by PON1 in 2 separate ways. Firstly, during the metal ion or cell induced oxidation, PON1 inhibits the accumulation of lipid peroxides and secondly, PON1 eliminates the pre-formed lipid peroxides (136). Moreover, PON1 attenuates the uptake of Ox-LDL by macrophages *via* SR CD36 (134), prevents macrophage cholesterol biosynthesis and cholesterol accumulation (137). Therefore, PON1 can be considered as the major contributory factor to the anti-atherogenic properties of HDL. Anti-oxidative activities of HDL are also associated with the functions of PAF-AH, LCAT, and several apolipoproteins such as apoA-I, apoA-II, apoA-IV, apoE and apoJ (129).

Modifications of structure and composition of HDL play a crucial role in the progression of several pathological conditions and the alterations of HDL are often associated with inflammation, oxidative stress, atherosclerosis and hyperlipidemia. One of the major modifications that severely affects the proper functioning of HDL is alteration apoA-I. During acute inflammation, the secretion of apoA-I from the liver is often decreased or apoA-I on HDL is replaced by SAA (47, 138).

Previous studies by several groups indicated that the replacement of apoA-I by SAA significantly impairs the cholesterol efflux capacity of HDL. Although, the native HDL present during acute inflammatory response or SAA containing HDL seemed to bind to macrophages with a higher affinity, they are less effective to induce free cholesterol efflux from the macrophages under these conditions (138-140). Furthermore, it has been observed that the acute phase HDL or SAA containing HDL promote CE uptake by monocytes and macrophages and thus enhance the process of foam cell formation (139). Acute phase HDL or SAA containing HDL also have reduced capacity to bind to hepatocytes and deliver cholesterol for clearance from the body (141). Therefore, during acute inflammation or due to replacement of apoA-I by SAA, HDL become pro-atherogenic as they promote CE influx to peripheral tissues, become less effective to induce cholesterol efflux from macrophages and demonstrate reduced association with hepatocytes which is essential for the final steps of RCT. ApoA-I is also susceptible to oxidation by metal ions *in vitro* and by MPO enzyme *in vivo*. Recently the oxidation of HDL associated with MPO has received a lot of attention. MPO is a neutrophil granulocyte derived peroxidase enzyme that catalyzes the formation of hypochlorous acid from chloride ions and nitrogen dioxide radicals by using hydrogen peroxide and nitric oxide as oxidizing agents. *In vitro* studies have showed that MPO catalyzes the chlorination and nitration of specific tyrosine residues on apoA-I to form chlorotyrosine and nitrotyrosine respectively (142, 143). Moreover, plasma HDL isolated from patients with CAD revealed higher concentrations of chlorotyrosine and nitrotyrosine per apoA-I than those present in

subjects without the history of CAD (144, 145). Oxidation of apoA-I impairs its ability to induce free cholesterol efflux from macrophages. Previous studies have suggested chlorination of tyrosine residue 192 on apoA-I disables the strong association between apoA-I and ABCA1 transporter which is essential for the efflux of cholesterol to lipid poor or lipid free apoA-I and nascent HDL (88, 142). Additionally, chlorination of apoA-I prevents the activation of LCAT (88) and thus oxidation of HDL affects RCT in a 2-fold way. It has also been suggested that oxidation of apoA-II impairs the antioxidant properties of HDL (102).

HF is one of the most common, disabling and costly public health problem and is complex in nature. HF is associated with high rate of morbidity and mortality. Population studies show that 30-40% of the patients diagnosed with heart failure, die within a year while 60-70% of the mortality happens within 5 years. HF happens following the gradual and severe muscle weakness/ stiffening in the heart and inefficient pumping of the blood. As the muscle weakness progresses, the organs and peripheral tissues receive less amount of oxygenated blood and the pressure in the heart gradually increases. EF refers to the percent of the total amount of blood in the left ventricle, which is pumped out with each heartbeat. A normal heart's EF varies between 55-70% and EF below 40% is a strong indicator of HF.

Our preliminary studies indicated that the peripheral blood (PB) plasma from the human HF patients with low EF had high levels of Ox-LDL accumulation when compared with the PB plasma from the HF patients with normal EF. The goal of this study was to determine if the accumulation of higher amount of Ox-LDL is a reflection of the quality of the HDL. The quality and the functionality of HDL was

investigated by performing cholesterol efflux assays with foam cells, generated *in vitro* using cholesterol/Lyso PtdCho mixed micelles (28). Additionally, PON1 arylesterase activity of the plasma samples was assessed to evaluate the functionality of the patients' HDL as an anti-atherogenic lipoprotein.

## **Materials and Methods**

### *Reagents:*

RPMI 1640, FBS, sodium pyruvate, L-glutamine, PS, 1X PBS, NBD-cholesterol and Hank's balanced salt solution (HBSS) were bought from Invitrogen Life Technologies (Carlsbad, CA). Cholesterol, calcium chloride (CaCl<sub>2</sub>), magnesium chloride (MgCl<sub>2</sub>), Lyso PtdCho from egg yolk, oleic acid, 0.9% saline and p-Nitrophenyl acetate (p-NPA) were purchased from Sigma-Aldrich (St. Louis, MO). Acetone, isopropyl alcohol and ethyl alcohol were purchased from VWR international (Randor, PA). [1, 2- <sup>3</sup>H (N)] cholesterol was bought from American Radiolabeled Chemicals (St. Louis, MO).

### *Cell Culture:*

RAW 264.7 macrophages were cultured in RPMI 1640 medium containing 10% FBS, 1% PS, 1% sodium pyruvate and 1% L-glutamine. Cells were seeded at 3 X 10<sup>5</sup>/ 250 µl density in the 48-well plate and incubated at 37° C in a 5 % CO<sub>2</sub> incubator to reach 65-70% confluence overnight. For the experiments involving foam cell development RPMI 1640 with 0.1% LPDS was used as incubation medium.

*Generation of Foam Cells in vitro:*

Foam cells were generated *in vitro* as described previously (146). Briefly, RAW 264.7 macrophages were incubated with cholesterol (unlabeled and NBD or <sup>3</sup>H-cholesterol)/Lyso PtdCho mixed micelles for 18 hours in presence of 40 μM oleic acid.

*Human Subjects:*

Following the Ohio State University School of Medicine Institutional Review Board (IRB) approval, blood was collected from 48 patients (consented) undergoing routine cardiovascular surgery [coronary artery bypass grafting (CABG), valve repair/replacement (AVR, MVR, TVR: aortic, mitral, or tricuspid valve replacements) or other open heart surgeries] at The Ohio State University Medical Center. Simpson's bi-plane technique was used to determine left ventricular ejection fractions. Two ml of left ventricular blood (LVB) was collected by aspirating blood using a 23 gauge needle and 10cc syringe placed through the ventricular apex. Simultaneously 5 ml of PB was collected from a peripheral venous line. Blood was collected in heparinized tubes and labeled with number associated with the study. Plasma was separated immediately after collection by centrifugation at 3000 rpm for 20 minutes. Isolated plasma were stored at -80°C for further analysis. No patients had clinical evidence of active ischemia at the time of blood sampling.

For the control group, peripheral blood was collected from the subjects undergoing overnight fasting. Blood was collected from the left-hand index finger

using blood lancet in a heparinized tube and plasma was isolated as described previously.

*Exclusion Criteria:*

Subjects who were on antioxidant supplements (vitamin E, C, beta carotene and lipoic acid) were excluded from the study. All the patients were optimized with medical management prior to surgery and these included the use of  $\beta$ -blockers, ACE inhibitors, statins and diuretic therapy. The use of such therapies themselves could affect the built-up of Ox-LDL. As per the clinical criteria HF subjects not older than 85 years and EF less than or equal to 70% were included for this study.

For the control group, the subjects were between 30-65 years of age and did not have any history of HF or never underwent any routine cardiovascular surgery.

*Cholesterol Efflux Study:*

Raw 264.7 macrophages were washed with sterile PBS twice after overnight incubation with cholesterol/Lyso PtdCho mixed micelles. Macrophage foam cells were incubated with 400  $\mu$ l of HBSS and 5  $\mu$ l plasma samples for 4 hours. At the end of 4 hour incubation, HBSS was collected from each well and efflux of labeled cholesterol was measured. HBSS containing no plasma and incubated with foam cells (NBD or  $^3$ H-cholesterol labeled) was used for background value in the cholesterol efflux experiments.

The HBSS and plasma samples incubated with foam cells containing  $^3\text{H}$ -cholesterol were collected and efflux of  $^3\text{H}$ -cholesterol was measured in 100  $\mu\text{l}$  aliquots using liquid scintillation and luminescence Counters (MicroBeta2 Plate Counter, PerkinElmer, Waltham, MA). For the cholesterol efflux study involving  $^3\text{H}$ -cholesterol, CPM value was converted into nmoles of cholesterol and expressed as the unit of measurement. When the cholesterol efflux study was performed using NBD-cholesterol containing foam cells, HBSS containing plasma samples were subjected to de-proteination steps.

*De-proteination of Samples:*

The HBSS and plasma samples used for cholesterol efflux study involving NBD-cholesterol was not suitable for direct fluorescence measurement. The high concentration of protein present in the plasma samples interferes with the fluorescence measurement and samples have to be subjected to de-proteination.. For the de-proteination procedure, 400  $\mu\text{l}$  of HBSS with or without the plasma samples were collected in glass test tubes at the end of the 4 hour incubation with foam cells. 1 ml of ice-cold acetone and isopropyl alcohol (vol: vol, 1:1) was added to HBSS with or without the plasma samples. The solution mixture was incubated at 4°C for 30 minutes and centrifuged at 400 g for 10 minutes. Precipitated protein pellet was visible at the bottom of the glass tube. Acetone: isopropyl alcohol phase of the supernatant was evaporated under nitrogen gas at 30°C without disturbing the protein precipitate. After evaporating the organic solvent phase, 100  $\mu\text{l}$  aliquots of the supernatants were taken and fluorescence was measured using



fluorescence plate reader (Envision 2014 Multilabel Plate Reader, PerkinElmer, Waltham, MA). Fluorescence of effluxed NBD-cholesterol was measured at emission spectra of 535 nm upon excitation at 485 nm.

*PON1 Assay:*

The plasma PON1 arylesterase activity was measured by following the method of Jaichander et al (147). Briefly, 10  $\mu$ l aliquots of human plasma was incubated with 1 mM p-NPA in 100  $\mu$ l phosphate buffer with 2 mM CaCl<sub>2</sub> and MgCl<sub>2</sub> at 37°C for 30 minutes. The reaction was set up in 96-well plate and the reaction for each patient plasma sample was set up in triplicates. p-NPA was used as the substrate for PON1 enzyme activity and the resultant product p-Nitrophenol conversion was measured at 410 nm.

*Statistical Analysis:*

Each experiment was performed more than three times and each experimental condition was set up in triplicates. All values were presented as mean  $\pm$  standard deviation. Analysis of variance (ANOVA) was applied with Bonferroni's correction for multiple comparisons using GraphPad Prism 5.0 software (San Diego, CA). The minimum level of significance in all tests was  $p < 0.05$ .

## Results

### *PB Plasma from Patients with Lower EF Showed Reduced Cholesterol Efflux:*

Foam cells generated with cholesterol/Lyso PtdCho mixed micelles were used for cholesterol efflux studies in presence of PB plasma from patients. The patient group with EF >60% showed almost 3-fold higher cholesterol efflux than the group with EF <40% (Fig. 18). This pattern was observed when the cholesterol efflux studies were performed with either NBD-cholesterol (Fig. 18a) or <sup>3</sup>H-cholesterol (Fig. 18b).

### *LVB and PB from the Same Patient Showed Different Level of Cholesterol Efflux:*

The cholesterol efflux study was done using PB and LVB from the same groups of patients. As mentioned earlier, the cholesterol efflux assay performed with PB demonstrated that the group of patients with lower EF showed less efficient cholesterol efflux when compared with the group of patients with normal EF. However, the cholesterol efflux studies using LVB from both of these groups showed very similar cholesterol efflux values [both with NBD-cholesterol (Fig. 18a) and <sup>3</sup>H-cholesterol (Fig. 18b)] and these values were very close to the efflux pattern observed with the PB plasma of the low EF group. Interestingly, the group with EF >60% showed higher levels of the cholesterol efflux with their PB plasma compared to the LVB. In contrast, the group of patients with EF <40% showed similar levels of cholesterol efflux with both, the PB and LVB blood, and the values of cholesterol efflux were distinctively much lower than the observed with the PB plasma of the normal EF group. There was no significant difference between the

cholesterol efflux capacity of LVB plasma used from patients with lower and normal EF.

*Plasma Samples with Reduced Cholesterol Efflux Capacity Also Showed Reduced PON1 Activity:*

PB and LVB plasma samples from the same groups of patients were used to assay plasma PON1 arylesterase activity. Conversion rate of p-NPA to p-Nitrophenol in a time period of 30 minutes was regarded as measurement of PON1 activity. The results obtained with the PB plasma showed that patients with normal EF had significantly higher PON1 activity compared to patients with lower EF. However, similar to the results obtained with the RCT assays, both groups showed a very similar pattern of PON1 activity when LVB plasma was used. On the other hand, the comparison of PB and LVB plasma from the same group of patients showed that PON1 activity was much lower in LVB plasma than in PB plasma in the group with EF >60% (Fig.19), whereas PON1 activity varied very little between the PB and LVB plasma samples from the group of patients with EF <40%.

*PB Plasma from Control Subjects Showed Much Higher Cholesterol Efflux than Both Groups of Patients:*

Cholesterol efflux study was done using plasma from PB plasma collected from the control subject group. Following the same method used with the patients' plasma samples, control plasma samples were incubated with foam cells containing <sup>3</sup>H-cholesterol for 4 hours. The results obtained with the assay showed that there was a wide variation in the values of cholesterol efflux among the control

group. However, even with the high standard deviation the control group showed a significantly higher cholesterol efflux value when compared with the cholesterol efflux values obtained with the PB plasma from HF patients with lower EF (Fig. 22). The cholesterol efflux capacity observed in the PB plasma from the control group and the patient group with normal EF (>60%) were not very different.

### **Discussion**

The loss of anti-atherogenic functions of HDL has been studied extensively in the last few decades. One of the major consequences of the dysfunctionality of HDL is impairment of HDL induced cholesterol efflux, clearance of free cholesterol and thus RCT. While aging is thought to be responsible for the reduced antioxidant activity and RCT capacity of HDL (148), oxidation of HDL and modification of HDL during acute inflammatory response have received tremendous attention. Among different subpopulations of HDL, HDL<sub>3</sub> is believed to have most of the cardioprotective functions associated with HDL. HDL<sub>3</sub> is most effective in stimulating cholesterol efflux from macrophages. Independent studies have showed that age-related reduced efficiency and metal-ion induced oxidation, hugely impaired the capacity of HDL<sub>3</sub> to induce cholesterol efflux (148-150). Loss of functionality of HDL is also known to be associated with MPO mediated oxidation of apoA-I. Oxidative modification of apoA-I impairs the interaction between apoA-I and ABCA1 and the cholesterol efflux is affected.

Dysfunctionality of HDL often refers to the loss of its antioxidative function. Antioxidant properties of HDL, in the context of protecting LDL from oxidative

modification, are closely related to the enzymes harbored by or associated with HDL. PON1 is an HDL associated enzyme and plays an essential role as an antioxidant enzyme. PON1 is secreted from the liver and its interaction with apoA-I provides stability to this enzyme as HDL acts as the hydrophobic harbor. PON1 catalyzes hydrolysis of lipid peroxides, fatty acid peroxides and hydrogen peroxides to prevent LDL modification (151). HDL associated PON1 prevents the oxidation of LDL in several ways- by inhibiting the formation of conjugated dienes, peroxides and by decomposing highly cytotoxic aldehydes. Additionally, PON1 protects HDL itself from oxidative stress (136, 152) and helps HDL to maintain its function associated with RCT (136). PON1 is also believed to prevent oxidized phospholipid mediated monocyte chemotaxis and macrophage activation (86), thiolactone associated endothelial injury and fatty streak formation and improves the capacity of HDL to induce cholesterol efflux from macrophages (47, 86). Plasma concentration of PON1 is markedly reduced under several pathological conditions such as diabetes, hypercholesterolemia and myocardial infarction (136). Recent study by Deakin et al (152), showed that oxidation of apoA-I associated with HDL impairs the capacity of HDL to stimulate the secretion of PON1 from liver and activation of the enzyme. This generates a vicious cycle in which PON1 fails to protect HDL from oxidative stress and makes it particularly prone to chlorination (152). As a result, HDL becomes less effective as an antioxidant and fails to protect LDL from oxidative modification.

All the patients included in this study were undergoing various routine cardiovascular surgeries and were categorized into 2 groups based on their left-

ventricular EF. Our preliminary results showed that the patients with low left-ventricular EF had higher Ox-LDL content in their PB plasma when compared with the patients with normal EF values (unpublished data). This observation led to the hypothesis that HDL functionality would vary between these two groups, as oxidative modification of LDL is attenuated by the antioxidative functions of HDL. The goal of this study was to evaluate the quality of HDL by performing cholesterol efflux assay.

Foam cells containing fluorescently labeled or radiolabeled cholesterol were generated by incubating RAW 264.7 macrophages with cholesterol (unlabeled and NBD or <sup>3</sup>H-Cholesterol)/ Lyso PtdCho mixed micelles for 18 hours in the presence of 40  $\mu$ M oleic acid (146). Foam cells were incubated with PB plasma from 2 different groups of patients with low and normal EF for 4 hours and efflux of cholesterol was measured. When the assay was performed with foam cells containing NBD-cholesterol, we observed that proteins present in the plasma were interfering with the measurement of the effluxed NBD-cholesterol. These samples were subjected to a deproteination procedure in order to precipitate the protein and then the amount of effluxed NBD-cholesterol was measured. As expected, cholesterol efflux assay performed with foam cells containing <sup>3</sup>H-cholesterol did not require any additional treatment before the amount of effluxed cholesterol was measured in the medium. However, the results obtained from both of these assays were very similar in terms of amount of effluxed cholesterol. The PB plasma from the patients with normal range of EF induced approximately 1.5-fold more cholesterol efflux than that from the patients with low range of EF (Fig.18). This

observation corroborated our previous observation regarding the content of Ox-LDL present in the PB plasma.

EF is an indicator of normal functioning of the heart as the EF value refers to the amount blood being pumped out of the blood with each contraction. The capacity of the heart to pump out the optimum amount of blood can be diminished for various reasons and current studies show that HF is common even with preserved systolic function or normal EF (153). Chronic heart disease conditions are often associated with increased oxidative stress biomarkers such as lipid peroxides and malondialdehyde in the blood (154, 155). In accordance to these previous findings we observed that PB plasma from the HF patients with low EF showed increased sign of oxidative stress than the patients with normal EF. The presence of high level of Ox-LDL in the PB of patients with low EF could be explained by the compromised quality of HDL. Reduced cholesterol efflux capacity of the HDL present in the PB plasma of the group with lower EF indicated the possibility of and oxidative modification of HDL. Furthermore, to evaluate the quality of HDL a standard PON1 assay was performed. As expected, the PB from the group with normal EF showed more than 2-fold increased PON1 activity than that from patients with low EF (Fig. 19). This observation not only confirmed our initial evaluation regarding the quality of HDL present in the PB, it also explained why different amounts of Ox-LDL were present in the blood of these 2 groups. As PON1 is known to be the major HDL associated enzyme that protects LDL from oxidative modification, the low PON1 activity observed in the PB plasma of patients with low EF validated the higher content of Ox-LDL. On the other hand, the PB

plasma from the patients with normal EF showed higher cholesterol efflux capacity indicating better HDL functionality, and higher PON1 activity in the corresponding plasma corroborated the lower content of Ox-LDL.

As we expected, the concentration of HDL observed in the plasma of these patients did not represent the functionality of HDL. In fact, the average HDL concentration found in the patients with low EF (36.3 mg/dL) was slightly higher than that found in the patients with normal EF (36 mg/dL). However, when the cholesterol efflux capacity and PON1 activity associated with HDL were compared, it became clear that patients with normal EF have higher amount of functional HDL than that found in the patients with low EF (Table 1.). From this observation it can be concluded that the measurement of cholesterol efflux capacity or PON1 activity is a much better indicator of HDL quality and functionality than the concentration of HDL alone.



**Table 1. Cholesterol Efflux Capacity and PON1 Activity Associated with HDL from Patients with Low and Normal EF.**

| Subject identifier | Ejection Fraction (%) | Plasma HDL concentration (mg/dL) | Cholesterol efflux (nmoles) | PON1 activity (nmoles/ min/ ml) |
|--------------------|-----------------------|----------------------------------|-----------------------------|---------------------------------|
| Sample 22          | 25-30                 | 34                               | 2.23                        | 24.78                           |
| Sample 23          | 30-35                 | 51                               | 2.37                        | 32.77                           |
| Sample 26          | < 20                  | Unknown                          | 2.57                        | 31.65                           |
| Sample 27          | < 20                  | 47                               | 2.89                        | 26.40                           |
| Sample 43          | 35                    | 28                               | 3.37                        | 26.90                           |
| Sample 46          | 23                    | 43                               | 2.83                        | 30.96                           |
| Sample 52          | 20-25                 | 25                               | 3.05                        | 27.53                           |
| Sample 53          | 17                    | 39                               | 3.17                        | 34.71                           |
| Sample 56          | 25                    | 7                                | 2.60                        | 33.15                           |
| Sample 58          | < 20                  | Unknown                          | 2.96                        | 29.28                           |
| Sample 70          | 30-35                 | 52                               | 2.76                        | 30.40                           |
| Sample 71          | 23                    | 37                               | 2.58                        | 29.03                           |
| Sample 35          | 60-65                 | 22                               | 5.42                        | 75.00                           |
| Sample 37          | 68                    | 45                               | 4.83                        | 65.45                           |
| Sample 42          | 67                    | 38                               | 4.66                        | 60.58                           |
| Sample 50          | 63                    | 69                               | 5.93                        | 67.29                           |
| Sample 44          | 61                    | 24                               | 5.21                        | 58.18                           |
| Sample 55          | 60-65                 | 25                               | 4.91                        | 71.82                           |
| Sample 61          | 60-65                 | Unknown                          | 4.78                        | 66.57                           |
| Sample 65          | 60-65                 | 33                               | 5.64                        | 91.95                           |
| Sample 67          | 60-65                 | 42                               | 4.87                        | 76.15                           |
| Sample 94          | 60                    | 47                               | 4.97                        | 75.09                           |
| Sample 111         | 60-65                 | 28                               | 4.88                        | 60.30                           |
| Sample 114         | 60-65                 | 23                               | 5.34                        | 59.43                           |

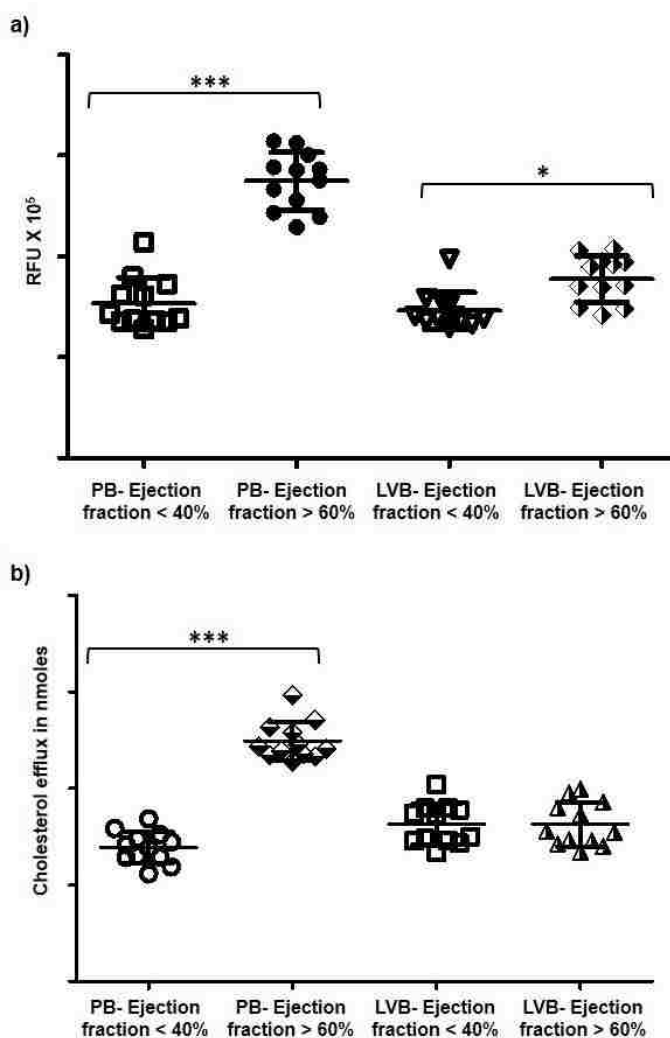
Surprisingly, the cholesterol efflux assay and the PON1 assay using the left-ventricular blood plasma collected from these 2 groups led to equivocal conclusions. LVB plasma from both groups showed low cholesterol efflux capacity and low PON1 activity. The cholesterol efflux capacity of PB and LVB plasma collected from the patient group with low EF were almost in the same range (Fig.

20a). The same was true for PON1 activity assay (Fig. 21a). However, the LVB plasma collected from the patients with normal EF gave very different profiles of cholesterol efflux capacity and PON1 activity when compared with the results obtained by using the PB plasma collected from the same group of patients. The cholesterol efflux capacity (Fig. 20b) and the PON1 activity (Fig. 21b) in the LVB plasma from the patients with normal EF were much lower than those observed in their PB plasma, which might indicate that there is more oxidative modification of the HDL in LVB plasma than in PB plasma. This unexpected observation arises several interesting questions. Use of PB plasma alone to study cholesterol efflux and associated quality of HDL might not be sufficient to predict the cardiovascular health of a person. Is there a repair mechanism present in our body that attenuates or reverses the oxidative stress related modifications of lipoproteins present in the blood circulating in the peripheral tissue?

It would be interesting to note here that the PB plasma collected from the control group subjects showed a much higher cholesterol efflux capacity than that from the groups of patients with low EF (Fig. 22). In accordance to our exclusion criteria this is not surprising as the control group subjects did not have any history of chronic heart diseases. However, to determine the quality of HDL and to evaluate the conditions associated with oxidative stress in the control group subjects, more studies needed to be performed. The average blood cholesterol level found in the control group was much higher (58.25 mg/dL) than either of the HF patient group included in the study and can be considered to be in the range to have cardioprotective functions. Nonetheless, the cholesterol efflux capacity of

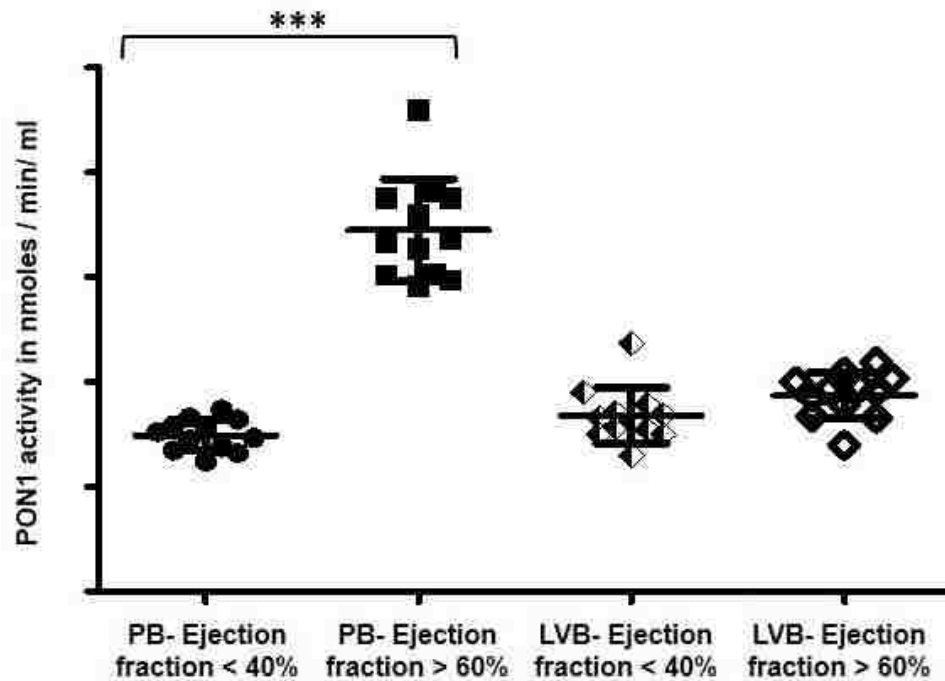
the PB plasma from the control group and the group with normal EF were very similar even though the concentration of HDL varied widely (average HDL concentration in the control group: 58.25 mg/dL and in the HF patient group with normal EF: 36 mg/dL).

Our initial studies indicated that the PB from the patients with low left-ventricular EF showed elevated sign of oxidative stress when compared with that from the patients with normal EF. Further investigation revealed that patients with low EF had more amount of dysfunctional HDL in the peripheral and LVB when compared with the PB collected from patients with normal EF. Functionality of HDL was evaluated by performing cholesterol efflux assay and by assaying PON1 enzyme activity. All the results taken together, we concluded that patient group with low EF had markedly reduced amount of functional HDL.



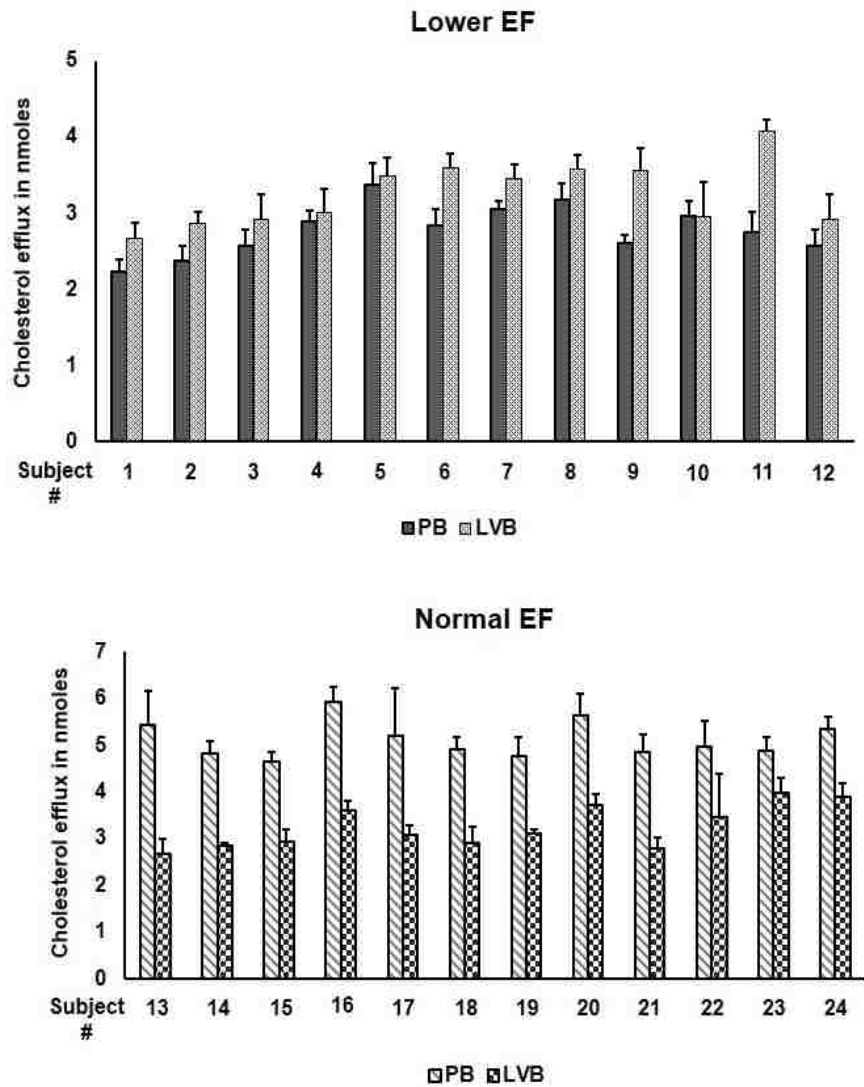
**Figure 18. PB Plasma from the Patients with Lower EF Showed Lower Cholesterol Efflux Capacity.**

a) PB and LVB plasma samples from patients with low and normal EF were incubated with foam cells loaded with NBD-cholesterol and the efflux of NBD-cholesterol was measured following deproteination of the samples; b) PB and LVB plasma samples from the 2 groups of patients were incubated with <sup>3</sup>H-cholesterol containing foam cells and the efflux of <sup>3</sup>H-cholesterol was measured, CPM values were converted to nmoles of effluxed cholesterol. Cholesterol efflux associated with PB plasma from patients with normal EF was compared with that from patients with low EF. Similarly, cholesterol efflux associated with the LVB plasma from the 2 groups were analyzed. All the samples were analyzed in triplicates and values are expressed as mean  $\pm$  SD (n  $\geq$  3). \*\*\* < 0.005 (one-way ANOVA with Bonferroni's multiple comparison test).



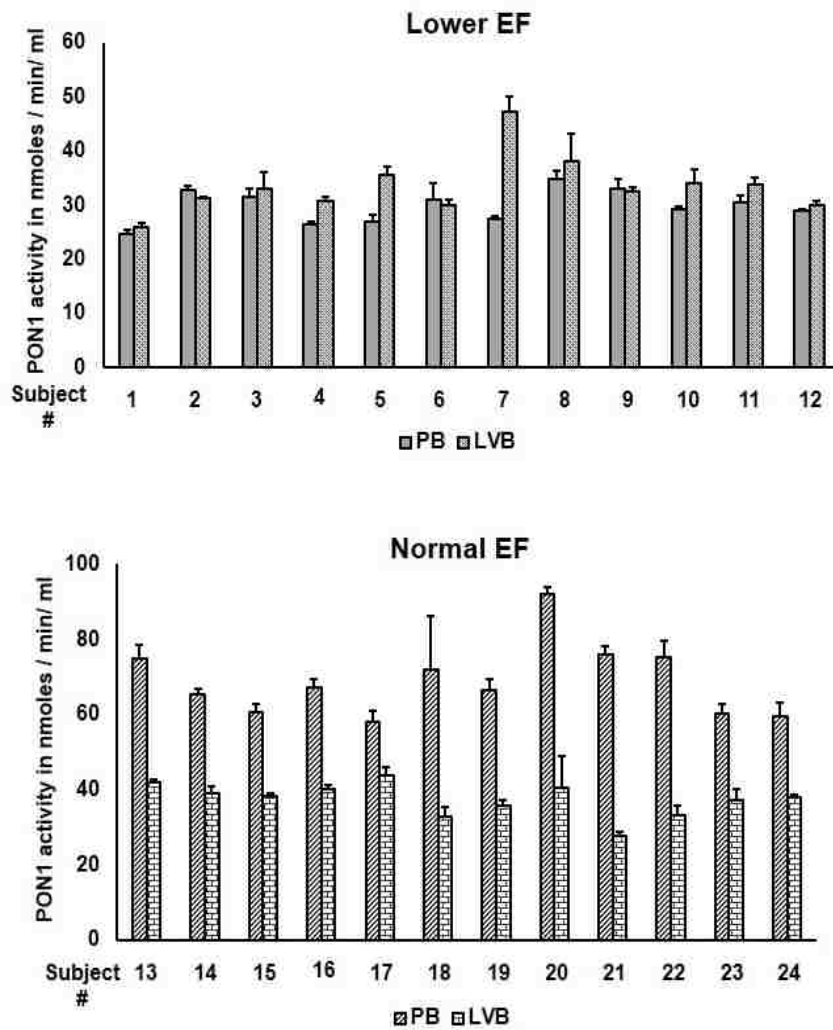
**Figure 19. PB Plasma from the Patients with Normal EF Showed Higher PON1 Activity.**

PB and LVB plasma samples from patients with low and normal EF were analyzed for HDL associated PON1 enzyme activity and the conversion of p-NPA to Nitrophenol was measured by reading absorbance at 410 nm. PON1 activity associated with PB plasma from patients with normal EF was compared with that from patients with low EF. The comparison of PON1 activity related to LVB plasma between the 2 groups was done similarly. All the samples were analyzed in triplicates and values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*\*  $< 0.005$  (one-way ANOVA with Bonferroni's multiple comparison test).



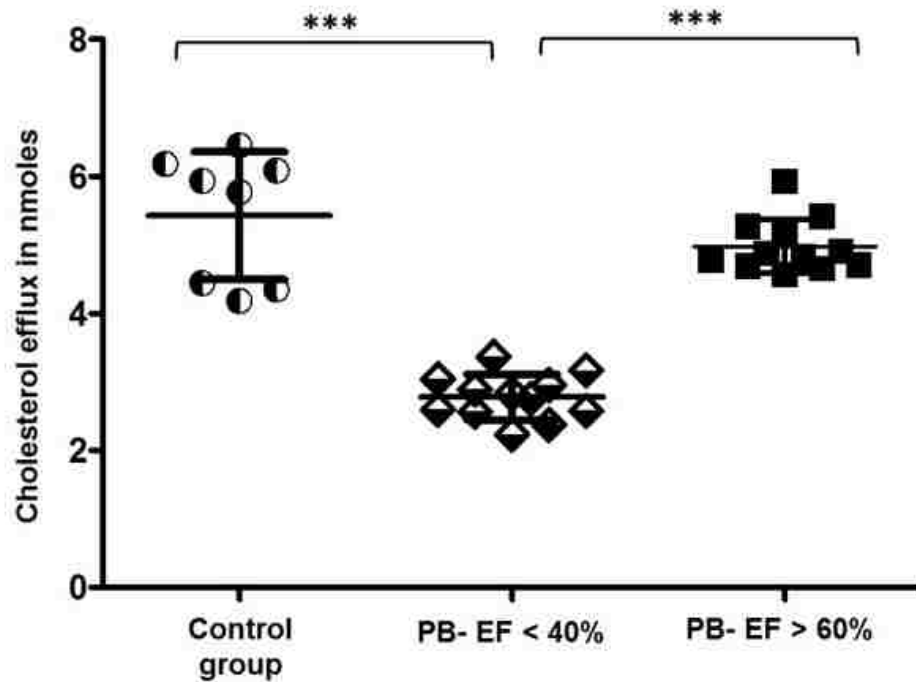
**Figure 20. Peripheral and LV Blood Plasma from the Same Patient with Low EF and/ or Normal EF were Compared in Terms of Capacity of Cholesterol Efflux.**

a) <sup>3</sup>H-cholesterol efflux associated with peripheral and LVB plasma from the patient group with low EF were measured and cholesterol efflux capacity of the PB plasma and the LVB plasma taken from the same patient was compared; b) Similarly, cholesterol efflux induced by the PB plasma and the LVB plasma from the patients in the normal EF group were compared. All the samples were analyzed in triplicates and values are expressed as mean  $\pm$  SD (n  $\geq$  3).



**Figure 21. PON1 Activity Associated with the PB Plasma and the LV Blood Plasma were Compared for each Patient Belonging to either Low or Normal EF Group.**

a) PON1 activity related to the PB plasma and the LVB plasma was compared for each patient in the low EF group; b) Similarly it was done for the patients belonging to the normal EF group. All the samples were analyzed in triplicates and values are expressed as mean  $\pm$  SD ( $n \geq 3$ ).



**Figure 22. Cholesterol Efflux Capacity Associated with Control Group with No History of Chronic Heart Disease and Patients Groups with Low and Normal EF were Compared.**

PB plasma from the control group and the peripheral and LV blood plasma samples from the 2 groups of patients were incubated with  $^3\text{H}$ -cholesterol containing foam cells and the efflux of  $^3\text{H}$ -cholesterol was measured. Cholesterol efflux associated with the PB plasma from the control group was compared with peripheral and LVB plasma from patients with low and normal EF respectively. All the samples were analyzed in triplicates and values are expressed as mean  $\pm$  SD ( $n \geq 3$ ). \*\*\* < 0.005 (one-way ANOVA with Bonferroni's multiple comparison test).



## CHAPTER 4: CONCLUSION AND DISCUSSIONS

Lipoproteins which are intricately associated with atherosclerosis play crucial roles in the formation or regression of atherosclerotic plaque. While high levels of plasma LDL are directly related to the risk of increased plaque burden, the relationship between HDL and the regression of the plaque burden is more complex. Increased concentration of plasma LDL leads to the initiation of a series of events which are pro-inflammatory in nature. Secretion of pro-inflammatory factors and cytokines stimulate differentiation of the monocytes to the adherent macrophages and expression of SRs. Modified LDLs, such as Ac-LDL or Ox-LDL act as ligands for these SRs and the uptake of modified LDLs by SRs or other mechanisms starts the events leading to atherosclerotic plaque formation. Macrophages loaded with unesterified and esterified cholesterol, known as foam cells, are the key components of these plaques. To study various aspects of atherosclerotic plaque development and RCT, foam cells are regularly generated *in vitro*. *In vitro* generation of foam cells traditionally involves incubation of macrophages with Ac-LDL or Ox-LDL probed with radiolabeled cholesterol. However, as described in the chapter 2, there are several drawbacks associated with the use of isolated and modified LDL, used for this purpose. One of the most important restraints of this technique is its limited use in the clinical set up. As described before, LDL is hard to isolate and even harder to store for long time without causing unwanted modifications to the lipoprotein. Thus, developing an

assay technique which can be used in the doctors' office or diagnostic clinic is really hard when LDL is used to generate foam cells.

RCT is a unique defense mechanism of our body that prevents or restricts atherosclerotic plaque formation. HDL is the key player involved in RCT. HDL with or without the help of transporters and receptors induce efflux of cholesterol from macrophage foam cells and delivers the cholesterol to the liver for excretion. Studies from several independent laboratories in the last decades of the 20<sup>th</sup> century strongly established the inverse relationship between HDL and cardiovascular diseases. Initially it had been thought that the quantity of plasma HDL is the most important indicator to diagnose CAD and the higher is the concentration of plasma HDL, less are the chances of atherosclerotic plaque development. However, the last few years of research indicated that the relationship between HDL and atherosclerosis is much more complex.

HDL can be considered as the 'guardian angel' which often multi-tasks to protect the cardiovascular health of our body. Apart from inducing cholesterol efflux and preventing plaque formation, HDL prevents oxidation of LDL and exerts anti-inflammatory, anti-apoptotic and anti-thrombotic effects. In other words, if LDL is called pro-atherogenic, HDL can definitely be referred as anti-atherogenic in function. Nonetheless, the anti-atherogenic properties of HDL are closely associated with the quality of this lipoprotein and not its quantity in the serum. Oxidation of HDL renders it dysfunctional and often pro-atherogenic. Ox-HDL fails to prevent LDL oxidation and is less effective in inducing cholesterol efflux from the foam cells. Oxidation of HDL is associated with the oxidation of its major

apolipoprotein, apoA-I, the oxidation of its anti-oxidant enzyme PON1 and often the displacement of apoA-I or PON1 with SAA. While the quantification of PON1 or SAA gives an indirect evaluation of the quality of HDL, measuring the efficacy of cholesterol efflux in the presence of HDL is the most direct approach to determine HDL quality.

For the last several years, different approaches have been taken to quantify cholesterol efflux or RCT and assess the quality of HDL from subjects with or without the history of CAD. No matter what the approach is, these studies start with the generation of *in vitro* foam cells. In this study, we intended to develop an assay which can quickly and effectively measure cholesterol efflux from foam cells using patients' plasma samples. Furthermore, our goal was to develop an assay which can be used in the doctors' office or in the diagnostic labs very efficiently without compromising the quality of the results.

The longest and very tedious step in foam cell generation is the isolation of LDL. Our goal was to enrich macrophages with free or esterified cholesterol in a faster and more efficient way. To overcome the biggest obstacle of solubilizing the highly hydrophobic cholesterol in aqueous solution, we have used Lyso PtdCho. Lyso PtdCho is an amphiphilic biological detergent which has been used by our laboratory before to enrich cells with highly non-polar  $\beta$ -carotene. We manipulated this technique further to deliver cholesterol or CE to the macrophages. We established that the foam cells generated by this technique were physiologically relevant by doing lipid droplet staining and quantifying the accumulation of cholesterol in the total lipid fraction and CE fraction. These foam cells were also

enriched with fluorescently labeled or radiolabeled cholesterol. We further established that micelle mediated foam cells generation is a highly reproducible technique.

One of the crucial drawbacks for Ac-LDL or Ox-LDL generated foam cells is the instability of the reagent. Ac-LDL or Ox-LDL are highly unstable and need to be used within 4-5 days since isolation. We showed that the micelles containing cholesterol (unlabeled and labeled) and Lyso PtdCho were highly stable at 4°C and -20°C for more than 4 weeks. It was further established that foam cells generated with stored micelles are qualitatively and quantitatively similar to the ones generated with freshly prepared micelles.

The generation of foam cells with mixed micelles was not a cytotoxic technique. Lyso PtdCho, which is a cytolytic detergent, was metabolized into non-cytotoxic PtdCho during micelle uptake by the macrophages. Unlike Ac-LDL or Ox-LDL mediated foam cell generation, micelle mediated foam cell development was completely independent of SR associated uptake. It is important to mention that generation of foam cells with mixed micelles is a very rapid technique and the cells were enriched with unlabeled and labeled cholesterol within 4-6 hours of incubation with the micelles. For the diagnostic screening purpose it would be crucial as the whole assay can be completed within 12 hours starting from the preparation of mixed micelles. We utilized the micelle generated foam cells to evaluate the cholesterol efflux induced by native and Ox-HDL. As expected, cholesterol efflux induced by native HDL was much higher in quantity when compared with the same concentrations of Ox-LDL associated cholesterol efflux.

Based on this observation, we hypothesized that these foam cells could be further utilized to screen patient samples to identify subjects with high or low level of Ox-HDL. Samples from human subjects with no history of CAD were used as the control group. The 2 other groups of patient samples belonged to human subjects undergoing routine cardiovascular surgeries [coronary artery bypass grafting (CABG), valve repair/replacement (AVR, MVR, TVR: aortic, mitral, or tricuspid valve replacements) or other open heart surgeries]. Among these, one group of patients had normal range (55-70%) of left-ventricular EF, while the other group had clinically defined low range (<40%) of left-ventricular EF.

Our preliminary studies revealed that the patient group with low EF showed higher level of Ox-LDL in the blood plasma when compared with that with normal EF. As HDL protects LDL from oxidation and oxidized status of LDL is closely associated with the quality of HDL, we decided to evaluate the quality of HDL by performing cholesterol efflux assay using micelle mediated foam cells. The cholesterol efflux study clearly showed that the amount of cholesterol effluxed from the foam cells in presence of plasma from patients with normal EF was much higher than in the presence of plasma samples from the group with low EF. On average the efflux of cholesterol upon incubation of foam cells with PB plasma from patients with normal EF was ~1.6 fold higher. However, the amount of effluxed cholesterol from the foam cells after incubating with PB plasma from the control group showed a significantly higher value than the rest of the groups. We concluded that the patients with low EF had a higher level of dysfunctional or Ox-HDL in their PB as the plasma failed to induce efficient cholesterol efflux when

compared with the control group or the patient group with normal EF. We further concluded that patients with normal range of EF have lower level of Ox-HDL in their PB.

To evaluate the quality of HDL we further performed PON1 assay. PON1 assay is another indirect method to investigate the quality and the functionality of HDL beside the measurement of Ox-LDL. The results from PON1 assay clearly reflected our previous observation. Patients with low EF showed lower PON1 activity in the PB plasma when compared with the patients with normal EF. As inactivation of PON1 can be a direct consequence of oxidation, which indicates oxidation of HDL, estimation of PON1 activity further elucidates the quality of HDL. However, left-ventricular blood plasma from both groups of patients showed low level of cholesterol efflux, low PON1 activity and high level of Ox-LDL. This finding was surprising for the group with normal EF range as the left-ventricular blood plasma appeared to have more Ox-HDL than their PB plasma.

This project was designed to develop a quick and efficient assay to measure and evaluate the quality and functionality of HDL. Our goal was to develop a screening assay which can be utilized by the doctors' office or by diagnostic clinics to screen a large number of patient samples to evaluate the efficiency of their RCT. We successfully generated physiologically relevant *in vitro* foam cells and utilized them to screen patient samples to evaluate the quality of HDL. This novel technique is highly reproducible and reliable.

This novel technique of delivering macromolecules to the cells can be further manipulated to deliver other hydrophobic large molecular cargos to the cells.

**APPENDIX A:  
IACUC APPROVAL LETTER**





Office of Research & Commercialization

10/24/2012

Dr Sampath Parthasarathy  
Burnett School of Biomedical Sciences  
Lake Nona  
6900 Lake Nona Blvd  
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr Sampath Parthasarathy:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #: 12-04

Title: Isolation and Studies on macrophages from the peritoneal cavity of mice

First Approval Date: 1/20/2012

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call me at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

A handwritten signature in blue ink that reads "Cristina Caamaño".

Cristina Caamaño  
Assistant Director

Copies: Facility Manager (when applicable.)

12201 Research Parkway • Suite 501 • Orlando, FL 32826-3246 • 407-823-3778 • Fax 407-823-3299

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THE UNIVERSITY OF CENTRAL FLORIDA  
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)  
*Re-Approval to Use Animals*

Dear Dr Sampath Parthasarathy,

Your application for IACUC Re-Approval has been reviewed and approved by the UCF IACUC Committee Reviewers.

Approval Date: 10/23/2012

Title: Isolation and Studies on macrophages from the peritoneal cavity of mice

Department: Burnett School of Biomedical Sciences

Animal Project #: 12-04

Expiration: 1/19/2014

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expiration date regarding your need to file another renewal.

Christopher Parkinson, Ph.D.  
IACUC Chair

Approved  Renewed

**APPENDIX B:  
IRB APPROVAL LETTER**



**Biomedical Institutional Review Board**

Office of Responsible Research Practices  
300 Research Foundation  
1960 Kenny Road  
Columbus, OH 43210-1063

Phone (614) 688-8457  
Fax (614) 688-0366  
[www.orrp.osu.edu](http://www.orrp.osu.edu)

October 30, 2008

Protocol Number: 2007H0139  
Protocol Title: STUDY TO DETERMINE THE PRESENCE OF OXIDIZED LOW DENSITY LIPOPROTEIN (OX-LDL) AND ITS DEGRADATION PRODUCTS IN THE BLOOD AND LEFT VENTRICULAR TISSUES OF PATIENTS WITH HEART FAILURE, Sampath Parthasarathy, Susan D. Moffatt-Bruce, Chittoor B. Sai-Sudhakar, Benjamin Sun, Thoracic Surgery

Request for change(s) to the protocol dated September 30, 2008 (Add Chang and Jones as key personnel, delete Talbot at key personnel, add 40 participants, and revise consent for blood to be obtained after patient is placed on cardiopulmonary bypass).

Type of Review: Amendment  
Approval Date: October 28, 2008  
IRB Staff Contact: Jennifer Spohn  
247-1562  
spohn.31@osu.edu

**Dear Dr. Parthasarathy,**

The Biomedical IRB **APPROVED** the above referenced protocol.

Note that if applicable, informed consent (and HIPAA research authorization) must be obtained from subjects or their legally authorized representatives and documented prior to research involvement. The IRB-approved consent form and process must be used. Changes in the research (e.g., recruitment procedures, advertisements, enrollment numbers, etc.) or informed consent process must be approved by the IRB before they are implemented (except where necessary to eliminate apparent immediate hazards to subjects).

It is the responsibility of all investigators and research staff to promptly report to the IRB any serious, unexpected and related adverse events and potential unanticipated problems involving risks to subjects or others.

This approval is issued under The Ohio State University's OHRP Federalwide Assurance #00006378.

All forms and procedures can be found on the ORRP website - [www.orrp.osu.edu](http://www.orrp.osu.edu). Please feel free to contact the IRB staff contact listed above with any questions or concerns.

Karla Zadnik, OD, PhD, Chair  
Biomedical Institutional Review Board

bs-001-03 Approval Amend  
Version 01/01/07

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