Triglyceride rich lipoproteins and Apolipoprotein C-III in Atherosclerosis

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By

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Abstract

While the causal role of low-density lipoprotein cholesterol (LDL-C) in atherosclerosis and the clinical benefit of lipid lowering with statins are well established, the impact of triglyceride rich lipoproteins (TRL) in cardiovascular disease is less well understood. Given the important association between hypertriglyceridaemia and both obesity and type 2 diabetes, mechanistic studies are required to further understand the role of TRL as both a causal factor and potential target for therapeutic modification.

This thesis aims to investigate the impact of both TRL and Apolipoprotein C-III (ApoC-III), an important factor that regulates TRL metabolism, in atherosclerosis. It demonstrated the adverse effect of oxidised TRL on endothelial cells following co-incubation studies *in vitro*. It also described the presence of ApoC-III within atherosclerotic lesions in an animal model of diabetes and dyslipidaemia, with evidence of a direct correlation between plaque levels of ApoC-III with both the burden and inflammatory composition of plaques. Additional studies demonstrated inverse correlations between hepatic levels of triglyceride and ApoC-III with expression of factors involved in the generation of high-density liporptoeins (HDL) and the promotion of reverse cholesterol transport.

Modification of LDL by myeloperoxidase (MPO), a peroxidase enzyme secreted by leukocytes, has been established to promote vascular inflammation and cholesterol uptake by macrophages, a critical step in foam cell formation. In cell studies, we demonstrated that MPO modified TRL (MPO-TRL) exerted an

adverse effect in human umbilical vein endothelial cells (HUVEC), as evidenced by an upregulation of mRNA expression of pro-inflammatory adhesion molecules. MPO-TRL co-incubation also resulted in a reduction in endothelial cell proliferation which can be restored by co-incubation with HDL. Cells treated with MPO-TRL also demonstrated an increase in expression of proteins involved in responses to hypoxia (HIF1α) and in angiogenesis (VEGF) and a reduced expression of the cholesterol transporter ABCG1.

We are further interested in whether triglyceride mediator ApoC-III exerts similar adverse effect in atherosclerosis in the settings of mouse models with dyslipidaemia and diabetes. We demonstrated that ApoC-III was present within plaque and the presence was positively associated with lesion size and inflammatory marker CD68. ApoC-III has been well characterised to induce endothelial cell inflammation, whether ApoC-III will induce inflammation in other vascular cells will be of additional interest in future studies.

We have also found that using patients' serum stratified with different levels of triglyceride, ApoC-III levels inversely correlated with HepG2 expression of both PPARα and cholesterol transporter ABCA1, important factors which involved in the synthesis of both ApoAI and HDL and subsequent effects on lipid transport. Further experiments are needed to demonstrate the correlation of ApoC-III in HDL metabolism in large, prospective cohorts.

In summary, these observations described potential adverse effects of triglycerides and ApoC-III on vascular cells, atherosclerosis and lipid metabolism. The findings support potential causal effets and novel targets for therapeutic targeting to prevent atherosclerotic disease.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree of diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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April 2019

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opportunity, work with excellent teams and have my strong bond of family and friendships. These enable me to confront the challenges and difficulties.

Abbreviations

1-Bromo-3-chloropropane	BCP
2-(N-morpholino) ethanesulfonic acid	MES
2-dimensional	2D
2-thiobarbituric acid	TBA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
4’,6-Diamidino-2-phenylindole dihydrochloride hydrate	DAPI
Acute coronary syndrome	ACS
Adelaide Hospital Research Ethics Committee/Royal Adelaide Hospital	CALHN/RAH
Amino acids	AA
Antisense oligonucleotide against ApoC-III	ASO-ApoC-III
Apolipoprotein A1	ApoA-I
Apolipoprotein A5	APOA5
Apolipoprotein B	ApoB
Apolipoprotein C3 gene	APOC3 gene
Apolipoprotein CIII	ApoC-III
Apolipoprotein E	ApoE
Arachidonic acid (AA)	AA
Arginine	Arg
Atherosclerotic cardiovascular diseases	ASCVD
ATP-binding cassette subfamily A member 1	ABCA1
ATP-binding cassette sub-family G member 1	ABCG1
Azidohomoalanine	AHA
B-cell lymphoma-extra large	Bcl-xL

Beta 3 adrenergic receptor	B3AR
Bis (2-hydroxyethyl) amino-tris(hydroxymethyl) methane	Bis-Tris
Bovine serum albumin	BSA
Calcium ions	Ca^{2+}
Carbon dioxide	CO_2
Carbohydrate response element–binding protein	ChREBP
Cardiovascular diseases	CVD
Carotid intima-media thickness	CIMT
Centimetres	cm
Chemoattractant protein 1	MCP-1
Chloride	Cl^-
Cholesteryl ester transfer protein	CETP
Chow diet	CD
Chronic kidney disease	CKD
Class A scavenger receptor	SRA1
Cluster of differentiation 36	CD36
Cluster of Differentiation 68	CD68
Coding domain	CD
Complementary DNA	cDNA
Control	Ctrl
Copper	Cu^{2+}
Coronary artery disease	CAD
Coronary heart disease	CHD
Cycle threshold	CT

Degree Celsius	C
Deoxyribonucleic acid	DNA
Dihydroxeicosatrienoic acids	DHETs
Dihydroxyoctadecenoic acids	DHOMEs
Dimethyl sulfoxide	DMSO
Distyrene, plasticizer, xylene	DPX
Dithiothreitol	DTT
Dulbecco's Modified Eagle's medium	DMEM
Endoplasmic reticulum	ER
Endothelial cell growth supplement	ECG
Endothelial cells	EC
Endothelial cells	EC
Endothelial nitric oxide synthase	eNOS
Endotoxin units per milliliter	EU/ml
Enzyme unit	U
Enzyme-linked immunosorbent assay	ELISA
Epoxyeicosatrienoic acids	EETs
Epoxyoctadecenoic acids	EpOMEs
Escherichia coli	E.Coli
Ethylene glycol tetra acetic acid	EGTA
Ethylenediaminetetraacetic acid	EDTA
Eukaryotic initiation factor	eIF2α
Extracellular regulated protein kinase phospho-p42/44	Erk 42/44
Familial combined hyperlipidaemia	FCHL

Fast protein liquid chromatography	FPLC
Fatty acid-binding protein 2	FABP2
Fetal bovine serum	FBS
Fetal calf serum	FCS
Fibroblast growth factor	FGF
Forkhead box protein O1	FoxO1
Free fatty acids	FFA
G Protein Subunit Beta 3	GNB3
Gain-of-function	Gof
Genome-wide association study	GWAS
Gram	g
Gram per millilitre	g/ml
Hemoglobin A1c	HbA1c
Hepatocyte nuclear factor 4	HNF4α
High cholesterol diet	HCD
High-density lipoproteins cholesterol	HDL-C
Histidine	His
Hours	h
Human APOC3 transgenic mice	hAPOC3 Tg mice
Human umbilical vein endothelial cells	HUVEC
Hydrogen peroxide	H2O2
Hypochlorous acids	HOCl
Hypoxia-inducible factor 1-alpha	HIF1α
Immunohistochemistry	IHC

Institute of Medical and Veterinary Science	IMVS
Insulin receptor substrate	IRS
intercellular adhesion molecule 1	ICAM-1
Interleukin 6	IL-6
Interleukin-8	IL-8
Intermediate density lipoproteins	IDL
Inter-quartile range	IQR
Intraperitoneal	IP
Intraperitoneal glucose tolerance test	IPGTT
Iodoacetamide	IAA
Isopropyl-b-d-thiogalactopyranosdie	IPTG
Isotope labelling using amino acids in cell culture	SILAC
Kilodalton	kDa
LDL receptor	LDLr
Lecithin-cholesterol acyltransferase	LCAT
Lectin-type oxidized LDL receptor 1	LOX-1
LepOb/Ob	ObOb
Leptin knockout obese mouse	LepOb/Ob
Limulus Amebocyte Lysate	LAL
Linoleic acid	LA
Lipoprotein lipase	LPL
Liver X receptor α	LXRα
Loss-of-function	Lof
Low-density lipoprotein cholesterol	LDL-C

Luria-Bertani	LB
Lysine	Lys
Malondialdehyde	MDA
mammalian Target of Rapamycin Complex 1	mTORC1
Medium 199	M199
Messenger ribonucleic acid	mRNA
Metabolic syndrome	MetS
Methylenetetrahydrofolate reductase	MTHFR
Micrometre	µm
Micromolar	mM
Milligram per millilitre	mg/ml
Millilitre	ml
Millimolar	mM
Minutes	min
Mitogen activated protein kinase	MAPK
Molar	M
Myeloperoxidase	MPO
Myocardial infarction	MI
Nanogram per liter	ng/l
Nanomole	nmol
Nickel	Ni
Nitrite ion	NO2-
Non-alcoholic fatty liver disease	NAFLD
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB

Oil red O	ORO
Optimal cutting temperature	OCT
Oxidised LDL	ox-LDL
Penicillin and streptomycin	P/S
Peroxisome proliferator-activated receptor gamma co-activator 1alpha	PGC-1α
Peroxisome proliferator-activated receptor α	PPARα
Phenylmethanesulfonylfluoride	PMSF
Phosphate buffered saline	PBS
Phosphoinositide 3-kinase	PI3K
Phospholipase A2	Lp-PLA2
Phospholipid transfer protein	PLTP
Pierce bicinchoninic acid	BCA
Platelet derived growth factor	PDGF
Platelet-derived growth factor	PDGF
Power of hydrogen	pH
Proliferating cell nuclear antigen	PCNA
Proprotein convertase subtilisin/kexin type 9	PCSK9
Protein kinase B (Akt)	Akt
Protein kinase C α/β	PKCα/β
Reactive oxygen species	ROS
Real-time (RT) quantitative polymerase chain reaction	qPCR
Ribonucleic acid	RNA
Room temperature	RT
Roswell Park Memorial Institute Medium -1640	RPMI-1640

Round per minute	rpm
Royal Adelaide Hospital	RAH
Scavenge receptor class B type 1	SRB1
SDS-polyacrylamide gel electrophoresis	SDS-PAGE
Small intestine	S. Intestine
Small nucleotide polymorphisms	SNPs
Smooth muscle cells	SMC
Sodium chloride	NaCl
Sodium deoxycholate	Na-Doc
Sodium dodecyl sulfate	SDS
South Australian Health and Medical Institute	SAHMRI
Standard deviation	STD
standard error mean	SEM
Streptozotocin	STZ
Super optimal broth	SOC
Svedberg flotation rate	Sf
Tetramethoxypropane	TMP
Thiobarbituric acid reactive substances	TBARS
Toll-like receptor 2	TLR2
Total cholesterol	TC
Trichloroacetic acid	TCA
Triglyceride rich lipoproteins	TRL
Triglycerides	TG
Tris (2-carboxyethyl) phosphine	TCEP

Tris hydrochloride	Tris-HCl
Type 1 diabetes	T1DM
Type 2 diabetes	T2DM
Vascular cell adhesion protein 1	VCAM-1
Vascular endothelial growth factor	VEGF
Very low- density lipoproteins	VLDL
Volts	V
Volume/volume	v/v
Weight/volume	w/v
Wildtype	WT

Chapter 1. General Introduction

1.1 Burden of cardiovascular disease

Cardiovascular disease (CVD), encompassing a range of disorders involving coronary, cerebrovascular and peripheral vascular territories, are among the leading causes of morbidity and mortality worldwide. In Australia, CVD caused 45,392 deaths in 2015, accounting for nearly 30% of all deaths nationally (1). CVD also produces a large financial and hospitalisation burden. In Australia, there are more than 500,000 cardiovascular hospitalisations per year, with a disproportionate representation of individuals from lower socio-economic groups, indigenous communities and those living in remote areas (2). In addition, there were 84 million CVD prescriptions in Australia in 2008, at a cost of $3 billion (3), making CVD the most expensive disease group in direct health care.

Extensive epidemiology investigations of large population cohort studies have identified a number of factors to associate with a greater prospective risk of CVD. Common CVD risk factors include diabetes, high blood pressure, dyslipidaemia, obesity, alcohol, smoking and family history of premature CVD events (4). The importance of obesity in CVD has been further emphasised on the basis of its association with a number of these established CVD risk factors, in addition to its role in promoting additional factors, such as inflammation and oxidative stress, which are also likely to exacerbate CVD risk. Given the rising prevalence of obesity in the community, the importance of these risk factors, with a particular focus on type 2 diabetes and the metabolic syndrome have received increasing importance (5) (6). Identifying risk factors associated with

CVD have been important in the development of risk prediction algorithms and new therapies aimed to lower CVD risk.

1.2 Lipid lowering treatments and CVD risk

Over the course of the last four decades, clinical trials have established the benefits of targeting a number of these risk factors to lower risk in both patients with clinically manifest atherosclerotic CVD (ASCVD) and in asymptomatic individuals who are deemed to be at high CVD risk. These studies have demonstrated the benefit of using lipid modifying agents, antihypertensives and anti-platelet/anti-thrombotic therapies, in addition to lifestyle modification. From a lipid perspective, the cornerstone for treatment of high CVD risk patients has focused on lowering levels of low-density lipoprotein cholesterol (LDL-C). Population (7) and genetic (8) studies have established the role of LDL-C in promotion of CVD. Large clinical trials have demonstrated that lowering LDL-C with statins by 18-55% associate with a reduction in clinical events by more than 30% (9, 10). Meta-analyses of these trials have demonstrated that each 1 mmol/L reduction in LDL-C associates with a 21% reduction in CVD risk (11). More recent studies with ezetimibe (12) and inhibitors of proprotein convertase subtilisin kexin type 9 (PCSK9) (13, 14) have also demonstrated that incremental lowering of LDL-C further reduces CVD risk in the statin-treated patient. As a result, treatment guidelines for the prevention of CVD risk highlight the importance of LDL-C lowering, with a specific emphasis on the use of statin therapy (15, 16).

However, a substantial residual risk of clinical events continues to be observed even in the setting of good LDL-C control with statins (6). In addition, many

patients fail to achieve LDL-C treatment goals with statins, while others experience difficulty with long term adherence due to intolerance (17). While these findings have supported the need to develop additional approaches to lowering LDL-C, they also highlight the potential importance of targeting alternative lipid factors. Population studies have demonstrated that hypertriglyceridaemia (18, 19) and low levels of high-density lipoprotein cholesterol (HDL-C) (20) also associate with CVD risk. Observations from genetic studies have suggested that factors leading to high triglyceride levels, but not HDL-C (21, 22), also associate with CVD risk. These observations have led to a number of clinical trials, yielding variable effects. Fibric acid derivatives (fibrates) lower triglycerides and raise HDL-C, with variable effects on CVD events (23, 24). Meta-analyses of these trials have suggested a borderline impact of fibrates on CVD risk, with the most striking benefit observed in patients with hypertriglyceridaemia at baseline (25). Reports of genetic polymorphisms of factors regulating triglyceride rich lipoprotein metabolism associating with CVD risk have led to increased interest in developing therapeutics directly targeting these factors (26, 27). This provides some hope that directly lowering triglyceride levels in the future may reduce CVD event rates in high risk patients.

In parallel, clinical trials of agents targeting HDL-C have proven to be disappointing. Studies of agents that raise HDL-C, including niacin and cholesteryl ester transfer protein (CETP) inhibitors, have not produced robust reductions in CVD risk in statin-treated patients (28, 29). While additional approaches involving the intravenous infusion of HDL mimetics have produced variable effects on atherosclerotic plaque in imaging studies (30-32), their

impact on CVD events remains unknown. Given the intimate association between obesity, diabetes and the combination of elevated triglycerides and low HDL-C levels (33, 34), there continues to be immense interest in understanding how these factors influence the artery wall and in the development of new therapies, with the potential to reduce CVD risk.

1.3 Role of LDL in atherosclerosis

Extensive preclinical investigation has delineated the role of LDL in the pathogenesis of ASCVD. Atherosclerosis starts early in life and represents a chronic, progressive process with the artery wall. A range of stages of the disease process have been identified, spanning from its initial development, long term progression and ultimate disruption leading to acute ischaemic events. In parallel, the role of LDL has been well established to associate with each of these processes, suggesting the potential to impact the disease across the life course by lowering LDL-C.

The earliest changes in the artery wall are evidenced by dysfunction of the endothelial layer of the artery wall. The endothelium plays an important role in maintenance of vascular homeostasis, regulating vascular tone and expressing factors that influence inflammation and thrombosis (35-37). Laboratory studies have demonstrated that elevated LDL concentrations promotes dysfunction of endothelial cells, including a reduction in nitric oxide production and increasing expression of proinflammatory adhesion molecules and chemokines and altering the balance of thrombotic and anti-thrombotic factors (38). Using surrogate markers of endothelial function in clinical studies, the presence of elevated LDL-C levels associates with abnormal vascular reactivity and greater

circulating levels of these inflammatory and pro-thrombotic markers (39, 40). Clinical trials of LDL-C lowering have reported favourable changes in these factors (41).

The development of endothelial dysfunction promotes adhesion of circulating leukocytes, with subsequent migration into artery wall (42). The combined migration of both LDL particles and monocyte-derived macrophages into the artery wall lead to the formation of atherosclerotic plaque. Within the artery wall, macrophages engulf modified forms of LDL to become foam cells, in a process that is facilitated by CD36 and class A scavenger receptor (SR-A1) on the macrophage surface (42) (Figure 1-1). The formation of the lipid laden macrophage (foam cell) within the artery wall is a pivotal step in the establishment of the fatty streak. The foam cell elaborates a range of factors promoting ongoing accumulation of both inflammatory cells and smooth muscle cells within the artery wall. Subsequent production of collagen by smooth muscle cells forms a fibrous cap overlying the cellular and lipid accumulation with the artery wall, with the combination of these features reflecting transition of an early fatty streak to a mature atheroma (43).

The majority of atheromatous lesions remain clinically silent, while some progress, resulting in a state of vulnerability or development of obstructive disease. The presence of free cholesterol within macrophages stimulates apoptosis, a form of programmed cell death, and appearance of necrotic cellular material within the artery wall (42). While the body has mechanisms designed to clear this necrotic material, this process can be overwhelmed, leading to the appearance of a necrotic material within a plaque, covered by a

thin fibrous cap, reflects transition of a lesion from a stable to vulnerable state. This vulnerable plaque is at a greater risk of undergoing rupture, exposing circulating blood to plaque contents (43). The vulnerable plaque core is a potent thrombogenic environment, which leads to the clot formation and potential occlusion of the artery lumen (44). It is this sudden, arterial occlusion that underscores the development of most episodes of acute ischaemia. The clinical manifestation of ASCVD involves the development of obstructive lesions within vascular territories. Progressively obstructive disease is likely reflect the outcomes of either (i) increasing accumulation of lipid and inflammatory material within a fibroatheroma or (ii) the result of repetitive episodes of plaque rupture and healing (45).

As a result, the presence of LDL lipid in the artery wall plays a critical role in the promotion of all of the stages of atherosclerosis from its formation and progression, through to the factors implicated in its transition to clinical complications. Intervention studies have consistently demonstrated that lowering LDL-C levels has a favourable impact on atherosclerotic plaque. This has been demonstrated in animal models of atherosclerosis, where both dietary reductions in cholesterol and use of statins have both been reported to favourably modulate both the size and composition of atherosclerosis (46). More recently, clinical trials using arterial wall imaging have demonstrated that lowering LDL-C with statins and PCSK9 inhibitors has beneficial effects on atherosclerotic plaque in patients with ASCVD (47, 48). The totality of these investigations provides a clear biological rationale for the role of LDL in the pathogenesis of ASCVD and provides an important model for the study of other lipid factors in the disease process.

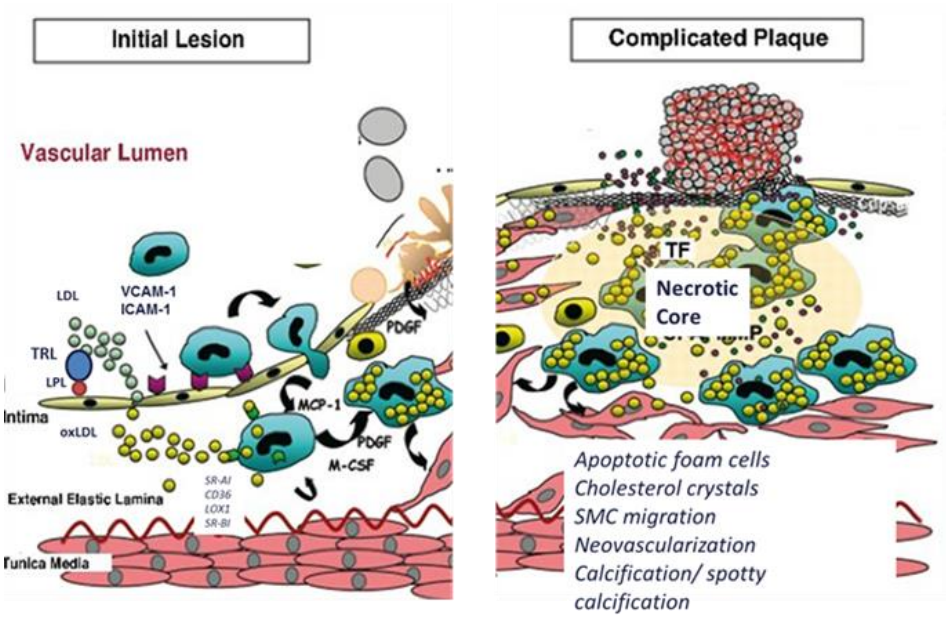


Figure 1-1 Molecular mechanisms of atherosclerosis from early atherosclerosis to later stage of advanced plaque formation [figure adapted from (42)].

1.4 Oxidative modification of lipoproteins

1.4.1 Oxidation of LDL and atherosclerosis

A number of chemical processes, including oxidation, glycation and carbamylation, have each been reported to modify LDL particles to render them more pro-atherosclerotic property such as increased uptake by macrophages (49-51). Population study has also demonstrated that plasma oxidised LDL (ox-LDL) were positively correlated with furture occurrence of CHD events, after multivariable adjustment in 88 healthy men from the general population (52). In addition, oxidation of LDL by copper (Cu^{2+}) activates the scavenger receptor and inhibits the formation of nitric oxide, an endothelium derived vessel relaxing factor, on endothelial cells (53).

1.4.2 Myeloperoxidase, LDL and HDL

LDL also can be oxidised by myeloperoxidase (MPO), which is a more physiological way. MPO is an enzyme originally secreted by neutrophils and macrophages for induction of antimicrobial/native immune response. Upon secretion, MPO uses hydrogen peroxide (H_2O_2) as a substrate to catalyse chloride (Cl^-) to hypochlorous acid (HOCl), the latter being one of the main host defenders against invading bacteria, viruses and tumor cells. Once the heme enzyme is detected within human atheroma (54), numerous studies emerged to focus on its detrimental effect on the histological (55-57), mechanistical (58-61) and epidemiological (62, 63) evolution of atherosclerosis. Circulating MPO independently predicts CVD (63) and recurrent acute coronary syndromes (ACS) (64). Importantly, recent identification of MPO positively correlated with

the occurrence of plaque erosion (62), which renewed our interests in studying MPO modified TRL; since during use of statin era, a potential triglyceride-related pathology has been hypothesised for superficial erosion (65).

MPO by working with H_2O_2 can either initiate lipid peroxidation, or perform an array of post-translational modifications to apolipoproteins, such as halogenation (66), nitration (58) and chlorination (67). MPO by H_2O_2/SCN system generates carbamylated LDL, a hallmark of lipoprotein modification in patients with chronic kidney disease (CKD) owing to extensive exposure to urea (68). LDL-Carbamyl-lysines, generated by MPO modification can usually be found on the lysine residues within Apolipoprotein B (ApoB), which result in the activation of the LOX-1 receptor, the production of reactive oxygen species (ROS) and diminished activity of endothelial nitric oxide synthase (eNOS) (61). Another MPO modification system H_2O_2/nitrite ion (NO_2^-) converts native LDL into NO_2-LDL, a highly pro-inflammatory form that can be uptaken by macrophages (58), leading to intracellular lipid deposition and foam cell formation. *In vitro* LDL modification, by MPO/H_2O_2/Cl^- via tyrosine chlorination and oxidation, has also been detected by fast protein liquid chromatography (FPLC) and mass spectrometry (69), though chlorinated LDL exhibiting lesser affinity than NO_2-LDL for macrophage uptake (70).

In HDL, HOCl and MPO preferentially target Apolipoprotein A1 (ApoA-I), leading to the formation of 3-chlorotyrosine-192 and methionine-148 oxidation in ApoA-I, and subsequently compromising ATP-binding cassette subfamily A member 1 (ABCA1) dependent cholesterol efflux, a pathway associated with

exacerbated coronary artery disease (CAD) status stratified as control, stable CAD and acute coronary syndrome (ACS) (60).

1.4.3 MPO and lipid peroxidation

MPO also initiates a cascade of peroxidative reactions with lipids such as double-bond phospholipids, cholesterol and fatty acids. By assessing the production levels of thiobarbituric acid reactive substances (TBARS), it is evidenced that MPO can oxidise phospholipids (66) and liposomes (egg yolk phosphatidylcholine), leading to enhanced oxidative damages in cells (71) and atherosclerotic tissues (67). Additionally, increasing evidence also highlighted a pivotal role of MPO in FFA oxidation. By comparing FFA types between wildtype and MPO deficient mice, it has been revealed that MPO is able to modify the most common FFA within the murine body— arachidonic acid (AA) and linoleic acid (LA) (72). MPO knockout mice exhibited a pronounced reduction in oxidative metabolites derived from AA and LA: epoxyeicosatrienoic acids (EETs) and epoxyoctadecenoic acids (EpOMEs) from AA; dihydroxeicosatrienoic acids (DHETs) and dihydroxyoctadecenoic acids (DHOMEs) from LA, respectively. All these oxidised products have been shown to be pro-inflammatory (72). The fact that triglycerides are composed of glycerol and fatty acids implies that oxidation of triglycerides, potentially within the sites of fatty acids may contribute to the progression of inflammation.

1.5 Role of triglycerides in atherosclerosis

1.5.1 Structure of triglycerides

In addition to cholesterol, triglyceride (TG) or triacylglyceride (TAG) is another form of lipid within the core of lipoproteins. Triglycerides are named as chemically structured, which is tri-esters of glycerol with three fatty acid molecules (Figure 1-2). Under normal conditions, triglycerides are the main body fat builders in mammals and the main components of vegetable fat. Blood triglycerides, mostly transported in very low-density lipoproteins (VLDL) and chylomicrons, are fundamental lipids in aiding the bi-directional transference between adipose fat and liver.

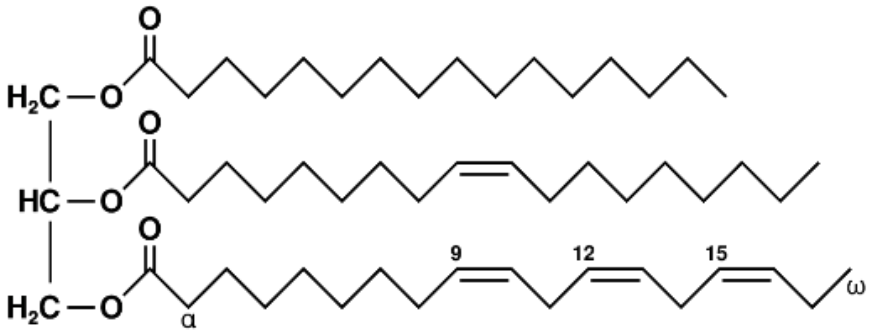


Figure 1-2 Chemical formula of a triglyceride molecule as an ester composed from ester reaction by one glycerol and three fatty acids. (https://en.wikipedia.org/wiki/Fatty_acid).

1.5.2 Triglycerides and ASCVD

Under pathological conditions, when the levels of circulated triglycerides go out of normal range, TRL are emerged as a factor in promoting CVD, though whether correlatively or causatively is under debating. Circulated triglyceride levels are considered as: normal: < 150 mg/dl (< 1.69 mmol/L), borderline high: 150-199 mg/dl (1.69-2.2 mmol/L), high: 200-499 mg/dl (2.3 mmol/L-5.6 mmol/L) and very high: > 500 mg/dl (> 5.6 mmol/L). Prospective studies suggested that non-fasting triglycerides are associated with ischemic stroke (73), cardiovascular events (18) and mortality (19). The parameter of nonfasting triglycerides also is observed to be a stronger predictor of CHD than fasting triglycerides, after multifactorial adjustment of other risk factors (18, 19), potentially on the account that postprandial triglycerides can reflect a metabolic dysfunction presented as intolerance of oral fat challenge. Among on-statin treat patients, persistent elevated triglycerides also remained as a residual risk of acute cardiovascular events independent of cholesterol: with LDL-C < 70 mg/dl, patients shows 1.6% increase in risk of the recurrent CHD as function of each 10 mg/dl raise in triglyceride levels (74).

However there remains a long-lasting habitually belief that the atherogenicity of TRL is due to their small cholesterol rich remnant, but triglyceride is not atherogenic. Genetic variants affecting remnant cholesterol alone are causally correlated with increased cardiovascular risk (75), whereas epidemiological observations found the attenuation of fasting triglycerides in capacity of predicting vascular diseases after adjustment for HDL-C, sex and fasting status in (18, 76). Mechanistically, these cholesterol rich remnants are able to induce

EC dysfunction and vascular inflammation (77). They are also able to enter the sub-endothelial layer and activate monocytes and macrophages. whereas large chylomicrons and TRL are unable to penetrate endothelium due to their size (78). Nevertheless small nucleotide polymorphisms (SNPs) identified from Genome-wide association study (GWAS), which harbor loci exclusively regulating triglycerides such as loss-of-function (Lof) Apolipoprotein A5 (APOA5), Lof TRIB1 and gain-of-function (Gof) apolipoprotein C3 (APOC3) gene, established a causal role of TRL in the development of CHD (22).

Therefore, it is still puzzling whether triglycerides directly contribute to the activation of atherogenesis in vascular cells. Indeed, the cardiovascular filed still awaits further mechanistic studies. Here, we hypothesised that oxidative/myeloperoxidase (MPO) modification of TRL has a direct adverse impact in EC.

1.6 **Apolipoprotein C-III and ASCVD**

1.6.1 ApoC-III structure

ApoC-III, identified over 40 years ago (79), recently becomes a hot spot in the field of lipidology and pathology, due to the findings of contributions to elevated triglycerides and risk of CVD (26, 27). Complete pre-cursor of ApoC-III as illustrated in Figure 1-3 is made up of 99 amino acids (AA) residues: signal peptide (AA 1-20) will be cleaved once ApoC-III is secreted and a mature ApoC-III main chain (AA 21-99) which has a lipid binding region (AA68-99). Within the lipid binding region, there were couple of known coding domain (CD) mutations taken place to influence its association with lipids. Mature ApoC-III, sized 8.8

kilodalton (kDa), is usually found in glycosylated forms (commonly at threonine74), and can reside on the surface of both ApoA (HDL) and ApoB [primary apolipoprotein of chylomicrons, VLDL, intermediate density lipoproteins (IDL) and LDL] lipoproteins (79). Mature ApoC-III wraps around the surface of micelles like a necklace via six helical regions, resulting in curved spanning and connecting to each other via semi-flexible hinges (80).

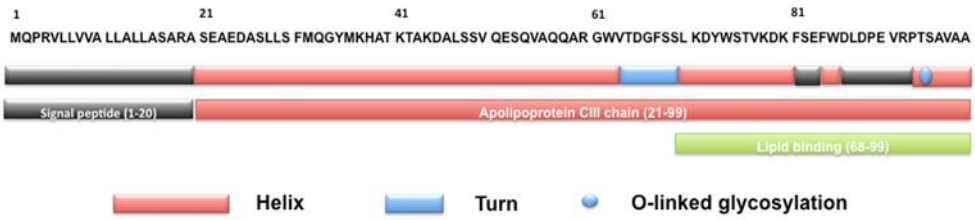


Figure 1-3 Schematic presentation of primary and secondary structures of ApoC-III.

1.6.2 The regulation of ApoC-III messenger ribonucleic acid (mRNA) and protein levels

Regulation of circulating ApoC-III level can be attributed to various factors. Fenofibrate, an activator of peroxisome proliferator activated receptor α (PPARα), can down-regulate ApoC-III levels (81, 82) by antagonising activity of Forkhead box protein O1 (FoxO1), an important APOC3 gene transcription factor (83). Patients treated with tesaglitazar, a dual activator of PPARα-PPARγ, also have a reduction in serum ApoC-III levels (84). A landmark mechanistic study has unravelled that insulin can attenuate APOC3 gene transcription through the activation of insulin receptor substrate (IRS)-phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) pathway, leading to FoxO1 phosphorylation and nuclear exclusion (85). ApoC-III levels can be up-regulated by activation of hepatic nuclear factor-4 (HNF-4), one of the transcription factors which binds and activates APOC3 promoter, in human hepatoma cells (HepG2) (86) as well as in intestinal carcinoma cells (CaCo2) (87). Hepatic *ApoC-III* mRNA production can also be stimulated by glucose, which involves the participation of HNF4 and carbohydrate response element–binding protein (ChREBP) (81). Levels of ApoC-III are elevated in serum from patients having type 1 diabetes (T1DM) and *in vitro* study demonstrated that aforementioned serum promoted pancreatic β cell apoptosis via deranged intracellular calcium ions (Ca^{2+}). Similar detrimental effect of serum ApoC-III on β cell also has been found in $Lep^{ob/ob}$ mice with type 2 diabetes (T2DM) (88, 89). To summarise, serum ApoC-III levels are regulated by several cellular metabolism related factors (e.g., PPARs, insulin and glucose), and elevated ApoC-III, in turn, can exacerbate T1DM (90) and T2DM (81, 89).

1.6.3 Regulation of lipid metabolism by ApoC-III

ApoC-III can affect CVD development through mediating lipid metabolism, such as the regulation of triglyceride levels (91-93) and HDL functionality (94). Exogenously expressing ApoC-III in cultured primary mouse hepatocytes promotes the assembly and secretion of $VLDL_1$ (91), a form of larger VLDL (Svedberg flotation rate, Sf >100) which positively correlated with insulin resistance and hypertriglyceridemia (95, 96). By comparing lipid levels produced from cells transfected with virus producing wildtype ApoC-III and ApoC-III mutants within lipid domain (A25T and K58E), it was further revealed that the $VLDL_1$ secretion highly demands the lipid binding capacity of ApoC-III (97, 98) and is under strict lipid rich condition (91, 97). However, the role of ApoC-III in $VLDL_1$ production does not convincingly explain the atherogenicity of ApoC-III in CVD, as large $VLDL_1$ scarcely penetrate under the endothelium but small LDL and TRL remnants do. Therefore, other mechanisms to supplement the story is required.

Researches also find that ApoC-III delays triglycerides clearance by either inhibiting the activity of lipoprotein lipase (LPL) (92) or preventing TRL remnants uptake (93, 99). The inhibitory effect of ApoC-III on the activity of LPL and HL, key enzymes hydrolyse VLDL into smaller remnants which are more easily removed from circulation, delays large TRL metabolism. In addition, ApoC-III also replaces ApoE and ApoB, proteins with a higher affinity to hepatic receptors including LDL receptor and proteoglycan receptor (100, 101), leading to the slow down of hepatic uptake of TRL remnants. Subsequent study also

shows that ApoC-III can completely abolish ApoE mediated LDL uptake by HepG2 cells (hepatocytes) through inhibition of LDL family receptors (93, 99).

In addition to its inhibitory role in triglycerides clearance, the presence of ApoC-III on HDL leads to a compromised cholesterol efflux capacity as well as delayed metabolism (102, 103). HDL isolated from patients with CAD having higher ApoC-III content demonstrated weaker cholesterol efflux capacity compared to non-CAD subjects (102). Metabolic analysis further reveals that ApoC-III slows HDL metabolic turnover including HDL hepatic clearance and HDL size expansion, resulting in small HDL particles which are believed to be less cardioprotective (103). Taken these together, it is postulated that lipoproteins (VLDL, LDL and HDL) harbouring ApoC-III, potentially due to ApoC-III's "inert" structural and biochemical properties to receptors and enzymes, are rendered delayed metabolism.

1.6.4 hAPOC3 transgenic mice

The structure and biochemical property of ApoC-III that inhibits the activity of LPL, possibly via displacing enzymes from the lipid droplets, enabled its capacity in regulating the levels of serum triglyceride (104). Correspondingly, ApoC-III is highly expressed in the liver and to some extent in the intestine, two major tissues where triglycerides are produced. Overexpression of ApoC-III in rabbits (105) and miniature pigs (106) leads to severely hypertriglyceridemia. Couple of studies further explored the importance of ApoC-III in lipidology and atherosclerosis by manipulating human APO3 transgenic (hAPOC3 Tg) mice, which are summarised in Table 1-1. Generally, all hAPOC3 Tg mice exhibited markedly raised serum ApoC-III and triglyceride levels, while with variations in

HDL-C and size of atherosclerotic lesion. When human APOC3 cross with LDL receptor (LDLr) knockout mice (hAPOC3 Tg/LDLr$^{-/-}$ mice) and hAPOC3 Tg/LDLr$^{-/-}$ mice cross with mice overexpress cholesterylester transfer protein (CETP), these mice showed the familial hyperlipidaemia combined with elevation of VLDL and LDL. These mice also exhibited 2-3 fold accelerated progression of atherosclerosis compared to the single LDLr$^{-/-}$ mice (107). However, when hAPOC3 Tg mice cross with ApoE$^{-/-}$ mice, virtually no difference in lesion size were demonstrated compared to ApoE$^{-/-}$ after 16 weeks on chow diet (108). Furthermore, Hu ApoA-I/CIII/AIVTg/ApoE$^{-/-}$ mice even had regressed plaque size compared to ApoE$^{-/-}$ mice, potentially due to substantially elevated HuApoA-I and HDL-C in transgenic species (109, 110). The role of ApoC-III in elevating TRL and TRL remnants has been well elicited in mice hAPOC3 Tg mice, nevertheless lefting the role of APOC3 overexpression in size of atherosclerotic lesion ambiguous.

Table 1-1 ApoC-III transgenic mice, hyperlipidemia and atherosclerosis

Animals	**Diet**	**Disease model**	**Lipids/ glucose profile**	**Others**	**Reference**
hAPOC3 Tg Line 2721(100 copies) Line 2674 (1-2 copies)	10% sucrose feeding/ bolus fat feeding	Hypertriglyceridemia	Increase triglycerides and ApoC-III concentrations	-	(111)
hAPOC3 Tg/ApoE$^{-/-}$	Chow diet	Hypertriglyceridemia	Increased VLDL and VLDL size, decreased HDL, decrease VLDL glycosaminoglycan binding	No difference in atherosclerotic lesion size	(108)
hAPOC3 Tg / LDLr$^{-/-}$ hAPOC3 Tg/ LDLr$^{-/-}$/ CETP Tg	Western diet	Familial combined hyperlipidaemia (FCHL)	Increased VLDL, LDL-C	Enhanced atherosclerotic lesion size compared to LDLr$^{-/-}$ mice	(107)
Hu ApoA-I/ CIII/ AIV transgenic	Atherogenic diet	-	Hyperlipidaemia, increased HDL-C	Reduced atherosclerotic lesion, increased scavenge receptor class B type 1 (SRB1), ABCA1	(109, 110)
hAPOC3 Tg	High-fat, cholesterol-rich diet	Restenosis model	Hyperlipidaemia	Increased neointimal formation	(112)

1.6.5 ApoC3 gene polymorphism and human CVD

Unlike in animals, APOC3 gene variants shows undoubtably positive relation to CVD, supported by studies ranged from clinical evidences (113, 114) to genetic polymorphism analysis (115, 116). Plasma levels of free ApoC-III and ApoC-III concentration in VLDL and LDL can independently predict the risk for coronary events with logistic regression analysis adjusted for age, sex and blood lipids (113, 114). ApoC-III also marked a subspecies of HDL, which predispose individuals with higher risk of CHD, whereas HDL without ApoC-III is associated with lower risk of coronary events, supported by meta-analysis from four prospective studies (94). Upon isolation of HDL from ACS patients, experiments further mechanistically explored that these HDL with increased ApoC-III content loses its protective capacity as it fails to activate anti-apoptotic B-cell lymphoma-extra large (Bcl-xL) pathway but stimulates pro-apoptotic p38-mitogen activated protein kinase (MAPK) in EC (117). Elevated levels of ApoC-III have also been found in young and aged survivors of myocardial infarction (MI), a symptom normally resulted from unstable plaque thrombosis (118-120).

Genetic studies identified several APOC3 polymorphisms including Gof mutations in APOC3 promoter region (115, 116) and Lof mutations (26, 27, 121), which link to pro-atherogenic and cardioprotective lipid profile respectively (summarised in Table 1-2). S2 alleles, with mutations found within the Sst-1 restriction site, are associated with MI (122), higher blood pressure (116) and insulin resistance (123). Additionally, Gof mutations in the APOC3 promoter region (-482C→ T, -455T → C) are correlated with MetS, hypertriglyceridemia (124, 125) and non-alcoholic fatty liver disease (NAFLD) (126). Nonetheless,

using a Mendelian randomization approach. the causal role of APOC3 in triglycerides has recently been established (26, 27). These Lof mutations including a nonsense mutation R19X (rs76353203) (121), missense A43T (127) and two splice-site mutations (IVS2+1G→A and IVS3+1G→T) (26, 27) are causally in relation with cardio-protective lipid profile, characterised by reduced levels of triglyceride and LDL-C.

Table 1-2 Summary of the roles of ApoC3 polymorphism in ApoC-III levels, serum lipid profile and CVD

ApoC3 polymorphism	**Participants**	**Associated disease**	**Lipids profile**	**Other**	**Reference**
Allele S2	73 type 2 diabetes mellitus/	MI	Hypertriglyceridemia	Greater systolic blood pressure, less hemoglobin A1c (HbA1c)	(116)
Promoter variants (-482C → T, -455T → C)	110 diabetes patients from Chennai Urban Population Study	MI and MetS	Dyslipidaemia (odds ratio = 10.03%)	Other gene polymorphism also mentioned Fatty acid-binding protein 2 (FABP2), ApoE, beta 3 adrenergic receptor (B3AR), G Protein Subunit Beta 3 (GNB3), LPL, PPARα, PPARγ	(124, 125)
Allele S2	48 post-myocardial infarction patients and 47 control	MI	Higher triglycerides	but no difference insulin gene linkage	(128)
Promoter variants (-482C → T, -455T → C)	7,983 prospective cohort	Lean carriers with type 2 diabetes	Hypertriglyceridemia	Associated with insulin treatment	(129)

ApoC3 polymorphism	Participants	Disease	Lipids profile	Other	Reference
Allele S2	115 male students	Healthy volunteers	-	Increased insulin response in oral glucose tolerance test indicating preposition of insulin resistance	(123)
1100C →T, 3175 C → G, 3206 T → G	77 nuclear families of Caucasian origin	Associated with carotid intima-media thickness (CIMT)	-	Associated with CETP, fibrinogen and Methylenetetrahydrofolate reductase (MTHFR)	(130)
R19X mutation	809 Lancaster Amish/1,267 Greek	Reduction in coronary artery calcification	Reduction in serum triglycerides, LDL-C and higher HDL-C	-	(121, 131)
Promoter variants (-482C → T, -455T → C)	163 healthy non-Asian Indian men	38% in NAFLD	30% increase in ApoC-III, 60% increase in triglycerides,46% reduction in triglycerides clearance	Associated with insulin resistance	(126)
Loss-of-function polymorphism (R19X, IVS2+1G→A and IVS3+1G→T), A43T, D65N, A10T	75,725 in Denmark 3734 of European or African ancestry 110,970 people	40-41% reductions in risk of Ischemic vascular disease (hazard ration, 0.59) and coronary heart disease (odds ratio, 0.60)	39% -44% reduction in triglyceride level	-	(26, 27)

1.6.6 The effect of ApoC-III on vascular cell signaling

Apart from ApoC-III mediated hypertriglyceridemia, intracellular signaling pathways (Figure 1-4) by which ApoC-III directly exerts its detrimental impact on vascular cells have also been elicited. In THP-1 monocytes, ApoC-III induces expression of inflammatory biomarker including phospholipase A2 (Lp-PLA2) and Vascular cell adhesion protein 1 (VCAM-1), through the activation of extracellular regulated protein kinase phospho-p42/44 (Erk 42/44)→ NF-κB (132) and protein kinase C α/β (PKCα/β)→NF-κB pathway (133) respectively. ApoC-III is also able to activate EC by driving NF-kB→VCAM-1 and *ICAM-1* expression, which can be suppressed by statin treatment (134). In myotubes, via activation of toll-like receptor 2 (TLR2)→Erk 42/44 pathway, ApoC-III induces endoplasmic reticulum (ER) stress and attenuates mitochondrial metabolism. This was demonstrated by reduced levels of PPARγ and its co-activator 1alpha (PGC-1α), which can subsequently slow down protein translation in ER via increased phosphorylation of eukaryotic initiation factor (eIF2α) (135). The fact that ApoC-III promotes cell inflammation and ER stress implies potential mechanisms that link intraplaque ApoC-III to the development of inflammatory plaque.

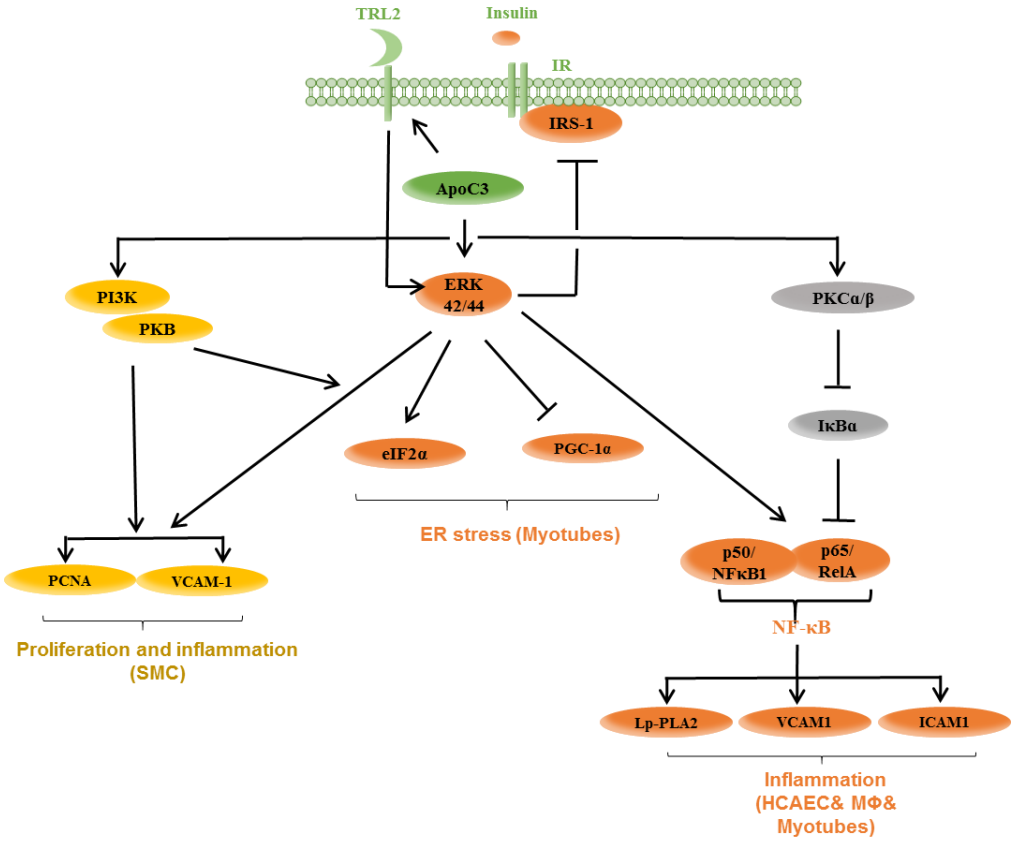


Figure 1-4 Schematic representation of cellular signaling pathways induced by ApoC-III. ERK serves as a central hub that senses ApoC-III to induce a variety of cellular processes, such as inflammation (Erk42/44-NF-κB), ER stress (Erk42/44-eIF2α) and mitochondrial metabolism (Erk42/44-PGC-1α). The ApoC-III promoted PI3K-proliferating cell nuclear antigen (PCNA) and PCKα/β-NF-κB pathways have also been reported. ApoC-III is also suggested to activate Erk through TLR2, a process which in turn downregulates insulin signaling pathway by attenuating IRS-1 signaling.

1.7 Thesis aims

As extensively described in this chapter, there are strong associations between triglycerides, ApoC-III and atherosclerotic CVD. However, the potential mechanism linking these factors are not fully understood. Accordingly, the aims of this doctorial studies are to perform a series of translational investigations of TRL and ApoC-III in the settings of atherosclerosis, diabetes, obesity and hypertriglyceridemia.

Diabetes mellitus and insulin resistance result in hypertriglyceridemia and low HDL-C levels, all of which are associated with increased incidence of CVD (136, 137). The mechanisms linking TRL and atherosclerosis remains unclear. Cellular studies have demonstrated that chemical modification of LDL, primarily by oxidation, is required to render LDL atherogenic. Recent study has revealed that oxidized phospholipids induced inflammatory signalling and promote ox-LDL uptake mainly in macrophages (138). While oxidized LDL have been extensively investigated, the impact of TRL oxidation has not. In addition, given the potential role for ApoC-III mediating atherosclerosis (139) and the metabolism of TRL (91), the expression of ApoC-III in animal models of atherosclerosis has not been well investigated.

In summary, this thesis aims to investigate 1) a potential direct impact of both oxidized TRL in endothelial cells; 2) expression of ApoC-III within vulnerable

atherosclerotic plaque and the liver in the settings of diabetes and obesity; 3) whether the presence of hypertriglyceridemia influences hepatic expression of factors involved in lipid metabolism and inflammation. It is hypothesised that TRL and ApoC-III exert adverse effect on vascular cells, atherosclerosis and lipid metabolism. These studies will provide further evidence linking triglyceride and ASCVD.

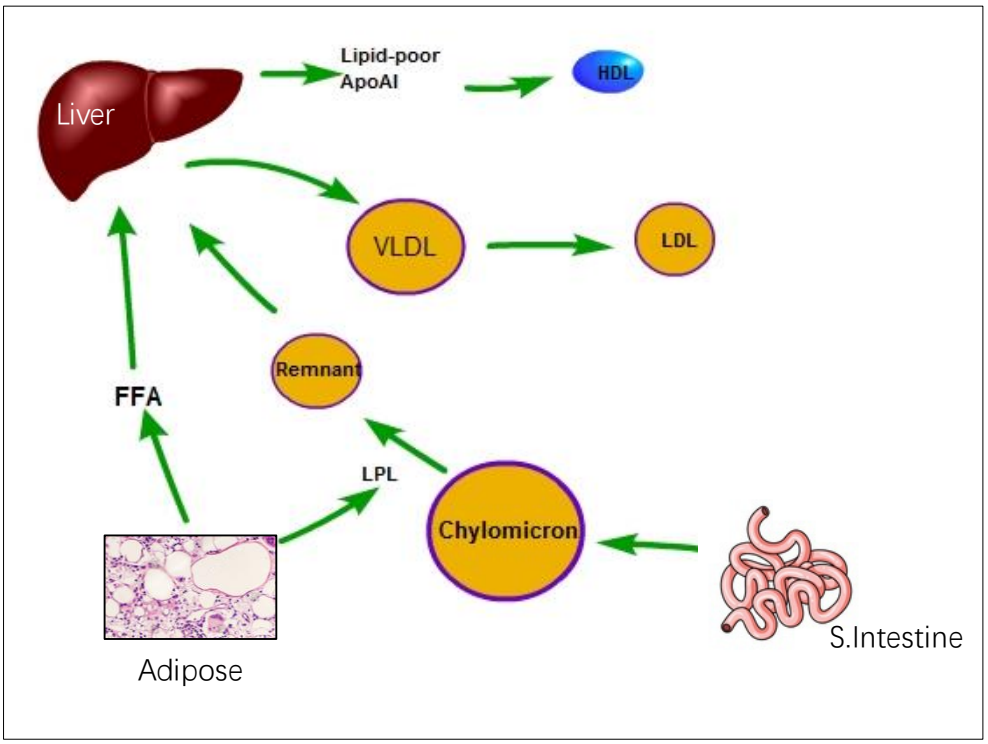


Figure 1-5 Triglyceride rich lipoproteins metabolism. VLDL, very low-density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoprotein; FFA, free fatty acids; S. Intestine, small intestine.

Chapter 2. Materials and Methods

2.1 **Methods**

Unless detailed below, all laboratory supplies were purchased from Sigma Aldrich (NSW, Australia). General methods are found in Chapter 2. Specific methods related in individual chapters are found in each chapter.

2.1.1 Human umbilical vein endothelial cells (HUVEC) cell culture

HUVEC were kindly provided by the Vascular Biology and Cell Trafficking Laboratory, Institute of Medical and Veterinary Science (IMVS, SA, Australia). Cells were routinely grown in T75 flasks in Mesoendo cell growth medium (Sigma, 212-500) containing equivalent 5% Fetal bovine serum (FBS). Cells underwent serum starvation by culturing either in 2% FBS or 0.2% bovine serum albumine (BSA) in medium 199 (M199) for 4 or 16 hours (h) before subsequent experiments. Cell functional assays and signalling assays were carried out in fresh M199 medium.

2.1.2 Cell Cryopreservation

Cells were harvested and resuspended in a cryopreservation mixture of 10% dimethyl sulfoxide (DMSO) in 90% fetal calf serum (FCS). 90% FCS was added to prevent cells being starved of nutrients. Cells in cryopreservation mixture were aliquoted [1 millilitre (ml)] into CryoTubes and kept into a Cryo 1-degree

Mr. Frosty freezing container for 48 h at -80 degree Celsius (°C). Cells were then transferred into ultra-low temperatures (-196 °C, liquid nitrogen).

2.1.3 Lipoproteins

Plasma was obtained from Australian Red Cross Blood Service. TRL [density 1.019 gram per millilitre (g/ml)] and HDL (density 1.21 g/ml) were isolated by sequential ultracentrifugation [50,000 round per minute (rpm), 18 h at 4 °C] using potassium bromide. Before using for cell culture, lipoproteins were dialysed against 1X phosphate buffered saline (PBS) for 24 h and then filter sterilised using a 0.45 micrometre (µm) (for TRL) and 0.22 µm (for HDL) filter. Protein concentrations in lipoproteins were measured by Pierce bicinchoninic acid (BCA) protein assay kit (Thermo scientific, 23225).

2.1.4 Determination of plasma lipoprotein and lipid levels

Measuring of total plasma triglycerides and cholesterol of these mice were followed by enzymatic analysis of cholerol E (WAKO LabAssay, NovaChem, 439-17501) and Triglyceride E (NovaChem, 432-40201). The non-ApoB portion was obtained by Dextran-sulfate-Mg^{2+} precipitation methods (140) and Non-ApoB cholesterol and triglycerides levels were measured. For hyperlipidaemic plasma samples, dextran precipitation was carried out twice to ensure all the ApoB lipids had precipitated out (140). Total serum ApoC-III levels were diluted

according to manufactory requirement using enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab217777).

2.1.5 RNA-extraction

Aiming to quantify mRNA levels and assess cell polysome status, mRNA were first extracted by TRI reagent, following manufacture's instructions. Briefly, cells were washed with PBS and harvested in TRI reagent (Sigma, T9424). Followed by 1-Bromo-3-chloropropane (BCP, Sigma, B9673-200ML), proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were resolved into lower organic phase, milky interphase and upper clear phase respectively. The RNAs were precipitated out by iso-propanol, dissolved in RNases free water and at -80 °C.

2.1.6 Real-time (RT) quantitative polymerase chain reaction (qPCR)

Following TRI reagent extraction, RNA samples were quantified and normalised using a NanoDrop (Thermo Fisher, Australia). Purified total RNA was then used as a template for complementary DNA (cDNA) synthesis, prepared by using iScriptTM cDNA synthesis kit (BioRad, 1708841). Transcripts were assessed by RT-qPCR amplification [50 ºC /2 minutes (min), 95 ºC /10 min, 95 ºC /15 sec, 60 ºC /30 sec, 72 ºC /10 sec — 39 cycles] using

the BioRad CFX RealTime Thermal Cycler. The cycle threshold (CT) values were used to calculate relative mRNA copy number. All results normalised to the housekeeping gene β-Actin content for each sample and reactions were performed in triplicate and validated by the presence of a single peak in the melt curve analysis.

2.1.7 Western Blot analysis

Cells and tissues were homogenised by ice incubation or Precellys 24 tissue homonogeniser (Bertin Technologies, Paris). All proteins were extracted in Triton X-100 lysis buffer [1% Triton X-100, 150 millimolar (mM) NaCl, 1 mM Ethylene glycol tetraacetic acid (EGTA), 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Dithiothreitol (DTT) in 20 mM Tris hydrochloride (Tris-HCl) (power of hydrogen (pH7.5)] and loaded into Invitrogen Bis (2-hydroxyethyl) amino-tris(hydroxymethyl) methane (Bis-Tris) 4% - 12% pre-cast gels (Thermofisher, NW04127BOX). Proteins were transferred to nitrocellulose membranes and probed with primary antibodies overnight at 4 ºC. For detection of ABCA1 expression in the HepG2 and liver lysate, protein samples were heated at 65 ºC and room temperature (RT) for 30 min, respectively in the presence of 1 mM DTT. Secondary antibodies anti-mouse alexa fluor 680 and anti-rabbit alexa fluor 680 were used for detection of bands by Li-Cor Odyssey CLX.

2.1.8 Statistics

Detailed statistical approaches can be found in each chapter. Generally, *in vivo* animal data were represented as mean ± standard deviation (STD) and *in vitro* cell data was performed in three biological replicates and shown as mean ± standard error mean (SEM), unless otherwise described. Statistical comparisons between two groups were performed using Student's t-test (Mann-Whitney test) or one-way ANOVA where appropriate (Kruskal-Wallis test). A linear regression model was used for correlation analysis. *$p < 0.05$ was considered as statistically significant for all analyses.

2.2 **Materials**

2.2.1 Equipment

Equipment	**Specifications and Supplier**
Centrifuges	Eppendorf, Centrifuge 5430R
ELISA plate reader 1	iMarkTM Microplate Absorbance Reader, BioRad (NSW, Australia)
Microscopes	Olympus innovation, USA
ELISA plate reader 2	GloMax® Explorer Multimode Microplate Reader, Promega (NSW, Australia)
Real Time Thermal Cycler	CFX connect real-time PCR detection system, BioRad (NSW, Australia)
Scales	XS105 Dual Range, Mettler Toledo (QLD, Australia)
Scintillation & Luminescence counter	Tri-Cab 2810R, Perkin Elmer (VIC, Australia)
Invitrogen Novex gel system	Invitrogen Bolt Mini Gel Tank, Thermofisher Sicentific (VIC, Australia)
iBlot 2 dry blotting system	iBlot 2 Gel Transfer Device, Thermofisher Sicentific (VIC, Australia)
Microtome blade	Premier blade MX35, Thermofisher Scientific 3051835 (VIC, Australia)
Western Blot Imaging	Odyssey CLX, Li-Cor Biosciences Millenium Sciences (VIC, Australia)
Shandon cryotome	Shandon cryotome E, Thermo Scientific (VIC, Australia)
iBlot 2 Transfer Stack	iBlot 2 NC regular stacks, thermoIB23001 (VIC, Australia)

Equipment	**Specifications and supplier**
Thermal Cycler	T100 thermocycler, BioRad (NSW, Australia)
Vecto PEP pen	ImmEdge® Hydrophobic Barrier PAP Pen H-4000, Vectorlaboratories (Abacus DX, QLD, Australia)
Cryomolds	Cyromolds biopsy style Y565, Tissue Tek (QLD, Australia)
IHC slides	SuperFrost Plus adhesion slides, Thermoscientific J1800AMNZ (VIC, Australia)
Ultracentrifuge Rotor	50.2Ti 337901, Beckman Coulter Life Sciences (NSW, Australia)
EDTA-containing tubes	Plastic K2EDTA Tube, BD 367839 (Australia)
Scintillation vials	Scintiliation vials, Sigma Z376817, (Merck, NSW, Australia)
Spectrophotometer	NanoDrop, Thermofisher, USA
Ultracentrifuge tubes	Quick-Seal, polyallomer, 39ml, Beckman, 342414 (NSW, Australia)
Ultracentrifuge	Ultracentrifuge, Beckman Optima XPN1 (NSW, Australia)
Mr. Frosty Freezing container	Nalgene Cryo, thermofisher 5100-001 (VIC, Australia)
Invitrogen Tris-Bis 4%-12% gel	NW04127BOX, Thermofisher (VIC, Australia)

2.2.2 Culture Medium

Medium	**Recipe**			
	Solutions	Stock Conc.	Final Conc./ vol.	Company catalog.
HUVEC medium				
	FCS	-	20%	CellSera1013113
	Sodium Bicarbonate	7.5%-	1.125%	Sigma, S8761
	1M HEPES	1 M	0.02 M	Sigma, H0887
	Non-essential AA	100X	1X	Sigma, M7145
	Sodium Pyruvate	100 mM	1 mM	Sigma, S8636
	P/S	100X	1:100 dilution	Sigma, P4333
	L-glutamine	200 mM	2 mM	Sigma, G7513
	ECG	15 mg/ml	15 µg/ml	Sigma, E9640
	M199 medium	-	500 ml	Sigma M4530-500ML
MedoEndo Cell Medium				
	MesoEndo Medium	-	500 ml	Cell applications, 212-500
THP-1 Cell Medium				
	FBS	-	10 %	CellSera1013113
	Antibiotic-antimycotic	100X	1X	ThermoFisher, 15240096
	RPMI-1640	-	500 ml	Sigma, R7388
HepG2 cell medium				
	FBS	-	10%	CellSera,1013113

	Antibiotic-antimycotic	100X	1X	ThermoFisher, 15240096
	L-glutamine	200 mM	2 mM	Sigma, G7513
	DMEM-low glucose	-	500 ml	Sigma, D6046

2.2.3 Buffers and solutions

Reagents	**Recipe**		
	Solutions	Final Conc./ vol.	Company catalog.
RIPA Buffer			
	NaCl	150 mM	Thermo, AJA465
	Triton X-100	1%	Sigma, T8787
	Sodium deoxycholate (Na-Doc)	0.5%	Sigma, D6750
	Sodium dodecyl sulfate (SDS)	0.1%	Sigma, L3771
	EDTA, pH8.0	1 mM	Sigma E5134
	Protease Inhibitor	1 tablet in 10 ml	Sigma, 4693159001
	Phenylmethanesulfonylfluoride (PMSF)	1 mM	Sigma, 93482
	Phosphatase Inhibitor	1 tablet in 10 ml	Sigma, 4906845001
	Tris-HCl ph8.0	50 mM	Sigma, T1503
Triton X-100 buffer			
	NaCl	150 mM	Thermo, AJA465
	Triton X-100	1%	Sigma, T8787
	EGTA ph8.0	1 mM	Sigma, E 3889
	EDTA ph8.0	1 mM	Sigma, E5134
	DTT	1 mM	BioRad, 1610611
	Protease. inhibitor	1 tablet in 10 ml	Sigma, 4693159001
	Tris-HCl, pH7.5	20 mM	Sigma, T1378

Reagents	**Recipe**		
	Solutions	Final Conc./ vol.	Company catalog.
Western blot running buffer			
	20X Bolt MES SDS	-	Life tech, B0002
Immunohisothcemistry (IHC) blocking buffer			
	Donkey serum	3%	Jackson, 017-000-121
	Bovine Serum Albumin	3%	Sigma, A7906-50g
	Triton X-100	0.3%	Sigma, T8787
	Tween- 20	0.2%	BioRad, 1706531
	PBS	-	Sigma, D8537
Scott's bluing solution			
	Sodium bicarbonate	0.35%	
	Magnesium sulphate	2%	Sigma, M7506
	H2O	-	-
3% Acid alcohol			
	Hydrochloric acid	3 ml	Sigma, H1758
	70% ethanol	97 ml	Fisher Scientific, A962P-4
0.25% Oil red O (ORO) solutions			
	ORO powder	1 g	Sigma, O0625-25G
	Propan-2-ol	250 ml	Fisher chemical, FSBP/7500/17
	ddH2O	150 ml	-

Reagents	**Recipe**		
	Solutions	Final Con./Vol.	Company log #
Trichrome Stain (Masson) Kit, Sigma, HT15-1KT			
	Biebrich Scarlet-Acid fusion solution	250 ml	Sigma, HT15-1
	Phosphomolybdic acid solution	250 ml	Sigma, HT 15-3
	Phosphotungstic acid solution	250 ml	Sigma, HT 15-2
	Anilline Blue solution	250 ml	HT15-1KT

2.2.4 Reagents

Reagents	**Information**
Iscript reverse transcriptase	BioRad, 1708841
Trizol	Sigma, T9424
BCP	Sigma, B9673-200ML
IQ SYBR green supermix	BioRad, 170-8886
WST-1	Roche, 11644807001
Urea	Ajax Finechem, AJA817
Acetone	Acetone (HPLC), fisher chemical, FSBA949-4
Endotoxin quantification kit	Pierce LAL chromogenic endotoxin quantitation kit, Thermo scientific, 88282
OCT	Optimal cutting temperature, Tissue Tek, IA018
Dextran [molecular weight (MW) 50,000]	Dextran sulfate sodium salt-50 kDa, Chem supply, GC2426
Lillie Mayer Haematoxylin	Australian Biostain, AHLM.2.5L
Bouin’s fixative reagent	Sigma, H10132-1L
Eosin	MP biomedicals, 17372-87-1
Weigert’s haematoxylin	Sigma, HT1079-1SET
Cholesterol Kit	WAKO LabAssay, NovaChem, 439-17501
Triglyceride Kit	Triglyceride E, NovaChem, 432-40201
Guanidine hydrochloride	Sigma, G4505-1KG
DMSO	Dimethyl sulphoxide hybri-MAX, Sigma, D2650
Agarose	Sigma, A9539-100g
Citric acid monohydrate	Sjgma, C1909-500g

Reagents	**Information**
Coomassie Blue	Coomassie blue R250, Fisher bioreagents, BP101-25
Tryptone	Sigma, 16922-500G
Yeast extract	Sigma, Y1625-250G
Formalin	Formalin, neutral buffered (10%), Sigma, HT501128-4L
Distyrene, plasticizer, xylene (DPX) mounting	DPX Mountant for histoloty, Sigma, 06522
IHC mounting with 4',6-Diamidino-2-phenylindole dihyrochloride hydrate (DAPI)	Invitrogen prolong gold antifade mountant with DAPI, P36935
0.25% Trypsin-EDTA (1X)	Life technologies, 25200-056
Streptozotocin	Sigma, S0130
Mouse ApoC-III ELISA kit	Abcam, ab217777
Human ApoC-III ELISA kit	Abcam, ab154131
Iso-pentane	Ajax Finechem, AJA 1521
Glycerol	Ajax Finechem, AJA242-500 ML
Potassium Bromide	Chem supply, PA006-5 KG

2.2.5 Antibodies

Antibody	**Catalogue No.**	**Opt. Conc.**
ABCA1	Abcam, ab18180	1:200
ABCG1	Abcam, ab52617	1:200
SRB1	Abcam, ab52629	1:200
ApoC-III (Immuno Staining)	Santa Cruz, sc-50378	1:200
ApoC-III (WB)	Cloud Clone, PAB890Mu01	1:200
CD31	Abcam, ab7619	1:200
CD68	BioRad, MCA1957GA	1:250
ApoB	Abcam, ab20737	1:1000
SMC-actin	Sigma, C6198	1:400
p-S6K1	Cell signalling, #9205	1:500
Actin	Cell signalling, #4970	1:1000
Anti-His Tag	Cell signalling, #2365	1:1000
Cy5 donkey anti-rat	Jackson immunoresearch, 712-175-153	1:400
Cy3 donkey anti-rabbit	Jackson immunoresearch, 711-165-152	1:400
Cy5 donkey anti-mouse	Jackson Immunoresearch, 715-605-150	1:400

Chapter 3. Cellular studies of the potential atherogenicity of TRL

3.1 Abstract

Introduction— Increasing evidence has implicated TRL in atherosclerotic CVD, although preclinical studies have not fully elucidated their impact on the artery wall.

Methods and Results—TRL were isolated from the plasma of healthy human subjects by ultracentrifugation and subsequently modified by MPO. The impact of incubation of native or MPO-TRL (50 μg/ml) with HUVEC, in the presence or absence of HDL (2 mg/ml), was assessed. MPO resulted in oxidation of ApoC-III and lipids within TRL. MPO-TRL were unable to maintain EC survival and early wound closure capacity, which was restored by HDL co-incubation. An increase in proinflammatory adhesion molecules and chemokines by EC was also observed in the setting of MPO-TRL co-incubation. Further investigation revealed a translation switch, whereby co-incubation with MPO-TRL resulted in greater EC expression of angiogenesis factors (HIF-1α and VEGF) and reduced expression of ABCG1 and mTORC1 activity.

Conclusions—MPO modification generates TRL that have adverse effects on factors implicated in vascular repair and inflammation, suggesting that oxidative modification may be one factor involved in potential atherogenicity of TRL.

3.2 Introduction

The association of LDL-C and CVD has been well established by population (7, 141, 142) and genomic studies (8, 143, 144). Mechanistic studies have demonstrated that modification of LDL, by a range of factors including oxidation and carbamylation, increases their ability to promote vascular inflammation (145, 146), foam cell formation (58, 147) and impairment of vascular repair (148). In parallel, hypertriglyceridaemia has also been demonstrated to associate with cardiovascular risk, a finding which persists after controlling for the presence of additional metabolic risk factors (18, 19). However, the mechanisms that directly link TRL to vascular dysfunction have not been well elucidated. While some groups have postulated that cholesterol content of these particles may underscore any adverse effect on the arterial wall (75, 149, 150), limited studies have directly investigated their impact on the endothelium (78). Furthermore, it is unkown whether chemical modification of TRL, such as observed with LDL, alters their impact on the vasculature.

MPO has been identified to play a biological role in both lipoprotein modification and atherosclerosis *in vivo* (59). Chemical studies have revealed that MPO catalyses the formation of chlorinating oxidants from an MPO/Cl^-/H_2O_2 system by converting Cl^- into hypochlorous acid (OCl^-). The generation of tyrosyl radicals by hypothlorous acid or MPO directly plays a role in the initiation of

oxidation of lipid species within LDL particles. While systemic MPO levels associated with cardiovascular risk (63, 64). MPO has been identified within eroded plaque of ACS patients (62) and mechanistic studies have implicated a role for MPO in progression of atherosclerotic disease (58), the impact of MPO modification of TRL have their influence on the arterial wall is not known.

A potential role for HDL in the protection against atherosclerotic cardiovascular disease has been established (151, 152). In addition to population studies demonstrating an inverse association between HDL-C levels and cardiovascular risk (20, 153), preclinical studies have revealed that HDL possess a range of functional properties that may confer this benefit (154-156). In particular, cellular studies have demonstrated that co-incubation with HDL inhibits the deleterious effects of inflammatory and oxidative stimuli (157-159). However, the impact of HDL on the potential effects of TRL has not been fully investigated.

The aim of the studies described in this chapter were to investigate the impact of TRL, in the presence and absence of MPO modification on endothelial factors involved in the pathogenesis of vascular disease. The specific aims included:

1. To determine whether native and MPO-modified TRL promote pro-inflammatory changes in EC.

2. To determine whether native and MPO-modified TRL influence EC migration and proliferation and associated signalling pathways.
3. To determine whether co-incubation of HDL influences the impact of native and MPO-modified TRL on EC.

The hypothesis of these studies was that oxidation of TRL, as previously described with LDL, would results in more adverse effects on endothelial pathways involved in inflammation and vascular repair.

3.3 **Methods**

3.3.1 Myeloperoxidase modification of lipoproteins

Isolated and dialysed native-TRL were immediately used for MPO modification. A 4 h incubation period was selected on the account that the half-life of TRL in human plasma is 6 h (160). Human TRL (10 ml) were incubated with 1 U myeloperoxidase MPO: Sigma, M6908), 200 micromolar (μM) H_2O_2, 100 mM Cl^- at 37 °C for 4 h, at pH 7.4. The concentrations of the H_2O_2 was verified spectrophotometrically using molar extinction coefficients of 39.4 centimetres $(cm)^{-1}M^{-1}$ at 240 nM. MPO activity was confirmed by OCl^- generation using EnzCheck MPO activity assay kit (Thermofisher, 33856). All studies were performed by using paired lipoproteins (native-TRL and oxidised -TRL from the same pool of donors) that had been stored for less than 4 weeks. Apolipoprotein

modification and lipid oxidation were assessed by mass spectrometry and TBARS assay respectively.

3.3.2 TBARS assay

0, 0.1, 0.2, 0.4, 0.6 and 1 nanomole (nmol) of Malondialdehyde bis (dimethyl acetal) or Tetramethoxypropane (TMP) (Sigma, 10, 838-3) were used as standard. TMP standards and 20 μg of TRL were mixed with 25% trichloroacetic acid (TCA), 1% 2-thiobarbituric acid (TBA) and incubate for 45 min at < 95 °C. The reacted mixtures were centrifuged for 20 min at 2000 rpm and the supernatants were quantified by colorimetric spectrometer at 520 nm. Results were calculated and presented as nmol of malondialdehyde (MDA) in 100 μg of TRL.

3.3.3 Apolipoprotein 2-dimensional (2D) gel purification

Apolipoproteins (300 μg) were precipitated by acetone wash from native-TRL and MPO-TRL. Methods were modified and adapted from previously published methods (161). Briefly, samples were loaded onto isoelectric focusing gels from pH 3-11. After 8,000 volts (V)-hours, the gels were equilibrated with SDS and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an ALL-kDa precast gel (BioRad, 5671121). Ingel digestion was performed after protein detection by Coomassie-blue R250

staining (Coomassie blue R250, Fisher bioreagents, BP101-25). Aiming to isolate protein for proteomics analysis, Coomassie-stained spots were de-stained and washed with 100 mM ammonium bicarbonate and acetonitrile, reduced with either Tris (2-carboxyethyl) phosphine (TCEP) at room RT for 20 min or DTT at 60 ºC for 40 min, and then alkylated by iodoacetamide (IAA) in a dark place for 30 min. The gel was incubated in 50 µl of a 12 nanogram per liter (ng/l) modified trypsin solution in 50 mM ammonium bicarbonate, pH 8.6, and incubated at 60 ºC overnight. The resulting peptides were extracted first with a 1 : 1 solution of 25 mM ammonium bicarbonate and acetonitrile and then twice with a 1 : 1 solution of 5% formic acid and acetonitrile. The extracted tryptic peptides were lyophilised and resuspended with 15-20 µl of 5% formic acid for mass spectrometric analysis.

3.3.4 Wst-1 cell proliferation assay

EC (4,000 cells /well) were plated in 96-well plates. Following overnight incubation at 37 ºC in a 5% CO_2 incubator, the media was removed and replaced with various concentrations of native-TRL, MPO-TRL, HDL and control in M199 medium. Cultures were then co-incubated for further 24 h. Prior to final reading of the assay, the existing media was aspirated from each well and replaced with the WST-1 solution [10% volume/volume (v/v), Roche, Australia] in Roswell Park Memorial Institute Medium -1640 (RPMI-1640).

Plates were then incubated for 2 h at 37 ºC, 5% CO_2 and cell proliferation was assessed by measuring absorbance at 450 nM, using an ELISA microplate reader (BioRad iMarkTM Microplate Absorbance Reader).

3.3.5 Wound healing (scratch) assay for HUVEC

HUVEC wound healing ability was studied using an in vitro wound (scratch) assay. 200,000 HUVEC were seeded in 24-well plates. Once the cell monolayer was confluent, a cross / two lines were made in each well with a p200 pipette tip, to create consistent “wounds” (Figure 3-1). The cell debris was removed and fresh M199 medium was added with corresponding treatment. Images of the scratches were acquired under light microscopy at time 0 h, 6 h and 12 h, at 4X magnification (Olympus innovation, USA). Image J software (NIH, USA) was used to measure scratch areas. Wound healing abilities were calculated as the percentage reduction in scratch area relative to the wound area from starting time.

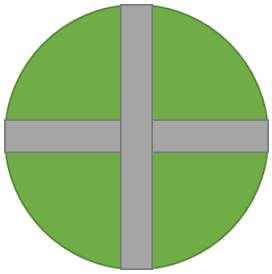


Figure 3-1 Scratch pattern. Wounds were introduced in a two-direction / cross pattern. Over time, images were captured, and wound area were quantified by ImageJ (NIH, USA).

3.3.6 Polysome analysis

Aiming to investigate the translational status of protein production, polysome analysis was assessed as described previously (162). Briefly, polyribosomes were resolved by sucrose-gradient-density centrifugation with ten fractions collected as per gradient. For RNA extraction, 1% (w/v) SDS and 0.15 mg/ml proteinase K were added to each fraction, and RNAs were extracted from upper phase of mixture of TRI reagent (TRI reagent, Sigma, T9424), and 1-Bromo-3-chloropropane (BCP, Sigma, B9673-200ML). The RNAs were then precipitated out by iso-propanol and dissolved in RNase free water.

3.3.7 Azidohomoalanine (AHA) and stable isotope labelling of amino acids in cell culture (SILAC)

Dulbecco's Modified Eagle's medium (DMEM) medium without methione, lysine and arginine (Dundee Cell Products, Dundee, UK) was used in cell isotope labeling. At plating, methione was added to the same concentration as in normal DMEM medium and lysine and arginine were substituted with either 'light', 'medium' Arginine (Arg, $^{13}C_6$) and Lysine (Lys, $^{2}H_4$) or heavy Arg ($^{13}C_6$+$^{15}N_4$) and Lys ($^{13}C_6$+$^{15}N_2$) and cells were cultured for 24 h for labeling in 10 cm dishes until confluent. HUVEC were then underwent serum starvation by culturing in 0.2% BSA DMEM medium for 4 h, and 30 min for methione starvation. Cells were then supplemented with AHA and corresponding

treatment (native-TRL, MPO-TRL or non-treatment) simutaneously in 20% FBS DMEM medium for 4 h. Isolation of newly synthesised proteins and preparation for mass spectrometry were performed as described (163, 164) (Figure 3-2).

3.3.8 Mass Spectrometry and protein analysis

Mass spectrometry was performed essentially as previously described (164). M /H values were used for analysis of protein profiles of MPO-TRL/native-TRL. Light Arg/Lys containing peptides were used only as reference values for analysis due to an overabundance of these peptides. The PANTHER database were employed to assign protein changes by MPO-TRL stimulation, according to the signalling pathways and biological processes (http://www.pantherdb.org/).

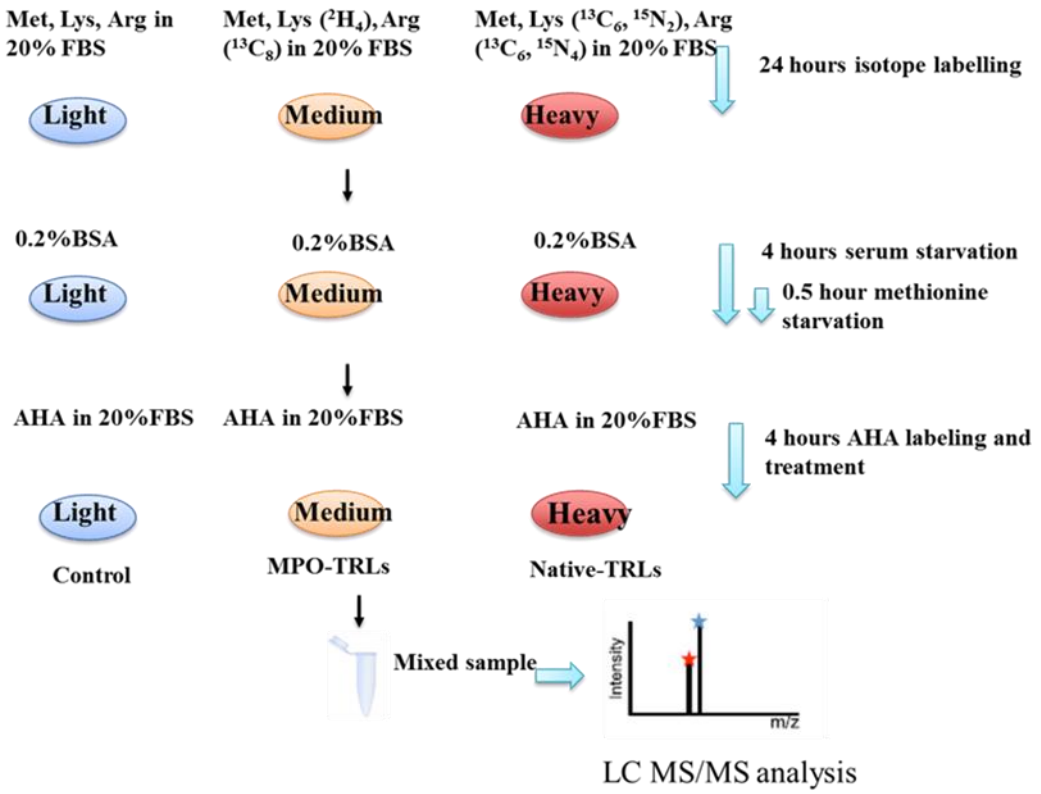


Figure 3-2 Schematic of method combining stable isotope labelling of amino acids in cell culture with azidohomoalanine.

3.4 **Results**

3.4.1 MPO modification increases protein oxidation and lipid peroxidation of TRL

MPO modifies lipoproteins, either via targeting AA residues such as tyrosine and methionine (59, 60) or initiating lipid peroxides (165). The impact of MPO modification on isolated TRL illustrated in Figure 3-3. After incubation with MPO/H_2O_2/Cl^-, ApoC-III and ApoE on the surface of MPO-TRL demonstrated change on 2D gel analysis (Figure 3-3 a), with an increase in the abundance of ApoC-III (Figure 3-3 b) and spots shift of ApoE towards low pH, compared to native-TRL. By measuring formation of peroxidative MDA (166), augmented oxidised lipids were observed in MPO-TRL compared to native-TRL (2.0 ± 0.03 vs. 0.4 nM ± 0.27/100 µg, *$p < 0.05$, Figure 3-3 c).

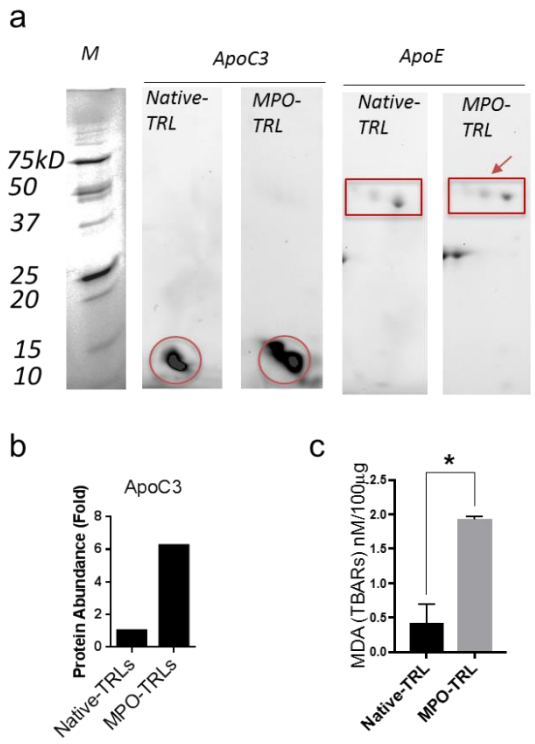


Figure 3-3 Characterisation of MPO modification. On TRL compared with their native form.(a) Oxidation of ApoC-III and ApoE, demonstrated by running 500 µg proteins precipitated from native-TRL and MPO-TRL in 2D gels. (b) Quantification of ApoC-III abundance in gel a. (c) Lipid oxidation levels of native-TRL and MPO-TRL, pooled from serum samples, n = 6. *p < 0.05 by Student's t-test. Expected molecular weight of ApoC-III: 8.8 kDa; ApoE: 36 kDa.

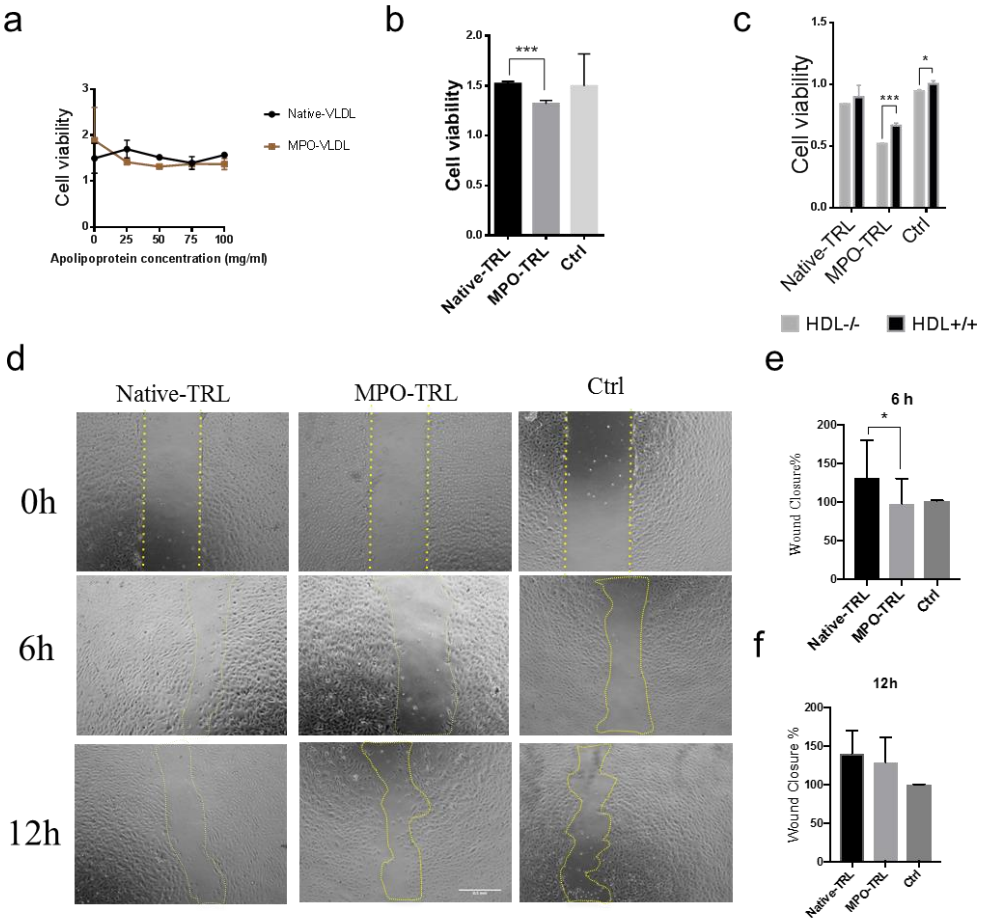
3.4.2 Impact of MPO modification of TRL on EC proliferation and and inflammatory activity

Local replication of mature EC has been recognised as an important mechanism involved in the maintenance of endothelial health (167) and protects against endothelium loss (168). This is evidenced by the capacity of EC to self-recover in denudated area overlying plaque erosion (169). Using cell viability WST-1 assay, native-TRL were able to sustain cell survival whereas MPO-TRL were not (Figure 3-4 a). For subsequent experiments, a concentration of 50 µg/ml of TRL was chosen based on prior literature (170). Additionally, at this concentration MPO-TRL exerted a reduction in live cell number compared to native-TRL (Figure 3-4 a, b), which was inhibited by co-incubation with 2 mg/ml HDL (Figure 3-4 c).

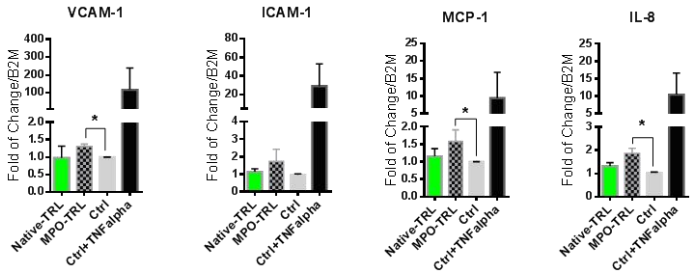
Using an established model of EC desquamative injury, we observed impairment of wound closure in the setting of incubation of MPO-TRL compared with native-TRL at 6 h (Figure 3-4 d, e). However, we did not observe a difference between TRL groups with regard to the rate of wound area closure after 12 h (Figure 3-4 d, f). The potential implications of early, but not later, differences in wound healing are uncertain and require futher investigation.

In addition to studies of EC and their role in wound closure, as a measure of vascular repair, we also investigated the impact of TRL co-incubation on EC

expression of inflammatory factors observed in the setting of endothelial dysfunction (169). Incubation of HUVEC with non-modified TRL (50 μg/ml) produced no significant increase in EC expression of inflammatory factors (Figure 3-4). In contrast, 4 h co-incubtation of HUVEC with MPO-TRL resulted in an increase in expression of *VCAM-1* by 1.30 fold (1.3 ± 0.04, p = 0.05, Figure 3-4 g), interleukin-8 (*IL-8*) by 2.00 fold (2 ± 0.125, p = 0.05) and monocyte chemosattractant protein -1 (*MCP-1*) by 1.58 fold (1.58 ± 0.19, p = 0.05), while no alteration in inercellular adhesion molecule 1 (*ICAM-1*) mRNA was observed (Figure 3-4 g). After 8 h of co-incubation with MPO-TRL, increasing expression of *IL-8* and *MCP-1*, but not *VCAM-1* persisted (Figure 3-4 h).



g 4 hour co-incubation



h 8 hour co-incubation

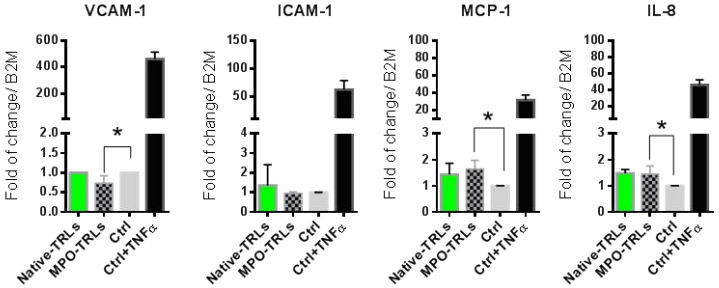


Figure 3-4 Assessment of HUVEC viability, wound closure capacity and expression of pro-inflammatory proteins with MPO-TRL co-incubation. (a, b) Cell viability following incubation with native or MPO modified TRL at indicated concentrations. (c) HDL recovery effect on cells co-incubated without or with HDL up to 24 h. (d) Scratch wound healing assays (n = 6) in response to TRL for incubation of (e) 6 h and up to (f) 12 h. Assessment of adhesion molecule expressions (n = 3) showing mRNA levels of *VCAM-1*, *ICAM-1, IL-8* and *MCP-1* by HUVEC co-incubated with serum for either (g) 4 h or (h) 8 h. Original magnification 4X. Control (Ctrl): vehicle-treated cell culture in M199 medium supplemented with 2% FBS. All data were expressed as mean ± SEM, *$p < 0.05$ by Student's t-test.

3.4.3 MPO-TRL and regulation of protein expression

To globally assess changes in EC proteome treated with MPO-TRL, stable isotope labelling using amino acids in cell culture (SILAC), coupled with mass spectrometry for protein identification and quantification was used. We substituted methionine with AHA and simultaneously treated cells with MPO-TRL. The AHA-containing synthesised proteins can be pulled down and analysed. Consistent with phenotypic assays, we observed that EC treated with MPO-TRL up-regulated factors involved in fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and aigogenesis while down-regulated proteins involved in cellular proliferation, compared to EC treated with native-TRL (Figure 3-5).

We subequently performed polysome analysis to assess changes of specific proteins, with a focus on a possible influence of MPO-TRL on angiogenesis and potentially protective effects of HDL. Hypoxia-inducible factor 1-alpha (HIF1α) regulates VEGF and platelet-derived growth factor (PDGF) (171, 172). Translational efficiency was determined by the P/M ratio between polysome fractions (fraction 6-10) and monosome fractions (fraction 1-5) (Figure 3-6 a). We observed that native-TRL promoted general translation (P/M ratio: 1.50), whereas co-incubation with MPO-TRL and serum starved control demonstrated similar low translation rate (P/M ratio: 1.08 vs. P/M ratio: 1.07) (Figure 3-6 a).

Co-incubation of EC with MPO-TRL demonstrated a delayed translation of ATP-binding cassette sub-family G member 1 (ABCG1) (Figure 3-6 b, P/M: 0.42) and accelerated translation of HIF1α (Figure 3-6 d, P/M: 18.39) and VEGF (Figure 3-6 e, P/M: 5.38) compared to control (ABCG1 P/M:2.46; HIF1α: 3.04; VEGF P/M: 0.5). MPO-TRL co-incubation failed to activate mammalian target of rapamycin complex 1 (mTORC1) activity, as assessed by levels of phospho-ribosomal protein S6 kinase beta-1 (p-S6K1) (Figure 3-6 f, g), reflecting factors regulating cell protein synthesis and cell cycle progression when it is active (173).

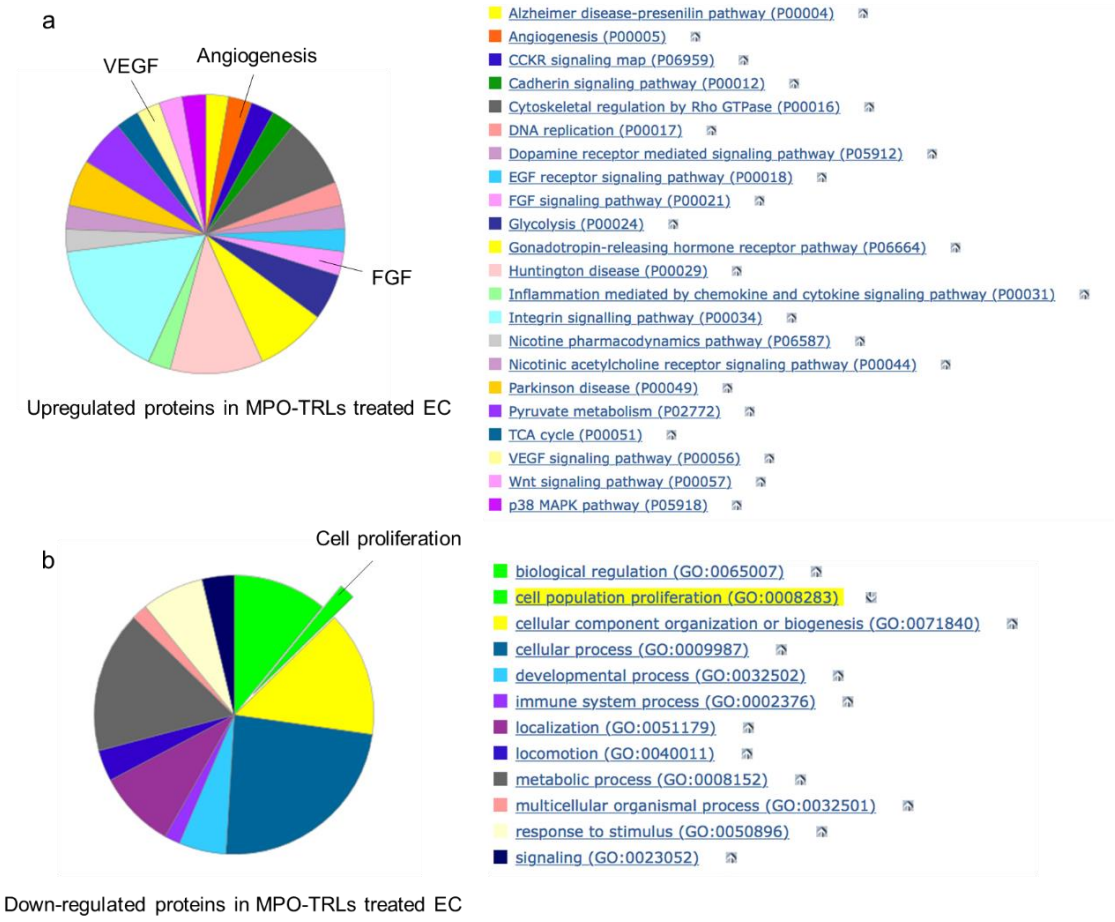


Figure 3-5 MPO-TRL treated EC protein profiles up to 4 hours, compared to incubation with native-TRL, indicating (a) upregulated proteins involved in angiogenesis, VEGF and FGF signaling pathway, and (b) down-regulated proteins involved in cell proliferation. Ctrl: vehicle-treated cell culture in M199 medium supplemented with 20% FBS.

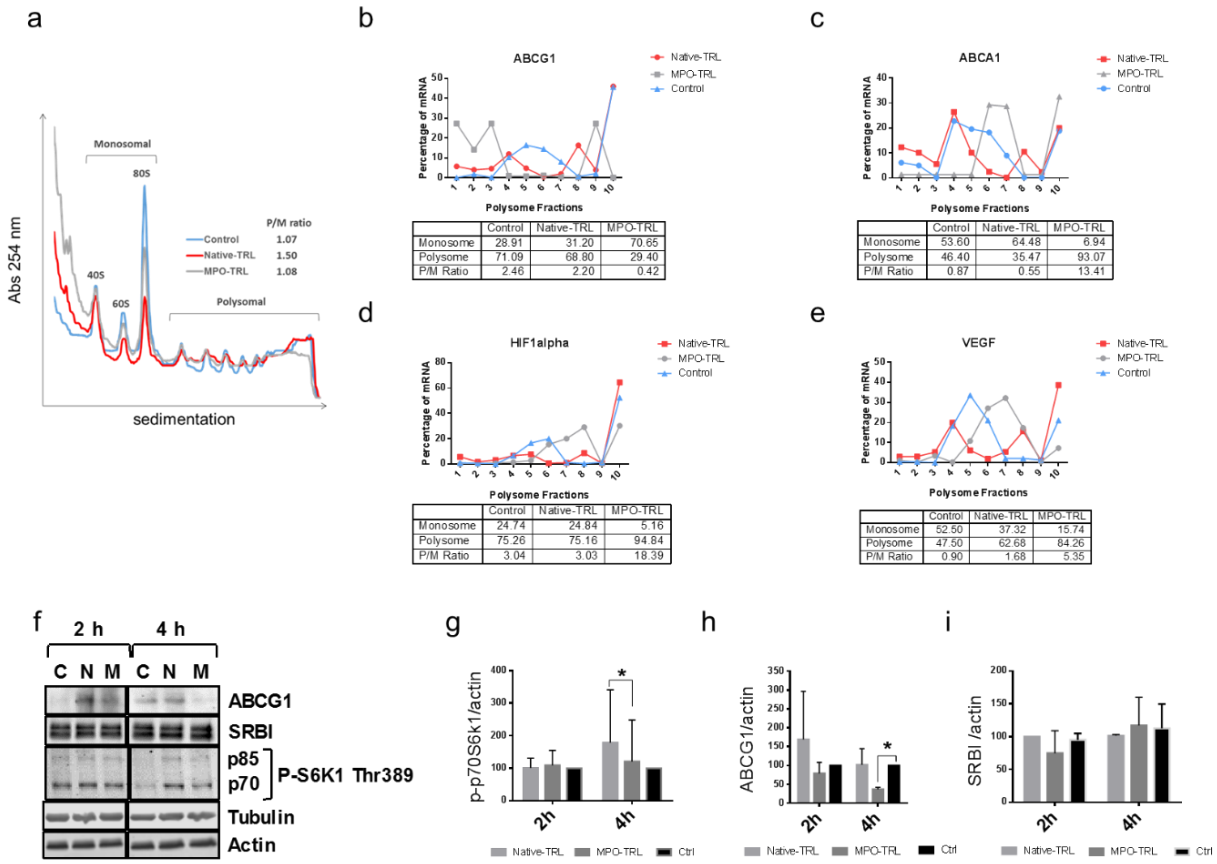


Figure 3-6 The effects of MPO-TRL on EC mTORC1 activity and mRNA translation under its incubation for up to 4 h. HUVEC were lysed and subjected to (a) General translational efficiency polysome analysis and relative distributions of individual mRNAs of (b) ABCG1 (c) ABCA1, (d) HIF1α and (e) VEGF in each of the polysome fractions assessed by RT-qPCR; and (f) immunoblotting analysis of (g) p-SK61, (h) ABCG1 or (i) SRB1. Ctrl: vehicle-treated cell culture in M199 medium supplemented with 0.2% BSA. Data were shown as means ± SEM from n = 3 experiments; * $p < 0.05$ by Student's t-test.

3.5 Discussion

In this chapter, we have demonstrated that oxidative modification of TRL by MPO results in an increase in lipid peroxidation and when incubated with EC has a number of potential effects, including (i) impaired vascular repair and (ii) increased expression of pro-inflammatory factors, compared with TRL that have not undergone oxidative modification. Additional investigations revealed a potential restorative effect of HDL, a preliminary observation that requires further investigation. These findings parallel obaservations that chemical modification of LDL enhances its atherogenic properties and provides an additional mechanistic link between MPO and CVD risk.

Local EC cellular proliferation and migration are essential factors in protecting a healthy endothelium, which is critical in regulation of vascular function. Lipid species such as cholesterol and triglyceride are critical for maintaining EC vasodilation and signalling (174, 175). We observed that incubation of EC with TRL that have not undergone MPO catalysed oxidation have no adverse effects. It is well established that oxidative modification of LDL results in an increase in atherogenic effects (58, 61). In our studies, MPO modification not only resulted in generation of oxidative species on TRL, but also led to a range of effects implicated in the formation and progression of vascular disease.

These findings highlight not only potential mechanisms associating TRL and atherosclerosis, but also provide additional factors that underscore the association between MPO and CVD. Population studies have demonstrated a direct association between circulating MPO levels and CVD risk (62, 64), while animal models of atherosclerotic plaque have reported the presence of MPO and its oxidative products within lesions (176, 177). Mechanistic studies hae demonstrated that MPO has a number of properties that promote vascular disease, including its role as a catalytic sink for nitric oxide and promotion of inflammatory and thrombotic pathways (50, 61, 178). Increasing lipid peroxidation with MPO is likely to stimulate these effects, as evidence by increased atherogenicity of LDL in cellular studies (179, 180). Additional investigations have revealed that HDL and its major protein, ApoA-I, are also oxidatively modified by MPO, resulting in impairment in their ability to promote cholesterol efflux and lipid transport (60). Our findings extend these potential lipid effects to suggest that MPO induced modification of TRL may also result in adverse effects on the vasculature.

To further investigate proteins involved in the regulation of cell proliferation and migration, we applied AHA-SILAC which enables identification and quantification of newly synthesised proteins. In the setting of incubation with MPO-TRL, we observed a cellular metabolic switch similar to that seen in response to hypoxia (181), with a decrease in proteins involved in cell

proliferation but an increase in angiogenesis factors. We also observed downregulation of expression of ABCG1, a lipid transporting factor well recognised for its interaction with HDL and role in cholesterol efflux. This finding may be relevant from a number of perspectives. Firstly, downregulation of ABCG1 can deactivate mTORC1, a protein kinase serving as a central regulator of cell cycle and protein synthesis (173, 182). It is therefore of interest that we observed HDL to rescue EC proliferation in the setting of MPO-TRL incubation. Furthermore, HDL mediated removal of oxysterol from macrophages via a number of transporters, including ABCG1, may play an important role in minimising cellular apoptosis within the artery wall (150). While our HDL investigations in this study were minimal, they warrant further exporation in the future.

In summary, oxidative modification of TRL by MPO generates lipoproteins which confer a range of adverse cellular effects at the level of the endothelium. This provides supportive evidence for the potential mechanistic role of TRL in the early stages of atherosclerosis and its subsequent progression. These findings suggest that interventions looking to reduce circulating TRL levels may have the potential to protect against the early stages of atherosclerosis.

Chapter 4. Characterising Apolipoprotein C-III in cell and animal models of dyslipidemia, atherosclerosis and hyperglycaemia

4.1 Abstract

Introduction—ApoC-III is an important regulator of TRL metabolism. While genetic studies implicate ApoC-III in the pathogenesis of atherosclerosis of atherosclerosis, it is uncertain whether ApoC-III exerts additional effects beyong raising TRL levels.

Methods and Results—The plaque distribution of ApoC-III was investigated in mouse models of atherosclerosis in the presence and absence of diabetes. Compared with a chow diet, 16 weeks of a high cholesterol diet (HCD) resulted in greater serum levels of triglycerides and cholesterol in ApoE$^{-/-}$ mice, which was exacerbated in the setting of streptozotocin induced diabetes and associated with greater serum ApoC-III levels. These lipid changes associated with more extensive plaque burde and immunofluorescent staining of plaque ApoC-III in the atherogenic and diabetic mouse models. Within plaque, ApoC-III accumulation correlated direct with the extent of both ApoB and macrophages. Diabetic ApoE$^{-/-}$ also demonstrated grater hepatic accumulation of ApoC-III, which inversely correlated with liver expression of the lipid transporting factors ABCA1 and SRB1.

Conclusion—Dyslipidaemia and diabtetes associate with more extensive atherosclerotic plaque and vessel wall staining of ApoC-III. The functional consequences of ApoC-III within the artery wall requires further investigation.

4.2 Background

In the last chapter, we investigated the impact of incubation of EC with TRL. We observed that non-modified TRL had no adverse effect on a range of cellular assays. In contrast, when TRL underwent oxidative modification by MPO, co-incubation with HUVEC had an adverse effect on a validated wound closure model and resulted in an increase in cellular expression of pro-inflammatory chemokines and adhesion molecules. These findings suggest that TRL have the potential to exert direct atherogenic effects, following oxidative modification, analogous to previous reports with LDL.

In addition to understanding to the potential atherogenic properties of TRL, there is considerable interest in the influence of factors that regulate their metabolism. ApoC-III is a small 8.8 kDa protein, usually found in glycosylated forms (80), which resides on the surface of chylomicrons, VLDL and their remnants (79). Metabolic studies have revealed that ApoC-III delays TRL clearance by inhibiting lipoprotein lipase activity (92) and ApoE mediated VLDL uptake via LDL receptors (93, 99). Overexpression of ApoC-III in mice (183), rabbits (105), and pigs (106) results in severe hypertriglyceridemia and chylomicronemia.

In humans, circulating ApoC-III levels independently associate with CVD risk (74). Genome wide association studies of large cohorts revealed that loss-of-

function APOC3 variants including variants including R19X (nonsense) (121), A43T (missense) (127), K58E (missense) (184), IVS2+1G>A and IVS23+1G>T (splice sites) associate with lower triglyceride levels and CVD risk (26, 27). These findings suggest that ApoC-III plays an important role in the pathogenesis of ASCVD. Whether this is exclusively due to the elevation in triglyceride levels or if ApoC-III exerts direct atherogenic effects remains uncertain.

ApoC-III has been demonstrated to possess a number of additional properties in cellular studies. Co-incubation of HUVEC with ApoC-III results in an increase in cellular expression of the pro-inflammatory adhesion molecules, *VCAM-1* and *ICAM-1*, an effect that is inhibited by co-administration of statins (134). In studies of mouse derived adipocytes, incubation with ApoC-III induces expression of *MCP-1* and interleukin 6 (*IL-6*) via TLR2, in a process involving activation of the extracellular signal-regulated kinase p38 and NF-κB (185). To what degrees these pro-inflammatory effects of ApoC-III influence atherosclerosis in the in vivo setting remain to be characterised.

Additional cellular studies have suggested that ApoC-III has other properties that may also contribute to CVD risk. ApoC-III has been demonstrated to promotes β-cell apoptosis, suggesting a potential role in glucose homeostasis and generation of diabetes (90). In addition to residing on the surface of

atherogenic ApoC-III has also been demonstrated on the surface of HDL particles and identifies a subspecies of HDL, which may associate with a higer CVD risk (186). To what degree this reflects the presence of elevated TRL, further promotion of a pro-inflammatory state via HDL or generation of dysfunctional HDL particles is uncertain.

The aim of the studies describe in this chapter were to investigate the arterial wall expression and distribution of ApoC-III in dyslipidaemic and diabetes mouse models of atherosclerosis. The specific aims included:

1. To determine whether ApoC-III is present within atherosclerotic plaque
2. To determine whether plaque ApoC-III correlates with both lesion burden and inflammatory phenotype.
3. To determine the hepatic accumulation of ApoC-III in these models.

The hypothesis of these studies was that ApoC-III would be identified within more extensive and inflammatory atherosclerotic plaque, suggesting the potential to exert direct adverse effects within the artery wall.

4.3 Methods

4.3.1 Animals

Animal studies were approved by the Animal Ethics Committees of South Australia Health and Medical Institute (SAHMRI) (approval No. SAM186) and the University of Adelaide (approval No. M-2015-254).

The experimental designed is outline in Figure 4-1. All mice (n = 48) were fed chow diet (CD) as baseline control. At week 8, mice were divided into four experimental groups:

1. Wild type C57Bl6 mice (n = 12) continued CD until the end of the 26 week experimental period.

2. ApoE$^{-/-}$ mice (n = 12) continued CD until the end of the 26 week experimental period.

3. ApoE$^{-/-}$ mice (n = 12) were fed a high cholesterol diet (0.5% cholesterol, 21% total fat from week 10 to 26.

4. ApoE$^{-/-}$ mice (n = 12) underwent generation of diabetes at week 8 by treatment with 50 mg/kg/day streptozozotocin (STZ) injection for 5 consecutive days and subsequently fed a high cholesterol diet from week 10 to 26.

All mice were fed their assigned diet *ad libitum*. Mice were bled, weighted and underwent an intraperitoneal glucose tolerance test at week 10 (baseline), week 18 (mid) and week 26 (final). Tissues were collected at the end of the study.

The sample size for the study (n = 10 animals per group) was based on a 5% increase of atherosclerotic lesion area for $ApoE^{-/-}$ mice fed a HCD compared with $ApoE^{-/-}$ mice fed a CD (187), providing statistical power of 80% with $p < 0.05$. An additional two mice pergroup were included to account for any attrition during the entire study.

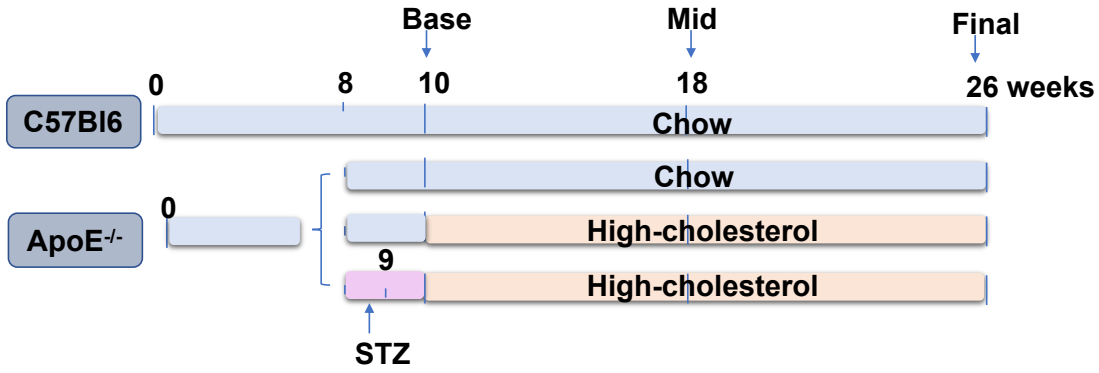


Figure 4-1 Schematic diagram of the disease mouse models. All mice were fed CD until week 8. At week 8, one arm of the ApoE$^{-/-}$ mice were treated with streptozotocin, inducing hyperglycaemia. All mice continued on CD until week 10. At week 10, two groups of ApoE$^{-/-}$ mice were transitioned onto a high cholesterol diet (including the STZ treated mice). Blood samples to test for hyperglycaemic and hyperlipiaemia were collected at week 10, 18 and 26. Mice were bled and weighed at week 10, 18 and 26. Mice were culled at the end of the study and a range of tissues were collected.

4.3.2 Streptozotocin injection to induce hyperglycaemia mice

At 8 weeks of age, mice underwent induction of diabetes by administration of 50 mg/kg body weight STZ (Sigma, S0130) for 5 consecutive days via intraperitoneal injection. STZ was prepared in 0.1 M sodium citrate buffer (pH 4.5, pH adjusted with citric acid monohydrate) prior to injection. Fasting blood glucose was assessed after 1 week of STZ injection before mice were placed on a high cholesterol diet (HCD: 0.15% cholesterol, 21% total fat).

4.3.3 Intraperitoneal glucose tolerance test (IPGTT)

Glucose tolerance was assessed by intraperitoneal glucose tolerance test (188). Briefly, mice were fasted overnight, and baseline (T0) glucose levels were determined before any administration of glucose. After intraperitoneal (IP) injection of glucose solution [20% weight/volume (w/v) glucose in 0.9% sodium chloride (NaCl) and injected 10 µl/body weight (g)], the blood glucose levels were measured at 0.5, 1 and 2 h (T0.5, T1 and T2).

4.3.4 Tissue processing and histological analysis

Animals were humanely sacrificed by carbon dioxide (CO_2) asphyxiation. Immediately after, blood samples were collected by cardiac puncture, using a syringe containing 10 ml PBS which was placed into the left ventricle for

perfusion. After perfusion, perivascular fat was cleaned, and heart, brachiocephalic artery, carotid artery and aorta were collected and fixed in neutral buffered formalin (10%, Sigma, HT501128-4L) overnight. Tissues then underwent sucrose drop for two days and imbedded in optimal cutting temperature compound (OCT, Tissue Tek, IA018), frozen over iso-pentane and stored at -80 ºC until sectioning. Another group of mice were dissected, and their organs were snap frozen in liquid nitrogen and stored at -80 ºC for Western blot analysis.

4.3.5 Immunofluorescent staining of atherosclerotic lesions

Immunofluorescent staining was performed on cryostat sections, using an indirect immunofluorescence technique. Mouse brachiocephalic artery, subclavian, common carotid artery and aortic arch were sliced. At least four sections of each animal were randomly picked for staining: three were experimental slices and one were stained with secondary antibody only as a background control. Briefly, sections on poly-l-lysine coated slides (Thermoscientific SuperFrost Plus adhesion slides, J1800AMNZ) were fixed with ice cold formalin [neutral buffered (10%), Sigma, HT501128-4L], followed by permeablisation/blockage in IHC blocking buffer (3% BSA, 0.3% of triton X-100 and 3% donkey serum in PBS) for 30 min. Sections were then incubated

with corresponding primary antibodies overnight at 4 ºC, followed by incubation with the appropriated secondary antibodies. Sections were excluded found with dirty lipid staining, high background fluorescence in control slides or uncertainty of protein presence, resulting in 1-5 optimal slides from each animal artery analysed and quantified. Images were acquired using fluorescent microscopy (Olympus, USA). Images were separated into single fluorescence channels and positive signal of Cy3/Cy5 were analysed by ImageJ (NIH, USA).

4.3.6 Cloning of mature ApoC-III into pET23b vector

The human mature APOC3 cDNA was obtained from the pANT7-cGST template (DNASU plasmid repository, HsCD00077989). The mature APOC3 cDNA was then digested with NdeI (NEB, R0111S) and XhoI (NEB, R0146S) and sub-cloned into the template vector pET23 (Millipore, 69771-3). The ligation mixtures were then transformed into competent cells and the transformed cells were selected with ampicillin (50 μg/ml) on Luria-Bertani (LB)-agar plates. The entire APOC3 cDNA was sequenced from both directions, by Flinders Sequencing Facility (Flinders University, Adelaide).

4.3.7 ApoC-III recombinant protein expression and purification

pET23b-wildtype (WT)-APOC3 plasmid was transformed into competent Escherichia coli (E.Coli) B834, which were sequentially used to inoculate a culture in super optimal broth (SOC) medium containing ampicillin at 100 μg/ml and shaken at 200 rpm/min at 37 °C. After 5 h incubation, recombinant ApoC-III expression was induced with 1 mM isopropyl-β-d-thiogalactopyranosdie (IPTG; Sigma) for 2 h. Cells were lysed in lysis buffer (8 M Urea, 10 mM Tris-HCl, 500 mM NaCl in 50 mM phosphate buffer), lysates were centrifuged at 16,000X g (10 min at 4 °C). The supernatant was purified through Talon cobalt metal affinity resin (TALON® Superflow™, GE Healthcare, 28-9575-02, pack of 50 mL) for 3 h at 4 °C.

4.3.8 Endotoxin remover by FLPC

After purification by Talon Cobalt, ApoC-III fusion proteins were loaded on an anion-exchange column, MonoQ (GE healthcare) and were eluted with a linear NaCl gradient (from 0.1 to 0.25 M) in 4 M urea, 5 mM NH4HCO3 (pH 8.0) buffer. The fractions containing the pure protein (eluted at a NaCl concentration of approximately 0.15 M) were pooled and the final purity of the product was verified on a 14.5% SDS-PAGE gel by Western blot. Endotoxin levels of the final product was measured using Limulus Amebocyte Lysate (LAL) endotoxin

quantification kit (Pierce LAL chromogenic endotoxin quantitation kit, Thermo scientific, 88282).

4.3.9 LPL assay

Methods were adapted from published protocols (189) to assess the influence of ApoC-III in LPL activity. LPL (Sigma, 62335) was mixed with ApoC-III (in 5 M Urea, 50 mM tris-HCl buffer) and lipids (10% intralipid, 1 μCi H^3-triolein, 0.1 M NaCl, 60 mg/ml BSA, 0.15 M Tris-HCl, pH 8.5, and 16.7 U/ml heparin). The mixture was pre-incubated with ApoC-III for at least 20 min before addition of LPL (0.25 μg/ml) in a final 200 μl mixture. After 30 min incubation under agitation at RT, the reaction was stopped by adding 2 ml of mixture of isopropanol, heptane and 1 M H_2SO_4 (40 : 48 : 3), plus 0.5 ml of water. FFA were separated using sequential centrifugations in glass tubes for 10 min at 1500 g to separate the mixture into two phases. After the first centrifugation, 800 μl of the upper phase was transferred and mixed with 1 ml alkaline and 3 ml heptane before centrifugation. The supernatant (heptane phase) was removed and 800 μl of the lower phase was transferred to a vial containing 4 ml of scintillation liquid to count radioactivity using scintillation counter (Perkin Elmer, USA). The LPL activity was then expressed as percentage of the H^3 - FFA (dpm) released in the absence of ApoC-III.

4.3.10 Adhesive molecule mRNA assessment (RT-qPCR)

After overnight serum starvation in 2% FBS M199 medium, HUVEC were treated with recombinant protein ApoC-III-WT, ApoC-III-58E or vehicle control for 4 h. Cells were then harvested and RNA was extracted in TRI reagent. Subsequent mRNA assessment was done as described in general methods (Chapter 2) subtitled real-time quantitative PCR.

4.3.11 Statistics

In vivo animal data were represented as mean ± STD and in vitro cell data was performed in three biological replicates and shown as mean ± SEM. Statistical comparisons between two groups were performed using Student's t-test (Mann-Whitney's test) or one-way ANOVA (Kruskal-Wallis test) where appropriate. A linear regression model was used for correlation analysis. *$p < 0.05$ was considered as statistically significant for all analyses.

4.4 Results

4.4.1 Biochemical parameters and conventional lipid parameters of the mice models

All ApoE$^{-/-}$ mice developed dyslipidemia compared with the C57Bl6 baseline control (Table 4-1). As expected, mice fed the high cholesterol diet had higher serum triglycerides by the middle of the study (18 weeks) and all ApoE$^{-/-}$ mice, independent of treatment and diet, had raised total triglycerides by the end of the study. By the final time point, the ApoE$^{-/-}$ mice on the high cholesterol diet had elevated total cholesterol, which interestingly, and similarly to triglycerides, was elevated prior to the diet intervention in the hyperglycemic ApoE$^{-/-}$ mice.

Serum ApoC-III levels were elevated in mice with hyperglycemia (996 ± 62 mg/dL) compared with ApoE$^{-/-}$ mice fed either a CD (385 ± 122 mg/dL) or high cholesterol diet (400 ± 132 mg/dL) achieving statistical significance ($p < 0.05$) for both comparisons (Figure 4-2).

As expected, the ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration exhibited impaired glucose tolerance at all stages of the experiments, compared to all other models (Figure 4-3).

Table 4-1 Characteristics of mouse serum biochemistry

	C57BL6 (n=12)	ApoE$^{-/-}$+CD (n=12)	ApoE$^{-/-}$+HCD (n=12)	ApoE$^{-/-}$ +HCD+STZ (n=12)	P Value
Weight (g)[B]	35 (32- 38)	33 (30- 37)*	42 (32- 45)	29 (25- 34)**	0.01
Total triglycerides (mg/dL)					
Base, week10	43± 22	94±11	94±11	162±29**	0.0003
Mid, week 18	78±11	150±20	383±111*	384±143*	0.002
Final, week 26	43±18	233±115**	336±171****	368±137****	<0.0001
Total cholesterol (mg/dL)					
Base, week10	327±82	360±122	360±122	576±27*	0.03
Mid, week 18	163±8.6	400±174	622±149	786±169**	0.0008
Final, week 26	93± 45	302±106	606±209***	809±241****	<0.0001
NonApoB-cholesterol (mg/dL)					
Base, week10	33±9.8	23±11	23±11	16±12	NS
Mid, week 18	92±1.1	103±19	34±34	3.2±3.9*	0.003
Final, week 26	85±27	34±4	33±1*	54±21	0.01
Glucose (mg/dL), final, week 26[C]	7.5±1.3	5.8±1.5	6.5±2.7	14±8.5**	0.008

Blood lipid results are presented as mean ± STD (n = 8-10 pooled plasma, n=3/pool) or individual mouse plasma compared to wild-type control, *p < 0.05, **p < 0.005 by ANOVA (Kruskal-Wallis test). [B] Results were median, IQR (n = 8-10) compared to ApoE$^{-/-}$ mice fed a high cholesterol diet by Student's t-test. [C] Weight data are presented as mean ± STD (n = 8-10) compared to *ApoE*$^{-/-}$ mice fed a CD by by Student's t-test.

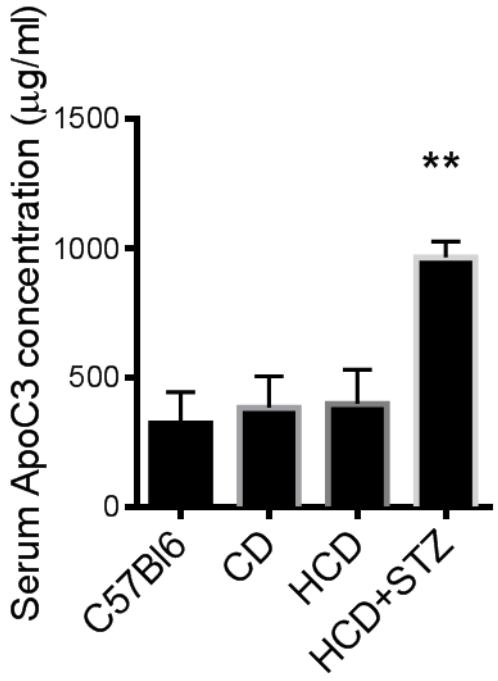


Figure 4-2 Serum ApoC-III levels wre measured at 26 weeks of age in control (C57Bl6), ApoE$^{-/-}$ mice fed a CD, ApoE$^{-/-}$ mice fed a high cholesterol diet (HCD) and ApoE$^{-/-}$ mice fed a high cholesterol diet after administration of STZ (HCD+STZ). ApoE$^{-/-}$ mice with diabetes fed a HCD demonstrated increased serum ApoC-III levels assessed by enzymatic analysis, at week 26. Results are presented as mean ± STD (n = 8-10), **p < 0.005 for comparison between C57Bl6 mice and ApoE$^{-/-}$ mice fed a high cholesterol diet after administration of STZ.

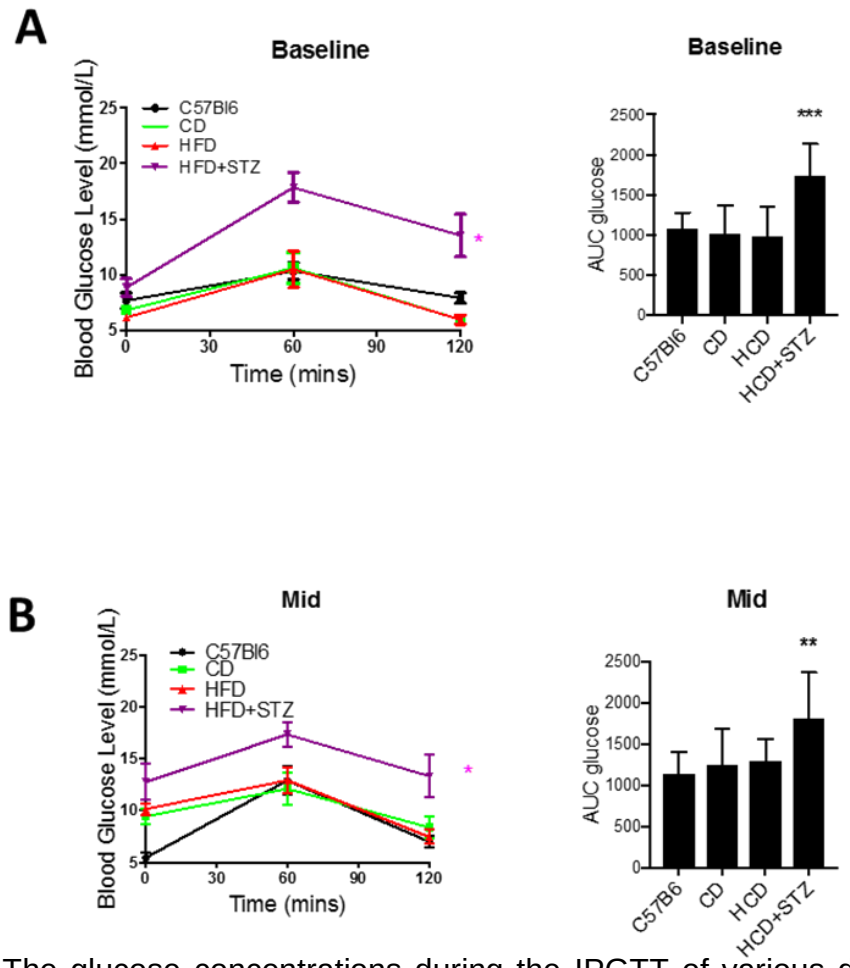


Figure 4-3 The glucose concentrations during the IPGTT of various disease mice models (A) week 10 (baseline) and, (B) week 18 (mid). Results are presented as mean ± STD (n = 8-10) *p < 0.05, **p < 0.005 by ANOVA (Kruskal-Wallis test).

4.4.2 ApoC-III is present in atherosclerotic plaque and is exacerbated in the setting of hyperglycemia

To explore whether ApoC-III is potentially implicated in atherogenesis within the artery wall, and whether it is exacerbated by hyperglycaemia, the brachiocephalic artery, subclavian, common carotid artery and aortic arch were dissected and assessed for the presence, size and composition of lesions, lipid accumulation and ApoC-III.

As expected, ApoE$^{-/-}$ mice fed a high cholesterol diet demonstrated lipid accumulation in aorta, as evidenced by *en face* ORO staining (Figure 4-4), which was extensive in the diabetic mice (23% compared to 9% in ApoE$^{-/-}$mice fed a high cholesterol diet only). Hyperglycaemic mice also demonstrated a 1.6 fold narrowed vessel lumen (ratio of intima to media), when compared to ApoE$^{-/-}$ mice only fed a high cholesterol diet.

To further characterise plaque composition, the aortic root was examined as cross section. ORO staining revealed that ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration developed lesions containing more lipid (24.3% v.s. 14.0%) in ApoE$^{-/-}$ mice fed a CD). Cluster of Differentiation 68 (CD68) staining revealed a more intense inflammatory composition, with 5-fold greater macrophage infiltration in the setting of high cholesterol diet and diabetes compared with CD. In association with greater lipid and macrophage content,

the mice fed a high cholesterol diet on a background of diabetes demonstrated 30-40% lower content of fibrous tissue on Masson's Trichrome staining. One STZ treated mouse also demonstrated intra plaque neovascularization, as evidenced by CD31 staining, while no mice in the other groups developed such intraplaque vascular changes. No difference n smooth muscle cell (SMC-α) content was observed between the groups (Figure 4-5).

Using Western blot analysis to characterise the presence of ApoC-III, we identified a greater presence of ApoC-III in the aorta of diabetic *Apoe*$^{-/-}$ mice fed a high cholesterol diet, compared with ApoE$^{-/-}$ mice fed either a high cholesterol or CD (Figure 4-6). Immunohistochemistry staining of cross sections of bracheochaplic artery demonstrated the presence of ApoC-III depositied within the plaques of the STZ-treated mice (Figure 4-7 A, B). There was no significant difference in ApoB positive area within plaques of any of the mouse models (Figure 4-7 A, C). To examine the potential relationship between presence of intraplaque ApoC-III and atherosclerosis, linear regression analysis was performed. The degree of ApoC-III deposition was positively associated with lesion size ($r = 0.59$, $p = 0.005$) and CD68 macrophage staining ($r = 0.63$, $p = 0.005$) in the dyslipidaemic ApoE$^{-/-}$ mice cohort. However, ApoB did not show the same correlation, although a non-significant trend of association between ApoB staining and plaque size ($r = 0.34$, $p = 0.13$) was observed (Figure 4-8 E).

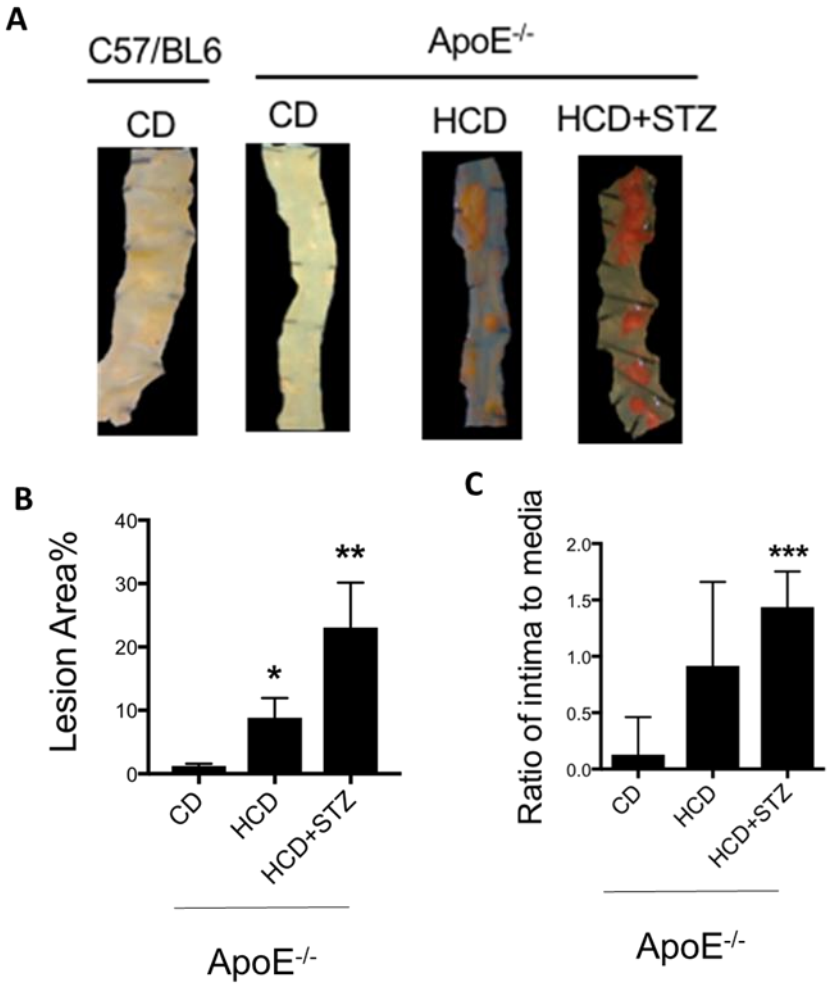


Figure 4-4 ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration demonstrated a greater lesion area in the aorta, assessed by (A) oil red o staining (B) Quantification of *en face* lesion area, n = 4/group, (C) cross sectional plaque burden analysis calculated by the ratio of intima to media area. *p < 0.05, **p < 0.005 by Mann-Whitney test.

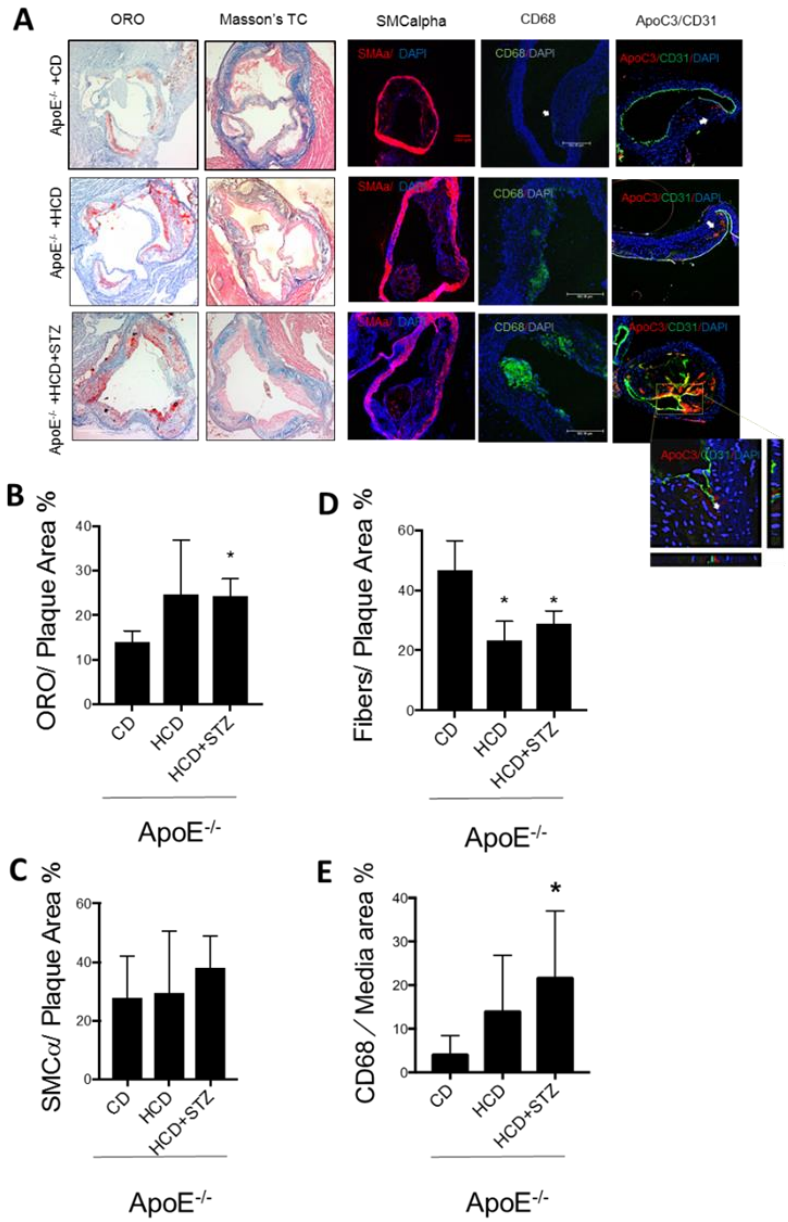


Figure 4-5 ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration developed evidence of a vulnerable plaque phenotype. (A) Histology and immunofluorescence of plaque cellular content and plaque morphology analysis of (B) lipid infiltration by ORO (C) smooth muscle content (SMC-actin) within the plaque, (D) fibrous content by Masson's thrichrome and CD68 content normalised to vessel media area. Plaque angiogensis assessed by

CD31 staining. Results are presented as mean ± STD (n = 4-10/group) *p < 0.05, **p < 0.005 by Mann-Whitney test.

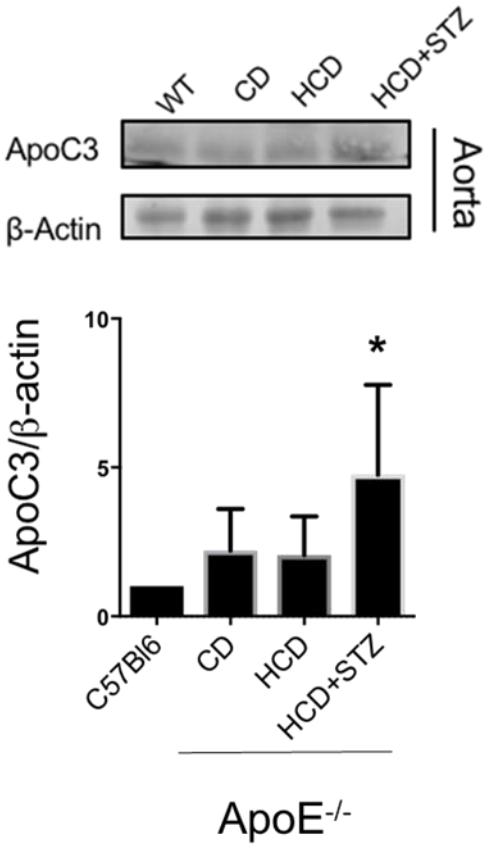


Figure 4-6 ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration have increased ApoC-III content in the whole aorta assessed by Western blot, n = 4/group, *p < 0.05, **p < 0.005 by Mann-Whitney test.

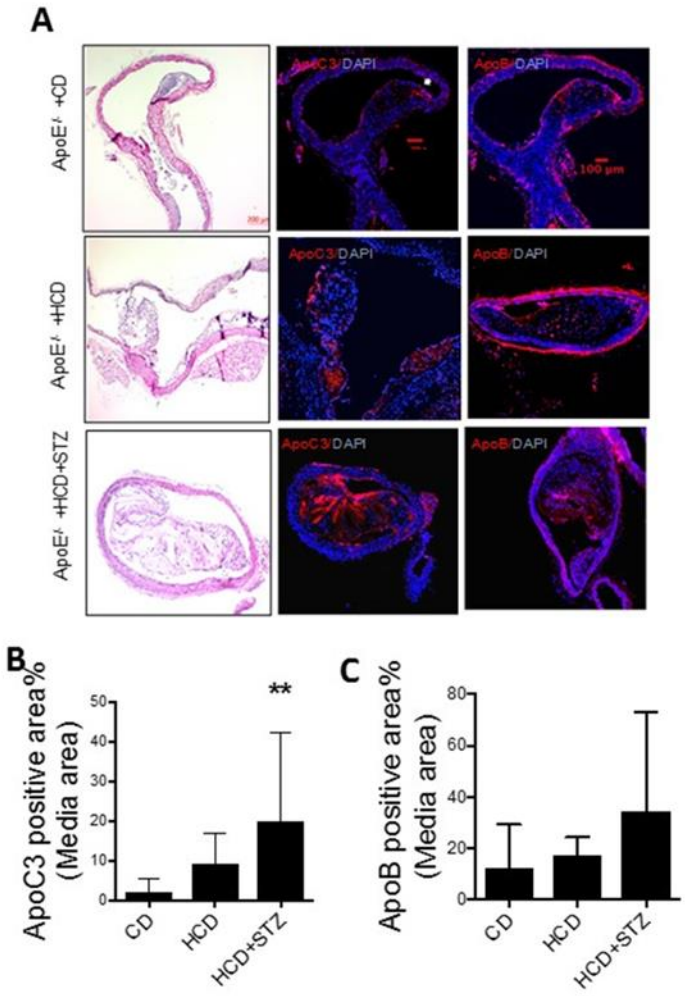


Figure 4-7 ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration have increased ApoC-III plaque infiltration, but not ApoB assessed by (A) immunofluorescent ApoC-III staining in ApoE$^{-/-}$ mice fed CD (CD, n = 6), ApoE$^{-/-}$ mice fed high cholesterol diet (HCD, n = 7), ApoE$^{-/-}$ mice fed a high cholesterol diet after STZ administration (HCD + STZ, n = 8). Analysis of proportion of plaque staining positive for (B) ApoC-III and (C) ApoB. Results are presented as mean ± STD (n = 4-10/group), *p < 0.05, **p < 0.005 by Mann-Whitney test.

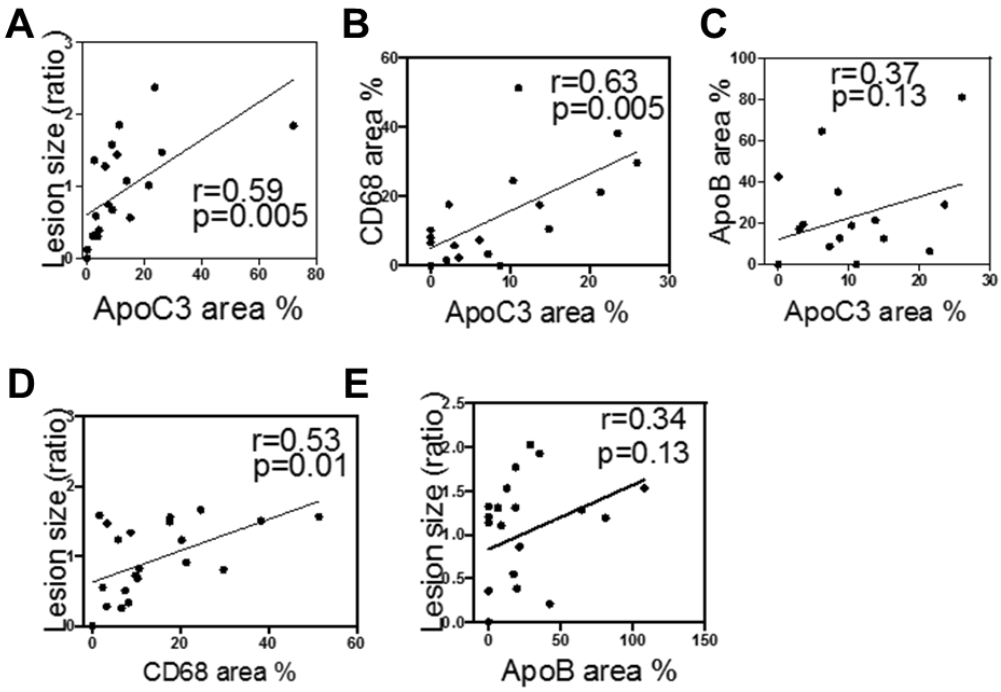


Figure 4-8 Incrased plaque ApoC-III staining was positively associated with more extensive and inflammatory atherosclerotic plaque. Linear regression analysis of plaque (A) ApoC-III v.s. lesion size, (B) ApoC-III v.s. CD68, (C) ApoC-III v.s. ApoB, (D) CD68 v.s. lesion size and (E) ApoB v.s. lesion size.

4.4.3 Liver and adipose analysis

To investigate the presence of ApoC-III in organs involved in lipid metabolism, liver and adipose tissue were homogenised and analysed by Western blot for the abundance of ApoC-III, ABCA1 and SRB1. $ApoE^{-/-}$ mice fed a high cholesterol diet after STZ administration demonstrated a 1.5 fold greater ApoC-III accumulation within liver tissue, which was inversely associated with hepatic ABCA1 expression ($r = 0.65$, $p = 0.003$). $ApoE^{-/-}$ mice fed a high cholesterol diet after STZ administration demonstrated lower hepatic content of both ABCA1 and SRB1. A non-significant trend towards an inverse association between hepatic content of ApoC-III and SRB1 ($r = 0.50$, $p = 0.11$) was observed (Figure 4-9, Figure 4-10).

In contrast, Western blot analysis of homogenised adipose tissue found very low or non-quantifiable amounts of ApoC-III within adipose tissue in all groups (data not shown). Furthermore, there was no difference between groups with regard to visceral adipose content of ABCA1 and SRB1. All $ApoE^{-/-}$ mice fed a high cholesterol diet did demonstrate greater SRB1 levels within subcutaneous adipose tissue (Figure 4-11).

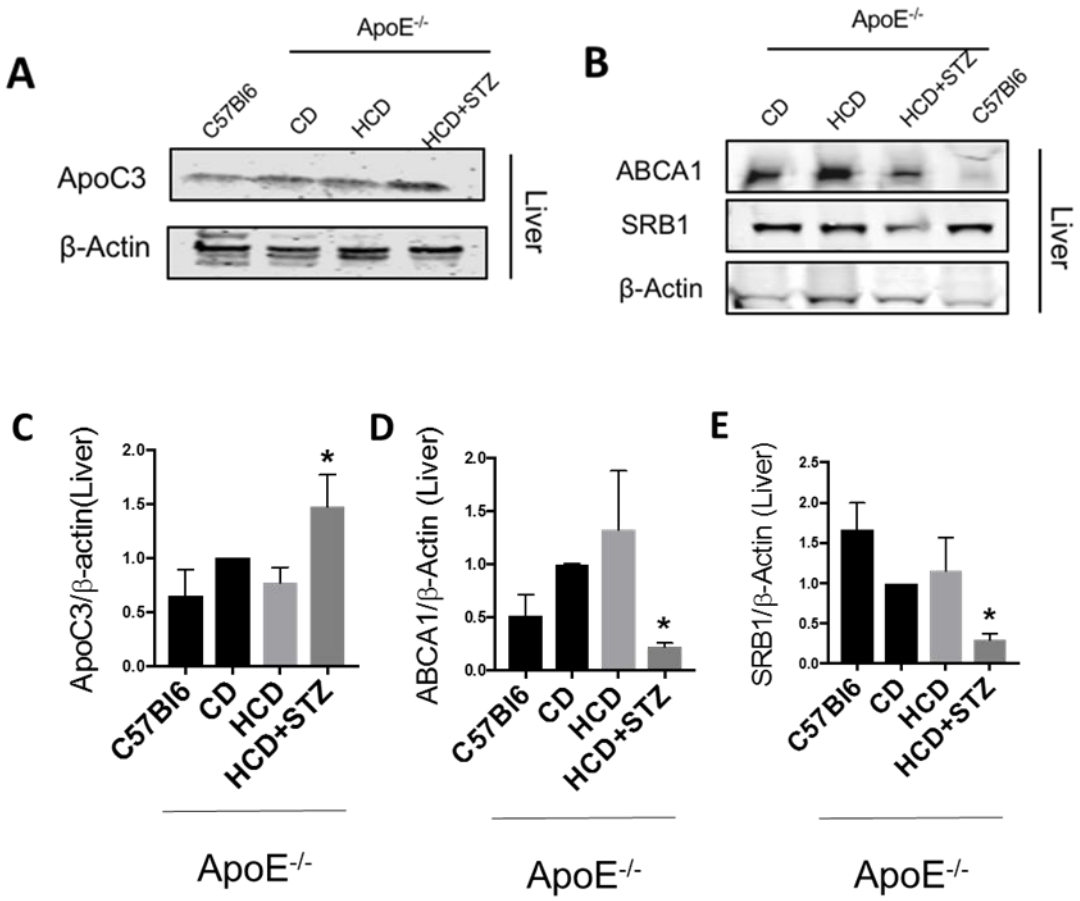


Figure 4-9 Hepatic ApoC-III, ABCA1 and SRB1 levels. Western blot analysis of hepatic levels of ApoC-III (A, C), ABCA1 (B, D) and SRB1 (B, E). Results are presented as mean ± STD (n = 4/group) *p < 0.05, **p < 0.005 by Mann-Whitney test.

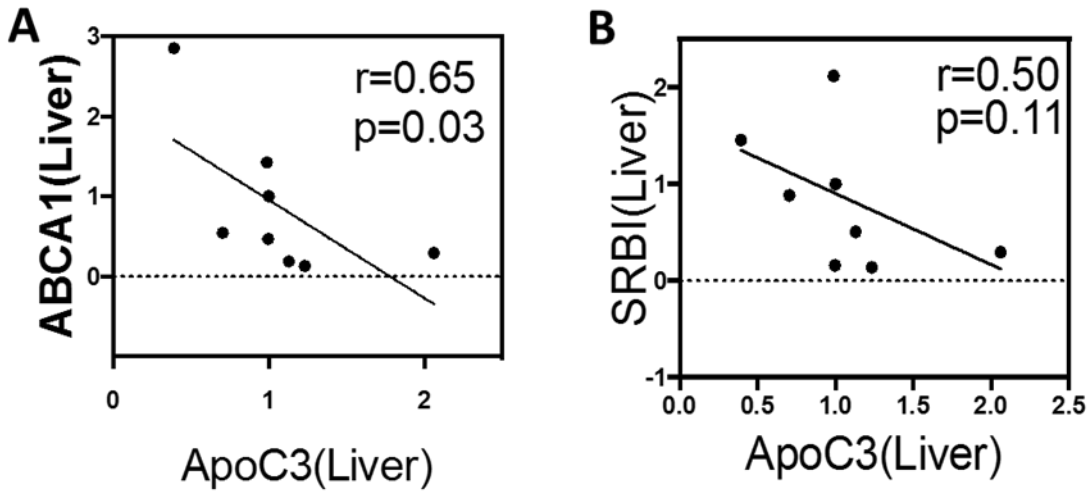


Figure 4-10 Abundance of hepatic ApoC-III was negatively associated with levels of ABCA1 but not SRB1 in liver. Linear regression analysis of (A) ApoC-III v.s. ABCA1, (B) ApoC-III v.s. SRB1 in the liver. Regression analysis were performed using pearson correlation coefficient.

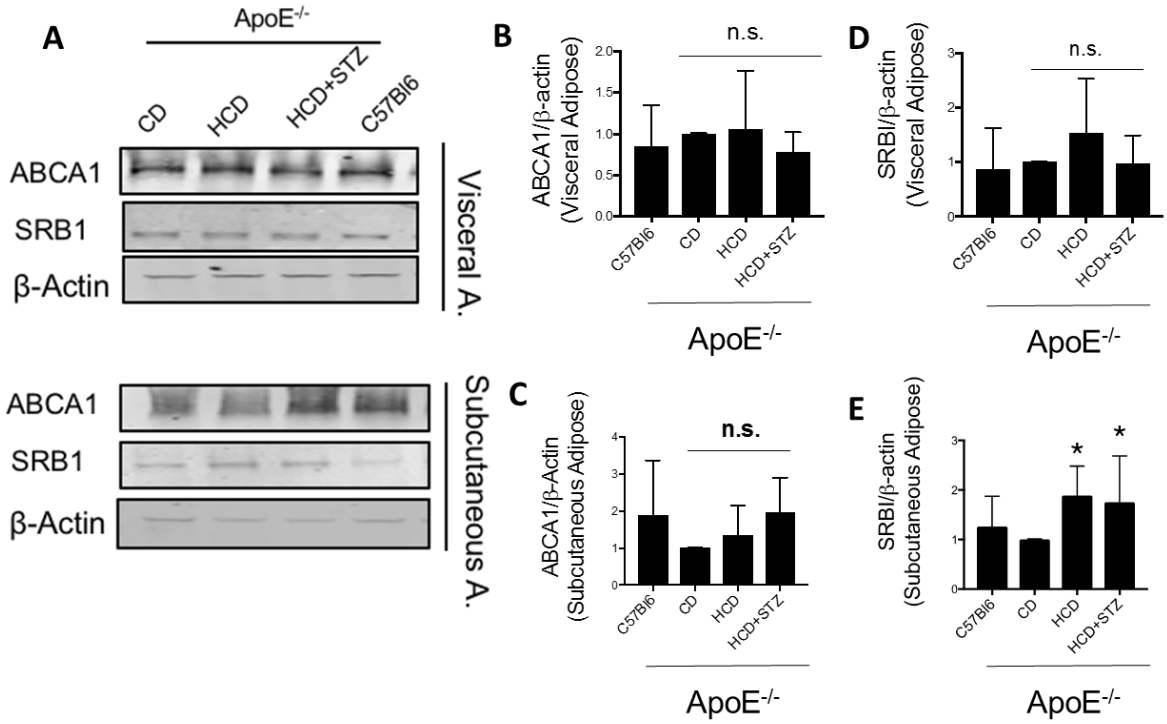


Figure 4-11 Abundance of ABCA1 (A, B, C) and SRB1(A, D, E) in visceral and subcutaneous adipose tissue. Results are presented as mean ± STD (n = 4/group) *p < 0.05, **p < 0.005 by Mann-Whitney test.

4.4.4 Recombinant APOC3-6x histidine (His) protein expression

Based on finding of ApoC-III within plaque, we sought to generate recombinant ApoC-III and assess its potential inflammatory activity. Given its presence within plaque, the ability to demonstrate that ApoC-III possesses pro-inflammatory activity might provide further evidence linking it to atherosclerotic CVD. Wildtype ApoC-III 6xHis recombinant protein was successfully expressed in B385 strain (Figure 4-12). The raw bacteria lysates were followed by nickel (Ni)-resin purification and proteins were resolved on 14.5% SDS-PAGE, deteted by Coomassie Blue (Figure 4-12 A). The expressed proteins were further confirmed by Western blot, as demonstrated by two clear and sharp bands approximately 10 kDa (Figure 4-12 B).

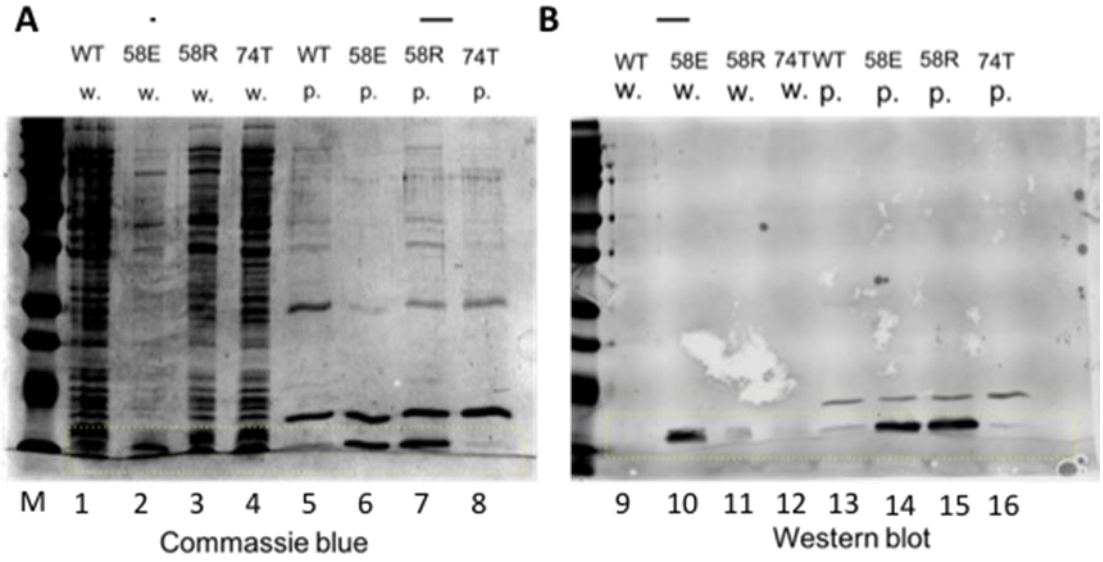


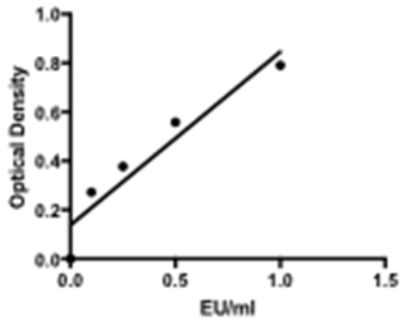
Figure 4-12 Recombinant ApoC-III-WT and its non-functional mutants were expressed in SOC (with 20 mM glucose) medium and inducted by 1 mM IPTG at 37 ºC for 2 h. Whole *E.coli* lysates were run in 14% SDS-PAGE and assessed by (A) Coomassie blue staining shown in lane 1-4 and (B) Western blot shown in lane 9-12 using anti-6 histidine antibody. *E.coli* lysate were then roughly purified through Talon cobalt metal affinity resin and assessed by (A) Commassie blue staining shown in lane 5-8 and (B) Western blot shown in lane 13-16.

4.4.5 Recombinant ApoC3-6x His inhibits lipoprotein lipase activity and induces EC inflammation in culture

After rough purification by Ni-resin, the protein needed to be further purified with a MonoQ column, in order to eliminate endotoxin contamination, which is a known stimulator of inflammation (190). Aiming to utilise the proteins for further experiments, ApoC-III-WT and endotoxins were separated based on their polarity with an ascending linear NaCl gradient (0-1000 mM) buffer. ApoC-III began to elute at around NaCl concentration 250-400 mM which corresponds to fraction 6-8 (Figure 4-13). The endotoxin concentrations were reduced to less than 1 endotoxin units per milliliter (EU/ml), which was suitable for use in subsequent cell studies (Figure 4-13 B).

The primary functional property of ApoC-III is the inhibition of lipoprotein lipase activity. It has also been reported that ApoC-III may also promote vascular expression of pro-inflammatory factors. To confirm the functionality of recombinant ApoC-III-WT, LPL activity assays were performed. As expected ApoC-III-WT inhibited LPL activity by up to 50%, at a concentration of 2 µM (**$p < 0.005$) (Figure 4-14). Purified ApoC-III-WT was also found to induce HUVEC expression of *VCAM-1* (1.5 fold, *$p < 0.05$) and *ICAM-1* (2 fold, *$p < 0.05$), but not *MCP-1* and *IL-8* compared to 2% FBS control (Figure 4-15).

A



EU/ml	Optical Density
1.00	0.790
0.50	0.558
0.25	0.377
0.10	0.272
0.00	0.000
	0.172

B

	EU/ml (Interpolated)	Optical Density (Entered)
Fr6	0.049	0.172
Fr7		0.043
Fr8	0.733	0.656

Figure 4-13 Endotoxin level assessment of FPLC purified/eluted recombinant ApoC-III-WT fractions assessed by Pierce LAL chromogenic endotoxin quantification kit. (A) standard curve of endotoxin EU/ml generated from LPS. (B) endotoxin level (EU/ml) of fraction 6-8 eluted from FPLC monoQ column, which are all < 1 EU/ml.

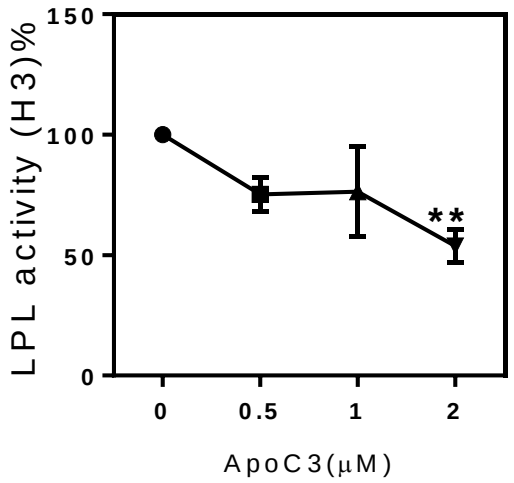


Figure 4-14 The inhibitory effect of purified ApoC-III on LPL hydrolysis of H^3-Triolein (triglycerides). LPL (3 ng/ml) was incubated for 30 min at 37 ºC in a total volume of 100 µl with ^{3}H-triolein labelled intralipid in the presence of various concentrations of ApoC-III. LPL activity is expressed as the percentage of ^{3}H-FFA released in the absence of ApoC-III. Results are mean ± SEM (n = 3) *$p < 0.05$, **$p < 0.005$ by ANOVA (Kruskal-Wallis' test).

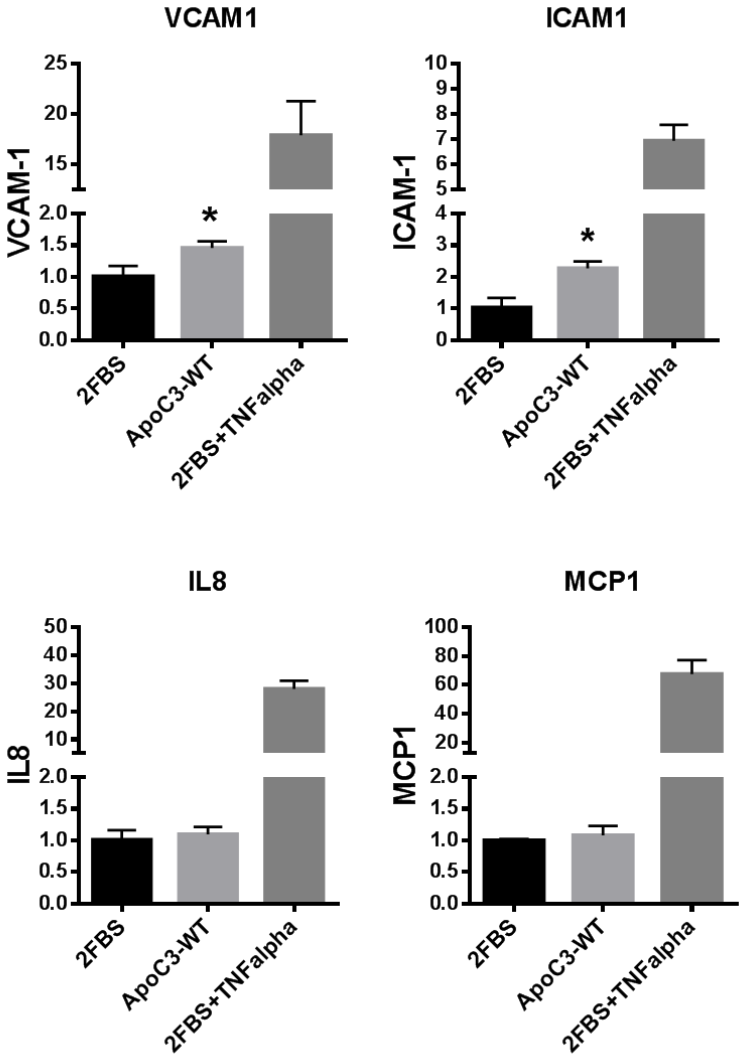


Figure 4-15 EC expression (RT-qPCR) of inflammatory markers (*VCAM-1, ICAM-1, MCP-1* and *IL-8*) by treating cells with purified ApoC-III-WT (n=3) after 4 h incubation with 2% FBS control, 10 µg/ml ApoC-III-WT and 2% FBS control + TNFα. All data are expressed as mean ± SEM, *p < 0.05 by Student's t-test.

4.5 Discussion

ApoC-III is well characterised as a regulator of TRL metabolism, via its role in the inhiting LPL activity (92, 104), delayed hepatic TRL uptake by LDL receptor family (93) and increase hepatic VLDL secretion (91). Additional studies have reported that ApoC-III may possess pro-inflammatory effects that may contribute to its potential role in promoting CVD risk. In the studies reported in this chapter, we investigated the distribution of ApoC-III in mouse models dyslipidaemia and diabetes. In the most atherogenic model, the $ApoE^{-/-}$ mouse fed a high cholesterol diet on a background of STZ-induced diabetes, we observed greater circulating levels of ApoC-III in association with elevations in both triglycerides, cholesterol and glucose levels. This associated with the presence of ApoC-III within plaque, the extent of which correlated with both the size and macrophage content of atherosclerotic lesions. These findings provide additional evidence implicating ApoC-III in atherosclerotic CVD.

Given the role of ApoC-III in the regulation of TRL metabolism and common presence of hypertriglyceridaemia in the setting of diabetes, we were particularly interested in studying the potential distribution of ApoC-III in a mouse model of diabetic dyslipidaemia. These animals demonstrated more extensive atherosclerosis and lesions with a vulnerable phenotype, as evidenced by more lipid and inflammatory components. This is consistent with

prior reports from human studies that diabetic atherosclerosis demonstrates greater lipid content, in association with adventitial inflammation and neovascularisation, hallmark features of plaque vulnerability (191). The demonstrated presence of ApoC-III within these lesions raises the question what pathogenic role does intra-plaque ApoC-III play?

It has been previously reported that ApoC-III may possess pro-inflammatory properties in addition to its role in TRL metabolism. These findings include the ability of ApoC-III to induces both endothelial expression of *VCAM-1* in an NF-κB mediated process (139) and endoplasmic reticulum stress in smooth muscle cells via the Akt pathway (192). In preliminary work, we expressed a recombinant form of wild type ApoC-III and demonstrated that it not only resulted in LPL inhibition, but also reproduced endothelial expression of pro-inflammatory adhesion molecules. While these findings are supportive of a potential role for ApoC-III within the artery wall, this will require further investigation to fully establish. Future work should also enable characterisation of the impact of ApoC-III on other inflammatory factors and to determine whether ApoC-III mutants, which do not inhibit lipoprotein lipase, similary lose their pro-inflammatory activity.

We also investigated the potential association of ApoC-III with diabetic dyslipidaemia through analysis of the liver and adipose tissue, two organs

involved in lipid metabolism (193, 194). A greater accumulation of hepatic ApoC-III was observed and associated inversely with hepatic ABCA1 expression. Given the role of ABCA1 in regulation of reverse cholesterol transport, these findings may provide support for additional factors promoting both hypertriglyceridaemia and low HDL-C levels in the setting of diabetes. Studies have reported that hepatic specific ABCA1 deletion resulted in higher plasma triglyceride levels, decreased HDL-C and accelerate renal clearance of HDL in mice (195). In contrast, we did not observe such a relationship in adipose tissue, in which our analytic approach failed to detect quantifiable levels of ApoC-III. While these findings suggest that the liver may play an important role in lipid homeostasis, future work can explore the specific impact on both lipid metabolism and HDL functionality.

In summary, our observational analysis revealed the presence of ApoC-III within both atherosclerotic plaque and the liver in a well validated model of diabetic dyslipidaemia. The presence of ApoC-III in association with features of more extensive and vulnerable atherosclerotic plaque, combined with preliminary observations of potential pro-inflammatory activity of ApoC-III suggest that there may be alternative links between ApoC-III and atherosclerosis, beyond its role in elevating triglyceride levels. Whether additional anti-atherosclerotic benefits may be derived from use of interventions aiming to reduce ApoC-III function require further investigation.

Chapter 5. Apolipoprotein C-III in an obese mouse model

5.1 Abstract

Introduction—The role of ApoC-III in the regulation of TRL metabolism may provide an important link between obesity, diabetes and atherosclerosis. In the preceding chapter, we have observed accumulation of ApoC-III within atherscletoric plaque and liver in mouse models of dyslipidaemia and diabetes. The tissue distribution of ApoC-III in the setting of obesity is a subjet of interest, with the potential to further influence lipid metabolism and atherogenesis.

Methods and Results—Obese mice ($Lep^{Ob/Ob}$) were fed either a chow or high cholesterol diet for 16 weeks. Plasma glucose, lipid parameters and ApoC-III were measured. Harvested tissue samples of aorta, liver and adipose tissue were analysed by immunocytochemistry and Western blot to characterise the expression of ApoC-III and lipid transporters. Compared with C57Bl6 controls, $Lep^{ob/ob}$ mice demonstrated higher serum ApoC-III concentrations. However, this did not associate with greater accumulation of ApoC-III within the liver. In contrast to our prior observations of diabetic mice, we did not observe an inverse correlation between hepatic levels of ApoC-III and ABCA1.

Conclusion—The increase in serum ApoC-III levels in $Lep^{Ob/Ob}$ mice, in contrast to the results observed in $ApoE^{-/-}$ mice, did not associate with greater hepatic ApoC-III accumulation.

5.2 Introduction

In the previous chapter, we observed that the setting of diabetic dyslipidaemia associated with elevated serum ApoC-III levels and expression of ApoC-III within atherosclerotic plaque. This vessel wall staining of ApoC-III associated with both the burden and macrophage content of plaque. Furthermore, greater accumulation of ApoC-III within the liver was observed, which inversely associated with its expression of the lipid transporter ABCA1. The studies in this chatper aim to further characterise the distribution of ApoC-III and its association with hepatic ABCA1 in a well validated mouse model of obesity.

Obesity and type 2 diabetes have become increasingly prevalent, resulting in major medical complications and mortality worldwide. The association of obesity and diabetes with atherogenic dyslipidaemia, hypertension and activation of inflammatory and oxidative pathways are likely to underscore the increase in CVD risk observed with these states (33, 34). Mechanistic studies have demonstrated that adipocyte biology is altered in the setting of abdominal obesity and diabetes, leading to a greater elaboration of factors that have the potential to activate inflammation, oxidative stress and thrombosis (196-198). These factors are likely to contribute to observations of more extensive and vulnerable atherosclerotic plaque on imaging in the setting of obesity (199, 200).

Obesity associates with an atherogenic dyslipidaemic phenotype, characterised by hypertriglyceridaemia, low levels of HDL cholesterol and a preponderance of low, dense LDL particles (34, 201, 202). While greater activity of ApoC-III is likely to contribute to less LPL activity and greater TRL concentrations (92), the relationship between adipose and ApoC-III has not been fully established. Recent studies using an antisense oligonucleotide against ApoC-III (ASO-ApoC-III) in $ApoE^{-/-}Ndst1^{fl/fl}Alb\text{-}Cre^{+}$ mice resulted in an increase in LPL activity within adipose tissue and a reduction in circulating triglyceride levels (203). While this supports the concept that ApoC-III may play an important role in the setting of obesity, further investigations are needed.

The aim of the studies described in this chapter were to investigate the expression and distribution of ApoC-III in a well validated mouse model of obesity. The specific aims included:

1. To determine expression of ApoC-III within the liver and adipose tissue.
2. To determine whether the inverse association of hepatic expression of ApoC-III and ABCA1 observed with diabetic dislipidaemia is also seen in obesity.

The hypothesis of these studies was that the pattern of ApoC-III expression and its association with cholesterol transporters regulating lipid metabolism in diabetic dyslipidaemia would also be observed in obesity.

5.3 **Methods**

5.3.1 Animals

The animal studies were approved by the Animal Ethics Committees of South Australian Health and Medical Institute (SAHMRI) (SAM186) and the University of Adelaide (M-2015-254). At 8 weeks of age, $Lep^{Ob/Ob}$ (ObOb) mice were allocated into two groups: (1) ObOb mice fed a CD for 18 weeks and (2) ObOb mice fed a high cholesterol diet (0.15% cholesterol, 21% total fat) *ad libitum* for 16 weeks. In parallel, wild-type C57BL6 mice were fed on a CD. High cholesterol diet feeding commenced at 10 weeks in the animals allocated to that group. Mice were bled and weighed at age of week 10 (baseline), week 18 (mid) and week 26 (final).

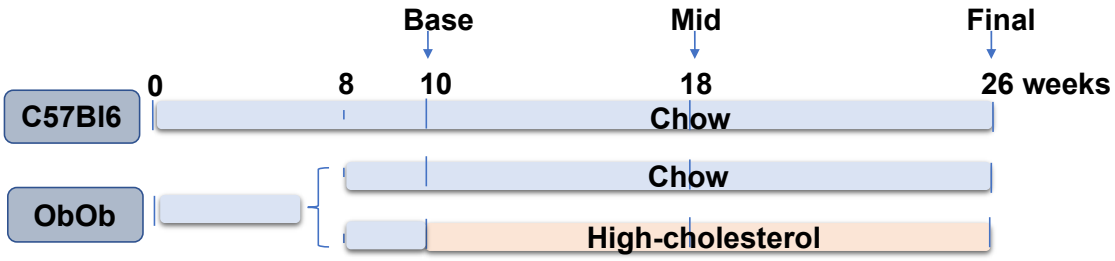


Figure 5-1. Schematic diagram of the experimental design of wild-type and Lep$^{ob/ob}$ mice fed a chow or high cholesterol diet. C57Bl6 mice (wild-type control) and Lep$^{ob/ob}$ at the age of 8 weeks were placed on either a chow or high cholesterol diet *ad libitum* until 26 weeks of age. Mice were weighed and bled at week 10 (baseline), week 18 (mid) and week 26 (final).

5.3.2 Dextran precipitation of HDL and determination of lipoprotein and lipid levels

Method for small volume HDL extraction was dapted from (140). Briefly, serum were incubated with Dextran-sulfate (MW: 50,000) in Mg^{2+} working solution for 10 min at room temperature and followed by a refrigenrate centrifugation (4 ºC) for 30 min. Large molecular protein ApoB were precipitated. The top clear supernant was used for assessment of total triglyceride, cholesterol and ApoC-III levels as described in Chapter 2 (general methods).

5.3.3 Statistics

In vivo animal data were presented as mean ± STD and *in vitro* cell experiments were performed in three biological replicates and the data were shown as mean ± SEM. Statistical comparisons among groups were performed using Student's t-test (Mann-Whitney's test) or one-way ANOVA where appropriate (Kruskal-Wallis' test). A linear regression model was used for correlation analysis. *$p < 0.05$ was considered as statistically significant for all analyses.

5.4 **Results**

5.4.1 Biochemical parameters in mice

Predicatably, $Lep^{ob/ob}$ mice demonstrated a greater weight in both dietary groups compared with the C57Bl6 control animals. Serum measures of lipid paramters, glucose and ApoC-III are summarised in Table 5-1. As previously reported, $Lep^{ob/ob}$ mice developed persistent hyperglycaemia from age of week 18, which was more pronounced in animals fed a high cholesterol diet. No significant difference in triglyceride levels was observed between the groups. In contrast, at week 26, $Lep^{ob/ob}$ mice demonstrated greater levels of total cholesterol and HDL cholesterol, more pronounced with high cholesterol feeding, compared with C57Bl6 mice. Serum ApoC-III levels were greater in $Lep^{ob/ob}$ mice fed a chow (949 ± 119 mg/dL) or high cholesterol (1023 ± 33.46 mg/dL) diet compared with C57Bl6 mice (323 ± 122 mg/dL, $p = 0.0003$).

Table 5-1 Biochemical and conventional lipid parameters of Lep$^{ob/ob}$ mice.

	C57BL6 (n=12)	Obese+CD (n=12)	Obese+HCD (n=12)	p value
Weight (g)[B]	35 (32- 38)***	67(65-68)	66(63-70)	0.0002
Total triglycerides (mg/dL)				
Base, week10	43± 22	66±6.7	63±8.3	NS
Mid, week 18	78±11	60±4.1	52±5.9	NS
Final, week 26	43±18	62±26	46±23	NS
Total cholesterol (mg/dL)				
Base, week10	327±82	173±7.0	175±8.2	NS
Mid, week 18	163±8.6	181±21	393±29*	0.03
Final, week 26	93± 45	174±7.5	366±16**	<0.0001
HDL-cholesterol (mg/dL)				
Base, week10	33±9.8	195±49	180±28	NS
Mid, week 18	92±1.1	427±34	508±79*	0.01
Final, week 26	85±27	148±12	292±70**	0.001
ApoC3 (μg/mL), final, week 26	323±122	949±119	1023±33	0.0003
Glucose (mg/dL), mid, week 18[C]	6.2±1.9	9.5±0.44**	11±3***	0.0027

[A] Results were expressed as mean ± STD (n = 8-12) from tests of pooled (n = 3/pool) or individual mouse plasma compared to the wild-type C57Bl6 control, *p < 0.05, **p < 0.005 by ANOVA (Kruskal-Wallis test). [B] Results were expressed as median, IQR compared to CD-fed Lep$^{ob/ob}$ mice by Student's t-test (Mann-Whitney's test). [C] Results were mean ± STD compared to the wild-type C57Bl6 mice by Student's t-test (Mann-Whitney's test).

5.4.2 Tissue expression of ApoC-III and ABCA1

In contrast to our observations in the preceding chapter of the impact of high cholesterol feeding in ApoE$^{-/-}$ mice rendered diabetic with STZ, We did not observe the development of aortic atherosclerosis with the same diet at week 26 (Figure 5-2 a). This was observed despite the obese mice demonstrating elevated levels of glucose, cholesterol and ApoC-III, yet paradoxically these animals also had higher HDL-C levels, which has been previously reported (204). Despite greater circulating ApoC-III levels, Lep$^{ob/ob}$ mice did not associate with greater hepatic accumulation. In fact, these obese mice demonstrated a reduction in both hepatic ApoC-III and SRB1 levels when fed a high cholesterol diet, compared with C57Bl6 controls (Figure 5-2 b). In a similar fashion, analysis of adipose tissue demonstrated no difference in ApoC-III, ABCA1 or SRB1 levels in the Lep$^{ob/ob}$ groups compared with non-obese control mice (Figure 5-2 j, k).

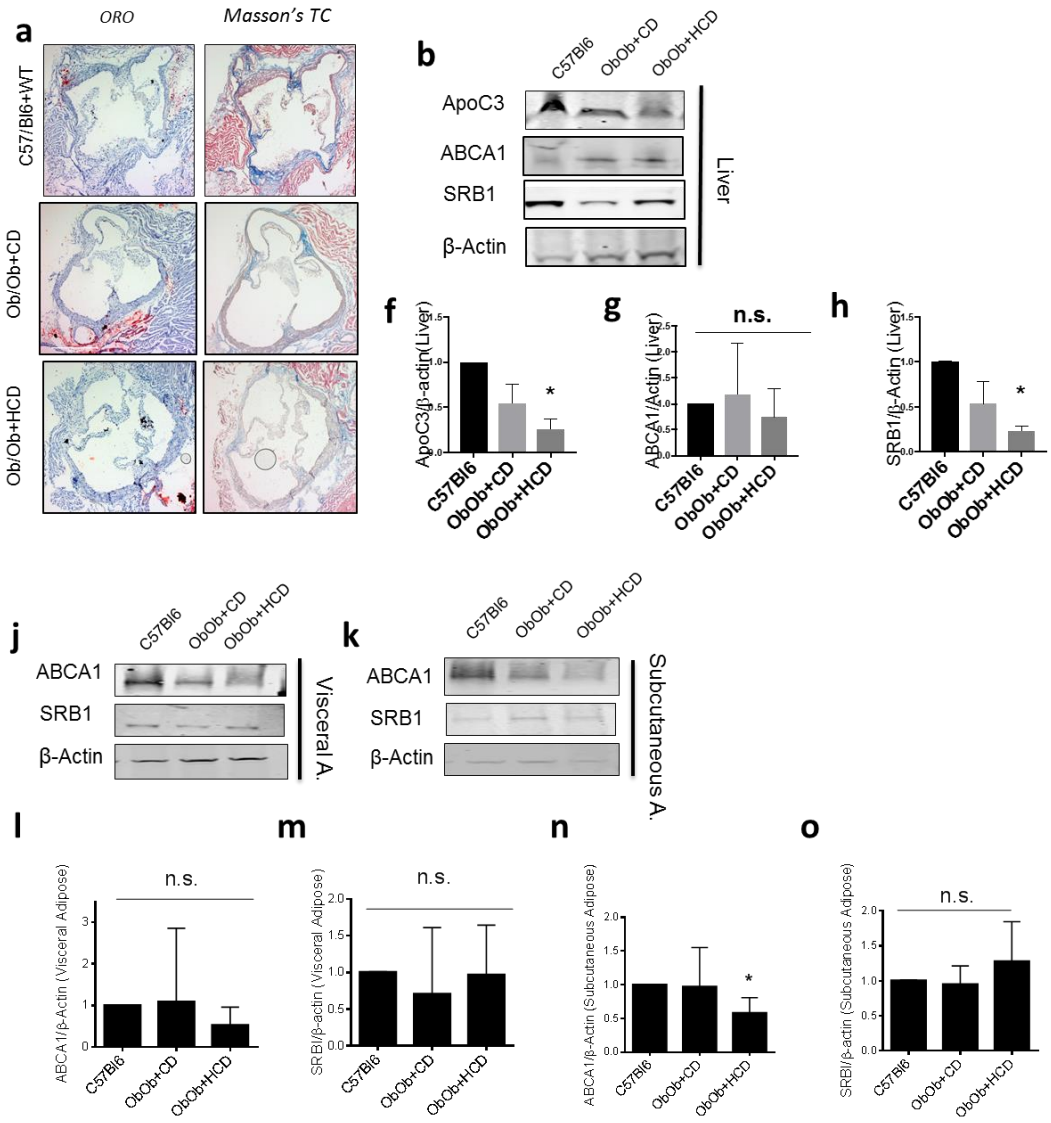


Figure 5-2 Increased serum ApoC-III levels did not associate with changes in hepatic accumulation of ApoC-III and was not inversely correlated with hepatic contnt of ABCA1 and SRB1 in Lep$^{ob/ob}$ mice. (a) ORO and Masson's trichrome staining to assess aortic root of Lep$^{Ob/Ob}$ mice. (b) Western blot analysis of hepatic (f) ApoC-III protein, (g) ABCA1 and (h) SRB1. Western blot analysis of (j) visceral adipose and (k) subcutaneous adipose in expression of (I, n) ABCA1 and (m, o) SRB1. Results were mean ±STD (n = 4/group), *p < 0.05, **p < 0.005 by one-way ANOVA (Kruskal-Wallis' test) or Mann-Whitney's test.

5.5 **Discussion**

In mouse models of dyslipidaemia and diabetes we had presented in Chapter 4 that elevated serum ApoC-III levels associated with accumulation of ApoC-III within atherosclerotic plaque and the liver. While the vascular ApoC-III directly correlated with the presence of more extensive and vulnerable atherosclerosis, its accumulation within the liver associated inversely with factors implicated in lipid transport. Given the association between obesity and diabetes, we sought to determine whether hepatic and adipose ApoC-III accumulation was altered in the setting of adiposity. While we observed greater ApoC-III levels within the serum of obese $Lep^{ob/ob}$ mice, this did not translate to greater content of ApoC-III within both the liver and adipose tissue. Furthermore, the inverse association between hepatic ApoC-III and ABCA1 observed in a diabetes model was not replicated in this mouse model of obesity.

A number of observations from this study highlights the distinction between the $Lep^{ob/ob}$ mouse and models of diabetes. The $Lep^{ob/ob}$ mice fed a high cholesterol diet demonstrated greater weight, glucose and total cholesterol levels. However, despite greater levels of ApoC-III, there were no differences in triglycerides compared with control mice. Furthermore, HDL-C levels were elevated in the $Lep^{ob/ob}$ mice, consistent with prior reports. These findings with regard to triglyceride and HDL-C levels contrast with typical findings of atherogenic

dyslipidemia in obesity in human studies. While this is a commonly used mouse model for the study of obesity, it does not reflect fundamental differences from humans that should be considered with regard to interpretation and potential implications of the findings.

In contrast to our findings of diabetic dyslipidaemia, the elevated serum ApoC-III levels in the $Lep^{ob/ob}$ mice did not associate with greater hepatic accumulation. We also failed to demonstrate an inverse correlation between ApoC-III and ABCA1 levels within the liver. Whether this is a consequence of elevated ApoA-I and HDL-C levels in these animals is unkown (204). As lipid transporter expression within the liver plays a role in reverse cholesterol transport, it is possible that effects on hepatic ABCA1 may influence circulating levels of HDL biomarkers. ABCA1 expression is regulated in various tissues in reponse to a range of transcription factors, predominantly PPARα and liver X receptor α (LXRα) (205, 206). Given the pivotal role of ApoC-III in the inhibition of lipoprotein lipase activity (92, 207) and the subsequent ability of TRL lipolysis to generate PPAR ligands (208), it is possible that ApoC-III could play a role in down-regulation of hepatic ABCA1 levels. Given the greater accumulation of hepatic ApoC-III in the diabetes model compared with the $Lep^{ob/ob}$ mice, this may contribute to the differences we observed in the two studies. It is also acknowledged that the relationship between humans and mouse models in terms of PPARα activity and ApoA-I metabolism are variable (204, 206, 209,

210), further confounding the ability to delineate the role of these factors in obesity. Additional studies will be required to determine the full impact of ApoC-III on PPARα and LXRα activity in the liver and its ultimate effect on lipid metabolism.

We also failed to observe the development of atherosclerotic plaque in these obese mouse models, even in the setting of high cholesterol feeding. To what degree this reflects the elevated HDL-C levels encountered in the $Lep^{ob/ob}$ mouse and the reported protective properties of HDL are unknown. It is also possible that longer term studies may ultimately results in the formation of plaque, which would contrast from the rapid atherosclerotic process observed with diabetic dyslipidaemia. Whether alternative models of obesity may produce difference results would also require further investigation.

In summary, an elevation in circulating ApoC-III levels in a well validated mouse model of obesity did not translate to vascular wall infiltration or hepatic accumulation. Furthermore, the distribution of ApoC-III in the setting of diabetes and its association with expression of lipid transporters was different. These findings suggest potential differences between obesity and diabetes with regard to tissue distribution of ApoC-III and subsequent influence on lipid metabolism.

Chapter 6. Effect of human hypertriglyceridaemic serum on hepatic expression of factors regulating lipid metabolism

6.1 Abstract

Introduction—While ApoC-III inhibits lipoprotein lipase and potentially impairs HDL cholesterol efflux capacity, its influence on other factors involved in HDL metabolism are not well defined. In Chapter 4, we observed that hepatic accumulation of ApoC-III in a mouse model of diabetic dyslipidaemia inversely correlated with ABCA1 levels. Accordingly, the study in this chapter aimed to evaluate the impact of co-incubating serum from individuals with different triglyceride levels on hepatic expression of factors regulating lipid metabolism.

Methods and Results— Serum from patients (n = 15) in a cardiology clinic, categorised as having normal (< 2 mmol/L), high (2 mmol/L-6 mmol/L) and very high (> 6 mmol/L) triglyceride levels, was co-incubated with HepG2 cells. Co-incubation of serum from the very high triglyceride group associated with lower expression of PPARα and cytoplasmic ABCA1. On correlation analysis, serum levels of ApoC-III, but not HDL-C, inversely correlated with expression of PPARα and cytoplasmic ABCA1.

Conclusion—Incubation of serum from hypertriglyceridaemic patients with HepG2 cells resulted in less expression of PPARα and cytoplasmic ABCA1, which associated with ApoC-III levels. This raises the possibility that ApoC-III may influene lipid metabolism beyond its effects on lipoprotein lipase.

6.2 Introduction

The major focus of the studies performed in this thesis have been to characterise the potential relationship between atherosclerosis and both TRL and ApoC-III. The findings have extended from observations that TRL modified by MPO have adverse effects on EC and that ApoC-III is found within more extensive and inflammatory atherosclerotic plaque in a mouse model of diabetic dyslipidaemia. In parallel, we have also observed that MPO-modified TRL incubation with EC associated with lower expression of ABCG1 and the diabetic dyslipidaemia mouse model demonstrated lower levels of hepatic ABCA1, which correlated inversely with increasing levels of ApoC-III. These findings suggest potential mehcanisms by which TRL and ApoC-III may modulate reverse cholesterol transport and lipid metabolism.

Cohort studies have commonly demonstrated the association between elevated triglycerides and low levels of HDL cholesterol (6, 25, 211, 212). This combinantion, along with the presence of small, dense LDL particles is typically referred to as atherogenic dyslipidaemia and associates with the presence of type 2 diabetes and an increased risk of atherosclerotic CVD (213-215). The finding that HDL cholesterol levels inversely associate with CVD risk (20, 152) and that HDL possess functional properties (154-156) that may confer a favourable effect on atherosclerosis have promoted interest in understanding

the factors that regulate HDL metabolism. Nascent, lipid-deplete forms of HDL are formed by the packing of ApoA-I with phospholipid into discoidal particles. ApoA-I is derived from both the liver and small intestine, produced in response to the activity of a number of transcription factors, including PPARα and LXRα (206). These lipid-deplete HDL particles are avid recipients of cholesterol effluxed from cells via ABCA1 (60, 216). The presence of free cholesterol on the HDL surface is esterified in a process facilitated by lecithin-cholesterol acyltransferase (LCAT) and subsequently stored within the particle core (217). The resulting larger, spherical HDL particles can continue to promote cholesterol efflux, albeit via different transporters such as ABCG1 and SRB1 (218, 219). Ultimately, cholesterol is delivered to the liver and other ograns, such as the adrenal glands, with uptake promoted via the same families of transporters (220). In parallel, some cholesterol ester is transferred from HDL to VLDL and LDL particles, in a process facilitated by CETP (221). This provides an alternative pathway for cholesterol delivery to the liver via the LDL receptor. Within the circulation, HDL particles are remodeled by a number of factors including the family of lipases (liporptein, hepatic, endothelial) (222) and phospholipid transfer protein (PLTP) (223, 224), which serve to influence the size and composition of HDL, with consequent effects on their metabolism. Given the impact of elevated triglyceride levels on a number of these factors, it

is therefore likely that the presence of hypertriglyceridaemia is likely to influence HDL metabolism.

In the preceding chapters, we have made a number of interesting observations involving factors implicated in HDL metabolism. The cellular studies in Chapter 3 demonstrated that incubation of EC with MPO modified TRL demonstrated reduced expression of ABCG1. The subsequent mouse studies, particularly in the setting of diabetic dyslipidaemia, revealed that hepatic accumulation of ApoC-III inversely correlated with expression of ABCA1. A similar trend was observed from hepatic SRB1 expression, although this just failed to meet statistical significance. These findings suggest potential mechanisms that may underscore links between hypertriglyceridaemia and HDL metabolism.

The aims of the study described in this chapter were to investigate the impact of incubating serum from patients with different triglyceride levels on hepatic expression of factors involved in lipid metabolism using a HepG2 cell model. The specific aims included:

1. To determine expression of transcription factors implicated in ApoA-I synthesis
2. To determine expression of transporters involved in reverse cholesterol transport.

The hypothesis of this study was that the presence of higher triglyceride levels would associated with reductions in expression of factors involved in the formation and remodeling of HDL particles.

6.3 **Methods**

6.3.1 Subjects

Serum samples were collected from patients aged between 18 years and 85 years found in the cardiology outpatient clinics at the Royal Adelaide Hospital (RAH). Medical history and concomitant medication use was recorded. Serum was isolated from blood samples for use in the cellular studies. Patients were categorised into three groups according to their levels of serum triglycerides. Patients treated with a fibrate, pregnant and breastfeeding women and those unable to provide written and informed consent were excluded from the study. All protocols were approved by Central Adelaide Local Health Network/Royal Adelaide Hospital Research Ethics Committee/Royal Adelaide Hospital (CALHN/RAH). For the purpose of the cellular experiments, serum from patients with normal (normal < 2 mmol/L), high (2 mmol/L-6 mmol/L) and very high (> 6 mmol/L) triglyceride levels (n = 5 per group) were assessed.

6.3.2 Cell culture

HepG2 cells were cultured in Low-glucose DMEM supplemented with 10% FBS and 10 mM L-glutamine. Cells were serum starved for 3 h to bring all the signal down and treated with serums from patients for 1 h. Cells were harvested, and cell lysates were analysed by Western blotting to assess expression of transporters involved in reverse cholesterol transport (SRB1, ABCA1) and transcription factors implicated in biosynthesis of ApoA-I (LXRα, PPARα, PPARγ).

6.3.3 Statistical analysis

Patients were stratified into three groups according to their serum triglyceride levels: normal triglycerides (normal TG, < 2 mmol/L, n = 5), high triglycerides (high TG, 2 mmol/L-6 mmol/L, n = 5) and very high triglycerides (very high TG, > 6 mmol/L, n = 5). Continuous clinical variables were presented as means and STD and categorical varibles are presented as integers. Age, height, weight and triglyceride levels were presented as median and interquartile range (IQR). To identify the relationship between ApoC-III, triglycerides and HDL-C, correlations were performed using spearman correlation coefficient as sample size was less than 10. Statistical significance was assessed using one way ANOVA (Kruskal-Wallis' test). All statistical analyses were performed with Prism.

6.4 **Results**

6.4.1 Biochemical and conventional lipids parameters

Demographic information, clinical and lipid parameters of subjects were summarised in Table 6-1. Patients with the highest triglyceride levels also demonstrated the highest serum level of total cholesterol ($p = 0.0009$) and known history of hypercheolesterolemia ($p = 0.04$). This was associated with non-significant trends towards higher levels of LDL cholesterol and lower levels of HDL cholesterol. Patients with the highest triglyceride levels also demonstrated the highest levels of ApoC-III ($p = 0.04$).

Table 6-1 Characteristics of the study population

	Normal TG (n=5)	High TG (n=5)	Very High TG (n=5)	P
Age	76 (65-83)	60 (52-70)	49 (48- 71)	NS
Height (cm)	163 (154-166)	166(162- 178)	175 (156-187)	NS
Weight (kg)	66 (51-86)	92 (84-97)	100 (62-123)	NS
Male	2	3	3	NS
Diabetes	2	2	3	NS
Hypertension	5	3	5	NS
Hypercholesteremia	1	3	5	0.04
Total triglycerides (mmol/l)	1.1(0.85-1.5)	2.7(2.45-3.8)	9.1(6.6-11.25)***	<0.0001
Total Cholesterol (mmol/l)	3.5± 0.8	4.7± 1.5	8.3± 4.8**	0.0009
LDL cholesterol (mmol/l)	1.8± 0.6	2.3± 1.4	3.3± 4.0	NS
HDL cholesterol (mmol/l)	1.1± 0.3	0.9± 0.1	0.9± 0.3	NS
ApoC3 (µg/mL)	320±600	760± 920	1560± 1340*	0.041
Medication				
Statin	3	4	4	NS
Fibrate	0	0	0	NS

Triglycerides, TG; LDL, low-density lipoprotein; HDL, high density lipoprotein.

Results were presented as mean ± STD or as median, IQR. *p < 0.05, **p < 0.005, ***p < 0.0005 by one-way ANOVA (Kruskal-Wallis' test).

6.4.2 HepG2 expression of lipid regulating factors

Human hepatocarcinoma cells HepG2 cells were treated with serum from patients with different triglyceride levels for 1 h. Cell lysates underwent analysis of expression of factors involved in the regulation of lipid metabolism by Western blotting. Serum with highest levels of triglyceride and ApoC-III demonstrated the lowest cellular expression of PPARα ($p = 0.04$, Figure 6-1 f) and cytoplasmic ABCA1 ($p = 0.02$, Figure 6-1 c), and in contrast, an increase in SRB1 ($p = 0.01$, Figure 6-1 d). While the reduced expression of LXRα when cells treated with serum from patients with high triglycerides ($p = 0.0004$), this was no longer observed in the presence of serum from those with very high triglyceride levels (Figure 6-1 a, e).

Predictably, a direct correlation was observed between HepG2 expression of PPARα and ABCA1 ($r = 0.83$, $p = 0.008$). Expression of LXRα, however, did not significantly associate with ABCA1 ($r = 0.55$, $p = 0.13$) (Figure 6-2). An inverse correlation was observed between serum ApoC-III levels and HepG2 expression of both PPARα ($r = -0.77$, $p = 0.02$) and cytoplasmic ABCA1 ($r = -0.86$, $p = 0.0045$), but not LXRα ($r = 0.18$, $p = 0.64$). As opposed to serum ApoC-III, HDL cholesterol levels did not significantly associate with HepG2 expression of any of the factors investigated (Figure 6-3).

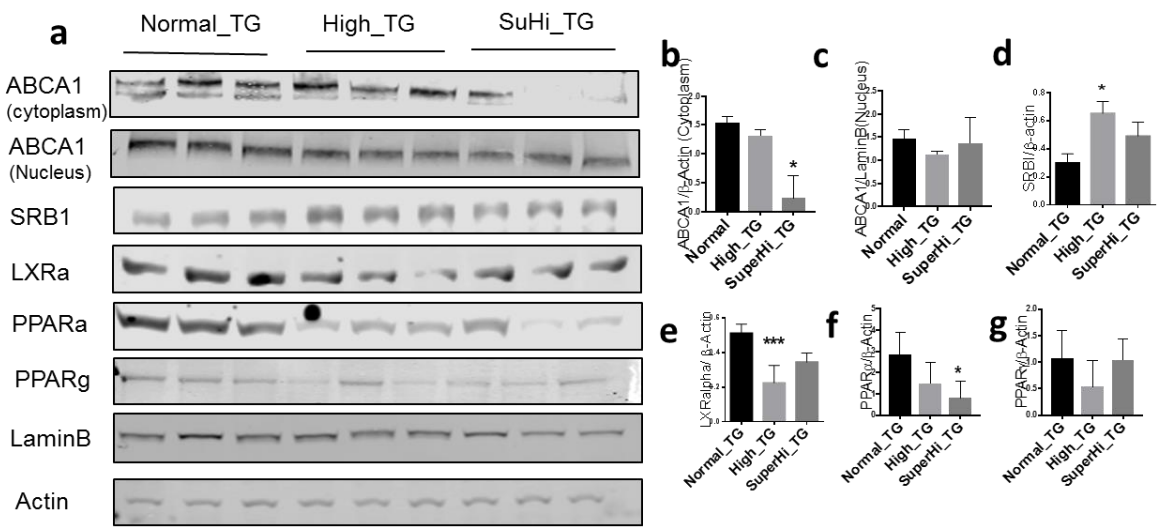


Figure 6-1 Inhibition of PPARα-cytoplasmic ABCA1 expression in HepG2 cells treated with serums with elevated TG and ApoC-III. (a) Western blot analysis and histograms represent the protein levels normalised to either laminB or actin: (b) nuclear ABCA1, (c) cytoplasmic ABCA1, (d) SRB1, (e) LXRα, (f) PPARα, (g) PPARγ. Results were means ± STD (n = 8-10) *p < 0.05, **p < 0.005 by one-way ANOVA (Kruskal-Wallis' test).

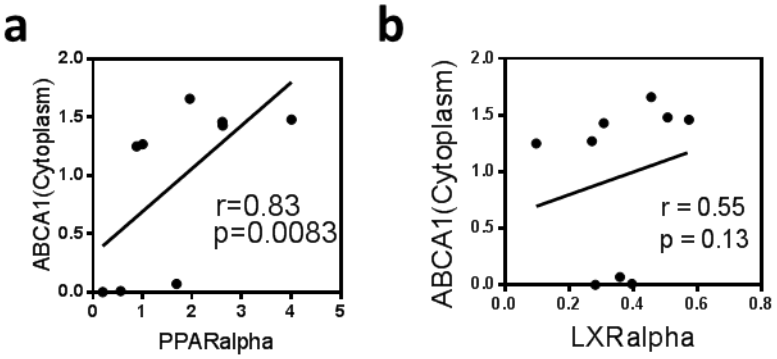


Figure 6-2 Linear regression analysis demonstrated that cytoplasmic ABCA1 expression was correlated with PPARα but not LXRα: (a) ABCA1 (cytoplasmic) v.s. PPARα, (b) ABCA1 (cytoplasmic) v.s. PPARα. Regression analysis were performed using non-parametric Spearman correlation coefficient as sample size was less than 10.

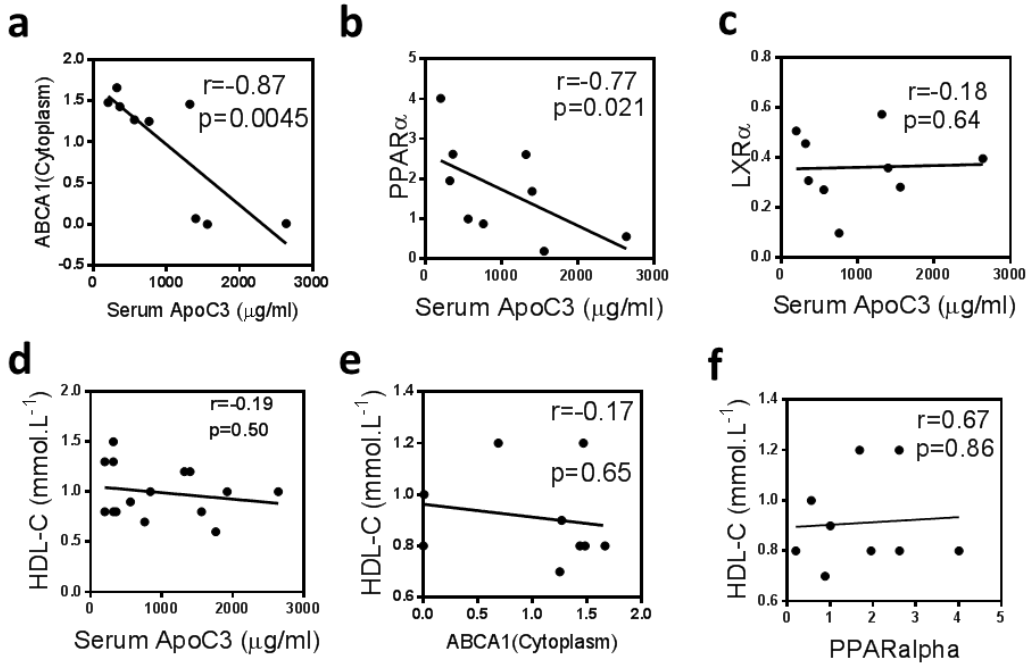


Figure 6-3 Serum ApoC-III was correlated with PPARα-ABCA1 but not LXRα, however HDL-C was not correlated with PPARα-ABCA1: (a) ApoC-III v.s. ABCA1, (b) ApoC-III v.s. PPARα, (c) ApoC-III v.s. LXRα, (d) HDL-C v.s. ApoC-III, (e) HDL-C v.s. ABCA1 and (f) HDL-C v.s. PPARα. Regression analyses were using non-parametric Spearman correlation coefficient as sample size was less than 10.

6.5 **Discussion**

While the primary objective of this thesis was to investigate the potential role of TRL and ApoC-III in the pathogenesis of atherosclerosis, a number of additional findings have suggested they may also have a relationship with expression of factors involved in HDL metabolism. We sought to investigate this further by incubating serum from patients with different triglyceride levels and hepatic cellular expression of transcription factors and lipid transporters. Serum from patients with the highest triglyceride levels associated with the lowest cellular expression of both PPARα and ABCA1, with further evidence that this correlated inversely with ApoC-III, but not HDL cholesterol levels.

While the clinical association of high triglycerides and low levels of HDL cholesterol is well recognised, particularly in the settings of obesity, insulin resistance and diabetes (33, 225, 226), the factors linking these lipid abnormalities are not completely established. The presence of hypertriglyceridaemia stimulates activity of a family of lipases, which remodel both LDL and HDL particles (227, 228). The generation of smaller HDL particles alters their catabolic rate, contributing to lower circulating HDL cholesterol levels (229, 230). To what degree, the presence of more TRL influences other factors that regulate the synthesis and metabolism of HDL particles remains uncertain. In addition, the impact of elevated triglyceride levels on a range of

HDL functional properties, particularly those involved in lipid transport, require further investigation.

A number of potential limitations of the current study should be noted. A small number of participants was enrolled, thus the findings can be interpreted as preliminary at best and warrant more extensive investigate in larger cohorts. Some patients were treated with a statin, which can influence regulation of factors implicated in cholesterol metabolism. The findings are observational and reflect a limited duration of cellular exposure to serum. To what degree these findings persist over longer periods of time are uncertain. As evidenced in the setting of postprandial elevation in TRL concentrations, transit changes in plasma lipids may have different effects than long term fasting levels. The findings also reflect observations involving one hepatic cellular line, whether they can be translated to the *in vivo* setting requires further investigation. Ultimately, whether these features can be modified with triglyceride lowering would require a serial study.

In summary, this pilot study demonstrated that serum from patients with very high triglyceride levels associates with reduced expression of some factors involved in the regulation of HDL synthesis and lipid transporting activity in a cellular study. While observational in nature, the findings suggest potentially additional mechanisms that may link the presence of high triglycerides and low

levels of HDL cholesterol. How these features contribute to cardiometabolic risk requires further investigation in large, prospective cohorts.

Chapter 7. Final discussion

7.1 General summary

With all increasing prevalence of obesity and type 2 diabetes, there is considerable interest in understanding the factors that underscore the heightened cardiovascular risk observed in these settings. While the role of dyslipidaemia in atherosclerosis has largely focused on the causal role of LDL and potential protective properties of HDL, triglycerides and associated factors have received recent attention. The studies in this thesis aimed to investigate the potential role of TRL and ApoC-III, a major factor regulating their metabolism, in cellular and animal models of metabolic disease and atherosclerosis. Three major observations were made in these studies, including (i) MPO-modified TRL exert adverse effects on entodhelial cells implicated in vascular repair and early stages of atherosclerosis, (ii) the presence of ApoC-III within extensive and inflammatory atherosclerotic plaque in a mouse model of diabetic dyslipiaemia and (iii) that triglycerides and ApoC-III may inversely correlated with factors that regulated HDL metabolism. These observational findings provide further potential mechanisms that may underscore hypertriglyceridaemia and atherosclerotic CVD.

7.2 Atherogenicity of TRL

Increasing evidence from both population studies and genomic analyses have demonstrated an association between TRL and atherosclerotic cardiovascular

disease. While the causal role of LDL in atherosclerosis has been well established, the impact of TRL has not been clearly established. In our studies we aimed to investigate the effect of TRL coincubation on EC properties implicated in cardiovascular disease. The studies in Chapter 3 demonstrated no adverse effect of TRL in their native state. However, when TRL had undergone oxidative modification by MPO, they resulted in endothelial changes characterised by an increase in expression of pro-inflammatory factors and impairment of cellular proliferation and migration pivotal in vascular repair. These findings support the conpcet of direct atherogenicity of oxidised forms of TRL, in a process that is analogous to that observed with LDL.

In parallel, we observed that the adverse effects of MPO modified TRL on EC were attenuated in the setting of co-incubation with HDL. This suggests the potential for additional protective properties of HDL that require further investigation. Contributing to the complexity of the relationship between triglycerides and HDL, we also observed that incubation of EC with TRL regulated in a reduction in expression of the lipid transporter, ABCG1, which plays an important role in regulation of HDL metabolism. Additional cellular studies should investigate the impact of alternative forms of TRL modification and their potential atherogenic effects. Furthermore, studies in hypertriglyceridemic mouse models will provide the opportunity to directly investigate their effect on vascular inflammation and repair in the *in vivo* setting.

7.3 ApoC-III and atherosclerotic plaque

With increasing evidence implicating TRL in atherosclerotic cardiovascular disease, there has been interest in the factors that regulate their metabolism. ApoC-III has been demonstrated to have a number of functional properties, including inhibition of LPL (92), preventing binding of lipoproteins to the LDL receptor (93) and promoting β-cell death within the pancreas, providing a potential common link between obesity, dyslipidemia and diabetes. In our mouse studies, we demonstrated that diabetic dyslipidemia associated with an increase in circulating ApoC-III levels, more extensive atherosclerotic plaque and the presence of ApoC-III within these lesions in the artery wall. Subsequent examination revealed that the extent of artery wall staining of ApoC-III directly associated with both the extent and macrophage composition of atherosclerotic plaque. These findings suggest not only the presence of ApoC-III within plaque, but also the potential for direct functional activity inside the vessel wall. Wether these findings would also be present in other mouse models of atherosclerosis is unknown.

Understanding the functional effects of ApoC-III within the artery wall can be realised by performing a number of different studies. In preliminary experiments, we investigated the functional properties of ApoC-III generated by an *E.coli* expression system. In additionl to inhibiting LPL, this form of ApoC-III resulted

in greater endothelial expression of pro-inflammatory adhesion molecules following incubation. Further studies will enable characterisation of additional inflammatory effects, not only on EC, but importantly on other cellular components of atherosclerotic plaque. This expression system also permits the generation of ApoC-III mutants, which lack the capapcity of inhibit LPL. Whether this will result in similar reduction in th potential pro-inflammtory effects remains to be determined, but may provide insights with regard to site-specific mutations and functional properties of ApoC-III.

7.4 ApoC-III and factors regulating HDL metabolism

In addition to the observations of a potential role of ApoC-III directly within atherosclerotic plaque accumulating within the artery wall, our studies also suggest a potential association with factors regulating HDL metabolism. In the setting of diabetic dyslipidaemia, we observed that greater circulating levels of ApoC-III associated with an increased presence of ApoC-III within the liver. Correlation analysis demonstrated an inverse association between hepatic levels of ApoC-III with both ABCA1 and PPARα, both factors involved in HDL metabolism. Subsequent incubation of serum from patients with very high triglyceride levels resulted in lower expression of both ABCA1 and PPARα by HepG2 cells in an inverse association with ApoC-III levels. These findings further suggest a potential link between ApoC-III, TRL and HDL metabolism. In

contrast, the greater circulating levels of ApoC-III in an oberse model moudel did not associate with changes in either levels of ApoC-III, ABCA1 or PPARα within the liver.

The findings suggest that ApoC-III and impaired TRL metabolism may contribute to an alterations in HDL metabolism via changes in both apolipoprotein synthesis and expression of co-transporters involved in lipid transport. In parallel, there are some reports that increasing ApoC-III content of HDL associates with an impairment of their ability to promote cholesterol efflux in cellular studies (102). The degree to which this influences reverse cholesterol transport and other functional properties of HDL remains to be determined in additional cellular and animal studies. The findings also raise the potential for therapies that either lower triglycerides or inhibit ApoC-III may influence the level or functional quality of HDL. The early experience with agents that target ApoC-III have yielded variable results, with a lack of HDL-C raising using ASO-ApoC-III (231) and a monoclonal antibody-ApoC-III (127), in most mouse models (93, 203), while in contrast a N-acetyle alactosamine-conjugated ASO-ApoC-III produced a dose-dependent elevation in HDL-C (232)

7.5 **Future directions**

The findings of our studies are observational and while they propose a number of mechanistic interactions between TRL and ApoC-III with atherosclerosis and

HDL metabolism, this will require more extensive investigate. This will involved elucidation of the specific factors that link TRL and ApoC-III with pathways involved in atherosclerosis in both cell and animal studies. The ability to express both wild type and mutant forms of ApoC-III and generate different forms of chemical modification of TRL, particularly when isolated from patients with different metabolic states, will provide further insight into their potential impact on cardiometabolic risk. More extensive studies of atherosclerotic plaque using both animal and human marterial will permit a greater understanding of the potential impact of TRL and ApoC-III within the artery wall. As increasing attention focuses on the development of a range of therapeutic interventions targeting triglycerides or ApoC-III, their impact on these biological properties will be of interest.

7.6 **Conclusion**

The increase in abdominal adiposity has seen a rise in the prevalence of diabetes, atherogenic dyslipidaemia and cardiovascular risk. Accordingly, there is considerable interest in determining optimal approaches to reducing this risk, which remains substantial despite use of evidence based therapies. Our studies suggesting a potential link between TRL, diabetes, HDL metabolism and atherosclerosis provides a number of mechanistic pathways that warrant further

investigation, in order to develop more effective approaches to treating cardiometabolic risk.

Appendix

ApoC-III WT and mutants sequencing

7.6.1 APOC3-WT-1 Combined Sequences

AGGACCCAACGCTGCCCGAGATCTCGATCCCGCGAAATTAATACGACTCAC
TATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAG
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TGCAGGGTTACATGAAGCACGCCACCAAGACCGCCAAGGATGCACTGAGCAGC
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GTTCCCTGAAAGACTACTGGAGCACCGTTAAGGACAAGTTCTCTGAGTTCTGGGA
TTTGGACCCTGAGGTCAGACCAACTTCAGCCGTGGCTGCCCTCGAGCACCACCACC
ACCACCAC**TGA**GATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGC
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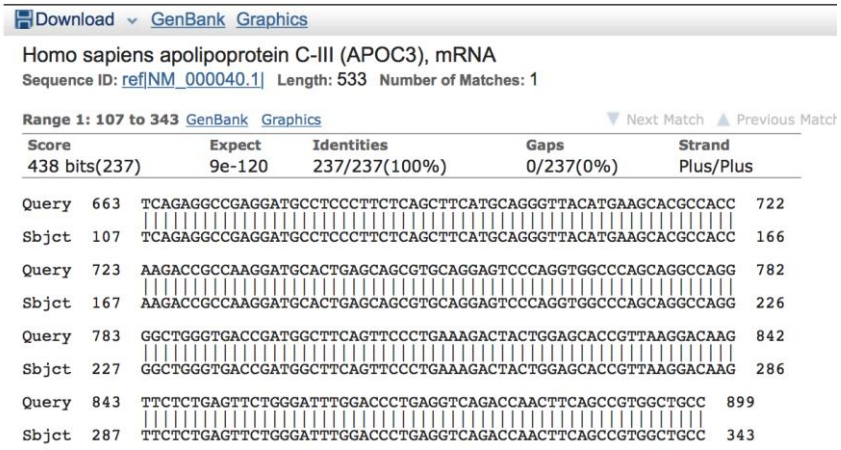

Translated sequences:

MSEAEDASLLSFMQGYMKHATKTAKDALSSVQESQVAQQARGWVTDGFSSLK
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7.6.2 APOC3-58E-1 Combined Sequences

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TAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCG
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TGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCG
CTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCC
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ACCCAACGCTGCCCGAGATCTCGATCCCGCGAAATTAATACGACTCACTAT
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GGAGATATACAT**ATG**TCAGAGGCCGAGGATGCCTCCCTTCTCAGCTTCATG
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GTGGCTGCCCTCGAGCACCACCACCACCACCAC**TGA**GATCCGGCTGCTAACAAAGCCCGAAAGG
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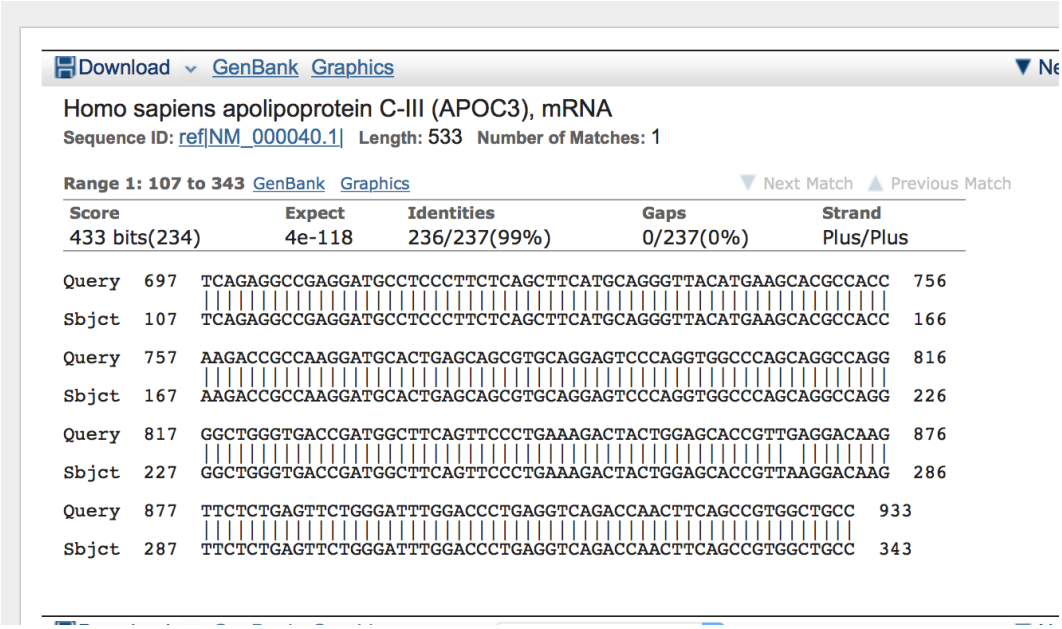

7.6.3 APOC3-58R-3 combined sequences

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Range 1: 107 to 343 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  227  GGCTGGGTGACCGATGGCTTCAGTTCCCTGAAAGACTACTGGAGCACCGTTAAGGACAAG  286

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7.6.4 APOC3-74T combined sequences

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Homo sapiens apolipoprotein C-III (APOC3), mRNA

Sequence ID: ref|NM_000040.1| **Length:** 533 **Number of Matches:** 1

Range 1: 107 to 343 GenBank Graphics ▼ Next Match ▲ Previous Match

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References

1. Statistics. ABo. Causes of Death 2015 (3303.0) September 2016. 2015.
2. 2015 AIoHaW. Australian hospital statistics 2014-15. Canberra: AIHW.
3. Welfare AIoHa. Australia's Health 2012. Canberra: Australian Institute of Health and Welfare; 2012.
4. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation. 2016;133(4):e38-360.
5. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. Jama. 1979;241(19):2035-8.
6. Chapman MJ, Ginsberg HN, Amarenco P, Andreotti F, Boren J, Catapano AL, et al. Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. Eur Heart J. 2011;32(11):1345-61.
7. Raal FJ, Pilcher GJ, Waisberg R, Buthelezi EP, Veller MG, Joffe BI. Low-density lipoprotein cholesterol bulk is the pivotal determinant of atherosclerosis in familial hypercholesterolemia. Am J Cardiol. 1999;83(9):1330-3.
8. Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006;354(12):1264-72.
9. Pignone M, Phillips C, Mulrow C. Use of lipid lowering drugs for primary prevention of coronary heart disease: meta-analysis of randomised trials. BMJ. 2000;321(7267):983-6.
10. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. Jama. 1998;279(20):1615-22.
11. Cholesterol Treatment Trialists C, Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, et al. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet. 2010;376(9753):1670-81.
12. Cannon CP, Blazing MA, Giugliano RP, McCagg A, White JA, Theroux P, et al. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. N Engl J Med. 2015;372(25):2387-97.
13. Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, et al. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. N Engl J Med. 2015;372(16):1489-99.
14. Sabatine MS, Giugliano RP, Wiviott SD, Raal FJ, Blom DJ, Robinson J, et al. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. N Engl J Med. 2015;372(16):1500-9.

15. Catapano AL, Graham I, De Backer G, Wiklund O, Chapman MJ, Drexel H, et al. [2016 ESC/EAS Guidelines for the Management of Dyslipidaemias]. Kardiol Pol. 2016;74(11):1234-318.
16. Grundy SM, Cleeman JI, Merz CN, Brewer HB, Jr., Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Arterioscler Thromb Vasc Biol. 2004;24(8):e149-61.
17. Pasternak RC, Smith SC, Jr., Bairey-Merz CN, Grundy SM, Cleeman JI, Lenfant C. ACC/AHA/NHLBI Clinical Advisory on the Use and Safety of Statins. Circulation. 2002;106(8):1024-8.
18. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. Jama. 2007;298(3):309-16.
19. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. Jama. 2007;298(3):299-308.
20. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM, et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. N Engl J Med. 2007;357(13):1301-10.
21. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet. 2012;380(9841):572-80.
22. Do R, Willer CJ, Schmidt EM, Sengupta S, Gao C, Peloso GM, et al. Common variants associated with plasma triglycerides and risk for coronary artery disease. Nat Genet. 2013;45(11):1345-52.
23. Morieri ML, Shah HS, Sjaarda J, Lenzini PA, Campbell H, Motsinger-Reif AA, et al. PPARA Polymorphism Influences the Cardiovascular Benefit of Fenofibrate in Type 2 Diabetes: Findings From ACCORD-Lipid. Diabetes. 2020;69(4):771-83.
24. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, et al. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. Lancet. 2005;366(9500):1849-61.
25. Jun M, Foote C, Lv J, Neal B, Patel A, Nicholls SJ, et al. Effects of fibrates on cardiovascular outcomes: a systematic review and meta-analysis. Lancet. 2010;375(9729):1875-84.
26. Jorgensen AB, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Loss-of-function mutations in APOC3 and risk of ischemic vascular disease. N Engl J Med. 2014;371(1):32-41.
27. Tg, Hdl Working Group of the Exome Sequencing Project NHL, Blood I, Crosby J, Peloso GM, Auer PL, et al. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. N Engl J Med. 2014;371(1):22-31.

28. Pitts R, Gunzburger E, Ballantyne CM, Barter PJ, Kallend D, Leiter LA, et al. Treatment With Dalcetrapib Modifies the Relationship Between High-Density Lipoprotein Cholesterol and C-Reactive Protein. J Am Coll Cardiol. 2016;68(22):2488-90.
29. Ference BA, Kastelein JJP, Ginsberg HN, Chapman MJ, Nicholls SJ, Ray KK, et al. Association of Genetic Variants Related to CETP Inhibitors and Statins With Lipoprotein Levels and Cardiovascular Risk. Jama. 2017;318(10):947-56.
30. Nicholls SJ, Andrews J, Kastelein JJP, Merkely B, Nissen SE, Ray KK, et al. Effect of Serial Infusions of CER-001, a Pre-beta High-Density Lipoprotein Mimetic, on Coronary Atherosclerosis in Patients Following Acute Coronary Syndromes in the CER-001 Atherosclerosis Regression Acute Coronary Syndrome Trial: A Randomized Clinical Trial. JAMA Cardiol. 2018;3(9):815-22.
31. Andrews J, Janssan A, Nguyen T, Pisaniello AD, Scherer DJ, Kastelein JJ, et al. Effect of serial infusions of reconstituted high-density lipoprotein (CER-001) on coronary atherosclerosis: rationale and design of the CARAT study. Cardiovasc Diagn Ther. 2017;7(1):45-51.
32. Nicholls SJ, Puri R, Ballantyne CM, Jukema JW, Kastelein JJP, Koenig W, et al. Effect of Infusion of High-Density Lipoprotein Mimetic Containing Recombinant Apolipoprotein A-I Milano on Coronary Disease in Patients With an Acute Coronary Syndrome in the MILANO-PILOT Trial: A Randomized Clinical Trial. JAMA Cardiol. 2018;3(9):806-14.
33. Ginsberg HN. Lipoprotein physiology in nondiabetic and diabetic states. Relationship to atherogenesis. Diabetes Care. 1991;14(9):839-55.
34. Bamba V, Rader DJ. Obesity and atherogenic dyslipidemia. Gastroenterology. 2007;132(6):2181-90.
35. Preston RJS, O'Sullivan JM, O'Donnell JS. Advances in understanding the molecular mechanisms of venous thrombosis. Br J Haematol. 2019;186(1):13-23.
36. O'Donnell JS, O'Sullivan JM, Preston RJS. Advances in understanding the molecular mechanisms that maintain normal haemostasis. Br J Haematol. 2019;186(1):24-36.
37. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med. 2012;209(4):819-35.
38. Vergnani L, Hatrik S, Ricci F, Passaro A, Manzoli N, Zuliani G, et al. Effect of native and oxidized low-density lipoprotein on endothelial nitric oxide and superoxide production : key role of L-arginine availability. Circulation. 2000;101(11):1261-6.
39. Nielsen MH, Irvine H, Vedel S, Raungaard B, Beck-Nielsen H, Handberg A. The Impact of Lipoprotein-Associated Oxidative Stress on Cell-Specific Microvesicle Release in Patients with Familial Hypercholesterolemia. Oxid Med Cell Longev. 2016;2016:2492858.
40. Chiva-Blanch G, Padro T, Alonso R, Crespo J, Perez de Isla L, Mata P, et al. Liquid Biopsy of Extracellular Microvesicles Maps Coronary Calcification and

Atherosclerotic Plaque in Asymptomatic Patients With Familial Hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2019;39(5):945-55.
41. Suades R, Padro T, Alonso R, Mata P, Badimon L. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. Thromb Haemost. 2013;110(2):366-77.
42. Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. Rev Esp Cardiol. 2009;62(10):1161-78.
43. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. Circ Res. 2014;114(12):1852-66.
44. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med. 2014;276(6):618-32.
45. Burke AP, Kolodgie FD, Farb A, Weber DK, Malcom GT, Smialek J, et al. Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. Circulation. 2001;103(7):934-40.
46. Tomey MI, Narula J, Kovacic JC. Advances in the understanding of plaque composition and treatment options: year in review. J Am Coll Cardiol. 2014;63(16):1604-16.
47. Nicholls SJ, Puri R, Anderson T, Ballantyne CM, Cho L, Kastelein JJP, et al. Effect of Evolocumab on Coronary Plaque Composition. J Am Coll Cardiol. 2018;72(17):2012-21.
48. Puri R, Nissen SE, Somaratne R, Cho L, Kastelein JJ, Ballantyne CM, et al. Impact of PCSK9 inhibition on coronary atheroma progression: Rationale and design of Global Assessment of Plaque Regression with a PCSK9 Antibody as Measured by Intravascular Ultrasound (GLAGOV). Am Heart J. 2016;176:83-92.
49. Chistiakov DA, Bobryshev YV, Orekhov AN. Macrophage-mediated cholesterol handling in atherosclerosis. J Cell Mol Med. 2016;20(1):17-28.
50. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med. 2007;13(10):1176-84.
51. Zimmermann R, Panzenbock U, Wintersperger A, Levak-Frank S, Graier W, Glatter O, et al. Lipoprotein lipase mediates the uptake of glycated LDL in fibroblasts, endothelial cells, and macrophages. Diabetes. 2001;50(7):1643-53.
52. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. Circulation. 2005;112(5):651-7.
53. Tanner FC, Noll G, Boulanger CM, Luscher TF. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries. Role of scavenger receptor and endothelium-derived nitric oxide. Circulation. 1991;83(6):2012-20.
54. Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest. 1994;94(1):437-44.

55. Thukkani AK, McHowat J, Hsu FF, Brennan ML, Hazen SL, Ford DA. Identification of alpha-chloro fatty aldehydes and unsaturated lysophosphatidylcholine molecular species in human atherosclerotic lesions. Circulation. 2003;108(25):3128-33.
56. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest. 1997;99(9):2075-81.
57. Beckmann JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM, et al. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol Chem Hoppe Seyler. 1994;375(2):81-8.
58. Podrez EA, Schmitt D, Hoff HF, Hazen SL. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. J Clin Invest. 1999;103(11):1547-60.
59. Nicholls SJ, Hazen SL. Myeloperoxidase, modified lipoproteins, and atherogenesis. J Lipid Res. 2009;50 Suppl:S346-51.
60. Shao B, Tang C, Sinha A, Mayer PS, Davenport GD, Brot N, et al. Humans with atherosclerosis have impaired ABCA1 cholesterol efflux and enhanced high-density lipoprotein oxidation by myeloperoxidase. Circ Res. 2014;114(11):1733-42.
61. Speer T, Owala FO, Holy EW, Zewinger S, Frenzel FL, Stahli BE, et al. Carbamylated low-density lipoprotein induces endothelial dysfunction. Eur Heart J. 2014;35(43):3021-32.
62. Ferrante G, Nakano M, Prati F, Niccoli G, Mallus MT, Ramazzotti V, et al. High levels of systemic myeloperoxidase are associated with coronary plaque erosion in patients with acute coronary syndromes: a clinicopathological study. Circulation. 2010;122(24):2505-13.
63. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, et al. Association between myeloperoxidase levels and risk of coronary artery disease. Jama. 2001;286(17):2136-42.
64. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation. 2003;108(12):1440-5.
65. Libby P, Pasterkamp G. Requiem for the 'vulnerable plaque'. Eur Heart J. 2015;36(43):2984-7.
66. Carlin G, Djursater R. Peroxidation of phospholipids promoted by myeloperoxidase. Free Radic Res Commun. 1988;4(4):251-7.
67. Spickett CM, Jerlich A, Panasenko OM, Arnhold J, Pitt AR, Stelmaszynska T, et al. The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids. Acta Biochim Pol. 2000;47(4):889-99.
68. Ok E, Basnakian AG, Apostolov EO, Barri YM, Shah SV. Carbamylated low-density lipoprotein induces death of endothelial cells: a link to atherosclerosis in patients with kidney disease. Kidney Int. 2005;68(1):173-8.

69. Hazen SL, Hsu FF, Mueller DM, Crowley JR, Heinecke JW. Human neutrophils employ chlorine gas as an oxidant during phagocytosis. J Clin Invest. 1996;98(6):1283-9.
70. Ismael FO, Proudfoot JM, Brown BE, van Reyk DM, Croft KD, Davies MJ, et al. Comparative reactivity of the myeloperoxidase-derived oxidants HOCl and HOSCN with low-density lipoprotein (LDL): Implications for foam cell formation in atherosclerosis. Arch Biochem Biophys. 2015;573:40-51.
71. Stelmaszynska T, Kukovetz E, Egger G, Schaur RJ. Possible involvement of myeloperoxidase in lipid peroxidation. Int J Biochem. 1992;24(1):121-8.
72. Kubala L, Schmelzer KR, Klinke A, Kolarova H, Baldus S, Hammock BD, et al. Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation. Free Radic Biol Med. 2010;48(10):1311-20.
73. Freiberg JJ, Tybjaerg-Hansen A, Jensen JS, Nordestgaard BG. Nonfasting triglycerides and risk of ischemic stroke in the general population. Jama. 2008;300(18):2142-52.
74. Miller M, Cannon CP, Murphy SA, Qin J, Ray KK, Braunwald E, et al. Impact of triglyceride levels beyond low-density lipoprotein cholesterol after acute coronary syndrome in the PROVE IT-TIMI 22 trial. J Am Coll Cardiol. 2008;51(7):724-30.
75. Varbo A, Benn M, Tybjaerg-Hansen A, Jorgensen AB, Frikke-Schmidt R, Nordestgaard BG. Remnant cholesterol as a causal risk factor for ischemic heart disease. J Am Coll Cardiol. 2013;61(4):427-36.
76. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, et al. Major lipids, apolipoproteins, and risk of vascular disease. Jama. 2009;302(18):1993-2000.
77. Watts GF, Ooi EM, Chan DC. Demystifying the management of hypertriglyceridaemia. Nat Rev Cardiol. 2013;10(11):648-61.
78. Nordestgaard BG, Zilversmit DB. Large lipoproteins are excluded from the arterial wall in diabetic cholesterol-fed rabbits. J Lipid Res. 1988;29(11):1491-500.
79. Brewer HB, Jr., Shulman R, Herbert P, Ronan R, Wehrly K. The complete amino acid sequence of alanine apolipoprotein (apoC-3), and apolipoprotein from human plasma very low density lipoproteins. J Biol Chem. 1974;249(15):4975-84.
80. Gangabadage CS, Zdunek J, Tessari M, Nilsson S, Olivecrona G, Wijmenga SS. Structure and dynamics of human apolipoprotein CIII. J Biol Chem. 2008;283(25):17416-27.
81. Caron S, Verrijken A, Mertens I, Samanez CH, Mautino G, Haas JT, et al. Transcriptional activation of apolipoprotein CIII expression by glucose may contribute to diabetic dyslipidemia. Arterioscler Thromb Vasc Biol. 2011;31(3):513-9.
82. Staels B, Vu-Dac N, Kosykh VA, Saladin R, Fruchart JC, Dallongeville J, et al. Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase. A potential mechanism for the hypolipidemic action of fibrates. J Clin Invest. 1995;95(2):705-12.

83. Qu S, Su D, Altomonte J, Kamagate A, He J, Perdomo G, et al. PPAR{alpha} mediates the hypolipidemic action of fibrates by antagonizing FoxO1. Am J Physiol Endocrinol Metab. 2007;292(2):E421-34.
84. Tonstad S, Retterstol K, Ose L, Ohman KP, Lindberg MB, Svensson M. The dual peroxisome proliferator-activated receptor alpha/gamma agonist tesaglitazar further improves the lipid profile in dyslipidemic subjects treated with atorvastatin. Metabolism: clinical and experimental. 2007;56(9):1285-92.
85. Altomonte J, Cong L, Harbaran S, Richter A, Xu J, Meseck M, et al. Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. J Clin Invest. 2004;114(10):1493-503.
86. Harish S, Khanam T, Mani S, Rangarajan P. Transcriptional activation by hepatocyte nuclear factor-4 in a cell-free system derived from rat liver nuclei. Nucleic Acids Res. 2001;29(5):1047-53.
87. Mietus-Snyder M, Sladek FM, Ginsburg GS, Kuo CF, Ladias JA, Darnell JE, Jr., et al. Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol Cell Biol. 1992;12(4):1708-18.
88. L J-B, Refai E Fau - Appelskog I, Appelskog I Fau - Andersson M, Andersson M Fau - Imreh G, Imreh G Fau - Dekki N, Dekki N Fau - Uhles S, et al. - Apolipoprotein CIII promotes Ca2+-dependent beta cell death in type 1 diabetes. Proc Natl Acad Sci U S A. 2004;101(27):10090-4.
89. Avall K, Ali Y, Leibiger IB, Leibiger B, Moede T, Paschen M, et al. Apolipoprotein CIII links islet insulin resistance to beta-cell failure in diabetes. Proc Natl Acad Sci U S A. 2015;112(20):E2611-9.
90. Juntti-Berggren L, Refai E, Appelskog I, Andersson M, Imreh G, Dekki N, et al. Apolipoprotein CIII promotes Ca2+-dependent beta cell death in type 1 diabetes. Proc Natl Acad Sci U S A. 2004;101(27):10090-4.
91. Sundaram M, Zhong S, Bou Khalil M, Links PH, Zhao Y, Iqbal J, et al. Expression of apolipoprotein C-III in McA-RH7777 cells enhances VLDL assembly and secretion under lipid-rich conditions. J Lipid Res. 2010;51(1):150-61.
92. Wang CS, McConathy WJ, Kloer HU, Alaupovic P. Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. J Clin Invest. 1985;75(2):384-90.
93. Gordts PL, Nock R, Son NH, Ramms B, Lew I, Gonzales JC, et al. ApoC-III inhibits clearance of triglyceride-rich lipoproteins through LDL family receptors. J Clin Invest. 2016;126(8):2855-66.
94. Jensen MK, Aroner SA, Mukamal KJ, Furtado JD, Post WS, Tsai MY, et al. HDL Subspecies Defined by Presence of Apolipoprotein C-III and Incident Coronary Heart Disease in Four Cohorts. Circulation. 2017.
95. Gill JM, Brown JC, Bedford D, Wright DM, Cooney J, Hughes DA, et al. Hepatic production of VLDL1 but not VLDL2 is related to insulin resistance in normoglycaemic middle-aged subjects. Atherosclerosis. 2004;176(1):49-56.

96. Duvillard L, Caslake MJ, Petit JM, Verges B, Gambert P, Packard CJ. Distinct patterns of heparin affinity chromatography VLDL1 and VLDL2 subfractions in the different dyslipidaemias. Atherosclerosis. 2008;199(1):27-33.
97. Sundaram M, Zhong S, Bou Khalil M, Zhou H, Jiang ZG, Zhao Y, et al. Functional analysis of the missense APOC3 mutation Ala23Thr associated with human hypotriglyceridemia. J Lipid Res. 2010;51(6):1524-34.
98. Qin W, Sundaram M, Wang Y, Zhou H, Zhong S, Chang CC, et al. Missense mutation in APOC3 within the C-terminal lipid binding domain of human ApoC-III results in impaired assembly and secretion of triacylglycerol-rich very low density lipoproteins: evidence that ApoC-III plays a major role in the formation of lipid precursors within the microsomal lumen. J Biol Chem. 2011;286(31):27769-80.
99. Morita SY, Sakurai A, Nakano M, Kitagawa S, Handa T. Presence of apolipoprotein C-III attenuates apolipoprotein E-mediated cellular uptake of cholesterol-containing lipid particles by HepG2 cells. Lipids. 2011;46(4):323-32.
100. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science. 1988;240(4852):622-30.
101. Mahley RW, Huang Y. Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing. J Clin Invest. 2007;117(1):94-8.
102. Luo M, Liu A, Wang S, Wang T, Hu D, Wu S, et al. ApoCIII enrichment in HDL impairs HDL-mediated cholesterol efflux capacity. Sci Rep. 2017;7(1):2312.
103. Morton AM, Koch M, Mendivil CO, Furtado JD, Tjonneland A, Overvad K, et al. Apolipoproteins E and CIII interact to regulate HDL metabolism and coronary heart disease risk. JCI Insight. 2018;3(4).
104. Larsson M, Vorrsjo E, Talmud P, Lookene A, Olivecrona G. Apolipoproteins C-I and C-III inhibit lipoprotein lipase activity by displacement of the enzyme from lipid droplets. J Biol Chem. 2013;288(47):33997-4008.
105. Ding Y, Wang Y, Zhu H, Fan J, Yu L, Liu G, et al. Hypertriglyceridemia and delayed clearance of fat load in transgenic rabbits expressing human apolipoprotein CIII. Transgenic Res. 2011;20(4):867-75.
106. Wei J, Ouyang H, Wang Y, Pang D, Cong NX, Wang T, et al. Characterization of a hypertriglyceridemic transgenic miniature pig model expressing human apolipoprotein CIII. FEBS J. 2012;279(1):91-9.
107. Masucci-Magoulas L, Goldberg IJ, Bisgaier CL, Serajuddin H, Francone OL, Breslow JL, et al. A mouse model with features of familial combined hyperlipidemia. Science. 1997;275(5298):391-4.
108. Ebara T, Ramakrishnan R, Steiner G, Shachter NS. Chylomicronemia due to apolipoprotein CIII overexpression in apolipoprotein E-null mice. Apolipoprotein CIII-induced hypertriglyceridemia is not mediated by effects on apolipoprotein E. J Clin Invest. 1997;99(11):2672-81.
109. Baroukh N, Ostos MA, Vergnes L, Recalde D, Staels B, Fruchart J, et al. Expression of human apolipoprotein A-I/C-III/A-IV gene cluster in mice reduces

atherogenesis in response to a high fat-high cholesterol diet. FEBS letters. 2001;502(1-2):16-20.
110. Vergnes L, Baroukh N, Ostos MA, Castro G, Duverger N, Nanjee MN, et al. Expression of human apolipoprotein A-I/C-III/A-IV gene cluster in mice induces hyperlipidemia but reduces atherogenesis. Arterioscler Thromb Vasc Biol. 2000;20(10):2267-74.
111. Ito Y, Azrolan N, O'Connell A, Walsh A, Breslow JL. Hypertriglyceridemia as a result of human apo CIII gene expression in transgenic mice. Science. 1990;249(4970):790-3.
112. Li H, Han Y, Qi R, Wang Y, Zhang X, Yu M, et al. Aggravated restenosis and atherogenesis in ApoCIII transgenic mice but lack of protection in ApoCIII knockouts: The effect of authentic triglyceride-rich lipoproteins with and without ApoCIII. Cardiovascular research. 2015.
113. Blankenhorn DH, Alaupovic P, Wickham E, Chin HP, Azen SP. Prediction of angiographic change in native human coronary arteries and aortocoronary bypass grafts. Lipid and nonlipid factors. Circulation. 1990;81(2):470-6.
114. Sacks FM, Alaupovic P, Moye LA, Cole TG, Sussex B, Stampfer MJ, et al. VLDL, apolipoproteins B, CIII, and E, and risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial. Circulation. 2000;102(16):1886-92.
115. Rodrigo E, Gonzalez-Lamuno D, Ruiz JC, Fernandez-Fresnedo G, Isla D, Gonzalez-Cotorruelo J, et al. Apolipoprotein C-III and E polymorphisms and cardiovascular syndrome, hyperlipidemia, and insulin resistance in renal transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2002;2(4):343-8.
116. Tas S, Abdella NA. Blood pressure, coronary artery disease, and glycaemic control in type 2 diabetes mellitus: relation to apolipoprotein-CIII gene polymorphism. Lancet. 1994;343(8907):1194-5.
117. Riwanto M, Rohrer L, Roschitzki B, Besler C, Mocharla P, Mueller M, et al. Altered activation of endothelial anti- and proapoptotic pathways by high-density lipoprotein from patients with coronary artery disease: role of high-density lipoprotein-proteome remodeling. Circulation. 2013;127(8):891-904.
118. Chivot L, Mainard F, Bigot E, Bard JM, Auget JL, Madec Y, et al. Logistic discriminant analysis of lipids and apolipoproteins in a population of coronary bypass patients and the significance of apolipoproteins C-III and E. Atherosclerosis. 1990;82(3):205-11.
119. Luc G, Fievet C, Arveiler D, Evans AE, Bard JM, Cambien F, et al. Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study. Etude Cas Temoins sur 'Infarctus du Myocarde. J Lipid Res. 1996;37(3):508-17.

120. Kavo AE, Rallidis LS, Sakellaropoulos GC, Lehr S, Hartwig S, Eckel J, et al. Qualitative characteristics of HDL in young patients of an acute myocardial infarction. Atherosclerosis. 2012;220(1):257-64.
121. Pollin TI, Damcott CM, Shen H, Ott SH, Shelton J, Horenstein RB, et al. A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. Science. 2008;322(5908):1702-5.
122. Tas S. Genetic predisposition to coronary heart disease and gene for apolipoprotein-CIII. Lancet. 1991;337(8733):113-4.
123. Salas J, Jansen S, Lopez-Miranda J, Ordovas JM, Castro P, Marin C, et al. The SstI polymorphism of the apolipoprotein C-III gene determines the insulin response to an oral-glucose-tolerance test after consumption of a diet rich in saturated fats. Am J Clin Nutr. 1998;68(2):396-401.
124. Guettier JM, Georgopoulos A, Tsai MY, Radha V, Shanthirani S, Deepa R, et al. Polymorphisms in the fatty acid-binding protein 2 and apolipoprotein C-III genes are associated with the metabolic syndrome and dyslipidemia in a South Indian population. J Clin Endocrinol Metab. 2005;90(3):1705-11.
125. Miller M, Rhyne J, Chen H, Beach V, Ericson R, Luthra K, et al. APOC3 promoter polymorphisms C-482T and T-455C are associated with the metabolic syndrome. Arch Med Res. 2007;38(4):444-51.
126. Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang XM, et al. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. N Engl J Med. 2010;362(12):1082-9.
127. Khetarpal SA, Zeng X, Millar JS, Vitali C, Somasundara AVH, Zanoni P, et al. A human APOC3 missense variant and monoclonal antibody accelerate apoC-III clearance and lower triglyceride-rich lipoprotein levels. Nat Med. 2017;23(9):1086-94.
128. Ferns GA, Stocks J, Ritchie C, Galton DJ. Genetic polymorphisms of apolipoprotein C-III and insulin in survivors of myocardial infarction. Lancet. 1985;2(8450):300-3.
129. van Hoek M, van Herpt TW, Dehghan A, Hofman A, Lieverse AG, van Duijn CM, et al. Association of an APOC3 promoter variant with type 2 diabetes risk and need for insulin treatment in lean persons. Diabetologia. 2011;54(6):1360-7.
130. Pallaud C, Sass C, Zannad F, Siest G, Visvikis S. APOC3, CETP, fibrinogen, and MTHFR are genetic determinants of carotid intima-media thickness in healthy men (the Stanislas cohort). Clinical genetics. 2001;59(5):316-24.
131. Crawford DC, Dumitrescu L, Goodloe R, Brown-Gentry K, Boston J, McClellan B, Jr., et al. Rare variant APOC3 R19X is associated with cardio-protective profiles in a diverse population-based survey as part of the Epidemiologic Architecture for Genes Linked to Environment Study. Circulation Cardiovascular genetics. 2014;7(6):848-53.

132. Han X, Wang T, Zhang J, Liu X, Li Z, Wang G, et al. Apolipoprotein CIII regulates lipoprotein-associated phospholipase A2 expression via the MAPK and NFkappaB pathways. Biology open. 2015;4(5):661-5.
133. Kawakami A, Aikawa M, Alcaide P, Luscinskas FW, Libby P, Sacks FM. Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells. Circulation. 2006;114(7):681-7.
134. C Z, Azcutia V Fau - Aikawa E, Aikawa E Fau - Figueiredo J-L, Figueiredo Jl Fau - Croce K, Croce K Fau - Sonoki H, Sonoki H Fau - Sacks FM, et al. - Statins suppress apolipoprotein CIII-induced vascular endothelial cell activation and monocyte adhesion. Eur Heart J. 2013;34(8):615-24.
135. Botteri G, Montori M, Guma A, Pizarro J, Cedo L, Escola-Gil JC, et al. VLDL and apolipoprotein CIII induce ER stress and inflammation and attenuate insulin signalling via Toll-like receptor 2 in mouse skeletal muscle cells. Diabetologia. 2017;60(11):2262-73.
136. Hoang A, Murphy AJ, Coughlan MT, Thomas MC, Forbes JM, O'Brien R, et al. Advanced glycation of apolipoprotein A-I impairs its anti-atherogenic properties. Diabetologia. 2007;50(8):1770-9.
137. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. Jama. 2002;287(3):356-9.
138. Que X, Hung MY, Yeang C, Gonen A, Prohaska TA, Sun X, et al. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. Nature. 2018;558(7709):301-6.
139. Zheng C, Azcutia V, Aikawa E, Figueiredo JL, Croce K, Sonoki H, et al. Statins suppress apolipoprotein CIII-induced vascular endothelial cell activation and monocyte adhesion. Eur Heart J. 2013;34(8):615-24.
140. Warnick GR, Benderson J, Albers JJ. Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. Clin Chem. 1982;28(6):1379-88.
141. Goldstein JL HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR BA, Sly WS, Valle D, eds, editor. The Metabolic and Molecular Bases of Inherited Disease. 8th ed ed. New York: McGraw-Hill Inc; 2001. p. 2863–913.
142. Prospective Studies C, Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, et al. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet. 2007;370(9602):1829-39.
143. Varret M, Rabes JP, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, et al. A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32. Am J Hum Genet. 1999;64(5):1378-87.
144. Sudhof TC, Goldstein JL, Brown MS, Russell DW. The LDL receptor gene: a mosaic of exons shared with different proteins. Science. 1985;228(4701):815-22.

145. Khan BV, Parthasarathy SS, Alexander RW, Medford RM. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J Clin Invest. 1995;95(3):1262-70.
146. Liao L, Starzyk RM, Granger DN. Molecular determinants of oxidized low-density lipoprotein-induced leukocyte adhesion and microvascular dysfunction. Arterioscler Thromb Vasc Biol. 1997;17(3):437-44.
147. Fogelman AM, Shechter I, Seager J, Hokom M, Child JS, Edwards PA. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. Proc Natl Acad Sci U S A. 1980;77(4):2214-8.
148. Chavakis E, Dernbach E, Hermann C, Mondorf UF, Zeiher AM, Dimmeler S. Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway. Circulation. 2001;103(16):2102-7.
149. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol. 2013;13(10):709-21.
150. Terasaka N, Yu S, Yvan-Charvet L, Wang N, Mzhavia N, Langlois R, et al. ABCG1 and HDL protect against endothelial dysfunction in mice fed a high-cholesterol diet. J Clin Invest. 2008;118(11):3701-13.
151. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med. 1977;62(5):707-14.
152. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. Jama. 1986;256(20):2835-8.
153. Gordon DJ, Rifkind BM. High-density lipoprotein--the clinical implications of recent studies. N Engl J Med. 1989;321(19):1311-6.
154. Nicholls SJ, Dusting GJ, Cutri B, Bao S, Drummond GR, Rye KA, et al. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. Circulation. 2005;111(12):1543-50.
155. Nicholls SJ, Cutri B, Worthley SG, Kee P, Rye KA, Bao S, et al. Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol. 2005;25(11):2416-21.
156. Feig JE, Rong JX, Shamir R, Sanson M, Vengrenyuk Y, Liu J, et al. HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. Proc Natl Acad Sci U S A. 2011;108(17):7166-71.
157. Xia P, Vadas MA, Rye KA, Barter PJ, Gamble JR. High density lipoproteins (HDL) interrupt the sphingosine kinase signaling pathway. A possible mechanism for protection against atherosclerosis by HDL. J Biol Chem. 1999;274(46):33143-7.

158. Navab M, Berliner JA, Subbanagounder G, Hama S, Lusis AJ, Castellani LW, et al. HDL and the inflammatory response induced by LDL-derived oxidized phospholipids. Arterioscler Thromb Vasc Biol. 2001;21(4):481-8.
159. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. Circ Res. 2004;95(8):764-72.
160. Nakajima K, Nakano T, Tokita Y, Nagamine T, Inazu A, Kobayashi J, et al. Postprandial lipoprotein metabolism: VLDL vs chylomicrons. Clin Chim Acta. 2011;412(15-16):1306-18.
161. Rahim A, Nafi-valencia E, Siddiqi S, Basha R, Runyon CC, Siddiqi SA. Proteomic analysis of the very low density lipoprotein (VLDL) transport vesicles. Journal of proteomics. 2012;75(7):2225-35.
162. Huo Y, Iadevaia V, Yao Z, Kelly I, Cosulich S, Guichard S, et al. Stable isotope-labelling analysis of the impact of inhibition of the mammalian target of rapamycin on protein synthesis. Biochem J. 2012;444(1):141-51.
163. Xie J, Van Damme P, Fang D, Proud CG. Ablation of elongation factor 2 kinase enhances heat-shock protein 90 chaperone expression and protects cells under proteotoxic stress. J Biol Chem. 2019;294(18):7169-76.
164. Liu R, Kenney JW, Manousopoulou A, Johnston HE, Kamei M, Woelk CH, et al. Quantitative Non-canonical Amino Acid Tagging (QuaNCAT) Proteomics Identifies Distinct Patterns of Protein Synthesis Rapidly Induced by Hypertrophic Agents in Cardiomyocytes, Revealing New Aspects of Metabolic Remodeling. Mol Cell Proteomics. 2016;15(10):3170-89.
165. Aviram M. Modified forms of low density lipoprotein and atherosclerosis. Atherosclerosis. 1993;98(1):1-9.
166. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302-10.
167. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 2007;115(10):1285-95.
168. Sugiyama S, Kugiyama K, Aikawa M, Nakamura S, Ogawa H, Libby P. Hypochlorous acid, a macrophage product, induces endothelial apoptosis and tissue factor expression: involvement of myeloperoxidase-mediated oxidant in plaque erosion and thrombogenesis. Arterioscler Thromb Vasc Biol. 2004;24(7):1309-14.
169. Quillard T, Araujo HA, Franck G, Shvartz E, Sukhova G, Libby P. TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. Eur Heart J. 2015;36(22):1394-404.
170. Jasmin SB, Pearson V, Lalonde D, Domenger D, Theroux L, Poirier J. Differential regulation of ABCA1 and ABCG1 gene expressions in the remodeling mouse hippocampus after entorhinal cortex lesion and liver-X receptor agonist treatment. Brain Res. 2014;1562:39-51.
171. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood. 2005;105(2):659-69.

172. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science. 1995;270(5234):296-9.
173. Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell. 1992;69(7):1227-36.
174. Ringseis R, Eder K. Fatty acids and signalling in endothelial cells. Prostaglandins Leukot Essent Fatty Acids. 2010;82(4-6):189-98.
175. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997;387(6633):569-72.
176. Zeng L, Mathew AV, Byun J, Atkins KB, Brosius FC, 3rd, Pennathur S. Myeloperoxidase-derived oxidants damage artery wall proteins in an animal model of chronic kidney disease-accelerated atherosclerosis. J Biol Chem. 2018;293(19):7238-49.
177. van Leeuwen M, Gijbels MJ, Duijvestijn A, Smook M, van de Gaar MJ, Heeringa P, et al. Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. Arterioscler Thromb Vasc Biol. 2008;28(1):84-9.
178. Downing LJ, Strieter RM, Kadell AM, Wilke CA, Brown SL, Wrobleski SK, et al. Neutrophils are the initial cell type identified in deep venous thrombosis induced vein wall inflammation. ASAIO J. 1996;42(5):M677-82.
179. Hayden JM, Brachova L, Higgins K, Obermiller L, Sevanian A, Khandrika S, et al. Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol. J Lipid Res. 2002;43(1):26-35.
180. Heery JM, Kozak M, Stafforini DM, Jones DA, Zimmerman GA, McIntyre TM, et al. Oxidatively modified LDL contains phospholipids with platelet-activating factor-like activity and stimulates the growth of smooth muscle cells. J Clin Invest. 1995;96(5):2322-30.
181. Kathagen A, Schulte A, Balcke G, Phillips HS, Martens T, Matschke J, et al. Hypoxia and oxygenation induce a metabolic switch between pentose phosphate pathway and glycolysis in glioma stem-like cells. Acta Neuropathol. 2013;126(5):763-80.
182. Xu J, Dang Y, Ren YR, Liu JO. Cholesterol trafficking is required for mTOR activation in endothelial cells. Proc Natl Acad Sci U S A. 2010;107(10):4764-9.
183. Raposo HF, Paiva AA, Kato LS, de Oliveira HC. Apolipoprotein CIII overexpression exacerbates diet-induced obesity due to adipose tissue higher exogenous lipid uptake and retention and lower lipolysis rates. Nutr Metab (Lond). 2015;12:61.
184. Sundaram M, Curtis KR, Amir Alipour M, LeBlond ND, Margison KD, Yaworski RA, et al. The apolipoprotein C-III (Gln38Lys) variant associated with human hypertriglyceridemia is a gain-of-function mutation. J Lipid Res. 2017;58(11):2188-96.

185. Abe Y, Kawakami A, Osaka M, Uematsu S, Akira S, Shimokado K, et al. Apolipoprotein CIII induces monocyte chemoattractant protein-1 and interleukin 6 expression via Toll-like receptor 2 pathway in mouse adipocytes. Arterioscler Thromb Vasc Biol. 2010;30(11):2242-8.
186. Jensen MK, Aroner SA, Mukamal KJ, Furtado JD, Post WS, Tsai MY, et al. High-Density Lipoprotein Subspecies Defined by Presence of Apolipoprotein C-III and Incident Coronary Heart Disease in Four Cohorts. Circulation. 2018;137(13):1364-73.
187. Koga M, Yamauchi A, Kanaoka Y, Jige R, Tsukamoto A, Teshima N, et al. BMP4 is increased in the aortas of diabetic ApoE knockout mice and enhances uptake of oxidized low density lipoprotein into peritoneal macrophages. Journal of inflammation (London, England). 2013;10(1):32.
188. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes. 2000;49(2):157-62.
189. Valleix S, Verona G, Jourde-Chiche N, Nedelec B, Mangione PP, Bridoux F, et al. D25V apolipoprotein C-III variant causes dominant hereditary systemic amyloidosis and confers cardiovascular protective lipoprotein profile. Nat Commun. 2016;7:10353.
190. Onda M. Recombinant immunotoxins with low endotoxins for clinical and animal studies. Methods Mol Biol. 2012;907:627-43.
191. Moreno PR, Fuster V. New aspects in the pathogenesis of diabetic atherothrombosis. J Am Coll Cardiol. 2004;44(12):2293-300.
192. Li H, Han Y, Qi R, Wang Y, Zhang X, Yu M, et al. Aggravated restenosis and atherogenesis in ApoCIII transgenic mice but lack of protection in ApoCIII knockouts: the effect of authentic triglyceride-rich lipoproteins with and without ApoCIII. Cardiovasc Res. 2015;107(4):579-89.
193. Liu M, Chung S, Shelness GS, Parks JS. Hepatic ABCA1 deficiency is associated with delayed apolipoprotein B secretory trafficking and augmented VLDL triglyceride secretion. Biochim Biophys Acta Mol Cell Biol Lipids. 2017;1862(10 Pt A):1035-43.
194. Yokoyama S. ABCA1 and biogenesis of HDL. J Atheroscler Thromb. 2006;13(1):1-15.
195. Assman GvE, A.; Brewer, HB, Jr. The metabolic and molecular bases of inherited disease. Scriver CB, AL.; Sly, WS.; Valle, D.; Childs, B.; Kinzler, KW.; Volkman, BF.,, editors., editors. New York: McGraw-Hill; 2001.
196. Merrick D, Sakers A, Irgebay Z, Okada C, Calvert C, Morley MP, et al. Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. Science. 2019;364(6438).
197. Samad F, Ruf W. Inflammation, obesity, and thrombosis. Blood. 2013;122(20):3415-22.

198. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004;114(12):1752-61.
199. Kang SJ, Mintz GS, Witzenbichler B, Metzger DC, Rinaldi MJ, Duffy PL, et al. Effect of obesity on coronary atherosclerosis and outcomes of percutaneous coronary intervention: grayscale and virtual histology intravascular ultrasound substudy of assessment of dual antiplatelet therapy with drug-eluting stents. Circ Cardiovasc Interv. 2015;8(1).
200. Kato M, Dote K, Naganuma T, Sasaki S, Ueda K, Okita M, et al. Clinical predictors of culprit plaque rupture assessed on intravascular ultrasound in acute coronary syndrome. Circ J. 2010;74(9):1936-42.
201. Arai T, Yamashita S, Hirano K, Sakai N, Kotani K, Fujioka S, et al. Increased plasma cholesteryl ester transfer protein in obese subjects. A possible mechanism for the reduction of serum HDL cholesterol levels in obesity. Arterioscler Thromb. 1994;14(7):1129-36.
202. Ashen MD, Blumenthal RS. Clinical practice. Low HDL cholesterol levels. N Engl J Med. 2005;353(12):1252-60.
203. Ramms B, Patel S, Nora C, Pessentheiner AR, Chang MW, Green CR, et al. ApoC-III ASO Promotes Tissue LPL Activity in Absence of ApoE-Mediated TRL Clearance. J Lipid Res. 2019.
204. Silver DL, Jiang XC, Tall AR. Increased high density lipoprotein (HDL), defective hepatic catabolism of ApoA-I and ApoA-II, and decreased ApoA-I mRNA in ob/ob mice. Possible role of leptin in stimulation of HDL turnover. J Biol Chem. 1999;274(7):4140-6.
205. Wang N, Chen W, Linsel-Nitschke P, Martinez LO, Agerholm-Larsen B, Silver DL, et al. A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. J Clin Invest. 2003;111(1):99-107.
206. Arakawa R, Tamehiro N, Nishimaki-Mogami T, Ueda K, Yokoyama S. Fenofibric acid, an active form of fenofibrate, increases apolipoprotein A-I-mediated high-density lipoprotein biogenesis by enhancing transcription of ATP-binding cassette transporter A1 gene in a liver X receptor-dependent manner. Arterioscler Thromb Vasc Biol. 2005;25(6):1193-7.
207. Liu H, Labeur C, Xu CF, Ferrell R, Lins L, Brasseur R, et al. Characterization of the lipid-binding properties and lipoprotein lipase inhibition of a novel apolipoprotein C-III variant Ala23Thr. J Lipid Res. 2000;41(11):1760-71.
208. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, et al. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. Proc Natl Acad Sci U S A. 2003;100(5):2730-5.
209. Vu-Dac N, Chopin-Delannoy S, Gervois P, Bonnelye E, Martin G, Fruchart JC, et al. The nuclear receptors peroxisome proliferator-activated receptor alpha and

Rev-erbalpha mediate the species-specific regulation of apolipoprotein A-I expression by fibrates. J Biol Chem. 1998;273(40):25713-20.
210. Peters JM, Hennuyer N, Staels B, Fruchart JC, Fievet C, Gonzalez FJ, et al. Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice. J Biol Chem. 1997;272(43):27307-12.
211. Nordestgaard BG, Varbo A. Triglycerides and cardiovascular disease. Lancet. 2014;384(9943):626-35.
212. Nicholls SJ, Tuzcu EM, Wolski K, Bayturan O, Lavoie A, Uno K, et al. Lowering the triglyceride/high-density lipoprotein cholesterol ratio is associated with the beneficial impact of pioglitazone on progression of coronary atherosclerosis in diabetic patients: insights from the PERISCOPE (Pioglitazone Effect on Regression of Intravascular Sonographic Coronary Obstruction Prospective Evaluation) study. J Am Coll Cardiol. 2011;57(2):153-9.
213. Austin MA, King MC, Vranizan KM, Krauss RM. Atherogenic lipoprotein phenotype. A proposed genetic marker for coronary heart disease risk. Circulation. 1990;82(2):495-506.
214. Reaven GM, Chen YD, Jeppesen J, Maheux P, Krauss RM. Insulin resistance and hyperinsulinemia in individuals with small, dense low density lipoprotein particles. J Clin Invest. 1993;92(1):141-6.
215. Packard CJ. Small dense low-density lipoprotein and its role as an independent predictor of cardiovascular disease. Curr Opin Lipidol. 2006;17(4):412-7.
216. Brunham LR, Kruit JK, Iqbal J, Fievet C, Timmins JM, Pape TD, et al. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J Clin Invest. 2006;116(4):1052-62.
217. Asztalos BF, Schaefer EJ, Horvath KV, Yamashita S, Miller M, Franceschini G, et al. Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. J Lipid Res. 2007;48(3):592-9.
218. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. Proc Natl Acad Sci U S A. 1997;94(23):12610-5.
219. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, Wang N. HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell Metab. 2008;7(5):365-75.
220. Azhar S, Reaven E. Scavenger receptor class BI and selective cholesteryl ester uptake: partners in the regulation of steroidogenesis. Mol Cell Endocrinol. 2002;195(1-2):1-26.
221. Nicholls SJ, Brewer HB, Kastelein JJ, Krueger KA, Wang MD, Shao M, et al. Effects of the CETP inhibitor evacetrapib administered as monotherapy or in combination with statins on HDL and LDL cholesterol: a randomized controlled trial. Jama. 2011;306(19):2099-109.

222. Tani M, Horvath KV, Lamarche B, Couture P, Burnett JR, Schaefer EJ, et al. High-density lipoprotein subpopulation profiles in lipoprotein lipase and hepatic lipase deficiency. Atherosclerosis. 2016;253:7-14.
223. Massey JB, Hickson D, She HS, Sparrow JT, Via DP, Gotto AM, Jr., et al. Measurement and prediction of the rates of spontaneous transfer of phospholipids between plasma lipoproteins. Biochim Biophys Acta. 1984;794(2):274-80.
224. Jiang H, Yazdanyar A, Lou B, Chen Y, Zhao X, Li R, et al. Adipocyte phospholipid transfer protein and lipoprotein metabolism. Arterioscler Thromb Vasc Biol. 2015;35(2):316-22.
225. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation. 2007;116(1):39-48.
226. Prevalence of abnormal lipid levels among youths --- United States, 1999-2006. MMWR Morbidity and mortality weekly report. 2010;59(2):29-33.
227. Kang I, Park M, Yang SJ, Lee M. Lipoprotein Lipase Inhibitor, Nordihydroguaiaretic Acid, Aggravates Metabolic Phenotypes and Alters HDL Particle Size in the Western Diet-Fed db/db Mice. Int J Mol Sci. 2019;20(12).
228. Chung BH, Segrest JP, Franklin F. In vitro production of beta-very low density lipoproteins and small, dense low density lipoproteins in mildly hypertriglyceridemic plasma: role of activities of lecithin:cholester acyltransferase, cholesterylester transfer proteins and lipoprotein lipase. Atherosclerosis. 1998;141(2):209-25.
229. Zeljkovic A, Vekic J, Spasojevic-Kalimanovska V, Jelic-Ivanovic Z, Bogavac-Stanojevic N, Gulan B, et al. LDL and HDL subclasses in acute ischemic stroke: prediction of risk and short-term mortality. Atherosclerosis. 2010;210(2):548-54.
230. Hussein H, Saheb S, Couturier M, Atassi M, Orsoni A, Carrie A, et al. Small, dense high-density lipoprotein 3 particles exhibit defective antioxidative and anti-inflammatory function in familial hypercholesterolemia: Partial correction by low-density lipoprotein apheresis. J Clin Lipidol. 2016;10(1):124-33.
231. Graham MJ, Lee RG, Bell TA, 3rd, Fu W, Mullick AE, Alexander VJ, et al. Antisense oligonucleotide inhibition of apolipoprotein C-III reduces plasma triglycerides in rodents, nonhuman primates, and humans. Circ Res. 2013;112(11):1479-90.
232. Alexander VJ, Xia S, Hurh E, Hughes SG, O'Dea L, Geary RS, et al. N-acetyl galactosamine-conjugated antisense drug to APOC3 mRNA, triglycerides and atherogenic lipoprotein levels. Eur Heart J. 2019.