2020

Effects of food additives and sugar substitutes on the biogenesis of high-density lipoproteins

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

EFFECTS OF FOOD ADDITIVES AND SUGAR SUBSTITUTES ON THE BIOGENESIS OF HIGH-DENSITY LIPOPROTEINS

by

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B.S., Boston University, 2018

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

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ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Haya Herscovitz, for giving me the opportunity to work with her and constantly pushing me to achieve greater heights. I would also like to thank my advisor, Dr. J Fernando Garcia-Diaz, for always believing in me.

EFFECTS OF FOOD ADDITIVES AND SUGAR SUBSTITUTES ON THE BIOGENESIS OF HIGH-DENSITY LIPOPROTEINS

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ABSTRACT

Lipoproteins are macromolecular assemblies of lipids and proteins that are essential for the transportation of the water insoluble lipids in the circulation. The major lipoproteins, are low density lipoproteins (LDL) and high-density lipoproteins (HDL). LDL, also called the "bad cholesterol", delivers cholesterol to peripheral tissues and is directly correlated with increased risk of cardiovascular disease, stroke and type II diabetes. HDL, on the other hand, is called the "good cholesterol" as it removes excess cholesterol from peripheral tissues for elimination by the liver in a process called reverse cholesterol transport (RCT). HDL is therefore, cardioprotective. The first and rate-limiting step in HDL biogenesis involves interaction of apolipoprotein A-I (apoA-I), the major protein of HDL, with the membrane transporter ABCA1 to promote cholesterol efflux to form nascent HDL.

Food additives and sugar substitutes have been widely consumed in recent years. While the FDA deems sweeteners such as stevia, sucralose, acesulfame and erythritol and food dyes such as Sunset Yellow and Allura Red safe for human consumption, their direct effects on HDL biogenesis are unknown.

In this study, we examined effects of the above-mentioned additives and sugar substitutes on HDL biogenesis using macrophage-derived cells, J774. We used a fluorescent analog, BODPIY-cholesterol, to label the cholesterol pool and to measured its efflux by

fluorescence. HDL biogenesis was determined by analyzing efflux media using native PAGE followed by immunoblotting using antibodies to apoA-I. Lipids were determined using cholera toxin subunit B to detect the ganglioside GM1.

We found that all sweeteners and food dyes exerted inhibitory effects on cholesterol efflux and HDL biogenesis to varying degrees under physiologically-relevant concentrations. Steviol, the metabolite of stevia, appeared to exert the largest inhibition. It reduced cholesterol efflux by > 50% and HDL biogenesis was reduced the most, as compared to the other sugar substitutes. Under control conditions nascent HDL particles varying from less than 7 to about 12 nm diameter produced. However, under most experimental conditions, additives appeared to have an impact on the larger particles (8-12 nm diameter) as they were either reduced substantially or absent). Interestingly, the sweetener Erythritol at low concentration (5mg/ml) was inhibitory but at higher concentration (15 mg/ml) appears to reverse this effect.

Since ABCA1 is essential for optimal cholesterol efflux and HDL biogenesis, we hypothesized that their inhibition by food additives and sugar substitutes was due to a decreased level of ABCA1. Surprisingly, we found that ABCA1 level under all conditions were either similar or higher than control levels. Therefore, we suggest that the additives used in this study presumably led to mislocalization of ABCA1 and/or caused a conformational change of the transporter. This could result in decreased binding of apoA-I thus leading to reduced cholesterol efflux resulting in decreased HDL biogenesis.

On the basis of this study we suggest that the FDA guidelines should be modified and sugar substitutes and foods containing artificial dyes should be consumed in moderation.

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LIST OF ABBREVIATIONS

ABCA1……. ATP-Binding Cassette Transporter A1

ABCG1……ATP Binding Cassette transporter G1

ACK.....Acesulfame Potassium

Apo A-I.....Apolipoprotein A-1

BDP..... Bodipy Cholesterol

CM…….Chylomicron

CPT…….. 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate

CVD……..……Cardiovascular Disease

DMSO.....Dimethyl Sulfoxide

ERY.....Erythritol

HDL .....High Density Lipoproteins

HRP…….Horse Radish peroxidase

IDL..... Intermediate Density Lipoproteins

LDL..... Low Density Lipoproteins

MEM.....Minimum Essential Media

RCT.....Reverse Cholesterol Transport

RPMI..... Roswell Park Memorial Institute

SDS ..... Sodium Dodecyl Sulfate

STV…….Steviol

SUC.....Sucralose

TAG……..……..Triacylglycerols

TBST..... Tris-Buffered Saline with 0.05% Tween

VLDL..... Very Low-Density Lipoproteins

1 INTRODUCTION

1.1 Lipids

Lipids are essentially hydrophobic molecules, i.e. insoluble in water. There are various different kinds of lipids such as cholesterol, triacylglycerol, phospholipids and fat-soluble vitamins.

1.1.1 Cholesterol

Cholesterol, which is the principal steroid alcohol in the human body, performs a number of essential functions. It is a component of cell membranes that modulates its fluidity [1]. The structure of cholesterol contributes to this function. It is a compound that consists of four fused hydrocarbon rings with an 8 carbon, branched hydrocarbon chain attached to carbon 17, making the entire structure highly hydrophobic. A fatty acid attached at carbon 3 forms a cholesteryl ester, which makes the structure even more hydrophobic than if a hydroxyl group was attached as shown in the structure of cholesterol (Fig. 1-1)

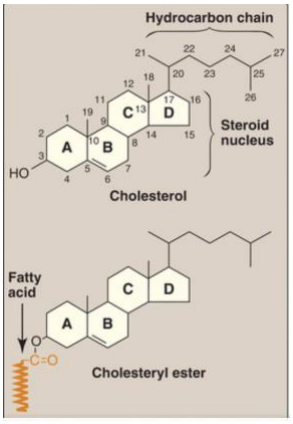


Fig. 1-1. The structure of cholesterol and cholesteryl ester. Figure taken from (Lippincott Illustrated Biochemistry Reviews, 2017)

Cholesterol is the precursor for various steroid hormones such as testosterone, estrogen, progesterone, aldosterone and cortisol. In specialized tissues, it is also a precursor of bile acids and Vitamin D [1].

1.1.2 Triacylglycerols

A triglyceride consists of long chain fatty acids, which are usually not of the same type, esterified to a molecule of glycerol. Fig. 1-2 shows that the fatty acid on carbon 1 is usually saturated, that on carbon 2 is usually unsaturated and the fatty acid on carbon 3 can be either. These molecules can be stored in either white adipose tissue or brown adipose tissue as they are mostly hydrophobic. The triacylglycerol in white adipose tissue is the major energy reserve of the body and the TAG in brown adipose tissue is used in preventing loss of heat from the body through the act of shivering [1].

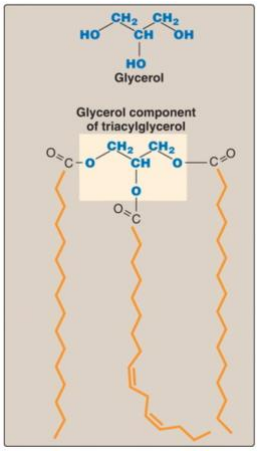


Fig 1-2. Structure of a triacylglycerol. Figure taken from (Lippincott Illustrated Biochemistry Reviews, 2017). Structure consists of three fatty acids esterified to a glycerol backbone.

1.1.3 Fat soluble Vitamins

There are four different kinds of fat-soluble vitamins: Vitamin A, Vitamin D, Vitamin E and Vitamin K.

Vitamin A comes primarily from the retinoid family, which is essential for growth, vision, maintenance of epithelial tissues, proper immune function and reproduction [1]. The natural form of Vitamin A is the retinol, a primary alcohol with an unsaturated side chain. Retinal is the result of oxidation of retinol and retinoic acid is the acid derived from the oxidation of retinal. Unlike retinoid acid, only retinal and retinol can be interconverted and used by the body. (Fig. 1-3.)

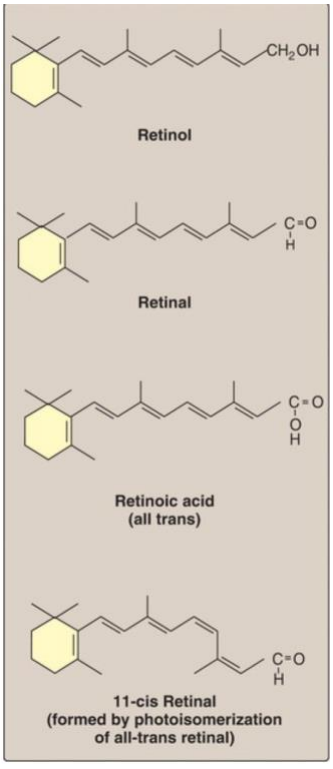


Fig 1-3. The retinoid family of proteins. Taken from (Lippincott Illustrated Biochemistry Reviews, 2017) The four major kinds are retinol, retinal, retinoic acid and 11-cis Retinal.

Vitamin D is essential for the regulation of serum levels of calcium and phosphorous. 7-Dehydrocholesterol, an intermediate in cholesterol synthesis, is converted to cholecalciferol in the dermis and epidermis of the skin after being exposed to UV rays. It then converts into the active form of Vitamin D3, 1-25 dihydroxycholecalciferol, or calcitriol, which can stimulate or repress gene transcription [1]. The most prominent action is absorption of calcium from the small intestine and excretion of phosphorus from the body. The inactive form of Vitamin D3 can also give rise to calcitriol upon consumption.

Vitamin E functions as a physiological antioxidant and prevents the formation of harmful free radicals, such as those formed in the oxidation of LDL. These vitamins consist of eight naturally occurring tocopherols of which a-tocopherol (Fig. 1-4) is the most active. It is also the least toxic of the fat-soluble vitamins and no toxicity has been observed at doses of 300 mg/day [1].

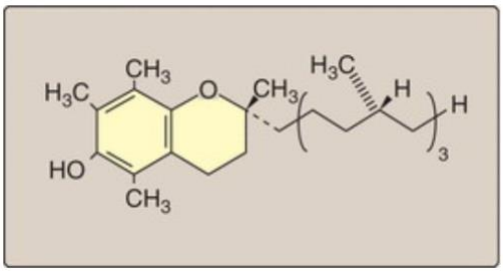


Fig 1-4. a-tocopherol. Figure taken from (Lippincott Illustrated Biochemistry Reviews, 2017). The most active form of tocopherol.

Vitamin K exists in several different forms, two of them as phylloquinone (Vitamin K1) in plants, menaquinone (Vitamin K2) in intestinal bacteria. Vitamin K is primarily

involved with blood clotting, in which it serves as a coenzyme in the carboxylation of certain glutamic acid residues. Vitamin K helps in the binding of the prothrombin-calcium complex to phospholipids in the membrane at the surface of damaged endothelial cells. Proteolytic conversion of prothrombin to thrombin can then occur, assisting with blood clotting [1].

1.2 Structure and types of Lipoproteins

Lipoproteins are complex particles composed of lipids and proteins. Their role is to transport the water insoluble lipids in the circulation. Lipoproteins contain a central, hydrophobic core consisting of mostly, cholesteryl esters and TAG. Occasionally, prostaglandins and fat-soluble vitamins might also be present [2]. The core is surrounded by a hydrophilic shell consisting of amphipathic phospholipids, free cholesterol and apolipoproteins which are essential for the formation and stabilization of lipoproteins (Fig. 1-5)

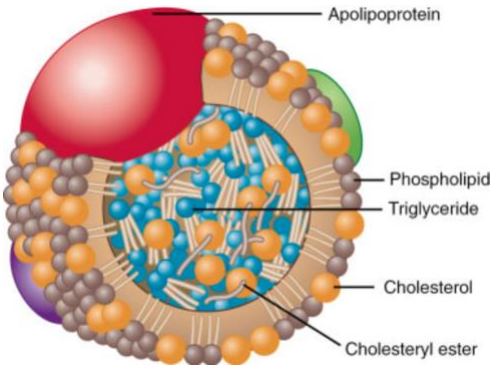


Fig. 1-5. Structure of a lipoprotein. Figure taken from (Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine, 2005).

Table 1-1: Density, size and constituents of lipoproteins. Table taken from (Feingold and Grunfeld, 2018). Major lipoproteins include Chylomicrons, Chylomicron Remnants, Very Low-Density Lipoproteins, Intermediate Density Lipoproteins, Low-Density Lipoproteins and High-Density Lipoproteins.

Lipoprotein	**Density (g/ml)**	**Size (nm)**	**Major Lipids**	**Major Proteins**
Chylomicrons	**<0.930**	**75-1200**	**Triglycerides**	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	**0.930-1.006**	**30-80**	**Triglycerides Cholesterol**	Apo B-48, Apo E
Very-Low Density Lipoproteins (VLDL)	**0.930-1.006**	**30-80**	**Triglycerides cholesterol**	Apo B-100, Apo E, Apo C
Intermediate Density Lipoprotein (IDL)	**1.006-1.019**	**25-35**	**Triglycerides Cholesterol**	Apo B-100, Apo E, Apo C
Low Density Lipoprotein (LDL)	**1.019-1.063**	**18-25**	**Cholesterol**	Apo B-100
High Density Lipoprotein (HDL)	**1.063-1.210**	**5-12**	**Cholesterol Phospholipids**	Apo A-I, Apo A-II, Apo C, Apo E

1.3 Formation and Metabolism of Lipoproteins

After digestion of dietary fat (e.g. TAG) and cholesteryl esters, the released free fatty acids, and free cholesterol are absorbed into the small intestine (enterocytes) from gastrointestinal tract (Fig. 1-6). These are re-esterified in the intestinal mucosal cells to form triglycerides and cholesteryl esters and then coupled primarily with apoB48, and other lipids, phospholipids and unesterified cholesterol into lipoprotein particles called chylomicrons [3]. ApoB-48, a truncated version of apoB100, is essential for chylomicron assembly [4]. These chylomicrons are large in size; thus, they are transported in the lymphatic system. They then enter the bloodstream through the thoracic duct and bind to walls of capillaries in skeletal muscle and adipose tissue. Upon breakdown by lipoprotein lipase, the free fatty acids, cholesterol and triglycerides are released to be used for energy metabolism [3].

After removal of most of the TAG core, the chylomicron remnants constitute mainly apoproteins B48 and E, along some TAG, and cholesteryl esters (Fig. 1-6). Chylomicron remnants are taken up by the liver via a receptor-mediated mechanism where apoE serves as a ligand. Part of the cholesterol pool together with triglycerides (both dietary and produced by the liver) are recruited by full-length apoB, apoB100, to form VLDL which are similar to chylomicrons in many respects. Upon secretion by the liver, VLDL like chylomicrons undergo metabolism by LPL to resulting in formation of VLDL remnants called intermediate density lipoproteins (IDL). IDLs are rich in TAG and cholesteryl esters [5].

IDLs are partially removed by the liver and the remainder continues to be metabolized to result in LDL. LDL are rich in cholesteryl esters and are the major carriers of plasma cholesterol as they contain almost three-fourths of all the cholesterol in human plasma [3]. HDL, the smallest lipoproteins, are rich in cholesterol and phospholipids and are stabilized by apoA-I, the major protein of HDL. HDL, unlike LDL, removes excess cholesterol from peripheral tissues and delivers it to the liver for elimination.

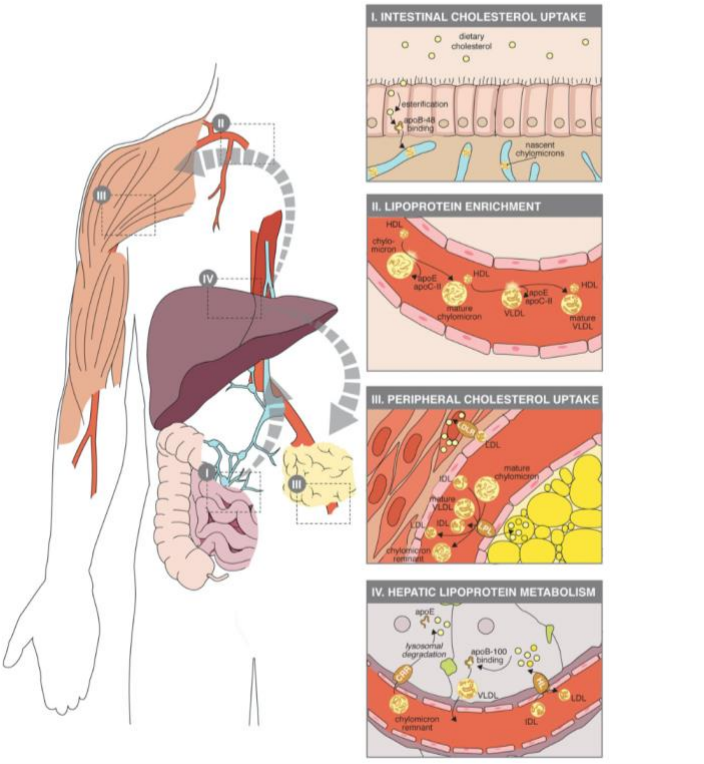


Fig. 1-6. Transport of chylomicrons from the small intestine to the liver, formation of LDL and peripheral cholesterol uptake. Figure taken from (Frambach, et al., 2020).

1.4 Reverse Cholesterol Transport

Cholesterol is an important component of cell membranes as it adjusts its fluidity during temperature changes. We also need cholesterol as a primary component to form

significant substances such as glucocorticoids, estrogen, testosterone and other hormones [6]. However, excess cholesterol in the body can contribute to diseases such as atherosclerosis, thereby increasing the need for elimination. Intestinal cells and skin cells such as sebocytes, keratinocytes etc. work by secreting smaller amounts of cholesterol into the intestinal lumen or onto the skin surface. However, because these are minute, the body relies on reverse cholesterol transport as the primary method of elimination. As mentioned earlier and according to Fig 1-9, ABCA1 plays a role in efflux of cholesterol to lipid poor apoA-1 particles, but ABCG1 plays a crucial role in efflux of cholesterol from cells to mature HDL particles [5].

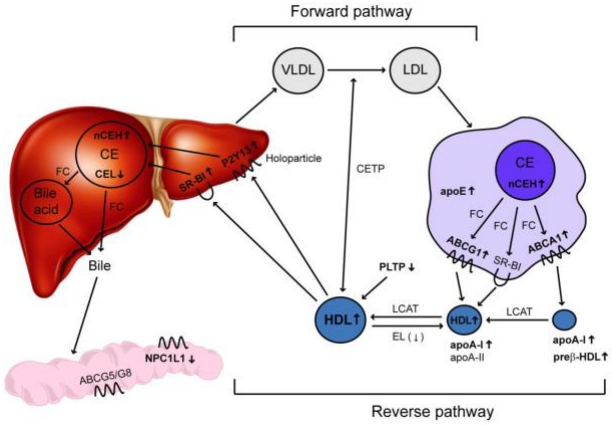


Fig. 1-7. Reverse Cholesterol Transport. Figure taken from (Annema and Tietge, 2012). ABCA1 and ABCG1 are shown, transferring cholesterol to apoA-I particles and HDL particles, respectively, overall decreasing cholesterol stores from body cells.

Lipoproteins have been linked to cardiovascular disease, stroke and type II diabetes as detailed below.

1.5 Formation of High-Density Lipoprotein

The formation of HDL via ABCA-1 (Fig. 1-8) mediated assembly is not as well understood as the formation of LDL particles. There are several proposed mechanisms. One of the most promising ones explain that ABCA-1 participates in two functions: transferring specific phospholipids from the inner to the outer membrane and binding to ApoA-1, thereby facilitating efflux of cholesterol and phospholipids. ApoA-1 is synthesized predominantly by the liver and the small intestine [7]. Its binding to ABCA1 is suggested to unfold a part of the N terminus so that it can insert into the cell membrane. ApoA-1 is lipidated by the effluxed lipids to form nascent HDL particle which is then spontaneously released from the cell membrane. [7]

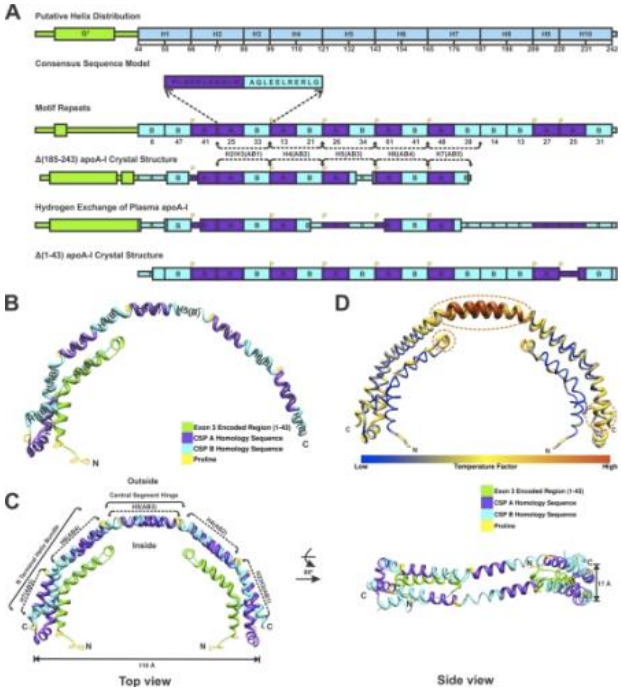


Fig. 1-8. Three-dimensional protein structure of apoA-I dimer. Figure taken from (Mei and Atkinson, 2011). The major protein component of HDL particles in plasma.

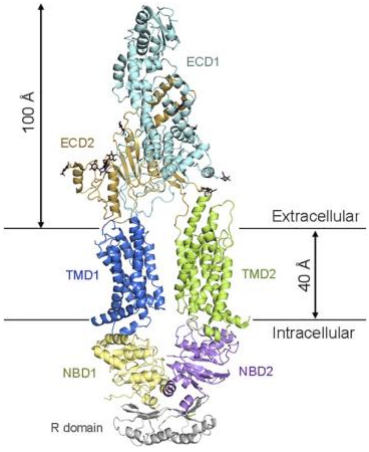


Fig. 1-9. Three-dimensional structure of ABCA1. Figure taken from (Qian H, et al., 2017). Shows the different domains such as ECD1 and ECD2 (extracellular), TMD1 and TMD2 (within the cell membrane) and NBD1 and NBD2 (intracellular)

Nascent HDL are discoidal particle as they contain apoA-I, and primarily surface lipids, free cholesterol and phospholipids. ApoA-I activates. The enzyme lecithin cholesteryl acyl transferase (LCAT) which hydrolyzes lecithin (phosphatidyl choline) to releases free fatty acid which is then used by the enzyme to esterify cholesterol to form cholesteryl ester which forms a neutral core and transforms the discoidal HDL into spherical particles representing mature HDL. [8]

1.6 Lipoproteins and Cardiovascular Disease

Non-communicable diseases are increasing at an ever-growing rate, accounting to 59% of global mortality (31.7 million deaths worldwide) and 43% of global burden disease. Out of those numbers, cardiovascular disease contributes the largest percent of mortality (30.9%) as compared to other non-communicable diseases such as cancer (13.4%), diabetes (1.1%) and chronic obstructive pulmonary diseases (4.2%) [9]. According to the

American Heart Association, cardiovascular disease remains the leading cause of death in the United States, accounting for 840,768 deaths in 2016. Approximately every 40 seconds, an American will experience a myocardial infarction. In the United States in 2019, coronary events were expected to occur in about 1,055,000 individuals, including 720,000 new and 335,000 recurrent coronary events. In addition, the prevalence of cardiovascular disease has increased in both men and women, with the highest percent population seen in ages 80+ (Fig. 1-10) The incidence of cardiovascular disease has also seen a rise in both men and women, with the highest detection of new cases again in ages 85-94. It is crucial to understand the major contributor to cardiovascular diseases [10]. In the United States, people with hyperlipidemia are at twice the risk of developing CVD than those without, specifically an imbalance in cholesterol levels, including low density lipoprotein and high-density lipoprotein in the blood. Approximately, 31 million Americans adults have total cholesterol levels that exceed 240 mg/dL, placing them at twice the risk of CVD as compared to those adults whose cholesterol levels are at baseline [10].

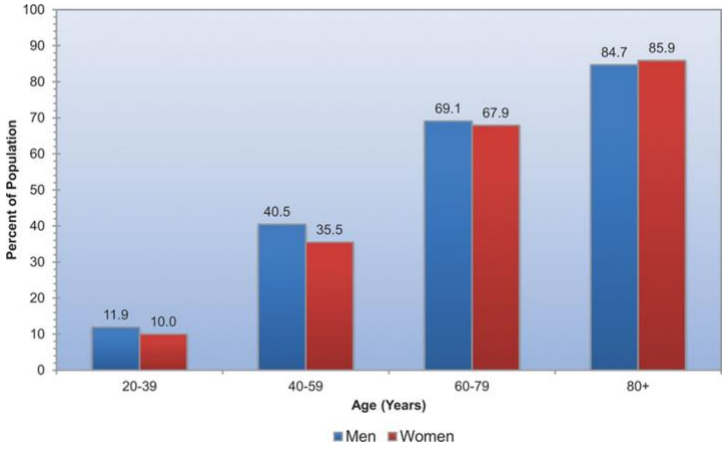


Figure 1-10. Prevalence of cardiovascular disease in adults aged >20 years by age and sex. Figure taken from (American Heart Associations, 2019)

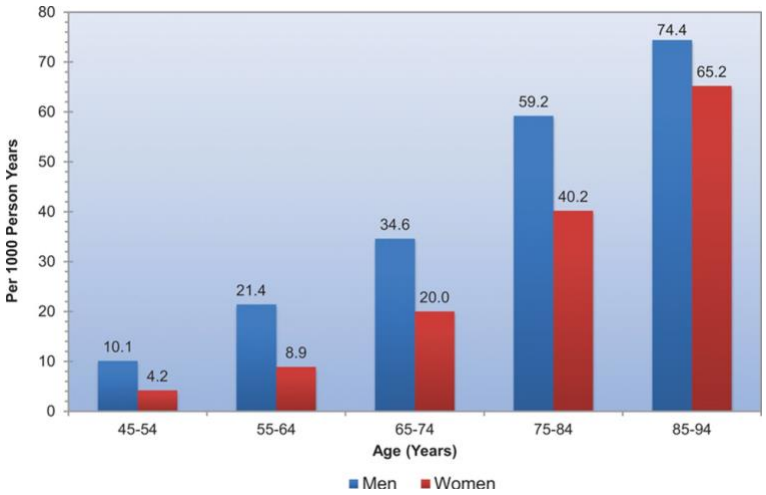


Fig. 1-11. Incidence of cardiovascular disease (coronary heart disease, heart failure and stroke; does not include hypertension alone) by age and sex.

1.8 Lipoproteins and Diabetes Mellitus

Diabetes Mellitus is commonly divided into two categories, Type I and Type II. Type I Diabetes Mellitus do not produce insulin and thus have elevated plasma glucose levels. In

contrast, individuals with Type II Diabetes Mellitus are unable to respond to the insulin produced by pancreatic beta islets, resulting in hyperglycemia. In both types, hyperglycemia has been shown to induce the formation of reactive oxidative species and activation of oxidative stress through glucose auto-oxidation, non-enzymatic glycation of proteins, activation of NADPH oxidase and protein kinase C and enhancement of superoxide radical production by the mitochondrial electron transport chain [12]. These harmful reactive oxygen species can induce alterations of endogenous cholesterol molecules, forming cholesterol oxides, which further induce apoptosis in vascular endothelial and smooth muscle cells. In addition, the cholesterol oxides due to hyperglycemia further exacerbate the diabetic condition by elevating proinflammatory and cytotoxic effects. Lastly, an increase in cholesterol oxides has been shown to worsen diabetic polyneuropathy by inhibiting nitric oxide synthase and as a result, the vasodilating agent nitric oxide [12]. Its diminished production has been linked to reduced endoneurial blood flow, resulting in cerebral ischemia, hypoxia and nerve degeneration. It is thus, essential, to control the elevation of cholesterol and by extension, cholesterol oxides [12].

1.7 Lipoproteins and Cerebral Infarction

Ischemic stroke, or ischemia, is a life-threatening condition where a blockage in an artery that supplies blood to the brain reduces oxygen and blood flow, leading to damage or death of neurons. Multiple meta-analysis done to predict the correlation between ischemic stroke and levels of cholesterol in the body have provided consistent results. In

cases of ischemic stroke, high levels of total cholesterol and low-density lipoproteins are often observed. Tirschewell et al. found that having total cholesterol levels in the top 20th quintile as compared to the bottom 20th quintile was associated with an increased risk of ischemic stroke (odds ratio 1.6; 95% CI).

Further studies done also show that within ischemic stroke, an elevated LDL cholesterol level was causally associated with large artery atherosclerotic stroke (odds ratio 1.28; CI 95%) but no correlation was observed between LDL levels and small artery occlusion [11].

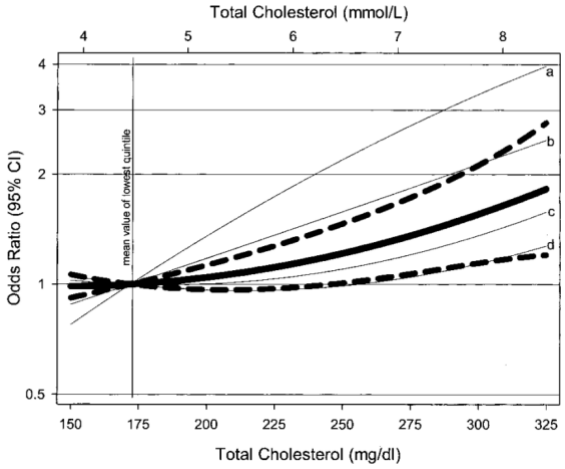


Fig. 1-12. Total cholesterol and risk of ischemic stroke. Figure taken from (Tirschewell, et al., 2004)

In contrast, the studies also showed that low levels of total cholesterol and low-density lipoprotein showed an increased risk of intracerebral hemorrhage (odds ratio 0.6, 95% CI), also a life-threatening condition in which a ruptured vessel causes excessive bleeding inside the brain [11].

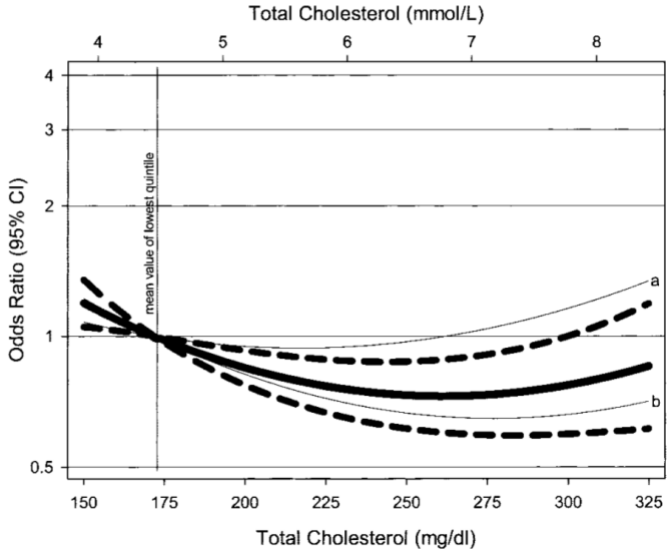


Fig. 1-13. Total cholesterol and risk of intracerebral hemorrhage. Figure taken from (Tirschewell, et al., 2004).

1.9 Contribution of Low-Density Lipoprotein to Cardiovascular Disease

The process of coronary artery disease and atherosclerosis, which is fundamental to the occurrence of cardiovascular disease, occurs via an interplay of genetics and environmental factors, but lipoproteins remain the foundation of its pathogenesis, specifically low-density lipoproteins and high-density lipoproteins [13]. The deposition of the oxidized LDL in the intima media is the first important step that leads to the formation of a nascent atheroma. (Fig. 1-14) These oxidized LDL particles promote the inflammatory process by forming adhesion molecules, which further disrupt the endothelial cell lining. The endothelium lining is responsible for regulating cardiovascular functions, including blood circulation, platelet aggression and vascular tone, thereby maintaining vascular homeostasis and blood pressure. Additionally, the

endothelial lining induces or modulates the expression of pro or anti-inflammatory factors. Oxidized LDL increases the expression of such inflammatory factors, thereby disrupting the endothelial function even further [14]. The oxidized LDL further cause monocytes to accumulate in the vessel wall, where they mature into tissue macrophages and subsequently into foam cells. These eventually develop into a necrotic core, exacerbating the endothelial lining and forming an atherosclerotic plaque (Fig. 1-14).

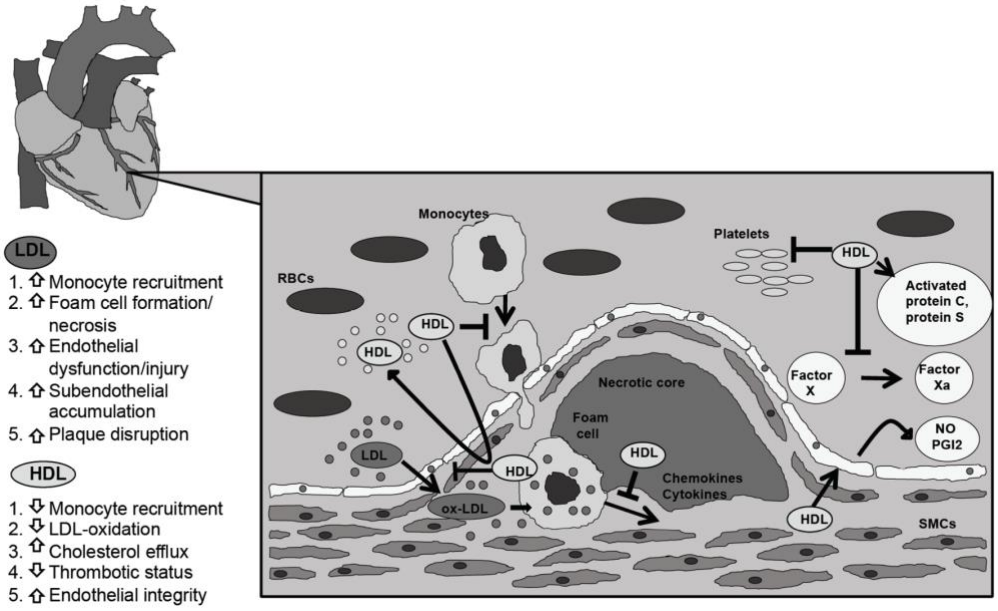


Fig. 1-14. LDL and HDL particles and their effects on the endothelial wall. Figure taken from (Androulakis, et al., 2017). Shows how the oxidized LDL particles cause eventual damage to the tunica intima of the coronary blood vessel and how HDL particles can prove to have a cardioprotective effect.

1.10 Contribution of High-Density Lipoprotein to Cardiovascular Disease

High Density Lipoproteins has been shown to have a cardioprotective effect on the body and reduce the risk and incidence of cardiovascular disease [15]. The credit is given to its role in reverse cholesterol transport as mentioned earlier [4]. ABCA1 and ABCG1 are membrane receptors that are found in almost all peripheral tissues, including on macrophages that engulf cholesterol from atherosclerotic plaques. During RCT, ABCA-1 mediated cholesterol efflux transfers cholesterol to lipid poor apo-1, leading to the formation of pre-β HDL particles. Similarly, ABCG1 proteins act as receptors and transfer cholesterol from tissues to HDL particles. Cholesterol efflux by ABCG1 is mediated via reorganization of cholesterol within the plasma membrane, which increases the plasma membrane cholesterol concentration. Thus, HDL does not necessarily need to bind to the plasma membrane to mediate efflux. Aqueous diffusion of cholesterol can aid in the process of cholesterol efflux. In this way, peripheral tissues contribute to the process of RCT, as mentioned earlier. [4] The cholesterol is carried to the liver, where SRB-1 mediates its influx into hepatocytes without uptake of the whole HDL particle, allowing unbound apoA-1 to enter a new cycle of RCT. Free hepatic cholesterol will enter the bile canaliculi either directly via ABCG5 and ABCG8 transporters or indirectly via conversion by cytochrome P450 into bile acid that can be subsequently transported into bile. The transport is carried out by MRP2/ABCC2 and BSEP/ABC11 transporters located on the canalicular membrane. This is then excreted out of the body in the fecal matter. In this way, HDL particles act as a shuttle to absorb free cholesterol from peripheral tissues and reduce the risk of cardiovascular disease [4].

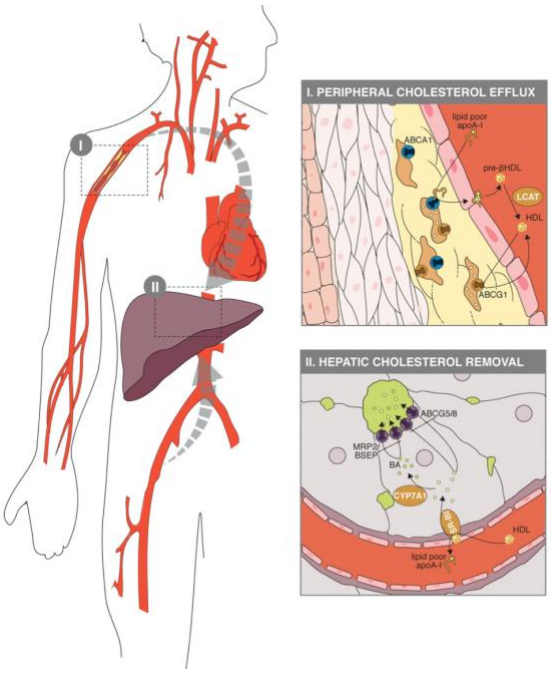


Fig. 1-15. Transport of HDL in the peripheral and hepatic circulation. Figure taken from (Frambach, et al., 2020).

1.11 HDL and CVD

As mentioned earlier, there is an inverse correlation between HDL and CVD. Low HDL is an independent risk for CVD and Type II diabetes [7]. A study done in patients with Tangier disease explains the critical role of ABCA1 in HDL biogenesis. A study done shows a link between Tangier disease, a rare autosomal recessive mutation that affects less than 100 people in the United States, and low HDL levels. [16] The study was done on a heterozygous patient who had an ABCA1 genetic mutation from a father who was normal and a mother who was heterozygous. Results showed that during reverse cholesterol transport, an ABCA1 mutation caused abnormalities in the outflow of

cholesterol and lack of transfer of the cholesterol and phospholipids to Apo A-1 as mentioned earlier. The reduced efflux of cholesterol would likely result in low HDL biogenesis, thereby reducing the cardioprotective effects of HDL on the heart [16]. In addition, a genetic disorder is one of the players involved in HDL biogenesis. This can lead to cholesterol outflow abnormalities from macrophages, thus leading to the formation of foam cells which can cause damage to the tunica intima and media, as mentioned earlier. Lastly, the study showed that lipid deposition and prevention of HDL-C can also destroy the reticular endothelial system, subsequently leading to atherosclerosis that can lead to cerebral infarction [16]. Since HDL is the main focus of this study, it should be discussed further.

1.12 Sugar Substitutes and Food Additives

Historically and even today, sugar is seen as something that causes weight gain or tooth decay and sweetness is preferred over bitterness and sourness. Sweeteners came into the industry in the 1800s and are now staple in many foodstuffs, either being used as additives or ingredients [17]. Natural or synthetic sweeteners are divided into two categories:

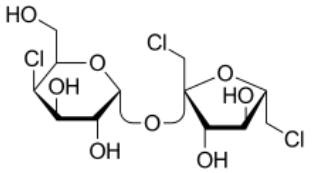
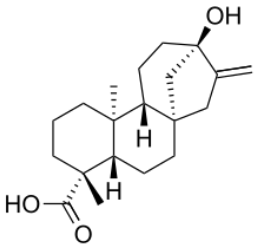
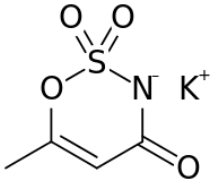
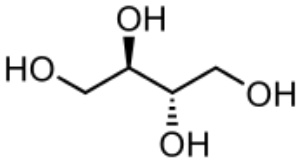
1) Nutritive sweeteners – These are the simple sugars such as glucose, fructose, sucrose etc. These also include sugar alcohols such as mannitol and sorbitol. They are classified in this category because they are considered food ingredients rather than additives. These can be starch derived, sucrose derived or polyols.

2) Intensive sweeteners – These sugars are considered additives rather than ingredients as they have negligible caloric contribution and high sweetening capacity, being used in low quantities in food. Generally, these do not trigger glycemic responses thus can be heavily used in the diets of diabetic patients and other cases where caloric intake must be controlled. Intensive synthetic substitutes were used in the experiments, which consisted of steviol, erythritol, acesulfame-K, Splenda and sucralose. For instance, acesulfame K is one of the most used synthetic sweeteners due to the lack of residual flavors and a sweetening power of over 200 times that of sucrose. With an ADI of 15 mg/kg and its high consumption by the population as baked goods, cereals, sweets etc. it is essential for it to be converted to its inactive compound. The problem there however, is that the inactivated residue is far more toxic than acesulfame-K itself.

   In addition, sucralose is about 600 times sweeter than sucrose and an ADI of 5 mg/kg, sucralose is metabolized via the cytochrome P450 physiological mechanism. Its principal application is in yogurt, ice cream, canned fruits and biscuits [17].

   However, the most controversial sweeteners are cyclamates. Although approved by the European Union at an ADI of 11 mg/kg, it was completely banned by the FDA in the 1970s in the United States. The base for the ban was a study that showed the metabolism of cyclamate produced a highly toxic metabolite called cyclohexylamine. It is now pending approval for readmission [17].

Table 1-2: Various types and structures of synthetic sweeteners used today.

Sugar substitute/Food additive	Structure
Sucralose	
Steviol	
Acesulfame Potassium	
Erythritol	

While there are reports documenting effects of sweeteners in vivo using mice and rats [18] as well as effects in humans, many used a combination of sweeteners defined as artificially-sweetened drinks [19]. Other studies documented effects on Type II diabetic patients [20]. Direct effects of these additives on HDL biogenesis and ABCA1 level have not been studied.

2 SPECIFIC AIMS

The FDA deems high intensity sugar substitutes such as sucralose, erythritol, acesulfame potassium and stevia and food additives such as red and yellow food coloring to be safe for human consumption in the United States.

The main objective of this project was to investigate whether these substitutes and additives negatively impact HDL biogenesis. If so, new guidelines should be issued given the critical role of HDL in promoting health, e.g. providing cardio protection and its inverse relationship with type II diabetes. We used cultured macrophages as a model. We measured cholesterol efflux using fluorescence techniques and determined HDL biogenesis and ABCA1 levels.

3 MATERIALS AND METHODS

3.1 Materials

Antibodies to AI were purchased for Clabiochem, antibodies to ABCA1 were purchased from Novus Biochemicals. HRP-conjugated antibodies to rabbit and mouse immunoglobulins were purchased from Jackson Immunochemicals, HRP-conjugated antibodies to goat, 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (CPT), Sunset Yellow, Allura Red, protease inhibitors cocktail and HRP-conjugated cholera toxin subunit B (CTB), were purchased from Sigma. The apoA-I protein used in the experiments was expressed in *E.coli*. It was purified and refolded by Dr. Mei.

3.2 Methods

3.2.1 Cell Culture

Mouse-derived macrophage cells, J774, were maintained in an incubator at 37 degrees, 95% air, 5% CO2, in growth media, Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were split twice a week.

For experiments, cells were plated in 24-well plates and incubated at 5% CO2 and 37°C for 24 to 48 hours. Cells were used at 70-80% confluency.

3.2.2 Labelling and Upregulation of ABCA1

Labeling and upregulation of ABCA1 was performed as described [21, 22] with slight modifications. Cells were washed in serum-free RPMI and cellular cholesterol pool was

labeled by 1-2 h incubation in Media 1 (RPMI supplemented with 0.2% albumin) containing 1 μM of a fluorescent analog of cholesterol, BODIPY-cholesterol (Topfluor). Complexed to methyl beta cyclodextrin to increase efficiency of BODIPY-cholesterol delivery. Cells were then washed and incubated with various sugar substitutes or food dyes (detailed in figure legends) in Media 1 with or without a cAMP analog, chlorophenyl thiol (CPT at 0.3 mM) for 20-24 hours. CPT was used to upregulate ABCA1. Cells were then washed in MEM and incubated in Media 2 (MEM supplemented with 0.01% albumin) containing or not CPT and 5 or 10 μg/ml of acceptor, apoA-I. This incubation media did not contain sugar substitutes or additives. Cells were then incubated at 5% CO2 and 37.5°C for 20-24 hours. MEM replaced RPMI since it provides a lower fluorescence background. Cells not incubated with CPT represented non ABCA1-mediated efflux. Cells incubated in media lacking CPT represented non ABCA1 mediated efflux. Cells incubated in media lacking the acceptor apoA-I were used to determine background fluorescence in media that was subtracted from fluorescence values obtained from media derived from cells incubated with acceptor. All experiments were run in duplicates or triplicates.

The following sugar substitutes were tested: sucralose (the active ingredient in Splenda) (0.1-5,000 μg/ml), steviol and DMSO, erythritol (1 μm – 15 μm) and acesulfame-K (0.02 μm – 0.5 μm). In addition, the following food dyes were also tested: Allura Red (21-105 μg/ml) and Sunset Yellow (concentrations ranging from 7.5 μm and 37.6 μg/ml).

3.2.3 Harvesting

The cells were monitored under a light microscope before harvesting was carried out. Detailed notes were written regarding cell density, morphology, and the evidence of stress and detached cells. The efflux media was removed and filtered through a 0.2 μm membrane to remove cell debris. Cells were solubilized by incubation in ice-cold lysis buffer consisting of 1% sodium cholate and a cocktail of protease inhibitors for 2 h on ice.

3.2.4 Efflux determination

Efflux was determined as described [22, 23]. Aliquots of media and lysates (50 μl) were transferred to 96-well black plates and clear bottoms and equilibrated to ambient temperature. Fluorescence was determined using Tecan plate reader (excitation 490 nm, emission of 520 nm; bandwidth 10 and 20 nm, respectively). Percent efflux was determined by fluorescence in media after subtraction of background (media from cells treated with CPT that were not incubated with acceptor apoA-I) divided by the sum of fluorescence in cell lysates and media multiplied by a 100.

% efflux = ((Background Fluorescence – Media Fluorescence)/(Fluorescence in cell lysates + Fluorescence in media))*100

3.2.5 HDL biogenesis and ABCA1 levels as determined by Western Blotting

Proteins and particles in media were resolved on gradient native PAGE. Samples were mixed with native loading buffer (BIORAD) and applied onto a 4-15% precast native PAGE. Native marker proteins (Amersham™ GE Healthcare) were also included. Gels were run for 38 min at 200V. Resolved proteins and particles were then electro transferred onto PVDF membranes, at a 100V for 1 h.

Membranes were stained using PonseauxS for 5-10 minutes to visualize marker proteins. Albumin present in samples was also detected by the stain.

Membranes were washed and incubated blocking buffer, Tris buffered saline 0.05% Tween (TBST), pH 8.2 containing 5% milk proteins to block areas on the membranes not containing proteins to prevent nonspecific binding of antibodies. Membranes were then incubated with rabbit, goat or mouse antibodies to ApoA-I in blocking buffer for up to 24 h. Membranes were then washed 3X 10 min in TBST and then incubated in HRP-conjugated anti-rabbit, goat or mouse antibodies in TBST containing 2.5% milk proteins for 30-60 min. ApoA-I bands were detected using the sensitive enhanced chemiluminescent (ECL) system. Bands were visualized after incubation with substrate Kwikquant by exposing membranes to an imager.

Membranes were then stripped by incubation in 0.1M glycine buffer at pH 2.5 for 1 h. Membranes were washed 3 X 10 times in TBST and incubated in blocking buffer. HRP-conjugated to cholera toxin subunit B (CTB) were then added and the membranes incubated for 30 min. GM1 was detected as described before. CTB specifically binds to GM1 and therefore, can be used to detect GM1 in nascent HDL [24].

ABCA1 level was determined using gradient SDS-PAGE. Cell lysates along with BIORAD Precision Plus Protein™ Dual Color Standards Prestained Marker were applied onto 4-15% Mini-PROTEAN® TGX™ Precast gels. Proteins were electro transferred onto PVDF membranes. Membranes were then incubated with rabbit antibodies to ABCA1 for 1-24 h followed by incubation with HRP-conjugated anti rabbit antibodies for 30-60 min. ABCA1 was visualized as described above.

4 RESULTS

4.1 ABCA1-Mediated Efflux and HDL biogenesis

Efflux of cholesterol and phospholipids can occur via several mechanisms including passive diffusion [25]. ABCA1 increases efflux substantially and is essential for RCT and HDL biogenesis [26, 27]. We used macrophage cells, J774, to determine ABCA1-mediated efflux. The cholesterol pool was labeled with the fluorescent probe, BODIPY-cholesterol complexed to methyl beta cyclodextrin. This method replaces the traditional radiolabeling approach and provides a high sensitivity [21]. Since these cells express very low level of ABCA1, the cells were incubated with a cAMP analog, CPT, to upregulate ABCA1. cAMP binds to the promoter of the gene encoding ABCA1 and this turns on ABCA1 gene expression [28]. CPT is more stable than cAMP and therefore it is typically used to measure cholesterol efflux [21]. Fig. 4-1A shows that under basal conditions, cholesterol efflux is relatively low but is not completely absent. This can be due to a combination of low activity of ABCA1 and/or passive diffusion. However, upregulation of ABCA1 resulted in about 6-fold increase in cholesterol efflux.

To determine whether the effluxed lipids bound to apoA-I to form nascent HDL we analyzed efflux media by Western blotting (Fig. 4-1 Panels B and C). Panel B shows a blot probed with antibodies to apoA-I. As shown in Lane 1, one major band (marked by red arrow) was detected under basal conditions (-CPT, low ABCA1). This is a dimeric form of apoA-I. The faster migrating band is the monomeric form of apoA-I. Importantly,

free, unlipidated apoA-I is found as a monomer than a dimer. This varies between various batches of apoA-I but has no impact on promoting ABCA1-mediated cholesterol efflux. In contrast, multiple distinct slower migrating bands can be detected in media recovered from cells treated with CPT (high ABCA1) (Lane 2). These slower migrating bands represent lipidated apoA-I corresponding to nascent HDL. These particles vary in size from less than 7 to about 12 nm diameter. Nascent HDL contain various lipids including cholesterol, phospholipids and the ganglioside GM1 (Fig. 4-1C). GM1 is also found in HDL derived from human plasma .While lipids cannot be detected by immunoblotting, GM1 can be detected by HRP-conjugated CTB [23, 24]. This reagent was developed on the basis of its high affinity to GM1. GM1 on the membrane of enterocytes is the receptor for cholera. Therefore, CTB is a very useful reagent for detecting lipids.

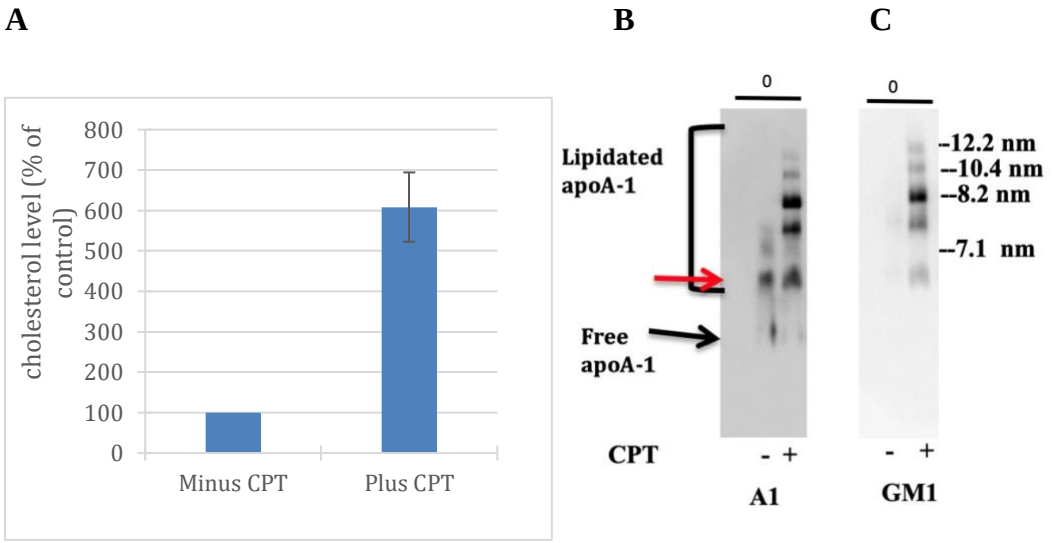


Fig. 4-1. Cholesterol efflux and HDL biogenesis. A. Cholesterol efflux. Cells were labeled with BODIPY-cholesterol and then incubated with or without CPT for 24h. Cells were then incubated with 5 µ/ml apoA-I for 24 h to promote efflux. Fluorescence in cells and media was determined using a plate reader.

Percent efflux was determined from the fluorescence in media expressed as % of total fluorescence in cells and media as described under the Methods section. Fluorescence in media (minus CPT) was set as a 100%. The bar on the right (plus CPT) is expressed as % of control (minus CPT). Panel B and C show HDL biogenesis. Effluxed media was resolved on gradient PAGE and transferred onto a membrane. Membrane was probed with antibodies to apoA-I (B) or incubated with CTB (C) Note that the red arrow indicates a dimer of lipid-free apoA-I. It comigrates with poorly lipidated apoA-I but does not contain lipids.

Panel C shows a blot probed with CTB. As expected, media recovered from cells not treated with CPT (low ABCA1) did not contain any detectable GM1 (Lane 1). Lane 2 which contains media derived from cells treated with CPT (high ABCA1) shows several bands containing GM1. These bands are the same bands identified by antibodies to apoA-I. Interestingly, the smallest particles (< 7 nm diameter) contained very little GM1. Thus, all apoA-I containing particles contain GM1.
In summary, this system is appropriate for studying the effects of sugar substitutes and additives on ABCA1-mediated cholesterol efflux and HDL biogenesis.

4.2 Effects of Sugar Substitutes or Food Dyes on Cholesterol Efflux and HDL biogenesis

4.2.1 Effects of Stevia

Extract of stevia leaves is naturally sweet. Therefore, it is used as a sugar substitute and is recommended for diabetic patients. While the FDA approved it as a food additive, not a sugar substitute, many use it as a safe sweetener. In this study we used steviol which is a metabolite formed by gut bacteria from stevioside, one of the molecules providing the sweetness.

We used one concentration, 0.1mM, to mimic the concentration of steviol found in the circulation following consumption of stevia**.** Since steviol is water insoluble, it was dissolved in DMSO. Therefore, DMSO (final concentration, 0.2%) was used as the vehicle control. Cells were incubated with steviol for the first 20-24h (Figure 4-2). Panel A shows that exposure of cells to DMSO did not produce a significant effect as efflux was 93.9% 10.6% of control non DMSO. Exposure of cells to steviol resulted in greater than 50% reduction in cholesterol efflux (45.9% ± 13.3% of control DMSO).

Next, we analyzed HDL biogenesis to determine whether decreased cholesterol efflux resulted in reduced HDL biogenesis. Panels B and C shows that exposure to stevia led to reduced nascent HDL biogenesis overall. ApoA-I-containing particles > than 8 nm diameter were more substantially reduced (Panel B). Similarly, the content of GM1 in these particles was also reduced or absent, respectively (Panel C). These findings are consistent with reduced availability of lipids.

Importantly, the inhibitory effects of steviol are long-lasting as cells were exposed to steviol for the first 20-24 h. During the following incubation period cells were incubated in media containing the acceptor, apoA-I but lacking DMSO or steviol. Thus, 24 h in the absence of steviol did not result in restoration of efflux to the control level. This finding has important implications and will be discussed further.

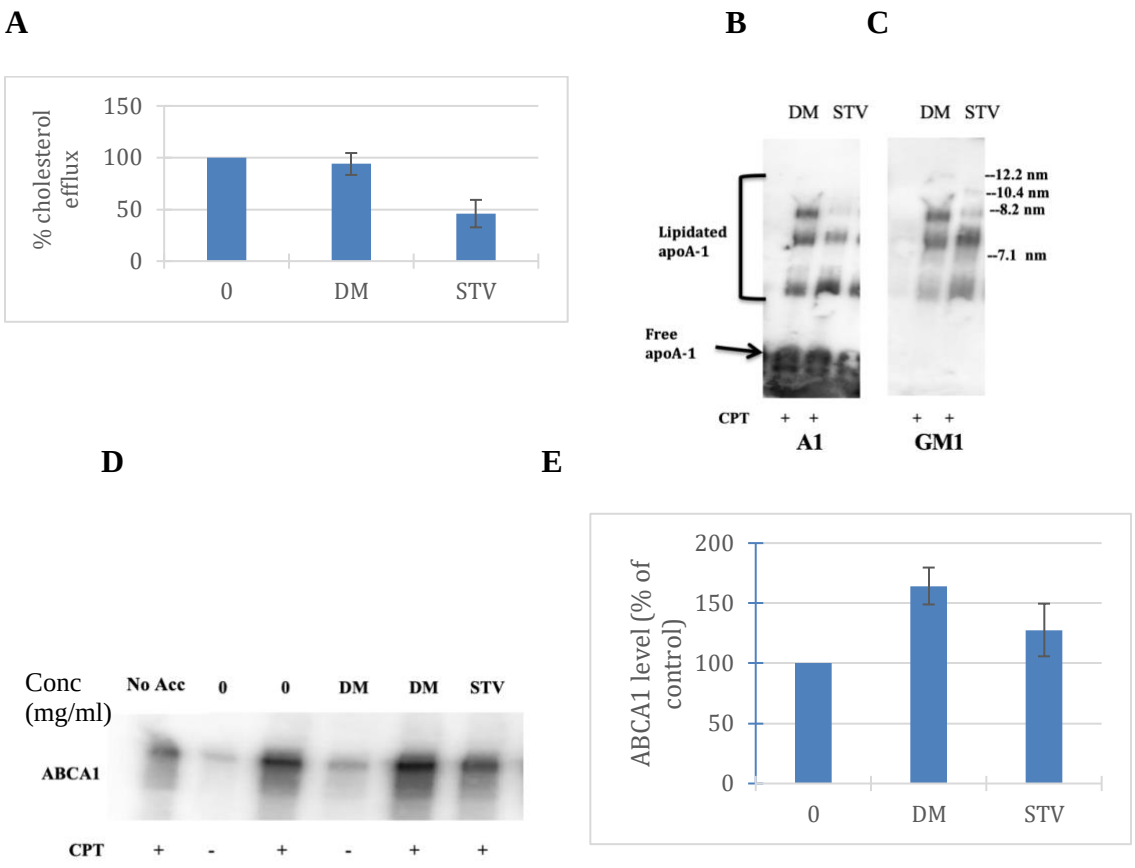


Fig. 4-2. Effects of steviol on efflux, HDL biogenesis and ABCA1 level. Cells were labeled with Bodipy-cholesterol and then incubated with 0.1 mM steviol or the vehicle control, DMSO, for the first 24 h. Cells were then incubated with or without the acceptor apoA-I in media lacking steviol or DMSO. Efflux was calculated from fluorescence. Bars represent the average of 3 independent experiments. Error bars represent the standard deviation of those 3 experiments. (A). Media were analyzed by native PAGE followed by immunoblotting. Membranes were probed with antibodies to apoA-I (B) or CTB (C). Cell lysates were analyzed by Western blotting. Membranes were probed with antibodies to ABCA1 (D) and quantified (Bars represent ABCA1 derived from cells treated with CPT and are the average of 3 independent experiments. Error bars represent the standard deviation of the range of those 3 experiments. (E)

Reduction in efflux and HDL biogenesis can result from reduced ABCA1 level. To determine whether steviol affected the level of ABCA1, cellular proteins were analyzed by Western Blotting. Panel D shows that cells not treated with CPT express very low

levels of ABCA1. Further, cells treated with CPT but incubated in media lacking apoA-I expressed higher levels of ABCA1 compared with no CPT treated cells but was lower than cells incubated with both CPT and ABCA1. As shown in panels D and E, exposure to steviol did not reduce ABCA1 level to a substantial level to account for the inhibitory effects on efflux and lipidation. These findings indicate that in the presence of steviol ABCA1 is not as functional perhaps due to a different, less active conformation and/or mislocalization.

4.2.2 Effects of Sucralose

Sucralose is a chlorinated form of sucrose and is the active ingredient in Splenda. In this set of experiments cells were exposed to various concentrations of sucralose (Fig. 4-3). Panel A shows that there is an inverse correlation between sucralose and efflux. Thus, sucralose inhibited cholesterol efflux in a concentration-dependent manner. Sucralose at 0.1 mg/ml reduced efflux to about 75% of control and further inhibited efflux to about 40% ±12.9% of control at concentrations between 1-2 mg/ml.

We then analyzed HDL biogenesis to determine whether decreased cholesterol efflux resulted in reduced HDL biogenesis. As observed in Panels B-G, particles greater than 8.2 nm were primarily reduced. Due to insufficient data we could not determine whether this is statistically significant.

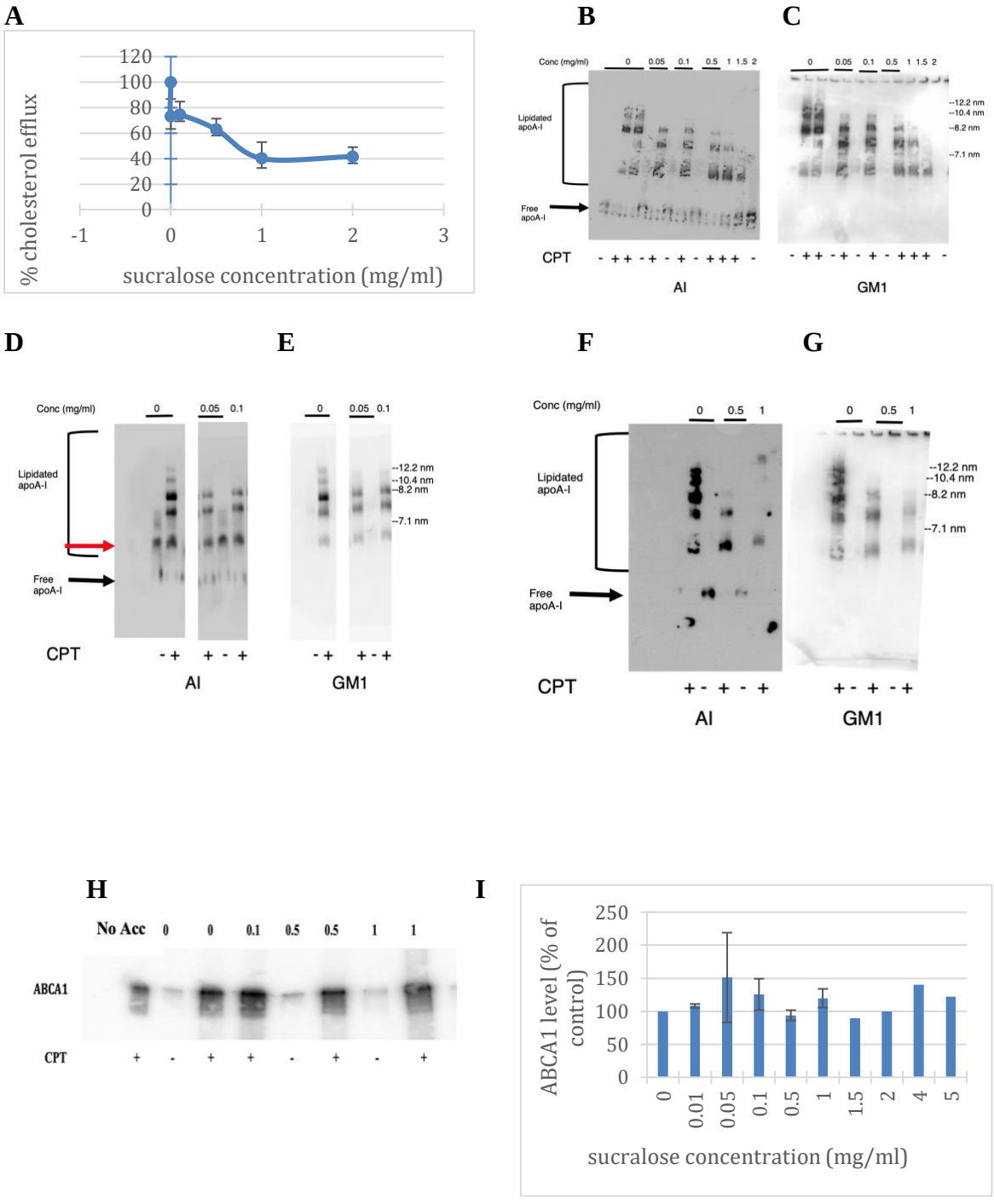


Fig. 4-3: Effects of sucralose on efflux, HDL biogenesis and ABCA1 level. Cells were labeled with Bodipy-cholesterol and then incubated with or without different concentrations of sucralose from 0.05 to 2 mg/ml. Cells were then incubated in media with or without apoA-1 in media lacking sucralose. Efflux was calculated

from fluorescence. Values represent the average of six independent experiments. Error bars shown are from the standard deviation of those experiments. (A). Media were analyzed by native PAGE followed by immunoblotting. Three experiments are shown. Membranes were probed with antibodies to apoA-I (B, D, F) or CTB (C, E, G). Note that in the experiment shown in D, the red arrow points to a dimer of free apoA-I that co-migrates with poorly-lipidated apoA-I. Cell lysates were analyzed by SDS-PAGE followed by Western blotting. Membranes were probed with antibodies to ABCA1 (H) and quantified (Bars represent ABCA1 derived from treated with CPT) (n=7) (Panel I)

In addition, as mentioned earlier, a decrease in cholesterol efflux and consequently reduced HDL biogenesis can result from a decrease in the level of ABCA1 protein. Panels H and I show that ABCA1 level was either similar or greater than control under all concentrations of sucralose. As mentioned before, these findings might indicate that sucralose may render ABCA1 less functional perhaps due to a different less active conformation. Similar to steviol, the inhibitory effects of sucralose are long lasting. Exposure of cells for up to 24 h lasted for at least 48 h.

4.2.3 Effects of Erythritol

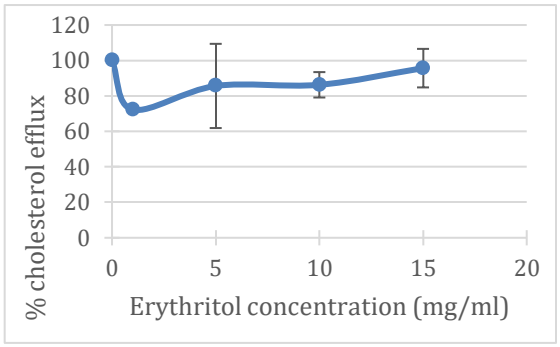
Erythritol, a sugar alcohol, is a naturally abundant sweetener. The cells were exposed to various concentrations of erythritol (Fig 4-4).

Panel A shows that at the lowest concentration, 1 mg/ml, erythritol reduced efflux to about 72% of control. Increasing the concentration from 5 to 15mg/ml resulted in a steady increase in efflux and at 15 mg/ml efflux was similar to control. Both concentrations of erythritol at 5 and 10 mg/ml inhibited efflux to the same extent. Consistent with reduced efflux, both led to a reduced production of apoA-I particles (Panels B, D, H and data not shown) and GM1 content (Panels C, E and I). However, in

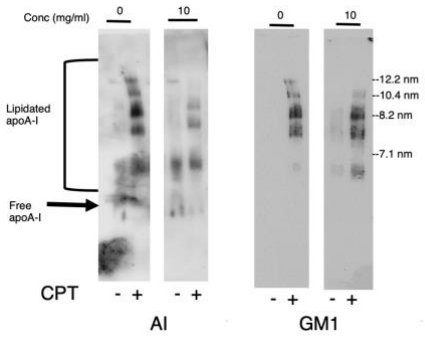
the experiment shown in Panels F and G, there was a small effect on HDL biogenesis. Exposure of cells to 15 mg/ml erythritol led to a small reduction in HDL biogenesis as determined by a small reduction in apoA-I containing particles. However, the content of the GM1 in these particles was similar to the control (Panels G and I).

The level of ABCA1 was either similar to control or higher (Panels J and K). The highest level of ABCA1 was observed for cells exposed to 1 mg/ml (202%).

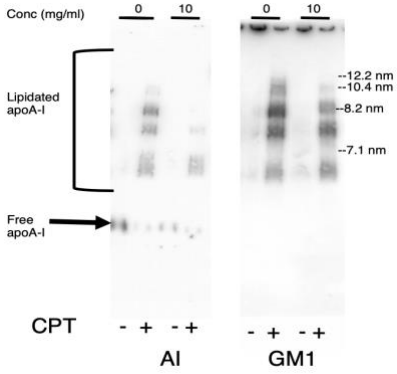
A



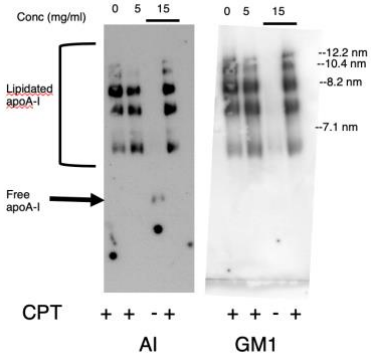
B **C**



D **E**



F **G**



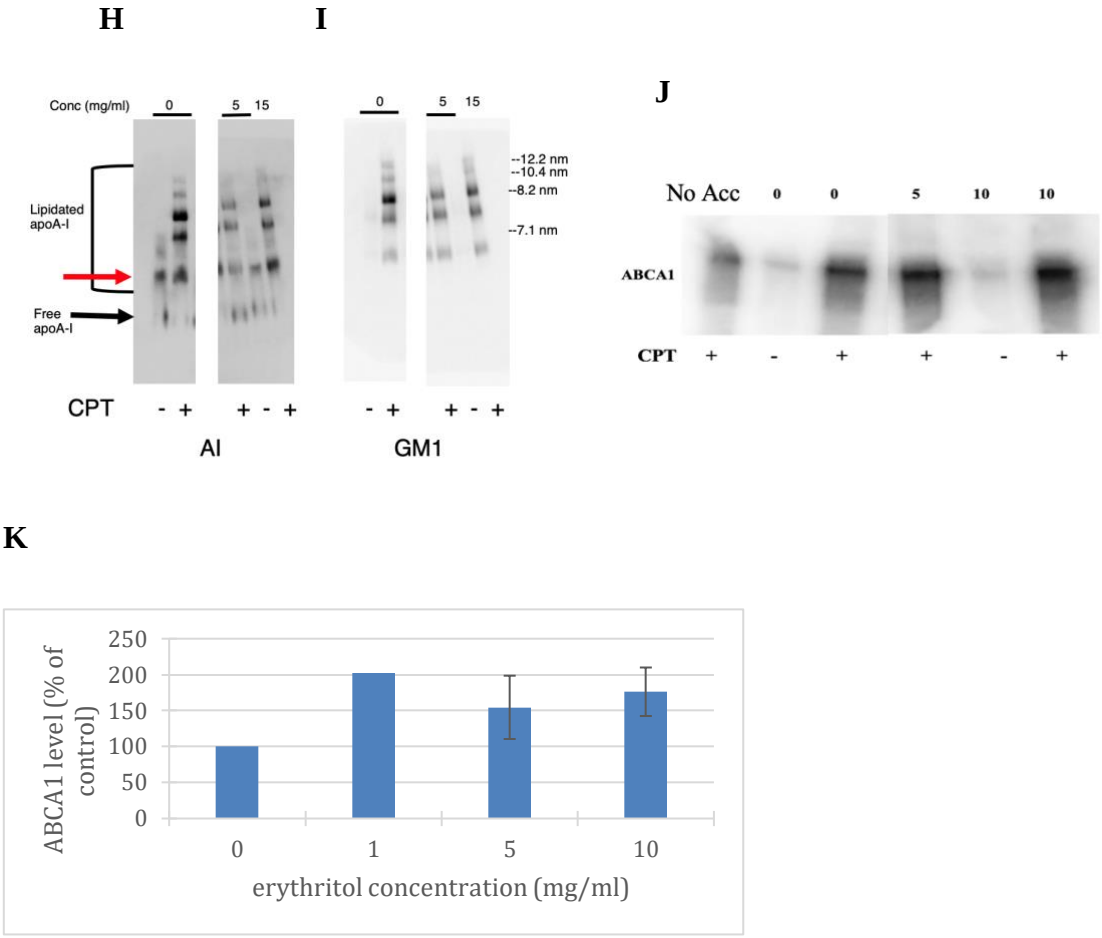
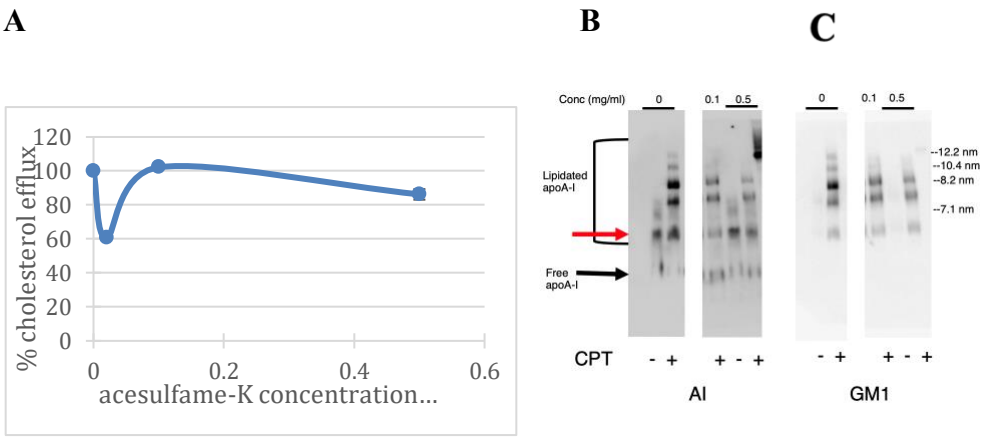


Fig. 4-4: Effects of erythritol on efflux, HDL biogenesis and ABCA1 level. Cells were labeled with Bodipy-cholesterol and then incubated with various concentrations of erythritol 1-15 mg/ml for 24 h. Cells were then incubated in with or without the acceptor, apoA-I in media lacking erythritol. Efflux was calculated from fluorescence (A) Media from 4 experiments were analyzed by native PAGE followed by immunoblotting. Membranes were probed with antibodies to apoA-I (B, D, F, H) or CTB (C, E, I). Note that in panel H the red arrow point to the dimer of apoA-I. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Membranes were probed with antibodies to ABCA1 (J) and bands representing cells treated with CPT were quantified (K)

4.2.4 Effects of Acesulfame-K

Fig. 4-5 shows that following exposure of cells to increasing concentrations of acesulfame-K cholesterol efflux was either unaffected (0.1 mg/ml) or reduced (0.5 mg/ml) to about 86% ± 3.1%. of control (Panel A). Interestingly, HDL biogenesis as determined by immunoblotting using antibodies to apoA-I was reduced following exposure to both 0.1 and 0.5 mg/ml (Panels B and D). As shown for sucralose and steviol, this reduction was primarily observed for particles > 8 nm-diameter consistent with reduced availability of lipids. However, similar to erythritol, the content of GM1 was less affected (Panels C and E).

Similar to sucralose and erythritol, ABCA1 levels were higher than control following exposure of cells to various concentrations of ACK. As concentration of ACK increases, the ABCA1 level decreases with ABCA1 level at 0.02 mg/ml and 0.1 mg/ml double that of control. The lowest ABCA1 level was observed for ACK 5 mg/ml (107.2% ±19.1%)



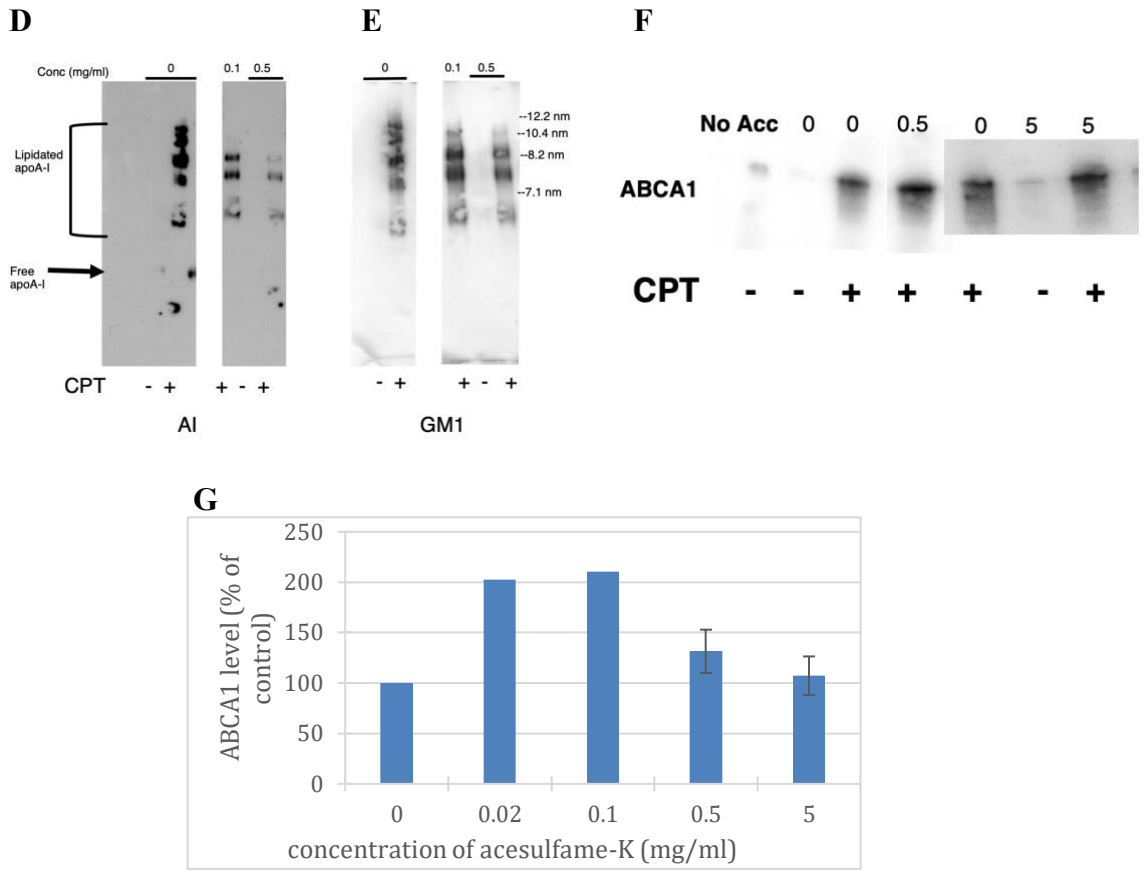


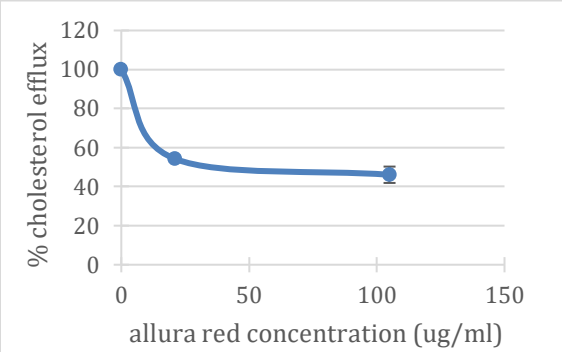
Fig. 4-5: Effects of acesulfame-K on efflux, HDL biogenesis and ABCA1 level. Cells were labeled with Bodipy-cholesterol and then incubated with various concentrations acesulfame-K from 0.02 50 5 mg/ml for 24 h. Then cells were incubated with or without apoA-I acceptor in media lacking acesulfame-K. Efflux was calculated from fluorescence (A). Media from 2 experiments were analyzed by native PAGE followed by immunoblotting. Membranes were probed with antibodies to apoA-I (B, D) or CTB (C, E). Note that in panel B the red arrow corresponds to dimers of free apoA-I which co-migrated with poorly lapidated apoA-I. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Membranes were probed with antibodies to ABCA1 (F) and bands derived from CPT-treated cells were quantified. (G)

4.2.5 Effects of Allura Red and Sunset Yellow Dyes

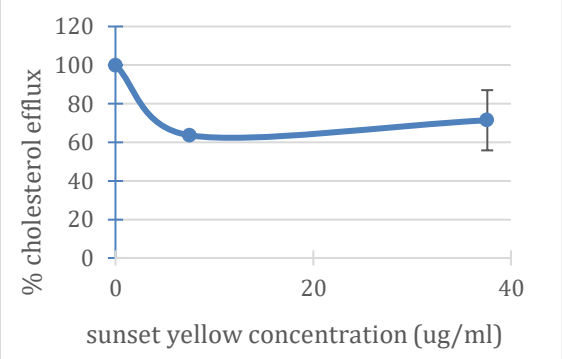
Exposure of cells to Allura red resulted in inverse correlation with cholesterol efflux (Fig. 4-6A). So that at the highest concentration, 105 µg/ml, cholesterol efflux was reduced by more than 50%.

Sunset Yellow also reduced cholesterol efflux but to a lesser degree. At the highest concentration, 37.6 µg/ml, efflux was reduced to 71.4% ±15.6% of control (Fig. 4-6B). Consistent with reduced efflux HDL biogenesis was reduced by both dyes. Allura red led to a more substantial reduction in both apoA-I-containing particles (Fig. 4-6C) and GM1 content (Fig. 4-6D). Quantification of apoA-I bands in panel C showed that Allura Red and Sunset Yellow reduced apoA-containing particles to about 20 and 52% of control. These effects are substantial.

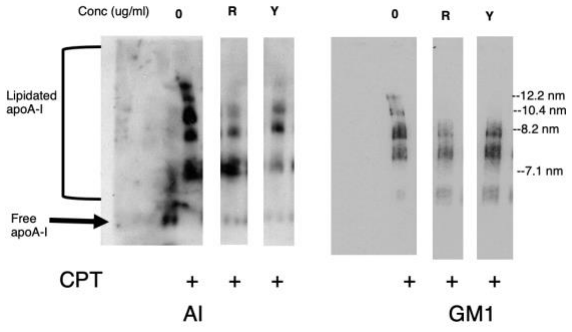
A



B

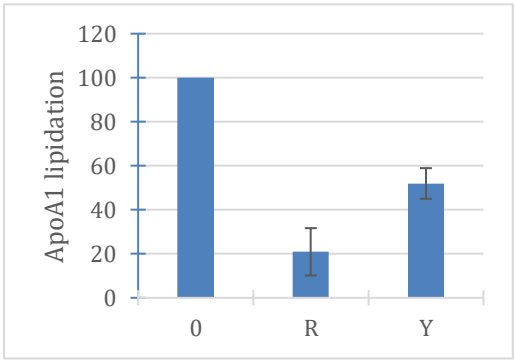


C



D

E



F **G**

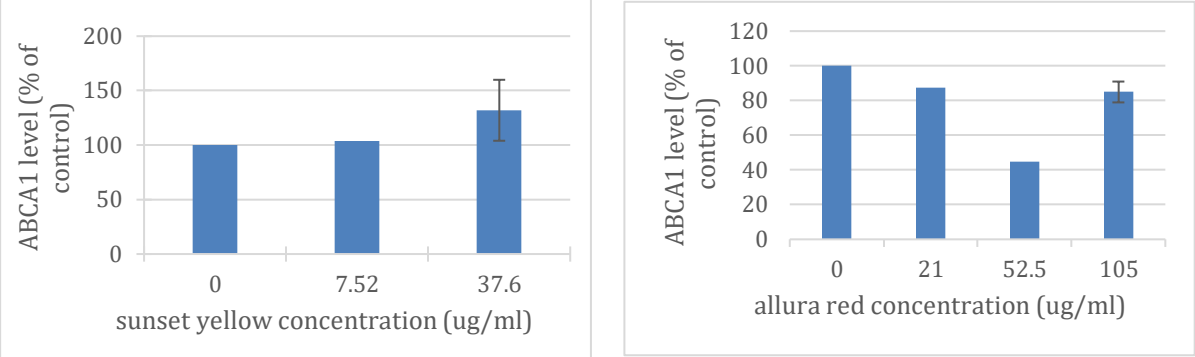


Fig. 4-6: Effects of allura red and sunset yellow on efflux, HDL biogenesis and ABCA1 level. Cells were labeled with Bodipy-cholesterol and then incubated with different concentrations red and yellow dyes for 24 h. Cells were then incubated with or without the acceptor apoA-I in media lacking the dyes. Efflux was calculated from fluorescence in media containing allura red (A) and sunset yellow (B). Media from cells exposed to allura red (105 µg/ml) and sunset yellow (37.6 µg/ml) were analyzed by native PAGE followed by immunoblotting. Membranes were probed with antibodies to apoA-I (C) or CTB (D). ApoA-I containing particles depicted in panel C were quantified and expressed as % of control (E). ABCA1 level in cells that were upregulated by CPT was quantified following immunoblot analysis sunset yellow (F) and allura red (G). The 105 ug/ml is the concentration for allura red and 37.6 ug/ml is sunset yellow concentration.

Panel E shows that ABCA1 level following exposure to Sunset Yellow was either similar to control or higher. In contrast, following exposure to Allura Red ABCA1 level was slightly lower than control. at 21 and 105 µg/ml but substantially lower corresponding to about 45% of control at 52 µg/ml (Panel F). This is the only condition that resulted in reduced level of ABCA1. This therefore, warrants further investigation.

5 DISCUSSION

The health benefits HDL are undisputable. Thus, it is important to identify potential factors that might impair their function or reduce their level. Sweeteners and food dyes are widely consumed to reduce obesity and type II diabetes. It was therefore, important to determine direct effects on HDL biogenesis at the molecular level. In the current study we used a well-established model system, macrophage cells, to determine whether these additives have negative effects on cholesterol efflux and HDL biogenesis. We examined the sweeteners steviol, sucralose, erythritol, acesulfame-K, and the food dyes, Allura Red and Sunset Yellow.

5.1 Steviol

Stevia is a natural sweetener and is therefore considered by many to be a safer sweetener. It is recommended for patients with Type II diabetes. Numerous studies have reported contradictory effects. A few studies showed that stevia has a number of beneficial effects including antihypercholeterolmia anti-inflamtory, antihyperglycemic, antihypertensive effects etc. [29, 30] Some of these studies included a small number of subjects and most included Type II diabetic patients. In general, human studies that reply upon questionnaires are hard to assess. Further, accurate information requires a very large number of subjects given the large heterogeneity. In addition, such studies should include proper controls. It is therefore, important to determine effects under healthy conditions and understand those effects at the molecular level. In the current study, we used cultured macrophages.

We exposed the cells to steviol which is the metabolite that is generated by gut bacteria and is absorbed into the circulation. The peak concentration in plasma is 0.2mM [31]. We demonstrated that half that concentration, 0.1 mM steviol, inhibited cholesterol efflux by more that 50% (Fig. 4-2A). Consistent with reduced availability of lipids, HDL biogenesis was also reduced. The apoA-I-containing particles, greater that 8 nm-diameter, were affected the most. This was also shown by the reduced content of GM1 in these particles (Fig.4-2B and C, respectively). Importantly, the cells were exposed for only one day. After an additional day of incubation in media lacking steviol, the inhibitory effects persisted. This finding has important implications and has to be analyzed in the context of the rate of metabolism and elimination of steviol. One study documented that it is metabolized and excreted relatively fast. If this is the case, steviol may not have enough time to exert its inhibitory effects. However, continuous consumption may still result in constant reduction in HDL biogenesis. Since we exposed the cells to steviol for 24 h, we plan to study its effects in the future after shorter exposures and determine the length of time required to restore HDL biogenesis to control levels. In addition, we will use media containing high glucose levels similar to those in diabetic patients to assess the effects of steviol under these conditions to determine if our *in vitro* system is consistent with effects reported in vivo.

Since ABCA1 is essential for optimal efflux and lipidation, we hypothesized that the inhibitory effects of steviol are due to reduced level of ABCA1. Fig. 4-2D and E demonstrates that surprisingly, ABCA1 level was not reduced. Therefore, we assumed that ABCA1 is either in intracellular membranes rather than the plasma membrane and/or

is has a non-active conformation. Tsujita et al [32] showed that
following incubation in high glucose HDL biogenesis was reduced and ABCA1 level was increased overall but its membrane localization was reduced. We performed a preliminary experiment using cell surface biotinylation [33]. We showed that similar to controls, ABCA1 was localized to the plasma membrane but its conformation was different than control (data not shown). This indicates that ABCA1 in steviol-treated cells was localized to the plasma membrane but was less active than controls. Thus, ABCA1 was less susceptible to degradation compared to controls. Future experiments will be conducted to confirm these findings.

5.2 Sucralose

Sucralose is 600 times sweeter than sucrose. Since only 10-15% of the ingested sucralose is absorbed into the circulation, its plasma concentration is low. Nonetheless, the cells along the GI tract are exposed to a much higher concentration. In addition, it was also found in breast milk. Therefore, we used a wide range of concentrations from 0.01 µg/ml to 2 mg/ml. As the concentration of sucralose increases, the cholesterol efflux decreases. Consistent with reduced efflux the level of the larger apoA-I-containing particles (e.g. greater than 8 nm diameter) decreased substantially (Fig. 4-2). Even at the lowest concentration there was a reduction in efflux along with reduced biogenesis. Importantly, the lowest concentration was tested on an older culture (high passage number). It is therefore critical to test the effects of 0.01 to 1 µg/ml on a younger culture (low passage number). While these cells are not considered a model for studying aging, they may be

appropriate for our studies as the controls seem to be consistent regarding efflux and lipidation so that they are not reduced with increasing passage number. It is possible that perhaps older adults may be more sensitive to inhibition by sucralose. Similar to effects of steviol, reduced efflux and HDL biogenesis did not result from reduced level of ABCA1. This will be resolved in future experiments as discussed for steviol.

5.3 Erythritol

Erythritol is a sugar alcohol that is a naturally occurring sugar. Its effect on human health are not clear since there are contradictory reports in the literature.

Some showed hypocholesterolemic effects [34] while others showed a negative impact on health demonstrating that it is in fact a pentose-phosphate pathway metabolite and that it is associated with adiposity [35].

In the current study we used various concentrations with interesting results.

Cholesterol efflux was reduced when low concentrations (0.1 mg/ml) were used. Increasing erythritol concentration to 15 mg/ml seemed to reverse the inhibition observed under lower concentration. In addition, the amount of apoA-I containing particles and GM1 content seemed similar to controls when 15 mg/ml was used. Both the larger and the smaller lipidated apoA-1 particles were present, as compared to previous additives where there were fewer larger lipidated apoA-1 particles. Lastly, levels of ABCA1 while greater than the control were similar to each other. These novel findings could result from effects on at least 2 pathways with opposing effects. At the lower concentration (up to 5 mg/ml) the inhibitory effects dominate. Higher concentrations may stimulate expression

of functional ABCA1 overcoming the inhibitory pathway. We are planning to conduct further studies using higher concentration of erythritol. While the highest concentration approved by FDA is about 12 mg/ml, higher concentrations of erythritol can be used to get insight into the detailed mechanism of ABCA1-mediated efflux and HDL biogenesis.

5.4 Acesulfame-K

ACK was discovered in 1967. It was shown to have a negative impact on cognitive function in mice [18]. We used various concentrations and found that similar to erythritol, ACK did not reduce cholesterol efflux to a great degree. At 0.5 mg/ml efflux was reduced to about 85% of control (Fig. 4-5A). While we don't know if this is statistically significant it appears that even such relatively small the reduction in lipid availability led to reduced resulted in amount of the larger A-I-containing particles (>8.2) and reduced GMI content. (Fig. 4-5 B, C).

Similar to the other sweeteners, the level of ABCA1 was either similar to control (at 0.5 or 5 mg/ml) or double the control level. In fact, it was double the control level at the lowest concentrations (0.01 and 0.5 mg/ml) (Fig. 4-F and G). It is unclear why very low concentration induces a doubling of ABCA1.

Cell surface biotinylation [33] will allow for assessing, localization and conformation of ABCA1. Since ACK is usually used in combination with other sweeteners, future experiments will address such combinations. A preliminary experiment involving co-incubation of ACK and Allura Red appeared to result in synergistic inhibition (data not

shown) while co-incubation with Sunset Yellow was not as detrimental. Further experiments using a wide range of ACK concentrations with dyes and other sweeteners will be carried out.

5.5 Allura Red and Sunset Yellow

The actual concentration in various products containing these dyes is not clear as it is not stated in food labels. Therefore, we used concentrations within the FDA allowable range. Both dyes excreted inhibitory effect on cholesterol efflux and HDL biogenesis. However, the red dye, Allura red, appears to have a much greater inhibitory effect than the yellow dye, sunset yellow (Fig. 4-6). After exposure of cells to both dyes, the level of ABCA1 was largely unaffected as it was similar to controls under most concentrations used similar to effects by sugar substitutes. In contrast, exposure of cells to 52 ug/ml Allura red resulted in a substantial reduction in the level of ABCA1 (Fig. 4-6) This is the only condition that led to reduced ABCA1 level. This could be either due to reduced expression or enhanced degradation of ABCA1. Further experiments involving a large range of concentrations and in combination with other additives will be carried out to gain a better understanding of the underlying mechanisms resulting in the findings reported here.

In summary, in this study we demonstrate that sweeteners, stevia, sucralose, erythritol and acesulfame-K as well as the dyes Sunset Yellow and Allura Red are mostly inhibitory when used under physiological concentrations. We show that these inhibitory effects did not result from reduced ABCA1 level. We hypothesize that the additives

either caused mislocalization of ABCA1 and/or induced a conformational change which is less functional. This could result in reduced binding of apoA-I and as a result, reduced efflux and HDL biogenesis. The fact that ABCA1 level is either similar to controls or higher could be due to the fact that these additives cause cellular stress which induces higher expression of ABCA1 might protect the cells from stress but may not promote optimal efflux. Potential role of ABCA1 in protecting the cells from stress has been proposed by others. Further experiments will be designed to gain a better understanding of the molecular mechanisms regulating ABCA1 protein level.

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CURRICULUM VITAE