AMERICAN UNIVERSITY OF BEIRUT

CHOLESTEROL AND LDL RECEPTORS IN UBIQUINONE TREATED PRIMARY HEPATOCYTES AND HEPG2 CELLS

by
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Life is a big challenge for us; it is full in obstacles and difficulties that can be overcame by our dreams, ambitions, patient and GOD's great people. We should not give up and sit down; we should always stand up and fight for our dreams, beliefs and ambitions. GOD will not leave us alone because we are fighting to be better people, to enrich our knowledge, to develop our society.

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MY ANGEL, MY GREAT MORNING IN ALL MY MORNINGS, TO MY MOTHER; YOUR PRAYERS PROTECTED ME AND NEVER FAILED. SHE ALWAYS SAYS "MAY GOD SEND YOU GREAT PEOPLE TO BE BESIDE YOU"

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AN ABSTRACT OF THE THESIS OF

Omar Shehade El-Rifai for Master of Science
Major: Biochemistry

Title: Cholesterol and LDL Receptors in Ubiquinone Treated Primary hepatocytes and HepG2 Cells.

Cholesterol is a vital component of cell membranes and a precursor of many biomolecules: cholesterol esters, steroid hormones and bile acids. Its synthesis involves the intermediate mevalonic acid (MVA) which serves also as a precursor for isoprenoids, dolichol and ubiquinone (UQ). UQ is a lipid soluble molecule that mediates the transfer of electrons in the respiratory chain. The role of its antioxidant property in enhancing mitochondrial function and in reducing LDL oxidation has been reported. In this study, we investigate the possible role of UQ in regulating MVA pathway and subsequently cholesterol level. Two types of ubiquinones were used UQ1 and the naturally occurring UQ10.

Isolated rat primary rat hepatocytes and HepG2 were pre-treated with UQ1 (25 and 50 µM) and UQ10 (50µM) followed by addition of 2-^{14}C MVA (for 24 hours), bypassing thus the HMGCoA reductase catalyzed step. Lipids were then extracted (Bligh and Dyer), evaporated, and separated by Thin Layer Chromatography using Heptane-Ethyl acetate as solvent system. The radioactivity incorporated into each of cholesterol, lanosterol, squalene and UQ10 was counted (Cpm) and were normalized to inorganic phosphate. Expression of LDL receptor in UQ1 and UQ10 treated cells was also examined using immuno-staining and western blotting. The effect of different inhibitors of MVA pathway in the presence or absence of UQ1/UQ10 was also examined. These include HMGCoA Reductase (Statin-50µM); Farnesyl/geranylgeranyl transferase (FPT-I-47µM); 4-hydroxybenzoate polyprenyl transferase (Bacitracin-50µM).

Our results showed a significant decrease in the radioactivity incorporated into cholesterol in both UQ1 and UQ10 treated cells, whereas it increased in lanosterol and squalene. UQ1 exhibited a more potent effect than UQ10 while it increased the incorporation of radioactivity in UQ10. Level of labeled cholesterol did not change in mevastatin treated primary hepatocytes while it decreased in HepG2 treated cells. Mevastatin co-treatment with either UQ1 or UQ10 did not alter the cholesterol profile obtained in UQ1 or UQ10 treated primary hepatocytes cells. Level of labeled cholesterol decreased in FPT-I treated primary hepatocytes, while it showed a synergistic effect when co-treated with UQ1, but not UQ10 suggesting different mode of action.

Bacitracin alone or co-administered with either UQ had no significant effect on cholesterol level in primary hepatocytes. Immuno-staining of LDL receptor in UQ1/UQ10 treated primary hepatocytes and HepG2 cells showed significant increase in the expression of LDL receptor. Western blot analysis verified this finding in HepG2 cells.

We hereby report for the first time the hypocholesterolemic effect of UQ1. This study identifies UQ1 as a potential compound that inhibits lanosterol conversion into cholesterol and increases LDL receptor expression. In addition, UQ1 favored UQ10 pathway thus ameliorating one of the side effects in statin treated patients.

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ABBREVIATIONS

HepG2	Human Liver cancer cells
DMEM	Dublecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PEN-Strep	Penicillin-Streptomycin mixture
ITES and ITS	Insulin, Transferrin, Ethanolamine and Selenium
Temed	N, N, N', N' tetra-methylethylenediamine
APS	Ammonium Per Sulfate
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Tween	Polyxyethylene-20-Sorbitan Monlaurate
SDS	Sodium Dodecyl Sulfate
ECL	Enhanced ChemiLuminescence's Reagent
^{14}C-MVA	DL-2-^{14}C-Mevalonic acid lactone
BSA	Bovine Serum Albumin
FPT I	Farnesyltransferase inhibitor
LDL-R	LDL Receptor
TCA	Trichloroaceticacid
Tris-HCl	TrizmaAcid
HClO	Per-chloric acid
MTT	3-[4, 5-dimethylthiazol-2, 5-diphenylTetrazolium bromide]
TLC	Thin Layer Chromatography
TBS	Tris Buffer Saline

CHAPTER I

INTRODUCTION

A. Cholesterol.

In 1815, Chevreul identified cholesterine in the human gallstone which was the first nomenclature of cholesterol (Gibbons, Mitropoulos, & Myant, 1982), in 1881 Ritenizir proposed the cholesterol molecular formula (Figure 1). In 1932, the complete structure of cholesterol was proposed using X-ray technology (Dane and Wieland). Three decades later (1964), Bloch was awarded the Nobel Prize for his pioneering work on cholesterol pathway He demonstrated that acetyl CoA is the precursor of the all cholesterol carbons. Final elucidation of cholesterol biosynthesis pathway was achieved in 1980 where both cytosolic and membrane bound enzymes were required. Further study on cholesterol regulation was conducted by Brown and Goldstein, a noble price was awarded for the later in 1985.

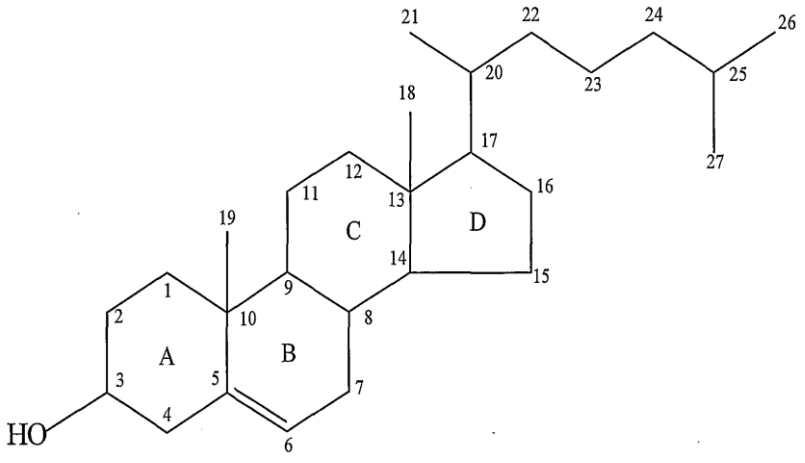


Figure 1.Cholesterol Structure

Cholesterol represents 20-25% of cellular lipids, heterogeneously distributed between cellular membranes and quite abundant in endocytic vesicles, golgi apparatus and endoplasmic reticulum. It is a main precursor of hormones, bile acids, cholesterol ester and vitamin D.

In human, cholesterol synthesis occurs in all nucleated cells, and represents more than half of cholesterol pool, with liver and kidney contributing for 20% of de-novo synthesized cholesterol. Its homeostasis is maintained via a balance between endogenous synthesis and uptake of circulating cholesterol.

1. Cholesterol synthesis Pathway.

De-novo synthesis of cholesterol occurs via a multi-enzymatic step reactions. Most enzymes are localized in the cytosolic fraction, whereas some are associated with membranes. Cholesterol synthesis may be summarized in four main consecutive steps involving: the conversion of Acetyl CoA to mevalonate; mevalonate to farnesyl pyrophosphate; farnesyl pyrophosphate to lanosterol; and lanosterol to cholesterol.

a. Conversion of Acetyl CoA to Mevalonate.

Three molecules of acetyl-CoA are converted by HMGCoA synthase into HMGCoA (Figure 2). The enzyme is localized in the cytosol and exists in two different forms; a mitochondrial form involved in ketogenesis, present in the liver and an extra hepatic form regulated by the cholesterol level.

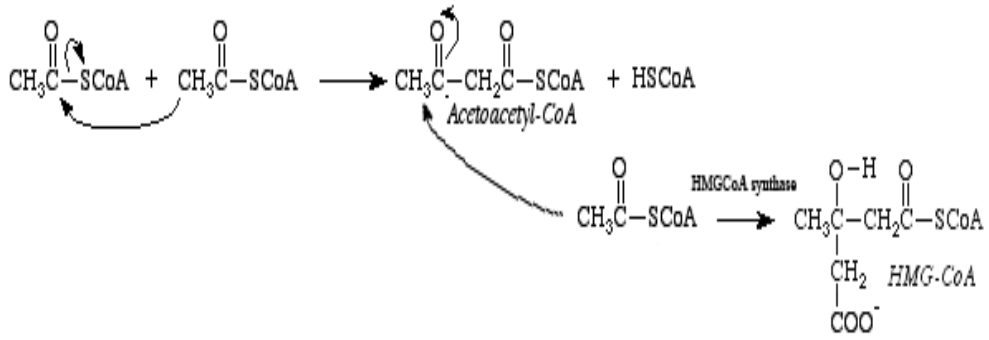


Figure 2. Condensation of AcetylCoA to form HMGCoA.

HMGCoA reductase, a 97 KDa glycoprotein, localized in the endoplasmic reticulum and perixosomes (Olivier & Krisans, 2000). It catalyzes the rate determining step reducing HMGCoA to mevalonate (Figure 3).

Figure 3. Conversion of HMGCoA to mevalonate.

Structurally, HMGCoA reductase is composed of three different domains: a membrane N-terminal domain linked to 8 transmembrane spans by a short linker, and a C-terminal domain composing the catalytic side (Figure 4) (Istvan & Deisenhofer, 2000). A flap domain localized in the C-terminus of the catalytic domain composed of 25-30 amino acids is the place where HMGCoA reduction to MVA occurs via multistep involving two NADPH molecules. The enzyme cofactor binding mediates co-localization of the His 866 in the flap domain to the active side; in addition, flap domain is the place where enzyme regulation and statin binding occurs, down regulation of HMGCR catalytic activity occurs via the phosphorylation of the Ser872 of the flap domain.

Spans 2-5 of the HMGCoA reductase represent the cholesterol sensing domain. These domains are conserved in many proteins involved in cholesterol synthesis. They are the place where N terminal of sterol regulatory element binding proteins bind mediating the over-expression of targeted genes.

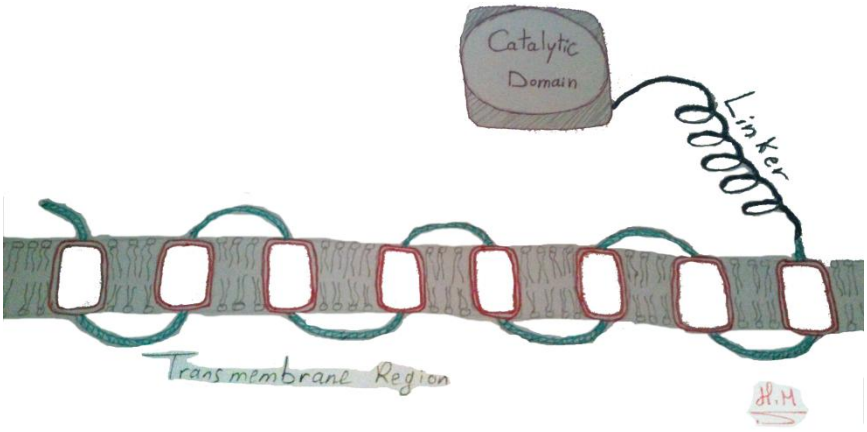


Figure 4. HMGCoA reductase Structure.

b. Conversion of Mevalonate to Farnesyl pyrophosphate.

Mevalonate conversion to farnesyl pyrophosphate occurs via successive steps involving 2 phosphorylation step of mevalonate catalyzed by kinases (Houten, Wanders, & Waterham, 2000), followed by a decarboxylation yielding isopentenyl-PP (IPP-5C) which is the precursor of geranyl (10C) and farnesyl pyrophosphate (15C). IPP serves as precursor of ubiquinone and dolicohol synthesis (Figure 5).

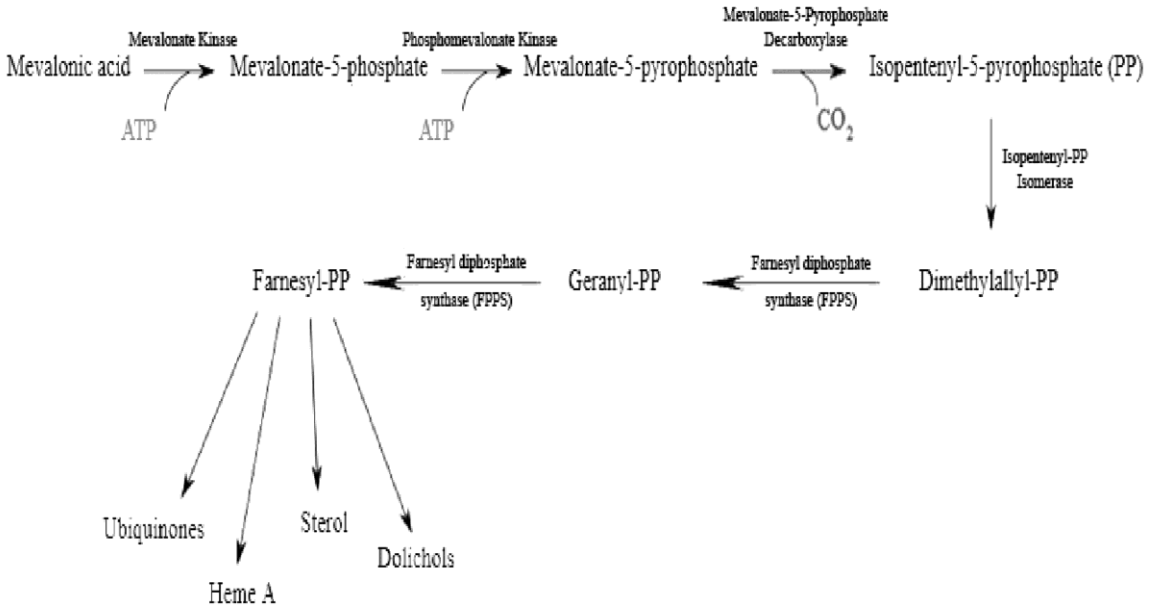


Figure 5. Conversion of mevalonate to different sterol and non-sterol intermediates.

In addition, farnesyl and geranyl are important in protein prenylation facilitating the protein-protein interaction, promoting protein-membrane interaction and cellular growth. Three different families of transferase are involved in the prenylation of the carboxy terminal of protein, farnesyl transferase, geranylgeranyl transferase and RAB geranylgeranyl transferase. Each enzyme has a specific recognized sequence where prenylation occurs. The different transferases and their recognition sequences are summarized in the table below (Table 1) (Yalovsky, Rodr Guez-Concepcion, & Gruissem, 1999).

Enzyme	Subunits	Recognition motifs	Protein substrates (examples)
FTase[a]	α β	CaaX	Ras, Gγ, a-factor, nuclear lamins, IP_3 5-phosphatase, cGMP phosphodiesterase
GGTase-I[a]	α β	CaaL	Rap, Rho, Rac
Rab-GGTase[b]	α, β, REP	CC, CXC, CCX, CCXX, CCXXX	Rab, Ypt

[a]FTase and GGTase-I are heterodimeric enzymes that share a common α-subunit but have distinct β-subunits[6,7].
[b]The subunits of the Rab-GGTase catalytic component are similar (but not identical) to those of FTase and GGTase-I. The catalytic component requires a third protein component, the Rab Escort Protein (REP), for full activity. REP binds non-prenylated Rab proteins, and presents them to the catalytic component[48].
Abbreviations: a, aliphatic amino acid; X, any amino acid.

Table 1. Enzymes prenylation and their recognition motifs.

c. Conversion of farnesyl pyrophosphate to lanosterol.

Farnesyl pyrophophate conversion into lanosterol involves squalene (30C) as an intermediate. Squalene synthase, another key enzyme in the cholesterol synthesis is a 47Kda protein composed of a C-terminal domain anchored to the endoplasmic reticulum membrane, and an N-terminal domain localized in the cytoplasm.it catalyzes the condensation of 2 farnesyl-PP units to squalene. Squalene synthase activity is regulated by

the level of cholesterol that determines efflux of farnesyl pyrophosphate toward cholesterol or other non-sterol pathway (Tansey & Shechter, 2000).

Squalene conversion to lanosterol is aerobic; it involves oxygen introduction yielding epoxide intermediate that is hydrolyzed by squalene-epoxidase. The oxido-squalene is cyclized to lanosterol by oxido-squalene cyclase (Figure 6).

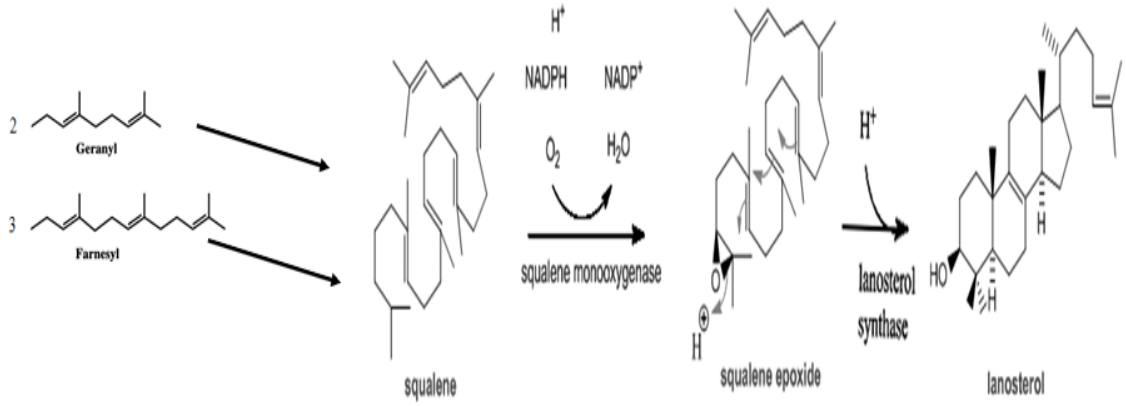


Figure 6.Conversion of squalene to lanosterol.

d. Conversion of lanosterol to cholesterol.

Lanosterol (30C) is converted to cholesterol via 19 reactions involving loss of 3 methyl groups (at C14 and C4), shift in double bond (C8-C9 to C5-C6) in ring B and reduction of double bond side chain involving isomerase, desaturase and reductase enzymes localized in the endoplasmic reticulum (Figure 7) (Fischer et al., 1991; Reinhart, Billheimer, Faust, & Gaylor, 1987; Schroepfer, 1982).

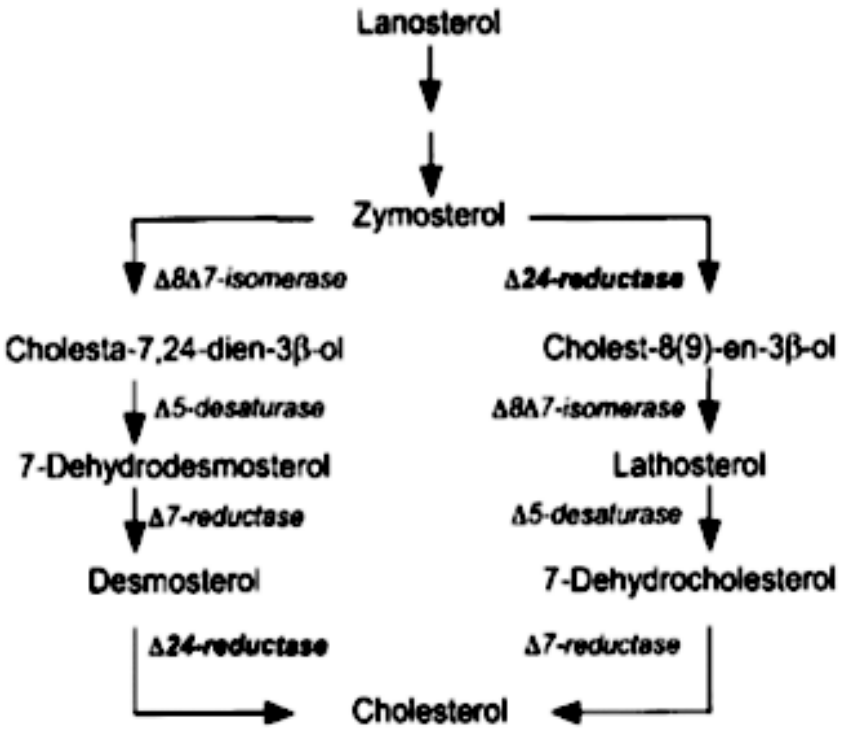


Figure 7. Lanosterol to cholesterol pathway.

2. Metabolism of cholesterol.

Cholesterol is an important precursor of many biologically important compounds serving important functional roles in human body. It is involved in the synthesis of steroid hormones via P450Scc, bile acids via cholesterol-7α-hydroxylase, oxysterol via 24, 25, 27 hydroxylase, vitamin D via cholesterol-7α-hydroxylase/UV light and cholesterol ester via Acyl-coA: cholesterol acyltransferase (ACAT) (Figure 8).

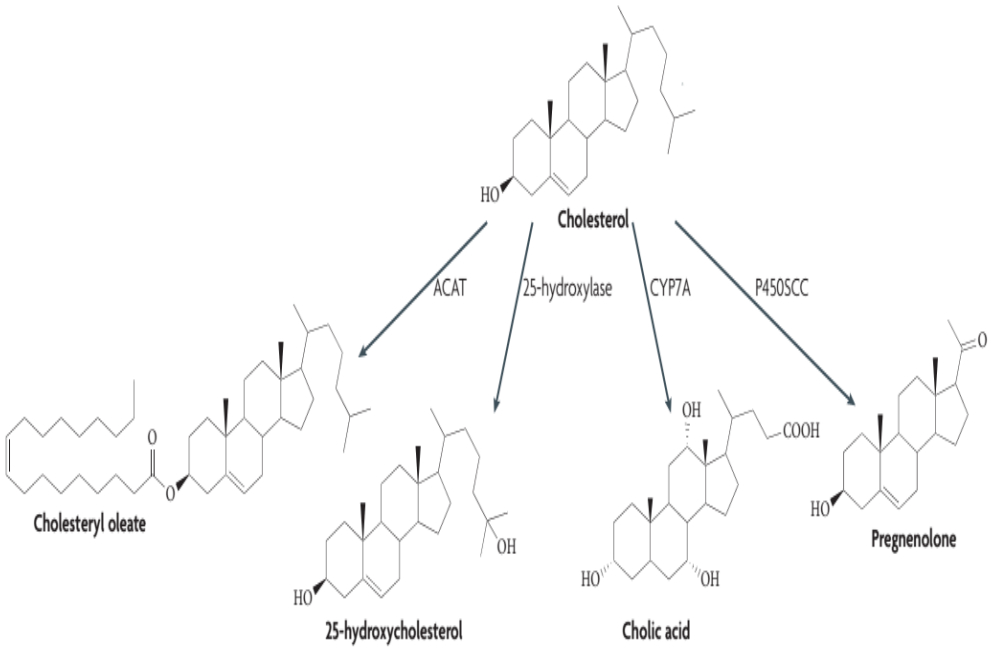


Figure8. Catabolism of cholesterol to cholesterol ester, oxysterols, Cholic acid and pregnenolone *(Ikonen, 2008).*

a. Synthesis of steroid hormones.

Steroid hormones are hydrophobic molecules capable of crossing the physiological membranes and binding to intra-cellular receptors. They play an important functional role in human body: sexual maturity, absorption of electrolytes, regulation of carbohydrate and amino acid metabolism. Cholesterol is initially transported to the mitochondria via steroidogenic acute regulatory (StAR) protein and converted to pregnenolone by P450Scc. Pregnenolone undergoes different reactions catalyzed by endoplasmic reticulum enzymes leading to glucocorticoid (cortisol) ,mineralocorticoid (Aldosterone) and androgens (Hanukoglu, 1992).

b. Bile acid Synthesis.

Cholesterol is the precursor of bile acids which facilitate intestinal absorption of dietary lipid. Cholesterol-7α-hydroxylase is the rate determining step in the synthesis of bile acids that occurs in the liver. Conversion of cholesterol to bile acid involves: epimerization of 3 hydroxyl group (β to α), desaturation of double bond, hydroxylation and trimming of the side chain. Two major primary bile acids are synthesized: cholic and chenodeoxycholic acid secreted as glycine or taurine conjugates they undergo further changes via the intestinal bacterial enzyme to deoxycholic acid and lithocholic acid. Most of bile acids are returned back to the liver via the portal circulation; however, lithocholic acid is not re-absorbed due to its insolubility. A small fraction (less than 1g/day) of bile acids is excreted by the feces.

c. Oxysterols.

Oxysterols are the hydroxylated form of cholesterol; they have the ability to diffuse via the plasma membrane and target lipid metabolism in the cytoplasm.
Oxysterol formation is catalyzed by three different enzymes: 24-hydroxylase that exists in the endoplasmic reticulum ER, 25-hydroxylase exists in the endoplasmic reticulum and golgi apparatus and 27-hydroxylase which is a mitochondrial enzyme (Russell, 2000). Oxysterols are potent suppressors of cholesterol synthesis (Kandutsch & Chen, 1974). 25-hydroxycholesterol regulates SREBP degradation that mediates cholesterol genesis regulation, whereas 27-hydroxycholesterol activates the transcription of enzymes involved in bile acid formation (Bjorkhem, 2001).

d. Vitamin D.

Vitamin D is a fat soluble molecule found in few foods and synthesized in the skin by ultraviolet light action on cholesterol derivative 7 dehydrocholesterol. It exists in two different major forms Vitamin D2 Ergocalciferol and D3 Cholecalciferol. Vitamin D plays an important role in maintaining the calcium and phosphorus concentration within the normal range by facilitating their absorption by the intestine.

e. Intracellular cholesterol ester synthesis.

Cholesterol esterification is catalyzed by Acyl-coA: Cholesterol Acyl Transferase (ACAT). It serves a role in cholesterol absorption in the intestine, in lipoproteins assembly and regulation of bile acids synthesis.

3. Cholesterol Homeostasis.

Cholesterol homeostasis maintains the balance between hepatic-extra hepatic tissue and blood circulation. It involves two main organs: the intestine responsible of dietary cholesterol absorption and its excretion as chylomicrons or HDL occurs to the blood circulation; the liver synthesizing VLDL is the precursor of other lipoprotein like IDL and LDL.

a. Enterohepatic circulation.

Cholesterol balance between the liver and intestine is maintained by the enterohepatic circulation. Liver secretes cholesterol either in VLDL form or converts it to bile salts secreted via ABCG5/G8 and bile salt export pump BSEP to the duct (Figure 9A). Intestinal cells absorb dietary cholesterol in the form of free cholesterol or hydrolyzed cholesterol ester. They are responsible of bile acid absorption and liver transport via the portal vein.

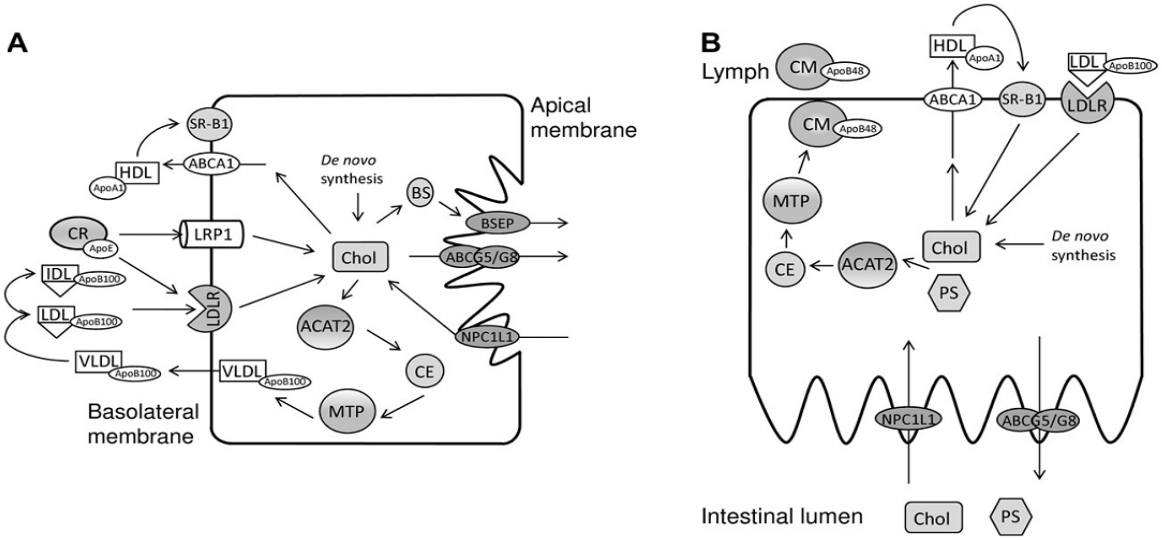


Figure 9. Cholesterol transporters, converting enzymes and lipoproteins in liver (A) and intestine (B) (van der Wulp, Verkade, & Groen, 2013).

Dietary cholesterol is absorbed by the intestinal Niemann-Pick C1 like receptor 1 (NPC1L1), esterified and packaged in ApoB48 containing lipoprotein known as chylomicrons, or secreted as free cholesterol in nascent HDL (ApoA1 HDL) by the ATP-binding cassette transporter A1 (ABCA1) (Figure 9B). In addition, intestinal cells can uptake circulating cholesterol in the form of Low Density Lipoprotein (LDL) via the LDL receptor or in the form of High Density Lipoprotein by the scavenger receptor SRB1.

b. Liporotein circulation.

Lipoproteins are responsible of hydrophobic lipids transports. They are classified into 4 different forms according to their densities. One structural component of lipoproteins is Apo protein that serves many function, another component is the nature of lipids transported, the table below summarize each lipoprotein with its lipid content and Apo protein (Table 2).

Human plasma lipoproteins					
	chylomicron	VLDL	IDL	LDL	HDL
Density (g/ml)	0.95	0.950–1.006	1.006–1.019	1.019–1.063	1.063–1.210
Components (% dry weight)					
protein	2	7	15	20	40–55
triglycerides	83	50	31	10	8
free cholesterol	2	7	7	8	4
cholesteryl esters	3	12	23	42	12–20
phospholipids	7	20	22	22	22
Apoprotein composition	A-I, A-II, B-48, C-I, C-II, C-III	B-100, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, C-I, C-II, C-III, D, E

Table 2. Human serum lipoprotein (Christopher K. Mathews, 2000).

Dietary cholesterol, cholesterol ester and triacylglycerol are packaged,or released into circulation by the enterocytes as nascent chylomicrons containing ApoB48 and ApoA1. In blood, chylomicrons may acquire ApoE and ApoC from circulating HDL. ApoC mediates the activation of lipoprotein lipase facilitating the hydrolysis of chylomicron triglyceride (TG) pool, releasing it as fatty acids and glycerol to the extra-hepatic tissue. The resulting chylomicron known as remnant chylomicrons is rich in cholesterol and cholesterol ester and is taken up by the liver.

Hepatic (endogenously synthesized) cholesterol is converted to bile salts or esterified via ACAT and is packaged in ApoB100 nascent VLDL particles that are secreted to the circulation where they gain ApoC and ApoE from circulating HDL. As nascent VLDL delivers its TG cargo to extra-hepatic tissue, the VLDL is then transformed to IDL characterized by ApoB100 & ApoE. Fates of IDL includes its uptake by liver by virtue of ApoE, loss of ApoE and get transformed into LDL with only ApoB100. Thus LDL is cholesterol rich particle whose main role is delivering cholesterol to extra-hepatic tissue (Figure 10).

HDL plays an important role in cholesterol influx and efflux from the cells. In addition, it facilitates exchange of Apo-proteins among lipoprotein ApoE and ApoC to chylomicron and VLDL. Nascent discoidal HDL (ApoA1) is free cholesterol rich molecule that is released by the intestine. The function of ApoA1 is to activate the lecithin: cholesterol acyl transferase that enriches nascent HDL with cholesterol ester yielding mature HDL that binds to scavenger receptor SRB1. ApoA1 mediates binding of HDL to SRB1, facilitating thus influx and efflux of cholesterol ester in the liver and other tissue in a process known as cholesterol reverse transport (Figure 10).

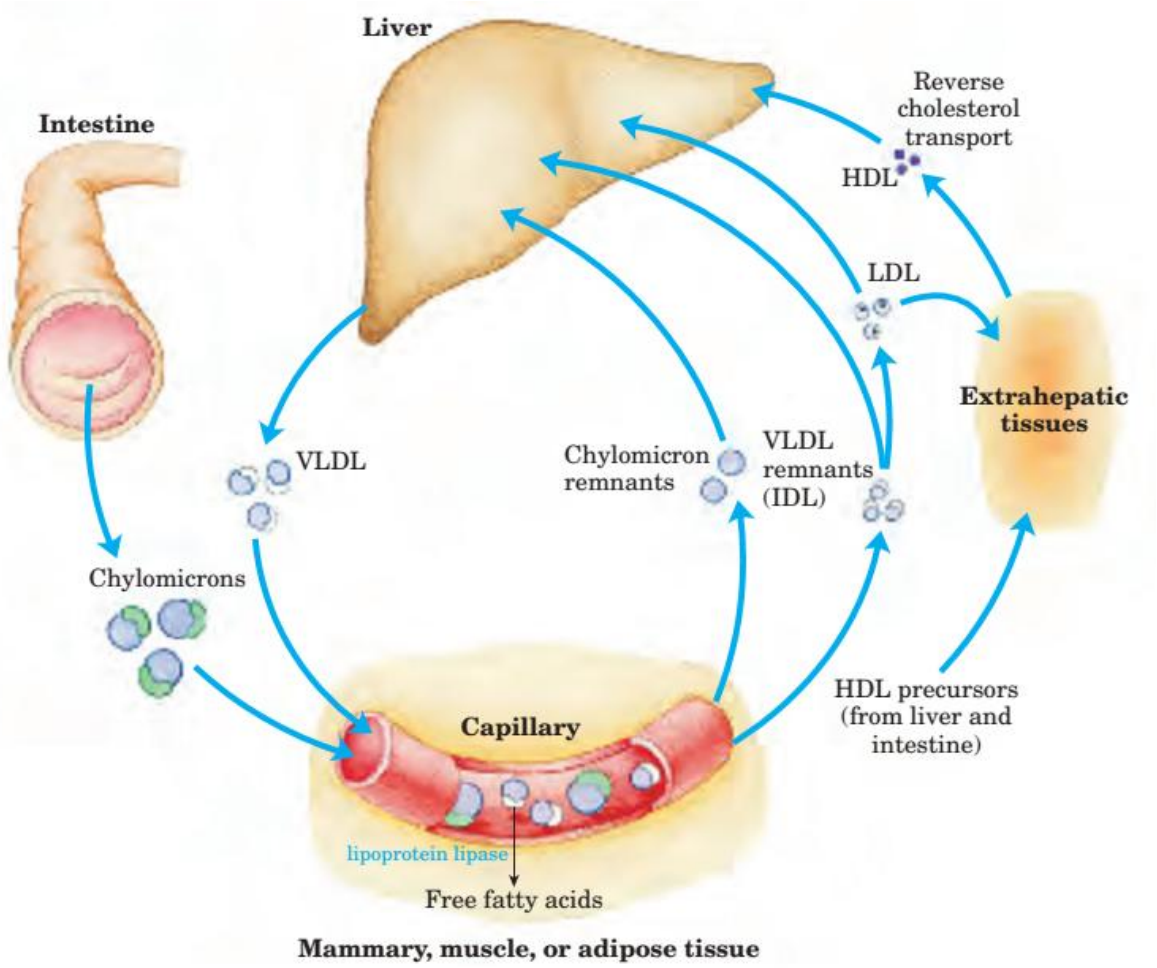


Figure 10. Lipoproteins and lipid transport (David L Nelson, 2004).

4. LDL Receptor.

a. Structure.

LDLR is a transmembrane protein composed of 839 amino acids folded into 5 domains: LDLR repeat domain, Epidermal Growth Factor (EGF) repeat domain, O linked glycosylation domain, trans-membrane domain and cytoplasmic domain (Figure 11).

- The LDLR repeat domain consists of seven homologous repeats (Yamamoto et al., 1984) where Apo B100 and Apo E binds (Mahley, Innerarity, Weisgraber, & Fry, 1977).
- The EGF repeat domain has 35% homology with its receptor. It is composed of three repeats (A,B,C), where A and B interact with the 4th and 5th repeats of the LDL repeat domain, this interaction is responsible of the ligand release following internalization of the receptor and exposure to low PH (Rudenko et al., 2002).
- The O-linked glycosylation domain is composed of 58 amino acids. It creates a separator region between the EGF repeat and the plasma membrane (Russell et al., 1984) which protects the extracellular domain from proteolytic cleavage (Kozarsky, Kingsley, & Krieger, 1988).
- Trans-membrane domain is composed of 25 amino acids rich in hydrophobic amino acids that anchors the LDLR with the plasma membrane (M. S. Brown, Herz, & Goldstein, 1997).

- The cytoplasmic domain is composed of 56 amino acids. It binds to the Autosomal Recessive Hypercholesterolemia protein ARH1 (Garcia et al., 2001) responsible for receptor internalization.

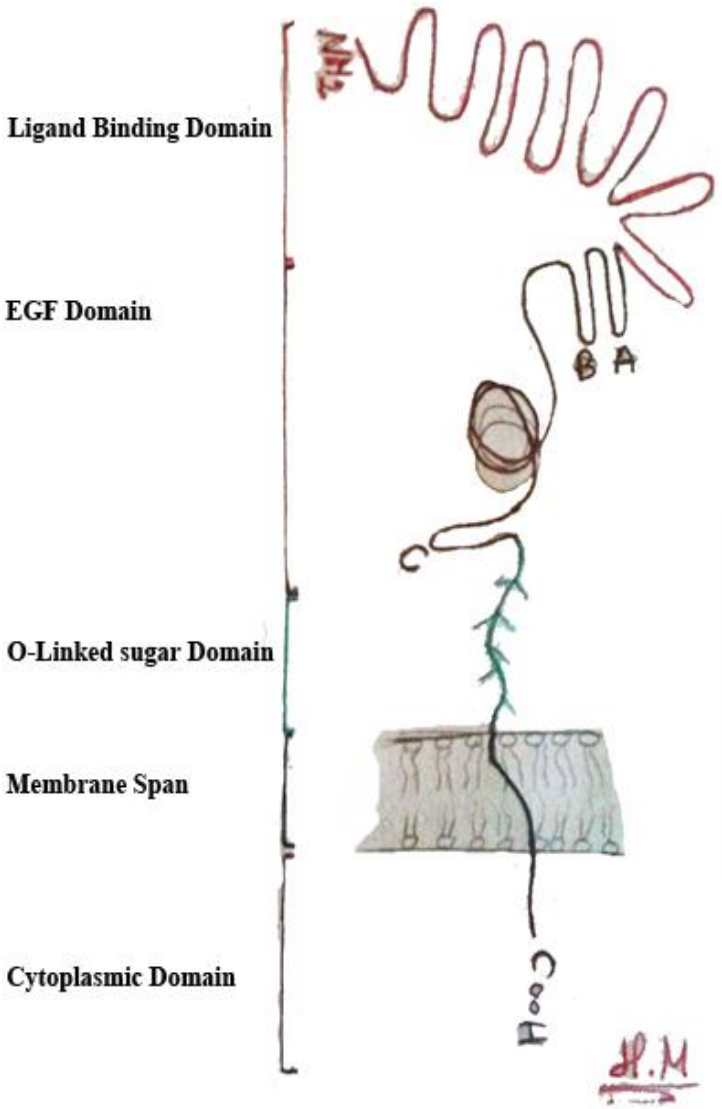


Figure 11. LDL-R Structure and domains.

b. Proteins involved in LDL-Receptor.

LDL receptor mode of action involves many proteins that regulates LDL receptor expression and mediates its internalization and degradation. Sterol regulatory element binding proteins (SREBP) regulate the expression of LDL receptor relatively to the cholesterol level while the Low Density lipoprotein adaptor receptor protein 1 (LDLRAP1) mediates LDL receptor internalization. Degradation of LDL receptor is mediated by two different proteins the inducible degrader of LDLR (Idol) and the Pro-protein convertase subtilisin-like kexin type 9 (PCSK9).

i. Sterol regulatory element binding proteins: SREBPs.

SREBPs are cytosolic 68KDa protein belonging to the family of helix-loop-helix leucine zipper (bHLH-Zip) that is present in other transcription factors. SREBPs are encoded by two different genes yielding three different iso-forms SREBP-1a,1c, SREBP-2 regulating fatty acids; and LDLR, HMGCoA reductase level respectively (Hua et al., 1993; Yokoyama et al., 1993). SREBps have 3 functional domains N-terminal fragment, two membrane spanning segment and C-terminal regulatory domain (Horton & Shimomura, 1999). The release of N-terminal domain is controlled by a chaperon protein SREBP cleavage-activating protein (SCAP) (Hua et al., 1993). When cholesterol level is low, SCAP interacts with SREBP inducing its translocation to golgi apparatus, cleavage and release the N-terminal sequence (Osborne, 2000). Subsequently the released SREBP sequence activates the transcription of cholesterol genesis enzyme and LDL-R.

ii. Low Density Lipoprotein Receptor Adaptor Protein 1: LDLRAP1.

Low density lipoprotein receptor adaptor protein 1 (LDLRAP1), known as ARH is a small protein composed of 308 amino acids that is highly expressed in the liver. It is folded into 3 different consecutive domains, the N-terminus domain, phosphotyrosine binding (PTB) domain and clathrin box consensus sequence. ARH mediates the internalization of LDL receptor facilitating the uptake of LDL. ARH mode of action involves dual binding: it binds by its PTB domain to the NPXY sequence in the cytoplasmic domain of the LDLR and by its LLDLE pentapeptide sequence to the clathrin then AP2 adaptin (He et al., 2002) mediating the internalization of the LDLR in liver cells (Cohen, Kimmel, Polanski, & Hobbs, 2003).

iii. Inducible degrader of the LDLR: Idol

Inducible degrader of the LDLR (Idol) is a protein that promotes degradation of LDLR receptor. IDOL is a 445 amino acids protein composed of two different domains: The N-terminal FERM domain and the C-terminal RING domain. FERM belongs to the ERM family protein that mediates interaction between membrane and membrane protein, the RING domain is similar to that found in the E3 ubiquitin ligases. Increase in cholesterol level leads to the activation of Liver X receptor (LXR) which mediates the over-expression of IDOL; thus decreasing LDL binding and LDL uptake by degrading the LDL receptor. IDOL facilitates the attachment of poly-K63 ubiquitin chain to the LDLR mediating its degradation which involves three different ubiquitin: E1 ubiquitin activation, E2 ubiquitin conjugation and E3 ubiquitin ligation; In addition, IDOL has an auto-proteolytic activity

via auto-ubiquitination that mediates its degradation (Figure 12) (Zelcer, Hong, Boyadjian, & Tontonoz, 2009).

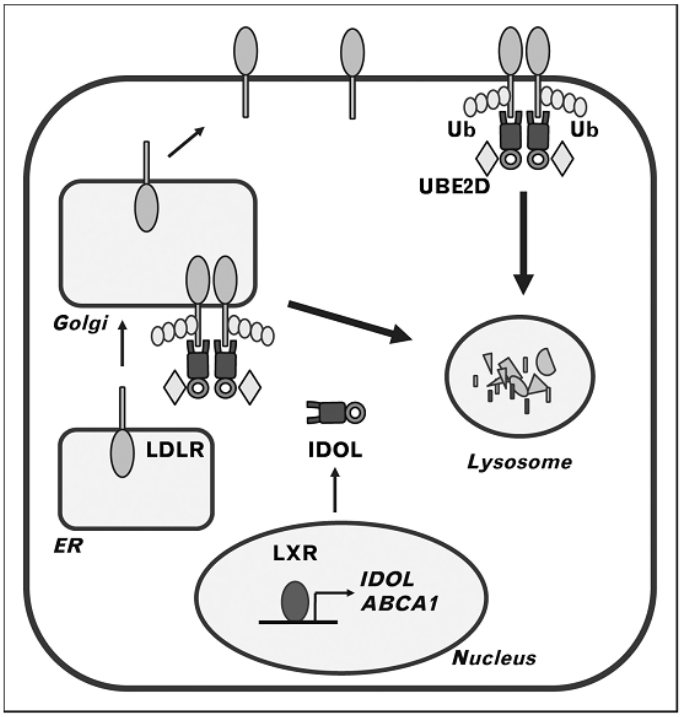


Figure 12. The LXR-IDOL-LDLR axis mediates ubiquitination and degradation of the LDLR (Sorrentino & Zelcer, 2012).

iv. Pro-proteinconvertasesubtilisin-like kexin type 9: PCSK9.

Pro-proteinconvertasesubtilisin-like kexin type 9 (PCSK9) is highly expressed as zymogen in the liver. The release of active pro-domain by autocatalytic activation of the inactive PCSK9 in golgi (Abifadel et al., 2003) assists the escorts of PCSK9 in the secretory pathway (Seidah et al., 2003). After secretion, PCSK9 binds to the EGF-A

domain of the LDL receptor and initiates the uptakes and degradation of the LDLR (D. W. Zhang et al., 2007); thus reducing LDLR number. PCSK9 is regulated by SREBPS (Figure 13) (Jeong et al., 2008), especially by SREBP2 (Maxwell, Soccio, Duncan, Sehayek, & Breslow, 2003; Shimano et al., 1997).

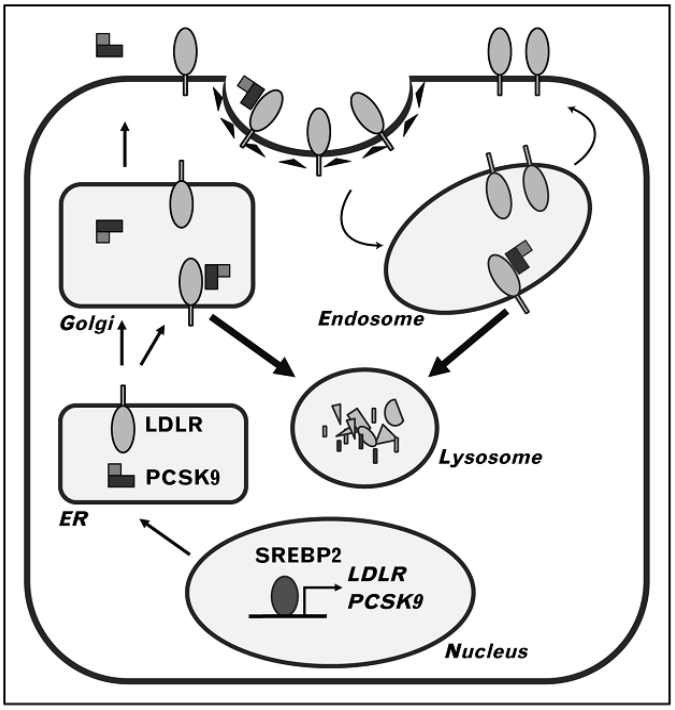


Figure 13. The SREBP–PCSK9 pathway controls abundance of the LDLR (Sorrentino & Zelcer, 2012).

c. Regulation of LDL Receptors

LDL receptor expression is regulated by SREBPs. When intracellular cholesterol is significantly decreased, SREBPs protein are cleaved in golgi apparatus and its N-terminus is transported to the nucleus activating the transcription of the LDLR, HMGCoA reductase gene and other enzymes related to cholesterol synthesis (Horton, Goldstein, & Brown, 2002). SREBPs cleavage is blocked by high cholesterol level, preventing the expression and internalization of LDL receptor (Figure 14).

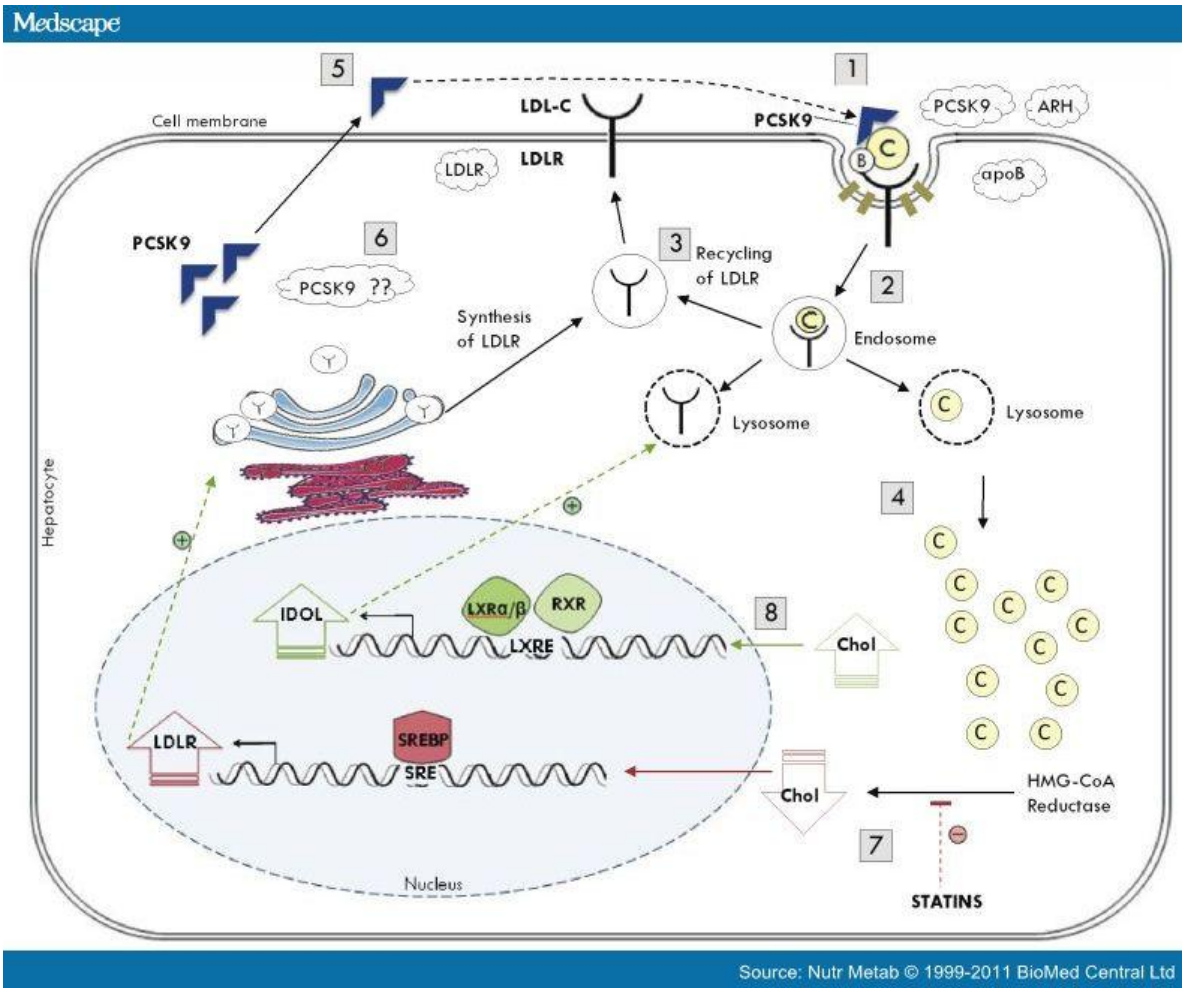


Figure 14. LDL-R signaling and regulation.

5. Regulation of cholesterol synthesis.

a. Transcriptional regulation and translational regulation.

HMGCoA reductase catalyzes the rate determining step in the cholesterol pathway. It shares with farnesyl-PP synthase, squalene synthase and LDL receptor a no palindromic sequence known as sterol regulatory element (SRE). SREBPs are transcription factors that bind to SRE domain; they are synthesized in their inactive form in the endoplasmic reticulum. They exist in different form SREBP-1a and c, and SREBP2 (Edwards, Tabor, Kast, & Venkateswaran, 2000). When level of cholesterol is low, SREBPs are trans-located to the golgi apparatus where the active N-terminal fragment is cleaved, released (M. S. Brown & Goldstein, 1997) and trans-located to the nucleus consequently activating transcriptions of target genes like the LDL receptor (Sato et al., 1994).

HMGCoA reductase is also regulated at the transcriptional level by mevalonate which reduces the mRNA level of HMGCoA reductase enzyme. Likewise, cholesterol feeding was shown to decrease the mRNA level of HMGCoA reductase (Nakanishi, Goldstein, & Brown, 1988; Peffley & Sinensky, 1985).

b. Covalent regulation (phosphorylation/de-phosphorylation).

HMGCoA reductase switches between active and inactive form via phosphorylation mediated by c-AMP-activated protein kinases. c-AMP increase as a consequent of ATP depletion and Glucagon/Insulin ratio increase. The increase in AMP levels activates the kinase dependent protein leading to the inactivation by phosphorylation of HMGCoA reductase and Acetyl CoA carboxylase, and consequently inhibition of cholesterol and fatty

acids synthesis; respectively. This inactivation provides a short term regulation and does not affect the cholesterol level but preserving the cellular energy [(Eds.), 2002)].

c. Proteolysis.

An increase in the intracellular cholesterol leads to the degradation of HMGCoA reductase consequently endogenous synthesis; isoprenoids such as farnesyl-PP, farnesol and mevalonate increase the rate of HMGCoA reductase degradation, down-regulating thus isoprene synthesis [(Eds.), 2002)]. Hence, the overexpression of squalene synthase or inhibition of HMGCoA reductase reduces the level of farnesyl-PP consequently preventing HMGCoA reductase degradation (Faust, Luskey, Chin, Goldstein, & Brown, 1982).

6. Disorders related to cholesterol pathway.

Different disorders were correlated with deficiency in enzymes involved in cholesterol synthesis such as mevalonic aciduria , characterized by an increase of mevalonic acid diffusion in the plasma (Sweetman, 2001), Smith-Lemli-Optiz syndrome (SLOS) due to deficiency in the 7-Dehydrocholesterol A7-reductase (R. I. a. H. Kelley, R.C.M. , 2001) and other metabolic disorders, correlated to deficiencies in cholesterol pathway enzymes, or cholesterol homeostasis component as summarized in the table below (Table 3).

Inborn errors of sterol biosynthesis

Syndrome	Metabolic defect
Mevalonic aciduria	Mevalonate kinase
Smith–Lemli–Opitz	Sterol Δ7-reductase
Desmosterolosis	Sterol Δ24-reductase
Rhizomelic chondrodysplasia punctata (CDP)	Pex7 peroxisomal enzyme import
CDP X-linked dominant (CDPX2)	Sterol Δ8,Δ7-isomerase
CHILD syndrome (congenital hemidysplasia with ichthyosis and limb defects)	Sterol Δ8,Δ7-isomerase Sterol C-4 demethylase
Greenberg skeletal dysplasia	Sterol Δ14-reductase

Table 3. Metabolic disorders related to cholesterol pathway(R. I. Kelley, 2000).

Other disorders related to cholesterol are due to defect in LDL receptor or protein involved in LDL receptor function, such as Familial Hypercholesterolemia (FH),

a. Familial Hypercholesterolemia (FH).

Familiar hypercholesterolemia (FH) is a hereditary disease characterized by an increase in cholesterol level and high serum LDL levels. It is due to mutations in the genes encoding for the proteins that facilitate the uptake of serum LDL and regulation of LDL receptor (Soutar & Naoumova, 2007)**.** Heterozygous FH is due to mutation in the LDL Receptor gene, ApoB100 gene or PCSK9 gene; it is transmitted in a dominant manner. However, homozygous FH is an autosomal recessive form characterized by mutation in ARH gene (Marais, 2004) . Clinical features include deposition of cholesterol in peripheral tissue leading to the formation of xanthomas and its deposition in the arteries which leads

to the formation of atherosclerosis and increases the risk of cardiovascular diseases (Table 4).

	Heterozygous	Homozygous
Clinical features	Tendon xanthoma	Tendon xanthoma Cutaneous xanthoma
	Coronary disease >25 years 5 mmol/L< LDLC <12 mmol/L	Coronary disease <25 years LDLC >12 mmol/L (Less in Phytosterolaemia and CTX)
Genetic disorders	LDL receptor: 1 allele apoB: 1 or 2 alleles NARC1: 1 allele	LDL receptor both alleles no gene dose effect not yet described ARH Phytosterolaemia CTX

Table 4.Clinical and genetic profile for FH.(Marais, 2004).

b. Treatment of Familial Hypercholesterolemia (FH).

For the last decades, Development of new therapeutic modalities has been a majortarget for researchers and drug industries. In addition, to dietary restriction, Different therapeutic agents, such as fibrate, cholestyramine, niacin and statins have been used to lower blood cholesterol levels

i. Cholestyramine.

Cholestyramine is an anion exchange resin that binds bile acids and prevents their re-absorption (Bergen, Van Itallie, Tennent, & Sebrell, 1959).it was expected to activate conversion of cholesterol into bile acid leading to a decrease in the intracellular cholesterol and up-regulation of LDL receptor (Ginsberg HN, 1990) Cholestyramine was reported to cause constipation, dizziness, nausea and many other side effect on liver, muscle.

ii. Niacin.

Niacin also known as nicotinic acid or nicotinamide is derived from vitamin B complex and known as precursor for nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NAD+ and NADP+).

Niacin was reported to reduce LDL, triglyceride levels and increase HDL level (Ginsberg HN, 1990). Niacin inhibits diacyl glycerol acyl transferase resulting in a decrease in lipolysis, triglyceride synthesis, and ApoB containing lipoprotein (Gille, Bodor, Ahmed, & Offermanns, 2008). Niacin was effective in lowering cholesterol, but it was reported to cause dermatological problems like flushing, stomach dyspepsia, and liver toxicity.

iii. Statins.

Statins are the most commonly used drugs in hypercholesterolemia treatment, (Inoue et al., 2000). Akira Endo was one of the leading scientists in the discovery of statins. In 1971, a mycotoxin,citrinin was found to inhibit HMGCoA reductase activity and lower serum cholesterol level in rats (Endo & Kuroda, 1976; Tanzawa, Kuroda, & Endo, 1977), with some toxicity to the kidney. In 1973, a blue mold component was discovered with structural similarity to compactin and HMGCoA molecule and reported to inhibit HMGCoA reductase activity (Figure 15) (Endo, 1992; Endo, Kuroda, & Tanzawa, 1976).

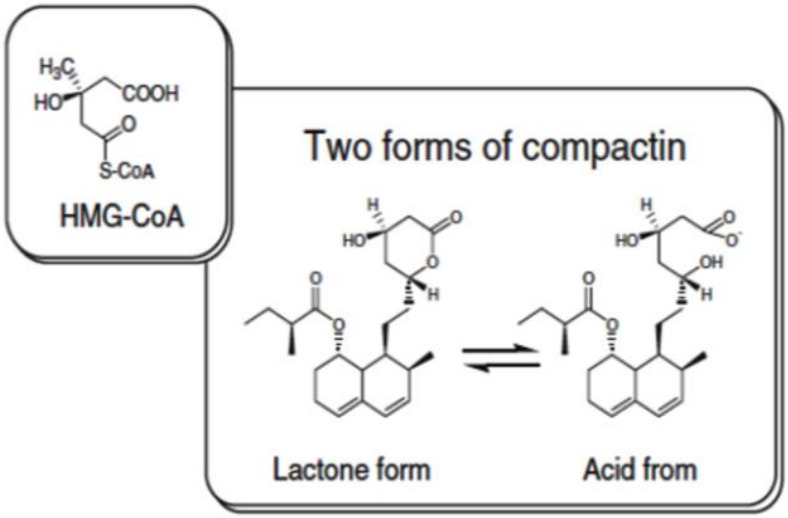


Figure 15. Structure similarity between HMGCoA and compactin.

Mevinolin discovered by Merck in 1976 was extracted from Aspergillust erreus (Alberts et al., 1980), was lately known as Lovastatin. Lovastatin was effective in increasing the LDLR level in dogs liver (M. S. Brown, Dana, & Goldstein, 1973)and efficient in treating patients with familial hypercholesterolemia.

In 1987, lovastatin was officially the first commercial statin used worldwide for the treatment of familiar hypercholesterolemia. Other semi synthetic statins that have structural similarity with lovastatin were introduced in the market like fluvastatin, atorvastatin, rosuvastatin and pitavastatin (Endo, 1992, 2008).

Statin decreases de novo synthesis of cholesterol by inhibiting HMGCoA reductase; consequently, the reduction in cholesterol level activates SREBP cleavage that activates expression of LDL-R and HMGCoA reductase. However, the increase in reductase has no significant effect on cholesterol since it will be inhibited by statin. Thus, the resulting increase in LDL-R will increase uptake of serum LDL and decrease serum cholesterol level (M. S. G. Brown, J.L 2004; Goldstein & Brown, 2009).

B. Ubiquinone

1. History

Ubiquinone (UQ) was discovered in 1975 as a lipid soluble molecule composed of a phenyl ring to which several isoprene units are attached (Figure 16).The number of isoprenoid units vary among species six isoprene units in Saccharomyces cerevisiae (CoQ6), eight in Escherichia coli (CoQ8), nine in mice (CoQ9), and ten in humans (CoQ10) (Crane, 2001; Ernster & Dallner, 1995).

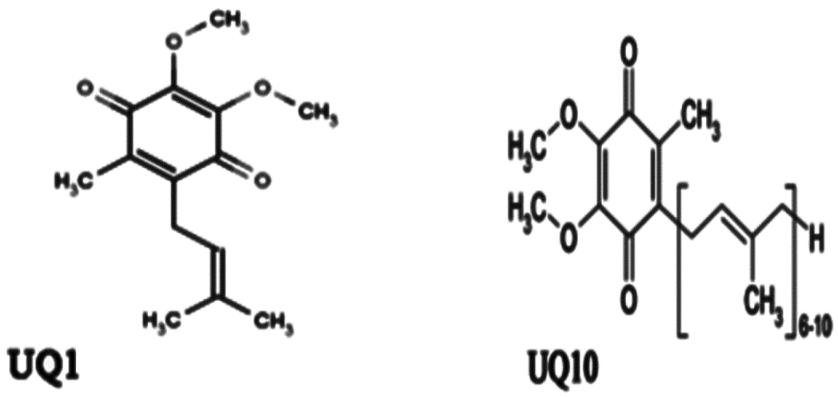


Figure 16. UQ1 & UQ10 Structure.

In addition to dietary intake, ubiquinone can be synthesized in all nucleated cells via mevalonate pathway (Figure 17) (Biotech, 2007-2014), the rate of synthesis of coenzyme Q is 100 times less than that of cholesterol (Bentinger, Turunen, Zhang, Wan, & Dallner, 2003). Ubiquinone synthesis pathway involves a main intermediates 4-OH-Benzoate, its prenylation requires transferase (Coq1 in yeast and Pdss1/Pdss2) which catalyze the formation of decaprenyl pyrophosphate that condenses with 4 OH-Benzoate via CoQ2 catalyzed reaction, followed by several modification involving (Tran & Clarke, 2007),

hydroxylation of C5 of the benzoquinone ring (Ozeir et al., 2011), decarboxylation, o and c-methylation mediated by Coq3, 5 and 7 (Baba et al., 2004; Marbois & Clarke, 1996; Poon et al., 1999).

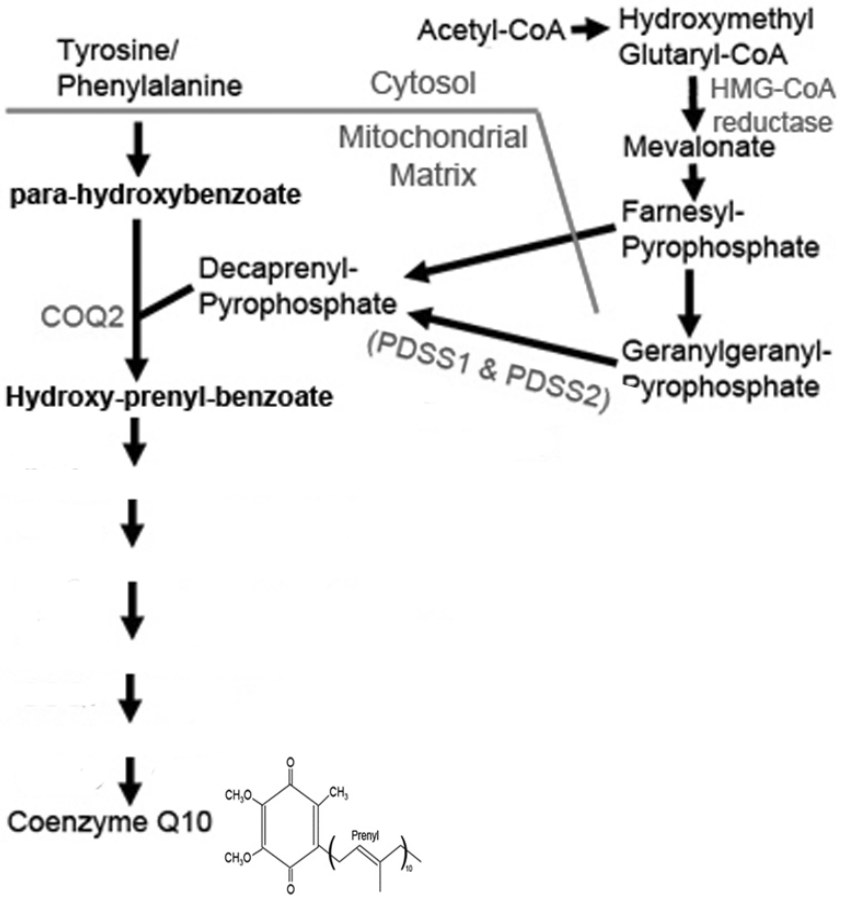


Figure 17.Ubiquinone synthesis pathway.

2. ***Function of Ubiquinone in Eltectron Transport Chain (ETC)***.

Function of UQs include, but is not limited to mitochondrial function in the electron transfer (ETC), it is a hydrophobic electron transport chain component that mediates transfer of two electrons from either of the complexes I and II to complex III. Its reduction gives rise to ubiquinol that acts as anti-oxidant (Figure 18) (Bentinger et al., 2003; Dai et al., 2011; Patel et al., 1995). In addition, it regulates mitochondrial permeability and proton transfer in un-coupler protein (Hamilton, Chew, & Watts, 2009).

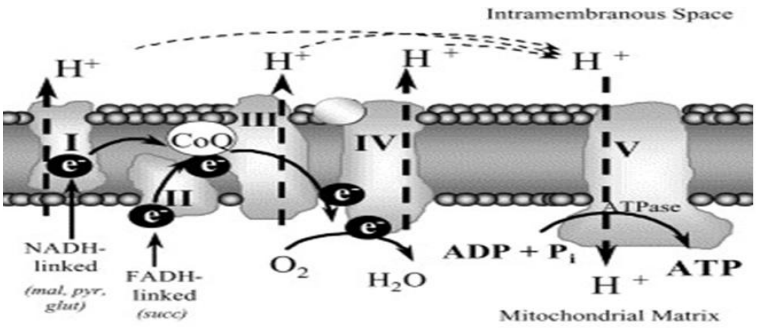


Figure 18. Electron transport chain (Cawtkon et al 2005).

Ubiquinone is reported to regulate cytosolic ratio of NAD/NADH,cell growth and differentiation (Gomez-Diaz et al., 1997); it counteracts apoptotic events like ATP depletion , caspase-9 activation depolarization of mitochondrial membrane potential and DNA fragmentation (Echtay, Winkler, Frischmuth, & Klingenberg, 2001; Papucci et al., 2003); Moreover, it conserves the enzymatic activity by maintaining the enzyme in its active reduced form (Y. Zhang, Aberg, Appelkvist, Dallner, & Ernster, 1995). Ubiquinone level was shown to decrease with the aging process while cholesterol increase (Kalen, Appelkvist, & Dallner, 1989); Deficiency in the ubiquinone level has been associated to many mitochondrial disorders with different clinical manifestation like encephalo-

myopathy, myoglobinuria and nephrotic syndroms (Emmanuele et al., 2012; Quinzii, DiMauro, & Hirano, 2007; Simonsen et al., 1991).

Ubiquinone is also found in all isolated lipoprotein fractions of human blood (Tomasetti, Alleva, Solenghi, & Littarru, 1999). Its anti-oxidant properties prevent the oxidation of LDL and increase a resistance of human low-density lipoprotein to the initiation of lipid peroxidation (Hassan Ahmadvand, 2013; Mohr, Bowry, & Stocker, 1992; Turunen et al., 2002) and stimulates endothelial release of nitric oxide which enhance endothelial functions (Hamilton, Chew, & Watts, 2007; Hamilton et al., 2009).

3. Results of Knock out Genes.

Recently, different knock out model was designed targeting enzymes involved in Ubiquinone synthesis. Pdss2 transferase knock-out mice showed a significant decrease in the ubiquinone 9 and 10 levels (Lyon & Hulse, 1971; Peng et al., 2008; Sibalic, Fan, & Wuthrich, 1997) with renal failure due to tubular dilatation, UQ10 supplementation was shown to improve some of the renal dysfunction (Saiki et al., 2008).

Pdss2, Coq3, Coq4 and Coq7 knock outs are lethal for mice; Coq7 mice exhibit a severe developmental delay and low level of ubiquinone 9 and 10 (Levavasseur et al., 2001; Nakai et al., 2001). Coq4 deficient mice show delayed embryonic development and a low level of ubiquinone, proving the importance of ubiquinone in embryonic development. Coq9 (R239X) knock-out mice are viable and show depletion in the level of ubiquinone 9 and 10, in addition to encephalo-myopatic signs (Duncan et al., 2009).

4. Clinical significance of Coenzyme Q10.

Studies have shown that increase in CoQ10 levels are subject to regulation by physiological factors such as oxidative stress, cold acclimation (Beyer, Noble, & Hirschfeld, 1962) and thyroid hormone treatment (Pedersen, Tata, & Ernster, 1963). On the other hand, a decrease in tissue CoQ10 levels have been reported under certain pathological conditions, in particular cardiomyopathy (Sugawara, Yamamoto, Shimizu, & Momose, 1990) and other types of muscle diseases

In addition, oral supplementation of CoQ10 has been tried in many genetic disorders in which CoQ10 synthesis is impaired such as Alzheimer's disease (Soderberg, Edlund, Alafuzoff, Kristensson, & Dallner, 1992) diabetes type2 (Palmeira, Santos, Seica, Moreno, & Santos, 2001) Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis (Barbiroli et al., 1997; Koroshetz, Jenkins, Rosen, & Beal, 1997; Shults, Haas, Passov, & Beal, 1997) with promising results.

C. Objectives of the study

Hypercholesterolemia is one of the major causes of atherosclerosis and increased risk of cardiovascular diseases. Statins, inhibitors of HMGCoA reductase, are efficient in decreasing serum cholesterol levels; however, their side effects and toxicity (De Pinieux et al., 1996; Gonzalez et al., 2009; La Guardia, Alberici, Ravagnani, Catharino, & Vercesi, 2013) increased the interest in finding alternative drugs that can lower lipid profile with possibly less side effects.

Ubiquinone one of the MVA pathway products beside cholesterol, was reported to play a role in lowering LDL oxidation, increasing HDL and reducing phospholipid and lipid profile in hypertriglyceridemia patients. Ubiquinone decreases with age while cholesterol level usually increases posing a question as to whether ubiquinone regulates MVA pathway and hence cholesterol level. The aim of this study therefore is:

1. To examine if UQ exhibits an effect on MVA pathway.
2. To identify, if possible, the site at which UQ exerts its effect.
3. To determine the effect of UQ on expression of LDL receptors.
4. To compare the effect of a known inhibitor of MVA pathway on UQ.
5. To investigate the effect of UQs co-administered with Statin (HMGCoA reductase inhibitor), FPT-I (FPP transferase and Geranylgeranyl transferase inhibitor) and Bacitracin (4-Hydrosybenzoate polyprenyltransferase) on cholesterol level.

CHAPTER II

MATERIALS AND METHODS

A. Materials.

1. Cancer cell lines.

Human Liver cancer cells (HepG2; Cat# HB-8065) were purchased originally from the American Type Culture Collection, Manassas, VA, USA.

2. Cell Culture Reagents.

Tissue culture reagents were purchased as listed below:

- **Lonza.**

Dublecco's Modified Eagle's Medium (DMEM; Cat# BE12-741F); Fetal Bovine Serum Heat Inactivated (FBS; Cat# DE14-801F); Penicillin-Streptomycin mixture (PEN-Strep; Cat# P4333-110M0790); Trypsin (BE 17-160E); Phosphate buffered saline 10X without calcium and magnesium (PBS; Cat# BE17-517Q); Insulin, Transferrin, Selenium (ITS; Cat# 17-8382); L-Glutamine (Cat# BE17-605E).

3. Disposable Lab Wares.

- Cell culture plastic wares were purchased from Corning
- Microscope cover glasses for immune-fluorescent assays (G15973C) were purchased from GAIGGER brand.

4. Kits.

- Roche

Cell Proliferation Kit I (MTT; Cat # 11699709001).

Enhanced Chemi Luminescence's Reagent ECL Kit (12015200001).

5. Chemicals and Reagents.

General chemicals and reagents were purchased as indicated below:

- BIO-Rad:

N, N, N', N' tetra-methylethylenediamine (Temed); Glycine; acrylamide/bisacrylamide; Nitrocellulose membrane (2µm); Ammonium Per Sulfate (APS).

- ICN

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes; Cat# 194550).

- Amersco.

Polyxyethylene-20-Sorbitan Monlaurate (Tween; Cat# 0777-1L); Sodium Dodecyl Sulfate (SDS).

- BDH laboratory reagent.

Sucrose (302997J).

- GE.

Rainbow molecular weight ladder (RPN800E).

- Acros organic.

Bacitracin(1405-87-4).

- Amersham.

DL-2-^{14}C-Mevalonic acid lactone CFA:194 Batch 64 (250uCi:1.03ml:22mCi/mmole) benzene solution.

- Sigma.

Sodium fumarate (Cat# F-1506); Gelatin; Trypan Blue; Methanol; Triton 100x; ß-Mercaptoethanol; Tris[hydroxymethyl]aminomethane Tris Base; Isobutanol, Coenzyme Q1 (C7956); Coenzyme Q10 (C9538); Mevalonolactone MVA (M4667); Ascorbic Acid (A0278)**;** Bovine Serum Albumin (BSA; Cat# A94180).

- AnalaR.

D-glucose (10117); Zinc chloride $ZnCl_2$; Copper (II) sulfate ($CuSO_4.5H_2O$); Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$).

- Fisher scientific.

Potassium chloride (KCl; Cat. # P-217); Sodium bicarbonate ($NaHCO_3$; Cat. # S233).

- Fluka.

D-Galactose (48260); Pyruvic acid Sodium salt (Mr110.04); Mevastatin (Cat#27696).

- Santa Cruz.

Farnesyltransferase inhibitor FPT Inhibitor I (sc-221625).

UltraCruz™ Hard-set Mounting Medium (sc-359850).

- Merck.

Sodium Chloride (NaCl; Cat# k: 35276104); Potassium dihydrogen phosphate KH_2PO_4; Magnesium sulfate $MgSO_4$; Ammonium Heptamolybdate.

- Worthington biochemical corporation.

Collagenase type II (4176).

- Research Products International Corp.

Econo-Safe ^{TM} Scintillation cocktail.

- Whatman.

Thin Layer Chromatography plates-Partisil LK6D, Silica Gel 60A, Catalogue number 4865-821

6. Antibodies.

Primary antibodies and Secondary antibodies were purchased as indicated:

- LDL Receptor (LDL-R) Antibody (ab52818) Abcam.
- Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) pre-adsorbed (ab150065) Abcam.
- GAPDH (6C5) mouse monoclonal antibody (Sc-32233) Santa Cruz.
- Anti-Rabbit IgG H+L Peroxidase Affinipure Goat (111-035-003) Jackson.
- Anti-Mouse IgG H+L peroxidase Affinipure goat (115-053-166) Jackson.

7. Equipments.

The following equipments were used in the study:

- Eppendorf: Mini Spin 22331.
- Thermo: MicroCL 17R.
- Fisher scientific: Hematocytometer.
- Axiovert 25: Inverted microscope.
- Zeiss: Confocal Microscope.
- KODAK: RP X-OMAT processor Model M6B.
- SHIMADZU: UV-VIS scanning Spectrophotometer UV-2101 PC.
- PerkinElmer: Liquid scintillation counter Tricarb 2100.

8. Buffer Preparations.

- Laemmli (4X) was prepared by mixing 8ml of 10% SDS , 1ml of glycerol 100%, 1ml of 10% ß-Mercaptoethanol and 100µl of 0.01% bromophenol blue.
- Acrylamide (30%) was prepared by dissolving 29.2g of acrylamide and 0.8g N, N-methylene-bisacrylamide in 100mL ddH_2O.
- APS 10% was prepared by dissolving 0.1g APS in 1mL ddH_2O.
- Resolving gel (8%): resolving gel was prepared by mixing 1.3 mL of 30% acrylamide; 1.3mL of Tris-HCl 1.5M (pH 8.8); 50µl of 10% SDS; 50µl of 10% APS and 3µl Temed. The volume was adjusted up to 5ml with double distilled water ddH_2O.
- Stacking gel (4%): stacking gel was prepared by mixing 330µl of 30% acrylamide, 250µl of 0.5M Tris-HCl pH6.8; 20µl of 10% SDS; 20µl of 10% APS and 2µl of TEMED.

- Running buffer 5X was prepared by dissolving 15.1 g of Tris-Base, 72 g of Glycine and 5 g of SDS in water up to 1 liter, where its pH is adjusted to 8.3
- Transfer buffer 1X was prepared by dissolving 3.03 g of Tris-Base, 14.4 g of Glycine and 10 mL of 10% SDS dissolved and diluted in ddH2O.
- Trichloroaceticacid (TCA) 0.3% solution was prepared by dissolving 0.3g TCA in 100ml ddH_2O.
- NaCl (5M) solution was prepared by dissolving 146.1g of sodium chloride in 500mL ddH_2O.
- TBS 1X was prepared by adding 10mL of Tris-HCl (1M; pH 8) and 30mL of NaCl (5M) diluted up to 1L ddH_2O.
- Blocking buffer was prepared by mixing 5g of fat free milk with 50 µl tween in 100 mL TBS 1X.
- Washing buffer is composed of 300 ml TBS with 300µL Tween20.
- Stripping buffer was prepared by dissolving 6.25ml of Tris-HCl (1M; PH 6.7); 20 ml of SDS 10 % in 100 ml distilled water
- Sucrose-Hepes-Tris base (SHT; 250mM -10mM -50mM) was prepared by dissolving 85.6g of sucrose, 2.83g of Hepes and 60g of Tris-Base in 1 liter ddH_2O, with pH adjusted to with HCl to 7.4.
- SHT-Triton was prepared by dissolving 10µl of triton 100% in 1ml of SHT.
- Heptane/Ethyl acetate prepared by mixing Ethyl acetate and Heptane at (1/2,v/v).
- Charring buffer was prepared by adding 9ml of 10N H_2SO_4, 1ml of 70% HClO and 40ml of ddH_2O.

- Ammonium Molybdate 0.9% solution was prepared by adding 0.9g of Ammonium Molybdate in 100 ml of ddH_2O.
- Ascorbic Acid 9% solution was prepared by dissolving 9g of Ascorbic acid in 100ml of ddH_2O.
- PBS 1X was prepared by diluting 100ml of 10X PBS up to 1 liter ddH_2O.
- BSA-PBS 2% was prepared by dissolving 0.2g of BSA in 10ml of PBS 1X.
- Triton-PBS 0.5% was prepared by dissolving 50ul of 100% triton in 10ml of PBS 1X.
- Primary Hepatocytes perfusion buffers (A&B):
    - Buffer A: NaCl (94mM); KCl (4.74mM); KH_2PO_4 (1.19mM); $MgSO_4$ (2.4mM); $NaHCO_3$ (25mM); Na-pyruvate (4.9mM); Na-fumarate (5.38mM) and D-glucose (11.5mM) , volume was then adjusted up to 650 ml with ddH_2O. A volume corresponding to 1000 units of heparin was added to 200ml of buffer A prior to its use in perfusion.
    - Buffer B was prepared by mixing 200ml of buffer A with 1000 units of heparin and 80mg of collagenase type2-364 μ/mg-CLS2.

9. Cell culture medium preparations.

Primary hepatocytes cells were cultured in DMEM media 500ml supplemented with glucose (4.5g/L), galactose (2 g/L), HEPES (2.83g/L), glutamine (1mM), $ZnCl_2$(0.4μM), $CuSO_4.5H_2O$ (0.08μM), $ZnSO_4.7H2O$ (0.26μM), ITS (1 ml/L), PEN-Strep (2.5 ml) and +/- FBS (50ml) were mixed and filtered sterile under the hood.

B. Methods.

1. $HepG_2$ cells: culture.

HepG2 cells were cultured in DMEM (500ml) containing FBS (50 ml) and PEN-Strep (2.5 ml) at a density as indicated for each experiment. Treatments were dissolved in FBS-free DMEM media.

2. Primary hepatocytes.

a. Isolation and culture.

Primary hepatocytes isolation was performed as described by *Mueller et al (Muller et al., 2004).* In brief, overnight fasting rat (250) gm was anesthetized by an intraperitonial injection of xylazine (100mg/1Kg rat weight): ketamine (10mg/1Kg rat weight). The abdomen was opened and a catheter was introduced in the hepatic portal vein. The liver was perfused at a rate of 35 ml/min with warm (37ºC) buffers A and B 200ml consecutively. The perfused liver was then transferred onto a sterile dish containing 1X-PBS solution, and scraped to shed the hepatocytes. The cells suspension was filtered through Mira cloth filter (pre-soaked in 1X PBS) into 50 ml sterile conical tube. A rapid qualitative cell viability test was performed using trypan blue exclusion assay (described below). Cells were discarded if viability was less than 70%; otherwise, they were allowed to sediment at room temperature, then washed consecutively 2 times with 50ml of 1xPBS and once with DMEM media containing FBS.

Isolated primary hepatocytes were counted, seeded (as indicated in each experiment) and cultured initially in FBS containing media onto plates coated with 0.2% gelatin, incubated in CO2 chamber at 37ºC to allow cell adherence; after 4 hours media was replaced by FBS-free DMEM media.

b. Trypan blue exclusion viability assay.

Hepatocyte viability was qualitatively assessed using Trypan blue exclusion assay. Cells mixed with Trypan blue, at ratio 1v/1v, were introduced into hemacytometer, visualized and counted under the light microscope. Blue stained cells indicate dead cells, whereas unstained opaque cells are viable. The % Viability was estimated by calculating the ratio of unstained cells/total number of cells (stained and unstained)

3. General assay.

a. MTT assay.

Quantitative determination of cell viability was performed on primary hepatocytes and $HepG_2$ cells using MTT assay as described by the instruction manual of the cell proliferation Kit I MTT from Roche. The assay depends on the ability of viable cells to reduce the yellow MTT (3-[4, 5-dimethylthiazol-2, 5-diphenylTetrazolium bromide]) into purple formazan crystals which were solubilized and incubated overnight. The intensity of the developed color was measured using ELISA reader at 595 nm.

Cells seeded in 96 wells plate at a density of 10,000 cells/well/100µl FBS/DMEM media, were incubated for 24 hours in CO2 incubator at 37ºC, following which they were

treated with various compounds and/or inhibitors dissolved in FBS free DMEM media. Viability of control versus treated cells was determined after 24 hours using MTT assay.

b. ^{14}C MVA incorporation into cholesterol.

Radiolabeled ^{14}C-mevalonate incorporation into cholesterol was determined in primary hepatocytes and HepG2 cells; In 6 well plates, cells were seeded at a density of 10^6cells/well/1ml, cultured in FBS-DMEM for 4 hours, then treated with ^{14}C mevalonolactone (^{14}C-10^6CPM/well) supplemented with non-labeled MVA-lactone (200µM). Following 24 hours incubation in CO_2 incubator, treated cells were scraped, collected, pelleted down, and re-suspended in 1ml PBS.

Lipids were extracted as described in part (3c), separated by TLC as described in part (3d) and exposed to X ray film for 2 days. Level of radioactivity incorporated into various intermediates of MVA pathway was determined and normalized to inorganic phosphate as described in (3e).

c. Lipid extraction.

Lipid extraction was carried on cell suspension (part 3b) as follow: Four ml of chloroform/methanol (1/2-v/v) were added to the 1ml PBS cell suspension (part3b), followed by successive addition of 1.3 ml of chloroform, 1.3 ml of water. Vigorous vortexing was performed for 1min after each addition.

Three different layers appeared: an upper layer containing polar lipids; mid white layer containing denatured protein and a lower chloroform layer. The chloroform layer was collected, dried using speed vacuum Centri-Vap for 4 hours. The residual oily lipid extract

was suspended in 250ul of chloroform and used for separation by TLC (210µl) and inorganic phosphate determination (40µl).

d. Thin Layer Chromatography (TLC).

Lipids were separated using silica gel thin layer chromatography (TLC) plates 20x20 divided into multiple lanes. TLC plate was initially washed in acetone and dried before samples application. Extracted lipids (210 µl) from part (3c) were applied on TLC, with each treatment on separate lane. Samples were then resolved by capillary migration using heptane/ethyl acetate (2; 1, v/v) pre-saturated chamber, dried and exposed to Xray film for 48 hours.

Separated lipids were identified by comparing their mobility relative to pure standards of cholesterol, lanosterol, Q10 and squalene, visualized by I_2 vapor. The amount of incorporated radioactivity in lipids intermediate was determined by scraping, collecting silica spot corresponding to any of above compounds; suspending it in methanol (1ml) for 1 hour followed by adding scintillation fluid and counting using liquid scintillator machine from Perkin Elmer. Results were normalized to inorganic phosphate and expressed as CPM/Pi.

e. Inorganic phosphate determination.

Extracted lipid (20µl) were introduced into glass tubes and were digested for an overnight (160º) in charring acid solution (600 µl). To the resulting solution, the following was then respectively added each followed by 10" vortexing: 900 µl of ddH_2O, 500 µl of 0.9% Ammonium Molybdate and 200 µl of 9% ascorbic acid. The mixture was further incubated for 30' at 45ºC and the intensity of blue color developed was measured at 820nm.

The amount of phosphate was evaluated relative to a standard curve KH_2PO_4 (5-100 nmoles).

f.Protein quantification:

The concentration of protein was determined using the Bradford assay as described by the supplier manual http://www.biorad.com/webroot/web/pdf/lsr/literature/Bulletin_9004.pdf). Developed color intensity was measured at 595 nm using a Schimadzu spectrophotometer and protein level was quantified compared to BSA standard (2-10µg).

g. Western blot analysis of LDL receptor.

The expression level of LDL-R in control and treated cells was determined using western blotting. Control/treated cells were lysed in 1% triton-SHT, a protein fraction corresponding to 100µg was then separated on 8% SDS polyacrylamide gel, separated, transferred onto nitrocellulose membrane. Immuno blotting of the membrane was carried on as follow: one hour blocking with 5% fat free milk, overnight incubation at 4ºC with primary antibody (anti-LDL receptor(1/1000) or anti-GAPDH (1/1000)), 3 washes with TBS-Tween (15 minutes each), incubation with secondary antibody (anti-rabbit (1/10000) or anti-mouse (1/10000) respectively) and a final washing (3 time) with TBS-Tween prior to the development using ECL reagent. Membranes were then exposed to Xray film and LDL-R and GAPDH bands were scanned and then quantified using image j software. Expression of LDL-R receptor was normalized to GAPDH.

h. Immuno fluorescent-staining of LDLR.

The expression of LDLR in control and treated cells was also assessed by immuno-staining; Cells ($25x10^4$cells/well/500ul) were seeded and treated on cover slips introduced in 12 well plates. After 24 hours, Cells were consecutively: fixed with Para-formaldehyde (4% solution in PBS) for 10 min, permeabilized (0.5% triton- PBS) for 4 min, treated with 2% BSA - PBS for 1hr; incubated overnight with primary antibody (anti-LDL receptor (1/100) diluted in 2%BSA/PBS (1/100,v/v)) and washed twice with PBS/tween (5 min). Secondary anti Rabbit antibody that is coupled to fluorescent probe was then added (1/100) for 1 hour, washed twice with PBS/tween (2 min). The coverslips were removed, allowed to dry, and then mounted on slides containing Heochest staining for the nuclei. Slides were kept for 24 hours at 4ºC, analyzed using Confocal Microscopy.

4. Effect of ubiquinone 1/10 and statin on the cholesterol, lanosterol, UQ10 and squalene level.

Effect of UQ1, UQ10 and statin on the incorporation of ^{14}C MVA to cholesterol, lanosterol, UQ10 and squalene was determined by pre-treating primary hepatocytes and HepG2cell for 1 hour each with UQ1 (25 µM/50 µM) or UQ10 (50 µM) or Statin (50 µM) prior to mevalonolactone addition (section3). Treatments were prepared in 1000 µl of FBS free DMEM hepatocytes media or FBS-free DMEM HepG2 media.

5. ***Effect of statin, FPT-I and bacitracin co-treatment with UQ1 or UQ10 on the cholesterol, lanosterol, and squalene level*****.**

The effect of specific inhibitors of mevalonate pathway enzymes co-administered with UQ1 and UQ10 was assessed on primary hepatocytes. Cells were pre-treated as described in the table below prior to mevalonolactone addition (Table1). Treatments were prepared in 1000 µl of FBS free DMEM hepatocytes media or FBS free DMEM HepG2 media.

Treatment 2 / Treatment 1	Control	25µM Q1	50µM Q10
Statin (50µM)	+/-	+/+	+/+
FPT (47µM)	+/-	+/+	+/+
Bacitracin (50 µM)	+/-	+/+	+/+

Table.1.summary of co-treatment of UQ1&UQ10 with different inhibitors

6. Toxicity of UQ1, UQ10 and different inhibitors.

The toxicity of UQ1, UQ10 and different inhibitors was assessed by determining the effect of each compound on cell viability using MTT assay expressed as % relative to control and treated cells.

Cells were treated to mimic treatment in 6 well plates respecting the cell dilution (50µM/ 1000cells/µl) which is equivalent to (5µM/ 100cells/µl) (Figure 1).

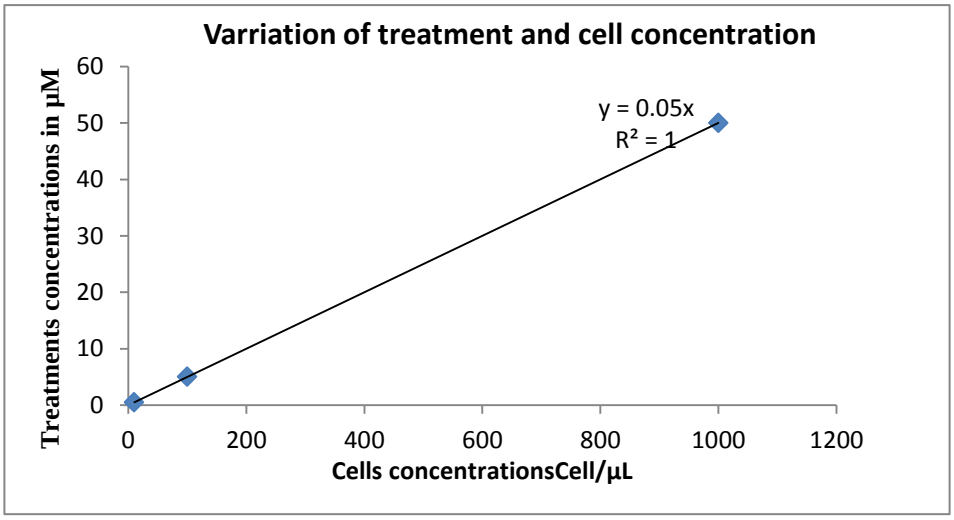


Figure 1. Variation of treatment concentration as function of cells dilution.

Treatments are described in the table below (Table2):

Treatment / Cell	2.5µM Q1	5µM Q1	5 µM Q10	5 µM Statin	5 µM Bacitracin	4,7 µM FPT
Hepatocytes	-	+	+	+	+	+
HePG2	+	+	+	+	-	-

Table 2. summary of treatments for toxicity assay in Hepatocytes and HepG2 cells

7. Effect of UQ1, UQ10 and statin on the expression of LDL-R by immuno-fluorescent staining and western blotting.

Effect of UQ1, UQ10 and statin on the expression of LDL-R was determined by immuno-fluorescent staining and immune blotting of LDL-R.Cells were pre-treated with each of UQ1 (25 µM), UQ10 (50 µM), Statin (50 µM) in presence or absence of MVA (200 µM) using suitable free FBS DMEM media, processing of cells was as described before under section

8. Statistical analysis:

Data are presented as average± Standard Error of the Mean (Yalovsky et al.) of ndeterminations of n experiments indicated in each figure. Statistical significance was analyzed using the independent sample t-test using SPSS software and the difference was considered significant whenever P-value was < 0.05.

CHAPTER III

RESULTS

A. UQ1 & UQ10 lower cholesterol level in primary hepatocytes.

The effect of UQ1 (25, 50μM) and UQ10 (50μM) on ^{14}C-MVA incorporation into cholesterol, lanosterol and squalene in primary hepatocytes was assessed. Both UQ1 & UQ10 showed a significant decrease in the cholesterol level; with UQ1 being more potent. A decrease of 58% and 94% was obtained at 25μM and 50μM UQ1, whereas only 14% reduction in cholesterol level was obtained with 50μM UQ10 (Fig1).

Furthermore, UQ1 & UQ10 treated primary cells caused an increase in lanosterol and squalene levels, UQ1 (25, 50μM) increased lanosterol level by 114% and 54%, and squalene level by 74% and 26%; respectively. UQ10 (50μM) increased both lanosterol and squalene level each by 84% (Figure 1); these findings suggest that the possible site of inhibition of these compounds is downstream the intermediate lanosterol.

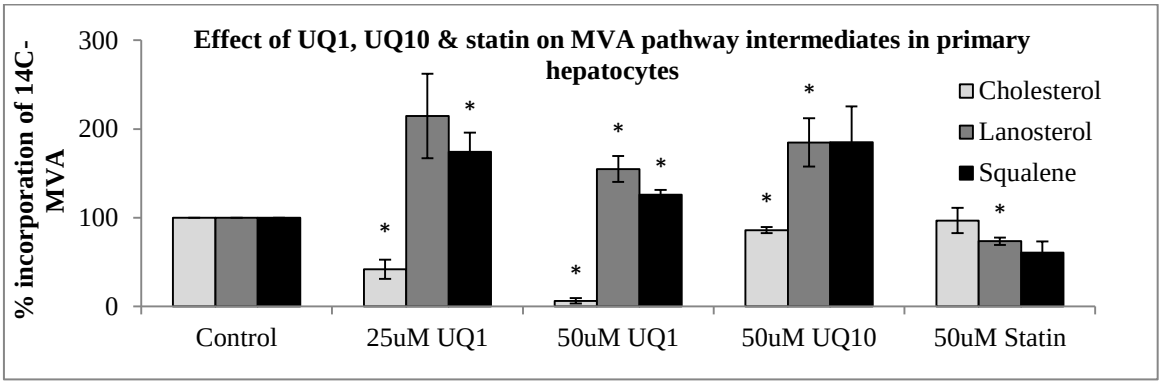


Figure 1. Variation of cholesterol, lanosterol and squalene level in treated primary hepatocytes. Results are average of n=3-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

In our experimental condition, the use of mevalonate bypassed HMGCoA reductase step. Hence, we tested the effect of statin (50μM), an inhibitor of HMGCoA reductase on cholesterol level. A decrease in lanosterol of 25% and squalene of 40% levels was obtained; while no significant effect on the cholesterol level was observed ($p>0.05$).

B. FPT-I but not statin or bacitracin reduces cholesterol level.

The effects of various individual inhibitors of the MVA pathway on cholesterol level were investigated. Statin, an inhibitor of HMGCoA reductase enzyme, FPT-I an inhibitor of farnesyl transferase and Bacitracin an inhibitor of 4-hydroxybenzoate polyprenyltransferase were investigated. Neither statin nor Bacitracin showed any significant variation ($p>0.05$) in the cholesterol level; however, a 40% decrease in cholesterol was obtained upon treatment with FPT-I (Figure 2). Statin and FPT-I decreased significantly ($p<0.05$) lanosterol by 27%, 50% and squalene by 40%, 27% respectively. However, Bacitracin had no significant ($p>0.05$) effect on either lanosterol or squalene level (Figure 2).

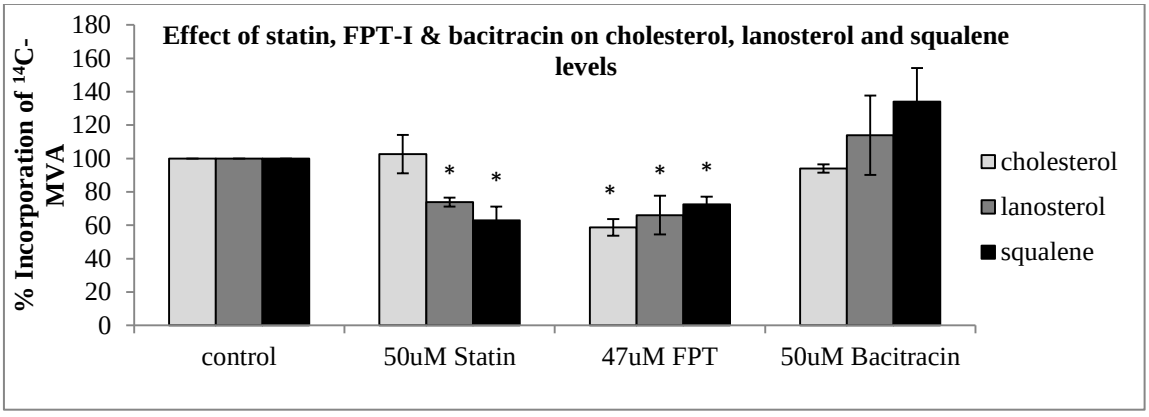


Figure 2. Effect of specific inhibitors of MVA pathway on level of cholesterol, lanosterol, and squalene. Results are average of n=4-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

C. Statin and UQ1/UQ10 co-treatment enhance the inhibitory effect on the mevalonate pathway.

In order to examine whether the effect of various inhibitors is synergistic or additive to UQ1orUQ10; we investigated the effect of statin UQ1/UQ10 co-treatment on cholesterol level. Our data show a decrease of 97% and 25% in MVA incorporation into cholesterol with statin -UQ1 and statin-UQ10 co-treatment; respectively (Figure 3). Moreover, lanosterol and squalene levels are increased by 67% and 139% in statin-UQ1 respectively.

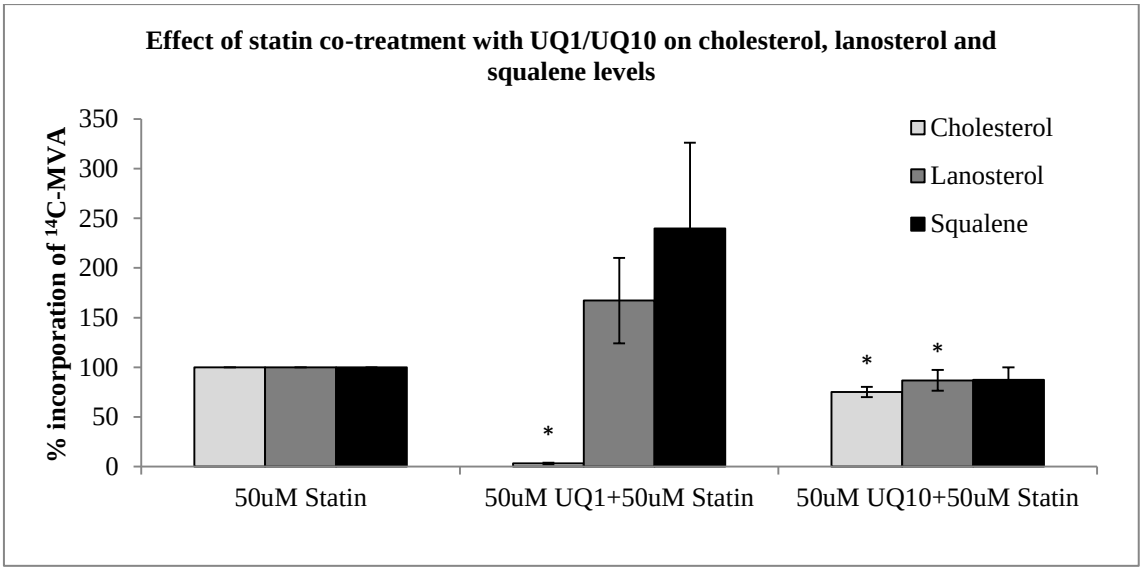


Figure 3. Effect of statin-UQ1 and statin-UQ10 co-treatment on level of cholesterol, lanosterol and squalene. Results are average of n=5-7. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

D. Bacitracin and UQ1/UQ10 co-treatment do not vary UQ1/UQ10 effect on mevalonate pathway.

Bacitracin an inhibitor of 4-hydroxybenozate polyprenyl transferase enzyme blocks the branched chain reaction that leads to ubiquinone.

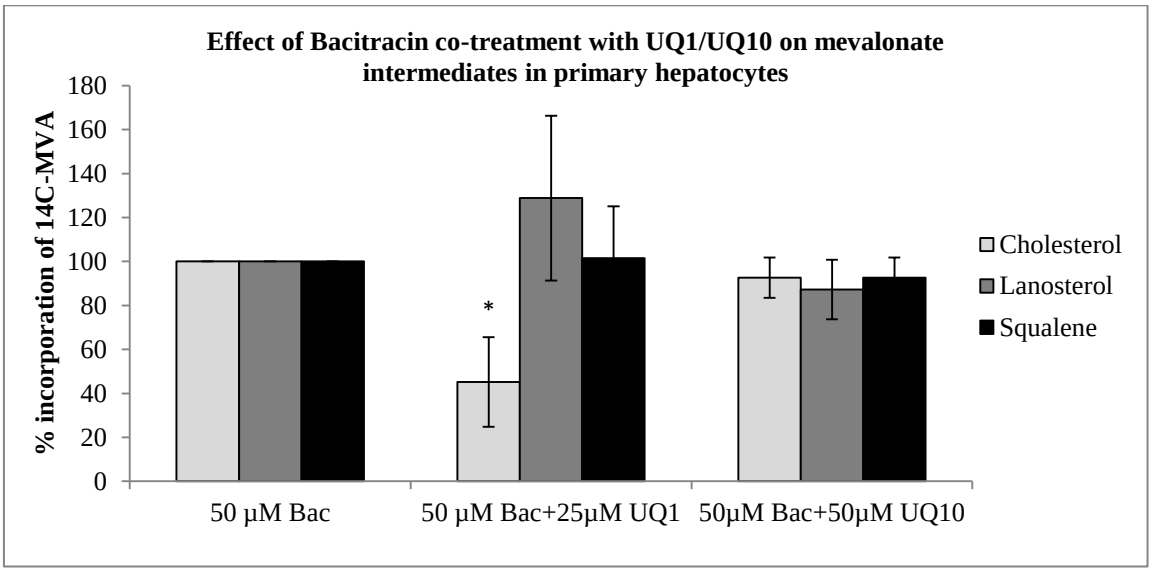


Figure 4. Effect of bacitracin (Bac)-UQ1 and bacitracin (Bac)-UQ10 co-treatment on level of cholesterol, lanosterol and squalene. Results are average of n=4. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

Primary cells co-treated with UQ1 (25µM) and Bacitracin (50µM) showed a similar profile to that of UQ1 (25µM). A decrease in cholesterol level by 55%, an increase lanosterol by 29% and no variation in squalene level was obtained; however, Cholesterol, lanosterol and squalene levels did not significantly vary upon co-treatment of 50uM bacitracin with 50uM UQ10 (Figure 4).

E. FPT-I inhibitors reverse UQ10 effect and enhance UQ1 effect on cholesterol level.

FPT-I is an inhibitor of farnesyl pyrophosphate transferase and geranygeranyl pyrophosphate transferase. Primary cells co-treated with FPT-I and UQ1 or UQ10 was assessed to determine the possible site at which inhibition occurred. Co-treatment with FPT-I- UQ1 co-treatment decreased significantly the cholesterol level by 75%; increased significantly lanosterol level by 75% and decreased squalene level by 6%; However, co-treatment with FPT-I- UQ10 had no significant effect on the cholesterol level, while lanosterol and squalene level increased by 35% and 16% respectively (Figure 5).

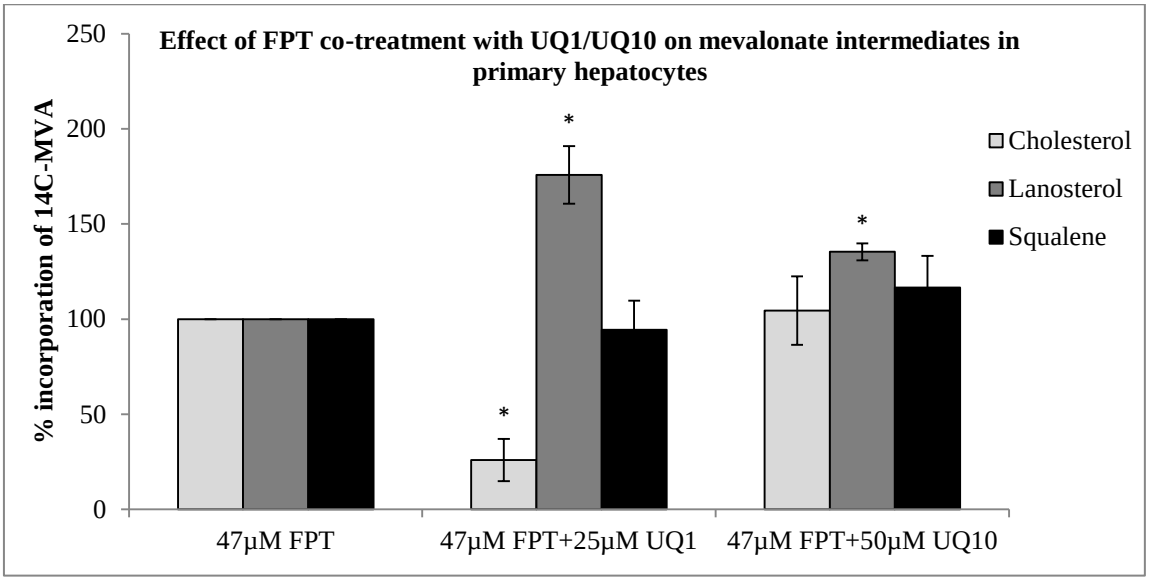
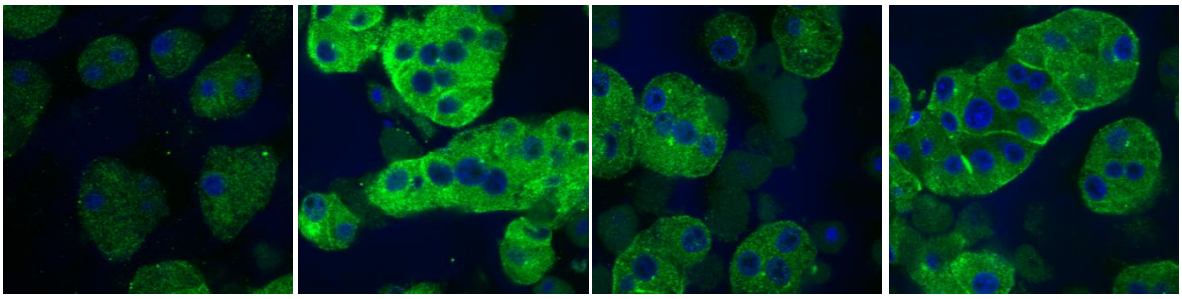


Figure 5. Effect of FPT-I-UQ1and FPT-I-UQ10 co -treatment on level of cholesterol, lanosterol and squalene. Results are average of n=4. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

FPT-I-UQ1 promotes the effect of UQ1. Further decrease in cholesterol level by 25% occurs while maintaining the increase in lanosterol level, emphasizing on previous suggestion that UQ1 acts after lanosterol formation. However, FPT-I/UQ10 co-treatment had no effect on cholesterol level while restoring a slight increase in lanosterol level, suggesting that UQ10 has FPT-I like effect.

F. UQ1 and UQ10 increase the expression of LDL receptor in primary hepatocytes.

The expression of LDL receptor was examined by immuno fluorescent staining using fluorescent probes. Results showed an increase in the intensity of the green color, thus reflecting an increase in the LDL receptor expression (Figure 6a). Quantification of the intensity of the green color in selected cells proves the increase in the LDL receptor (Figure 6b).



Control+0.2mM MVA | 25µMUQ1+0.2mM MVA | 50µM UQ10+0.2mM MVA | 50µM Statin +0.2mM MVA

Figure 6a. Representative figure of the immune staining of LDL-R in primary hepatocytes; control and following treatment with UQ1, UQ10, statin in presence of MVA. Green channel represent LDL-R, Blue channel represent nuclei.

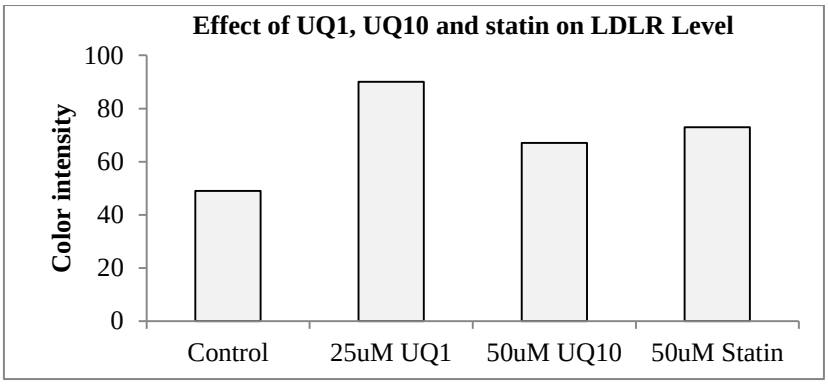


Figure 6b. Results of green color quantification in one cell after immuno staining of LDL-R in primary hepatocytes; control and following treatment with UQ1, UQ10 and statin in presence of MVA.

Our attempt for western blot analysis failed to detect LDL receptor since human antibody (Anti Human LDL-R) failed to interact with denatured rat proteins; however, LDL receptor was visualized by immune staining using fluorescent probes.

G. UQ1, UQ10 lower cholesterol level in HepG2 cells.

To determine whether cholesterol lowering effect by UQ1, UQ10 is specific to normal liver cells, we examined their effect on HepG2 cells. Results were in line to those obtained with primary heaptocytes. UQ1 significantly decreased cholestrerol level by 50 & 90% at 25 & 50 µM respectively, increased lanosterol (89 %) and squalene (51%) levels. On the other hand, UQ10 exerted more significant decrease (25%) in cholesterol level compared to primary hepatocytes, however it had no effect on lanosterol and squalene level, which mean that the inhibiton occurs at two different sites downstream lanosterol and upstream squalene. Statin treatment decreased significantly ($p<0.05$) cholesterol level by 40%, while it increased lanosterol and squalene level by 15% and 78% respectively (Figure 7). As a results UQ1 and UQ10 effect on primary hepatocytes is comparable to HepG2 cells; however, statin results were discordant to those obtained in primary cells with cholesterol level.

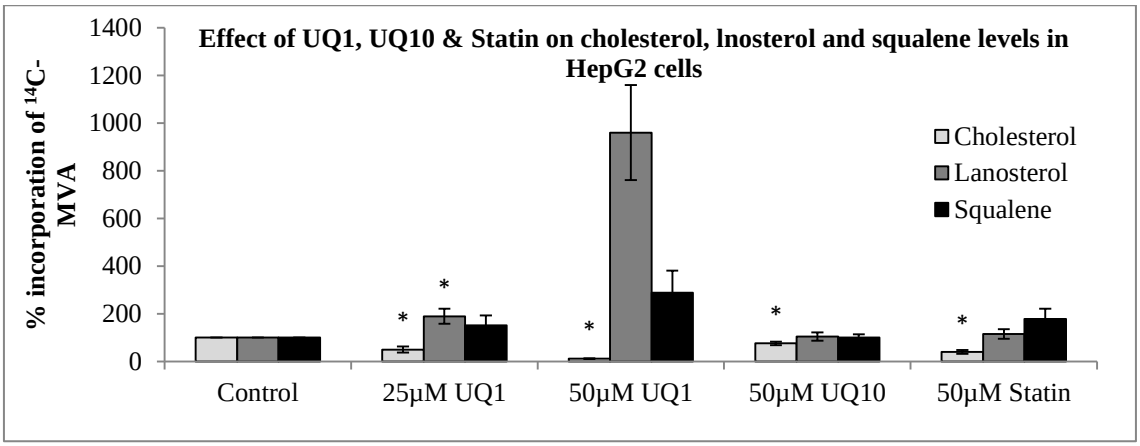
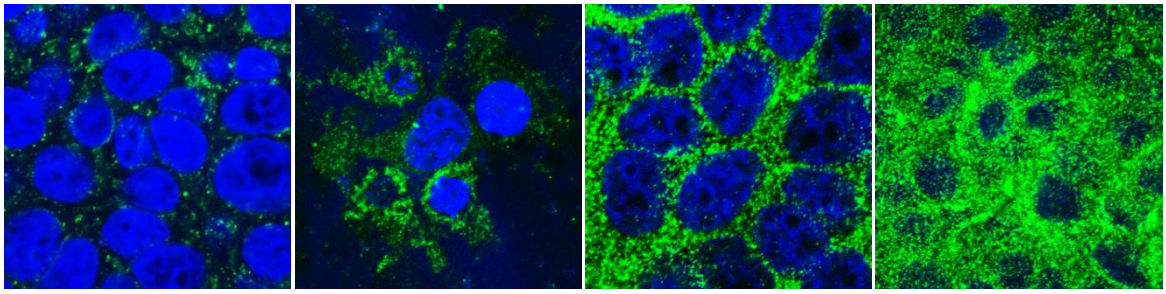


Figure 7. Effect of UQ1, UQ10 and statin on level of cholesterol, lanosterol and squalene. Results are average of n=3-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

H. LDL receptor level increases in UQ1 and UQ10 treated HepG2 cells.

The level of LDL receptor was assessed in UQ1, UQ10 treated HepG2 cells. Immune staining of treated cells in presence of MVA was performed. All treatment increased the intensity of green color when compared to control, reflecting the overexpression of LDL receptor (Figure 8a). Results were confirmed by western blot analysis, an increase in the expression of LDL-R by 2 folds was observed in UQ1 and UQ10 treated cells, statin increased LDL-R by 1.5fold (Figure 8b).

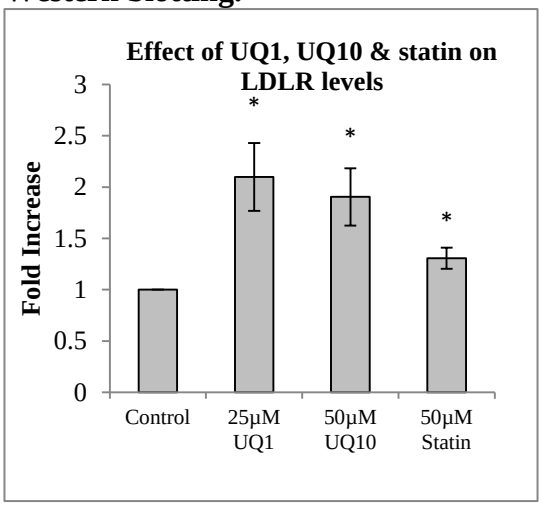
Immune Staining



Control+0.2mM MVA | 25µMUQ1+0.2mM MVA | 50µM UQ10+0.2mM MVA | 50µM statin +0.2mM MVA

Figure 8a. Representative figure of the immune staining of LDL-R in HepG2 cells; control and following treatment with UQ1, UQ10, statin in presence of MVA. Green channel represent LDL-R, Blue channel represent nuclei.

Western blotting.



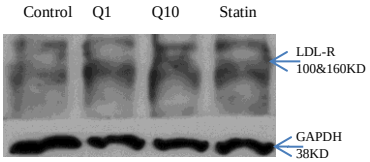


Figure 8b. Effect of UQ1, UQ10 and statin treatment on HepG2 cells in presence or absence of MVA. Results are average of n=4-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

I. UQ1 and UQ10 are not toxic on hepatocytes.

To examine if the cholesterol lowering effect is due to the inhibition in cholesterol synthesis and not to the toxicity of treatment, cells were treated with UQ1, UQ10 and different inhibitory following the corresponding dilution. UQ1, UQ10, FPT and bacitracin does not affect the viability of primary hepatocytes, while statin decrease the viability by 17% at (5µM treatment, 100cell/µl). Thus, the decrease in the cholesterol level is not due to cellular toxicity but to a direct inhibition of the synthesis pathway (Figure 10a).However, in HepG2 cells, UQ10 (5µM) and UQ1 (2.5µM) do not have any toxicity; UQ1 or statin at 5µM decrease the viability of HepG2 by 39% and 33% respectively (Figure 10b).

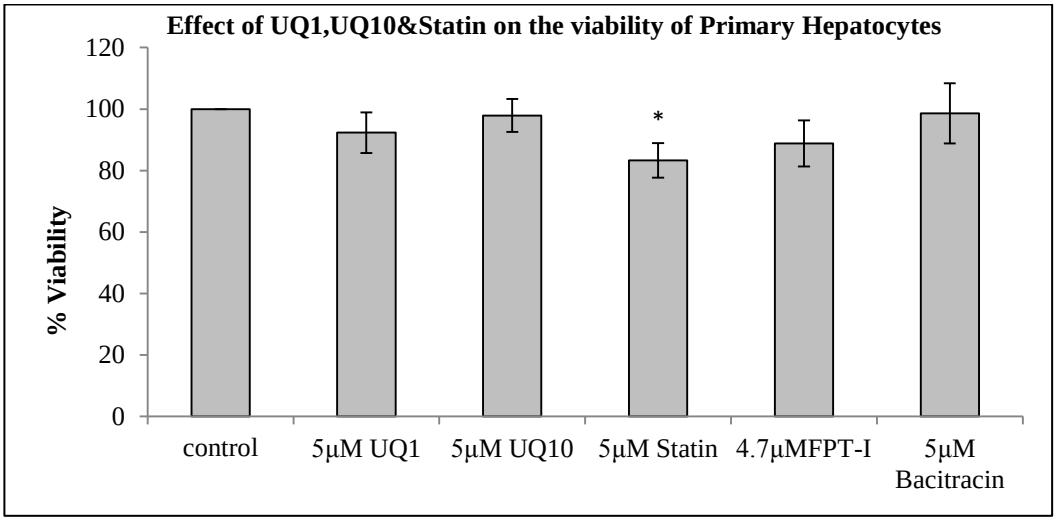


Figure 10a. Effect of UQ1, UQ10, statin, FPT-I and Bacitracin on the viability of primary hepatocytes. Results are average of n=4-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

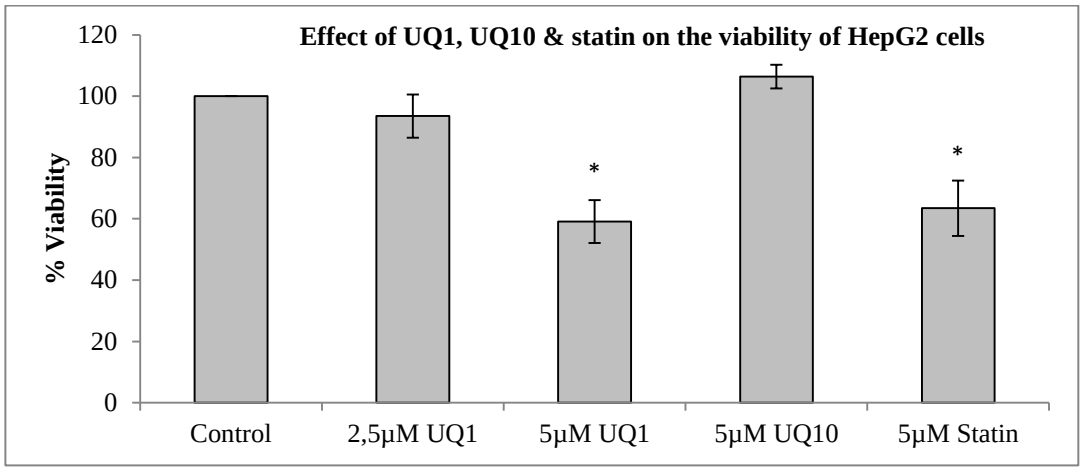


Figure 10b. Effect of UQ1, UQ10, statin, on the viability of HepG2 cells. Results are average of n=3-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

J. UQ1 increase UQ10 level in both primary hepatocytes and HepG2 cells.

To examine if the accumulation of squalene and lanosterol intermediates convert isoprene units toward UQ10 synthesis, the variation of UQ10 level was assessed in UQ1 treated primary and HepG2 cells. UQ1 increased the incorporation of ^{14}C-MVA into UQ10 in both primary and HepG2 cells by 46% and 106% respectively (Figure 11).

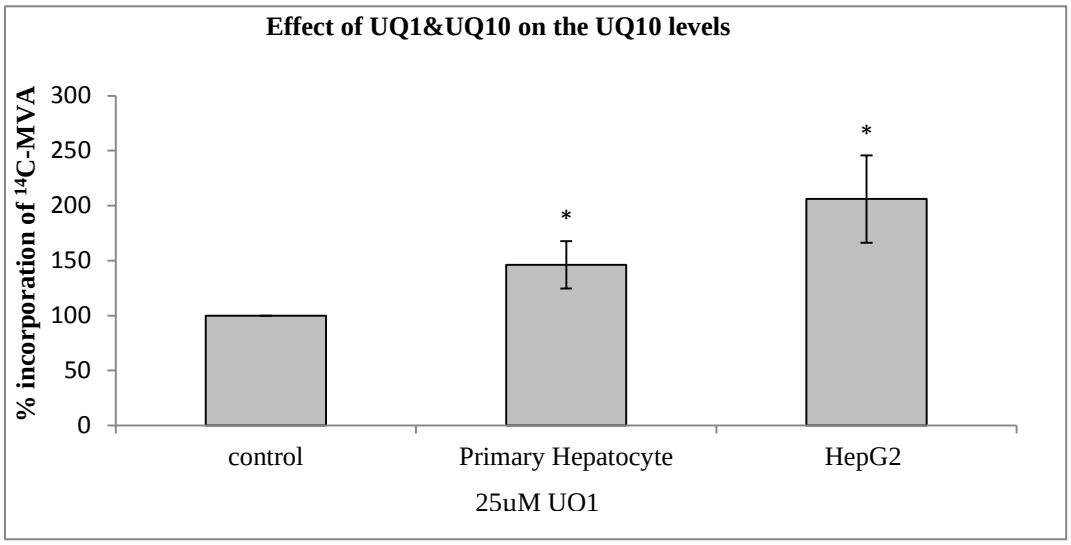


Figure 11.Effect of UQ1 on UQ10 level in both primary hepatocytes and HepG2 cells. Results are average of n=4-6. Asterisks on bars represent statistical significance relative to control (*) corresponding to $p<0.05$.

CHAPTER IV

DISCUSSION

A. Discussion

In this study, we provide for the first time evidence for the hypocholesterolemic effect of non-sterol isoprenoid, ubiquinone-1 (UQ-1). The incorporation of radio-labeled mevalonolactone into cholesterol decreased significantly in UQ-1 treated primary hepatocytes while the expression of LDL receptors increased concomitantly. On the other hand, the natural cellular component UQ-10 exhibited a profile similar to that of UQ-1. However, our findings with UQ-1/UQ-10 co-treatment with specific inhibitors of mevalonate pathway suggest distinct sites of action for each of UQ-1 or UQ-10 in their hypocholestrolemic effect with more potent effect for UQ-1 than UQ-10.

Mevalonate-isoprenoid pathway is among the metabolic pathways that generates vital biomolecules and intermediates essential in many cellular reactions or processes. Mevalonic acid is a common intermediate that diverges into sterol isoprenoids (cholesterol, bile acid, & steroid hormones) and nonsterol isoprenoids (farnesyl, geranylgeranyl, ubiquinone) serving thus important roles in: protein post translational modification, differentiation , cell growth, glycosylation and signaling (Buhaescu & Izzedine, 2007). The rate determining step in mevalonate pathway, generating MVA is catalyzed by HMGCoA reductase which regulates cholesterol level. This enzyme has been a therapeutic target for drug development of cholesterol lowering agents by world pharmaceutical companies,

since 1971 (Endo & Kuroda, 1976). However, inhibition of HMGCoA reductase decreases cholesterol, in addition to products of the MVA branching pathway, such as ubiquinone.

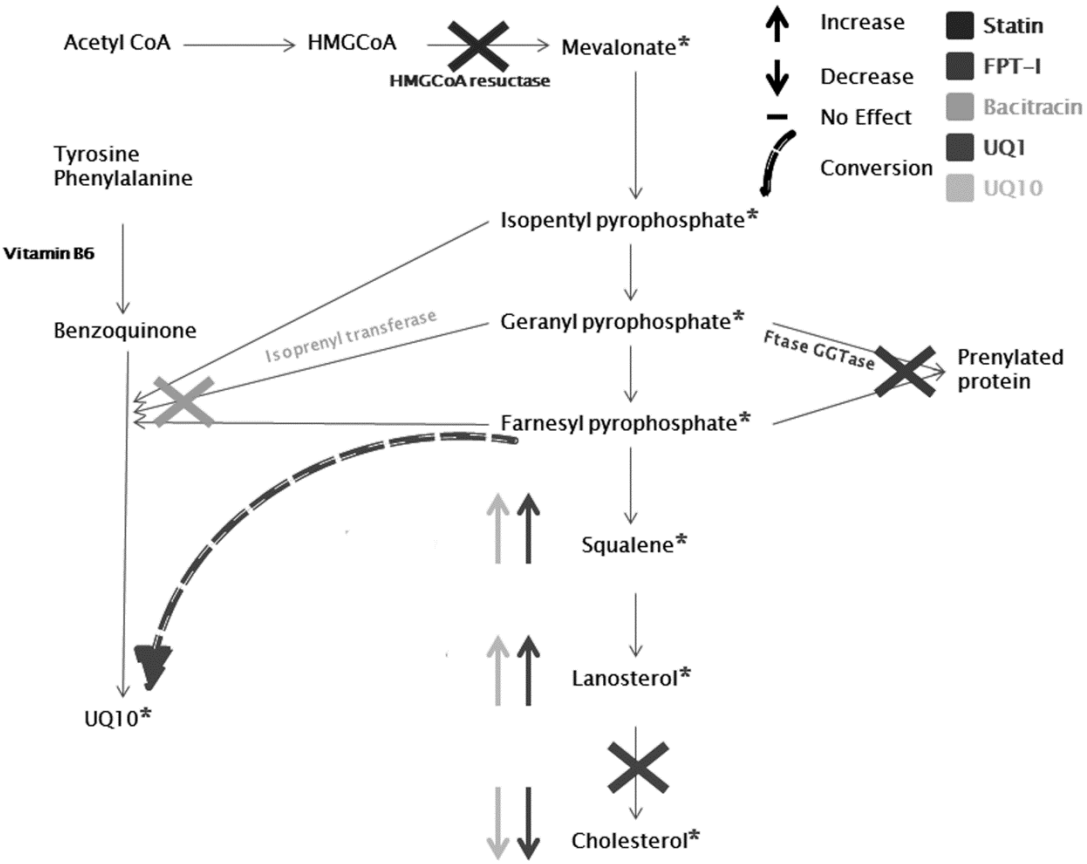
UQ-10 or coenzyme Q10, an endogenous product of the MVA pathway, plays an important role in cellular respiration and ATP production. Structurally, it is composed of a benzoquinone ring and an n-isoprene side chain (s).

The antioxidant property of UQ-10 has been reported to decrease ex-vivo LDL oxidizability which underlies the pathogenesis of atherosclerosis (Hassan Ahmadvand, 2013 #86). In addition it has been shown to improve vascular endothelial dysfunction (Hamilton et al., 2009; Watts et al., 2002). Variation in UQ-10 and cholesterol levels with age have been controversial; for instance previous studies reported a decrease in UQ level while cholesterol level increased with age (Kalen et al., 1989). Others showed that isoprenoid epoxides (Solanesol, Vitamin K2, CoQ10 1,2 or 3,4 epoxide) reduce cholesterol synthesis while Coenzyme Q-10 synthesis increases (Bentinger et al., 2008). These findings pose a valid question as to whether, the MVA branching pathway product, UQ-10 regulates cholesterol level.

Preliminary studies in our lab, using different isoprenylated UQ (UQn= 1, 2, 4, 6, &10) showed a decrease in the incorporation of labeled MVA into cholesterol. In this study, only UQ-1, the most effective in decreasing cholesterol level, was further investigated and compared to the naturally occurring UQ-10. The level of radioactivity incorporated into cholesterol, using labeled mevalonolactone was determined in presence and absence of UQ-1/10 at non-toxic levels (MTT assay). We opted to use the lactone form since free MVA has limited ability to permeate the membrane while hydrophobicity of mevalonolactone facilitates its transport across plasma membrane following which

intracellular esterase hydrolyzes it into mevalonic acid, bypassing thus the rate determining step reaction.

Our findings show a more potent effect for ubiquinones with shorter isoprenoid chain in lowering cholesterol level; for instance UQ-1 vs UQ-10. These results are in line with previous studies on thymocytes documenting a decrease in rate of cholesterol biosynthesis with ubiquinones having shorter isoprenoid chain (Novoselova, 1989; Novoselova, Kolomiitseva, Obol'nikova, Samokhvalov, & Kuzin, 1985). Moreover, the decrease in cholesterol level paralleled a significant increase in both squalene and lanosterol levels indicating that the effect of the UQs occurs at site (s) below the rate determining step yielding MVA. Further evidence in support of our findings is that obtained with lovastatin treated primary hepatocytes. Contrary to UQ-1/UQ-10, lovastatin had no significant effect on MVA incorporation into cholesterol indicating that UQ-1 effect is downstream the HMGCoA- reductase step, possibly involving the steps between lanosterol and cholesterol. The significant accumulation in lanosterol implies as well the piling up of intermediates upstream lanosterol including squalene (as obtained in our study) and farnesylpp (not examined) which is at the branching point of MVA pathway (Figure 1). The Km of the branch point enzymes for farnesylpp, leading to UQ synthesis or to prenyl intermediates (geranyl, geranylgeranyl) is much lower than that of squalene synthase. Previous reports, according to flux diversion hypothesis, have alluded to the pool size influence of farnesyl pp on transprenyltransferase involved in UQ synthesis (Dallner & Sindelar, 2000). Accumulation of farnesyl PP favors the degradation of HMGCoA reductase decreasing thus level of cholesterol [(Eds.), 2002)].



	Statin	UQ1/Statin	UQ10/Statin	FPT-I	UQ1/FPT-I	UQ10/FPT-I	Bacitracin	UQ1/Bacitracin	UQ10/Bacitracin
Cholesterol	NE	-97% (S)	-25% (S)	- 42% (S)	- 75%(S)	NE	NE	- 55%(S)	- 8% (NS)
Lanosterol	-27%(S)	+67% (NS)	-14% (S)	- 43%(S)	+ 75%(S)	+ 35%(S)	+ 13% (NS)	+ 28% (NS)	-13% (NS)
Squalene	-38%(S)	+139 (NS)	-13% (NS)	- 28%(S)	NE	+ 16%(NS)	+ 34%(NS)	NE	- 8%(NS)

Figure 1. Effect of MVA pathway inhibitors in +- of UQ1/UQ10 on cholesterol, lanosterol and squalene, + (increase), - (decrease), NE: No Effect, (S): significant, (NS): Not Significant.

We therefore examined the effect on cholesterol level, in primary hepatocytes co-treated with prenyltransferase inhibitor (FPT-1) and UQ1/or UQ10.

FPT-1 at 47uM inhibits both farnesyl-pp/geranylgeranyl pp transferases consequently protein prenylation (Biotech, 2007-2014), squalene synthase (IC50=516 µM) and mevalonate kinase (IC>200µM) (Patel et al., 1995). Primary hepatocytes treatment with non-toxic level of FPT- 1 (47µM) caused a decrease in labeled MVA incorporation in each of: squalene, lanosterol and cholesterol. Co-treatment however, of primary hepatocytes with FPT-1 and UQ1 promoted UQ-1 effect by: Decreasing further cholesterol level, increasing lanosterol while normalizing squalene level. These findings support our suggestion that UQ-1 acts somewhere between lanosterol and cholesterol possibly involving farnesylation of protein (s). Surprisingly, FPT-1 inhibitor co-administered with UQ-10 normalized cholesterol & squalene level while slightly increased lanosterol. Bacitracin, an inhibitor of 4-hydroxybenzoate polyprenyl transferase, showed no significant variation in all tested intermediates. No effect was obtained either with UQ-10 -bacitracin co-treatement. On the other hand, UQ-1 -bacitracin co-treatment maintained the significant decrease in cholesterol level with no significant increase in lanosterol or squalene. It is worth noting that UQ-1 effect on HepG2 followed a similar profile to that of primary hepatocytes with more reduction in cholesterol and further increase in lanosterol levels.

Our findings identifies the non-sterol isoprenoid UQ-1 as: a) an agent with potent hypocholesterolemic effect and b) a possible regulator of cholesterol level that acts between lanosterol and cholesterol involving protein farnesylation and /prenylation. Several studies have reported and dwelled upon the pleiotropic effect resulting from isoprenoid intermediates of MVA pathway such as farnesyl pp and geranyl geranyl- pp.

The function of these isoprenoids has been well documented in cell differentiation, signaling, and proliferation (Buhaescu & Izzedine, 2007).

Evidence in support of the role of isoprenoids' effect on cholesterol and in line with our findings was demonstrated as follows:

a) squalene epoxidase inhibited-system decreased cholesterol level and increased UQ-10 (Bentinger et al., 2008).

b) Clinical studies showing that UQ10 and carnitine administration decreased serum LDL level in hemodialyzed patients (Shojaei, Djalali, Khatami, Siassi, & Eshraghian, 2011)

c) Linalool, a monoterpene, administration to mice lowered total cholesterol, LDL levels and decreased HMGCoA reductase expression at the transcription and post-transcription level.

d) Synergistic anticholesterogenic effect was also reported upon linalool co/administration in absence or presence of simvastatin that was characterized by an increase as well in lanosterol, proposing it acts between lanosterol and cholesterol.

d) 1,8 –cineol, a mono-terpenoid inhibited cholesterol level in HepG2 cells by possibly acting between squalene and lanosterol decreasing the level of the latter consequently that of cholesterol (Rodenak Kladniew et al., 2014).

e) Geraniol a dietary monoterpene was reported to inhibit HMGCoA reductase. The incorporation of labeled acetate into cholesterol but not squalene and lanosterol was demonstrated indicating that it acts at a step between lanosterol and cholesterol (Polo & de Bravo, 2006).

Furthermore we compared the effects of UQ-1 to statins. Statins, competitive inhibitors of the HMGCoA reductase, decrease cholesterol synthesis and increase the

expression of LDL receptors. Although In our experiments, the HMGCoA reductase step was by-passed, yet incorporation of labeled mevalonate into cholesterol, in statin treated primary hepatocytes, was not affected while lanosterol and squalene levels were reduced. However the co-treatment of UQ-1 and statin decreased cholesterol significantly. Again statin co-administered with UQ-1 had a more potent effect than that of stain- UQ-10.

Qualitative assessment of LDL receptors by immune-fluorescence was performed in UQ1, UQ10 and statin treated primary hepatocytes showed an increase in LDL receptors. Unfortunately this could not be demonstrated with western blotting because of the primary human antibody used (against human LDLR) failed to interact with the denatured receptor protein band. Alternatively we performed immune-staining on HepG2 cells (human cancerous cells). A clear increase in plasma membrane localized LDL receptor in UQ1 and UQ10 treated HepG2 cells was observed (Results-Figure8b). Statin treated HepG2 cells showed significant increase in fluorescence intensity that was dispersed in the cytosolic region. Western blot analysis confirmed the increase in LDL receptors enforcing our findings of decreased cholesterol with UQ treatment. The mechanism as to how UQs increase LDL receptors requires further investigation.

The hypocholesterolemic effect of UQ-1 raises several questions among them whether it can serve as well as a precursor for UQ-10. Since the decrease in cholesterol level by UQ-1 may accumulate farnesyl intermediate, which will divert to branched pathway (Bentinger et al., 2003), we examined UQ-10 labelling in UQ-1 treated cells. An increase in radioactivity incorporated in UQ-10 suggests isoprenylation of UQ-1 to UQ-10.

To sum up, UQ-1 effect is downstream the HMGCoA reductase and has no effect on the mevalonate conversion into isoprene unit. This is in contradistinction to statins' effect, which inhibits HMGCoA reductase and decrease level of UQ-10 (Folkers et al., 1990; Ghirlanda et al., 1993; Krukemyer & Talbert, 1987; Laaksonen, Ojala, Tikkanen, & Himberg, 1994; Thompson, Clarkson, & Karas, 2003; Watts et al., 1993; Barbiroli et al., 1997; Koroshetz et al., 1997). Similar increase in UQ-10 level has been shown in polyisoprenoid treated HepG2 cells (Bentinger et al., 2008) and LDL supplemented cells (Faust, Goldstein, & Brown, 1979).

We hereby are showing for the first time that UQ-1 has a potential cholesterol lowering effect accompanied by an increase in LDL receptors. While its effect is similar to that of statin , its ability to get converted to UQ-10 might favor it as a substitute hypocholesterolemic agent without lowering UQ-10 level, one of the known side effects of statins.

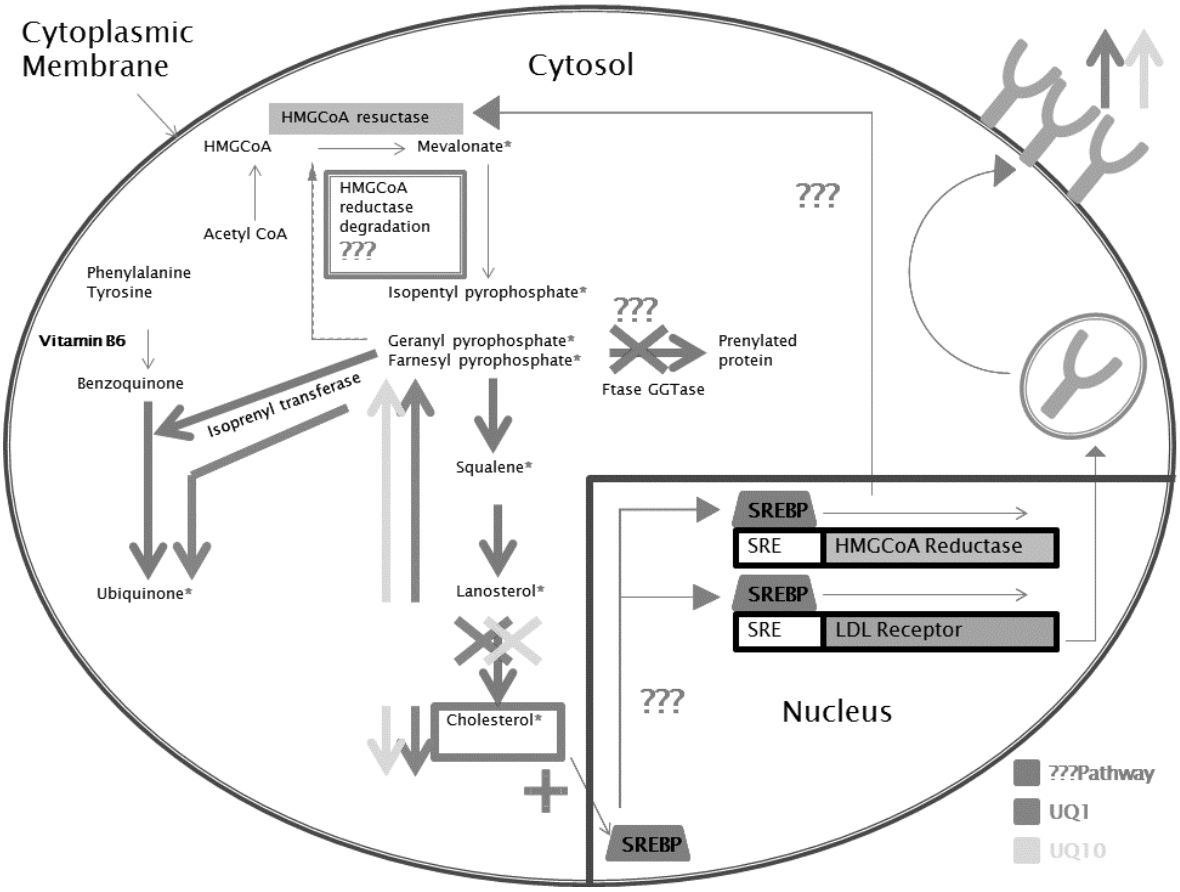


Figure 2. Summary of UQ1&UQ10 effect on MVA pathway and LDL receptor; ???s are pathways that needs further investigation.

B. Limitations of the study

The following limitations were identified in our study:

The activity of HMGCoA reductase and the expression of LDL receptor are regulated by intracellular cholesterol level. The endogenous pool of cholesterol and UQ10 was not determined. Fasting rats of same age and identical weight were used, assuming they would have same cholesterol and UQ10 level. In addition, all polar lipids intermediates were not assessed due to their extractability in water layer. Moreover, the anti- LDL receptor was a human specific antibody; although when purchased from ABCAM it was mentioned that it cross-reacted with rat and mouse LDL-Receptor. The human antibody partially worked in immunostaining of rat hepatocytes but not in western blotting with denatured proteins (SDS-PAGE).

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