GENETIC VARIATION AND STRUCTURE/FUNCTION STUDIES OF HUMAN APOLIPOPROTEIN A-IV IN RELATION TO ATHEROSCLEROSIS

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TO MY MOTHER AND FATHER.

DECLARATION

All of the work reported in this thesis is my own, or has been carried out as part of a collaboration in which I played a major part. All collaborative work has been acknowledged in the text (see page 312). No part of this thesis has been submitted for a degree, diploma or other qualification at any other University.

Wai-man R Wong

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PUBLICATIONS RESULTING FROM THIS THESIS

Full papers

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Wong,W.M., Stephens,J.W., Acharya,J., Hurel,S.J., Humphries,S.E., Talmud,P.J., 2004. The *APOA4* T347S variant is associated with reduced plasma TAOS in subjects with diabetes mellitus and cardiovascular disease. J. Lipid Res. 45, 1565-1571.

Submitted paper

Wai-man R. Wong, Andrew B Gerry , Wendy Putt , Jane L Roberts, Richard B. Weinberg, Steve E Humphries , David S Leake, Philippa J Talmud, 2005. Common variants of apolipoprotein A-IV differ in their ability to inhibit low density lipoprotein oxidation. Manuscript submitted to J. Lipid Res.

ABSTRACT

Apolipoprotein A-IV (apoA-IV) is an anti-atherogenic lipoprotein, involved in the reverse cholesterol transport pathway and postprandial triglyceride clearance. The aim of this PhD was to characterise further the function of apoA-IV.

Haplotype analysis of the *APOA5-A4-C3* gene cluster revealed that the *APOA4* T347S variant was associated with increased coronary heart disease (CHD) risk in the Northwick Park Heart II study [hazard ratio (HR) of 2.07 (95%CI 1.04 to 4.12)], independent of any effects on lipid levels. The S347 variant was also found to be associated with lower plasma apoA-IV levels (13.64+/-0.59 mg/dL) compared with carriers of the T347 allele (14.90+/-0.12 mg/dL, P=0.035) in the European Atherosclerosis Research Study I. Furthermore, amongst individuals with cardiovascluar disease (CVD) in the University College Diabetes and Cardiovascular Study, those homozygous for the S347 had significantly lower total anti-oxidant status compared to T347 carriers (31.2% ± 9.86 vs 42.5%±13.04, P=0,0024).

Functional studies indicated that the pro-atherogenic role of apoA-IV-S347 could be due to its reduced antioxidant activity. Compared to wild type apoA-IV, apoA-IV-S347 decreased the time of 50% conjugated diene formation ($T_{1/2}$) by 15% (P= 0.036) and apoA-IV-H360 increased $T_{1/2}$ by 18% (P= 0.046).

Potentially important residues of apoA-IV involved in LCAT activation were predicted by molecular modelling. Site directed mutagenesis was used to alter the polar face of helix 117-138 (R123Q, R134Q and N127R) imitating the three key arginine residues (R149, R153, R160) critical for apoA-I LCAT activation. On average the mutant apoA-IV proteins had reduced α-helicity compared to the wild type isoform in the lipid free state and limited differences in secondary structure once complexed with 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine. These mutations did not alter the ability of apoA-IV to activate LCAT, indicating that the three critical arginine LCAT activation motif of apoA-I does not reside within helix 117-138 of apoA-IV.

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ABBREVIATIONS

7K	7-ketocholesterol
[S]	Concentration of substrate (rHDL)
7α-OH	7α-hydroxycholesterol
7α-OOH	7α-hydroperoxycholesterol
7β-OH	7β-hydroxycholesterol
7β-OOH	7β-hydroperoxycholesterol
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABCA1	ATP-binding cassette transporter
AGE	Advanced glycation end products
ANOVA	Analysis of variance
Apo	Apolipoprotein
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
BHT	Beta-hydroxytoluene
BMI	Body mass index
bp	Base-pairs
BSA	Bovine serum albumin
CAD	Coronary artery disease
CAT	Cholesterol acyltransferase
CD	Circular dichroism
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
Cu^{2+}	Copper ion
DM	Diabetes mellitus
DMPC	1.2-dimyristoyl-sn-glycero-3-phosphatidylcholine
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EARS	European Atherosclerosis Research Study
EDTA	Ethylenediamine tetraacetic acid disodium salt
FA	Fatty acid
FFA	Free fatty acid
H1	Helix 1 (residues 117-138)
H2	Helix 2 (residues 146-161)
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol

HL	Hepatic lipase
HMW	High molecular weight
HR	Hazard ratio
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IEF	Isoelectric focusing
IL	Interleukin
IPTG	Isopropyl β-D-thiogalactose
IRE	Insulin response element
kb	Kilobase
kDa	Kilodalton
K_m	Michaelis constant
KO	Knockout
LAT	Lysolecithin acyltransferase
LCAT	Lecithin:cholesterol acyltranferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LRP	LDL receptor related protein
$LO^{\bullet}$	Alkoxyl radical
LOOH	Lipid hydroperoxide
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MI	Myocardial infarction
MLV	Multilamellar vesicles
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic acid
MW	Molecular weight
NIDDM	Non-insulin dependant diabetes mellitus
NPHSII	Northwich Park Heart Study II
nt	nucleotides
OD	Optical density
oligo	Oligonucleotide
OXLDL	Oxidised LDL
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PAPC	1-Palmitoyl-2-Arachidonoyl-*sn*-Glycero-3-Phosphocholine
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction

PDPC	l-Palmitoyl-2-Docosahexaenoyl-*sn*-Glycero-3-Phosphocholine
PEPC	l-Palmitoyl-2-Eicosapentaenoyl-*sn*-Glycero-3-Phosphocholine
PLPC	1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine
PLTP	Phospholipid transfer protein
POPC	1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
PUFA	Polyunsaturated fatty acid
REM	Relative Electrophoretic mobility
rHDL	Reconstituted high density lipoprotein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
S.E.M.	Standard error of the mean
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SR-A	Scavenger receptor A
SR-B1	Scavenger receptor B1
SSCP	Single strand conformation polymorphism
$T_{1/2}$	Time for 50% conjugated diene formation
TAOS	Total anti-oxidant status
TBS	Tris buffered saline
TBSTT	TBS-Tween-Triton
TEMED	N,N,N',N'-tetraethylethylenediamine
TG	Triglyceride
T_{max}	maximal absorbance at 234 nm
TRL	Triglyceride rich lipoprotein
UDACS	University College Diabetes and Cardiovascular Study
UTR	Untranslated region
UV	Ultra violet
V	Velocity
v/v	Volume/volume
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
V_{max}	Maximal velocity (maximum rate of reaction)
VNTR	Variable number tandem repeat
w/v	Weight/volume
WHO	World health organisation
WHR	Waist hip ratio
WT	Wild type

One and three letter codes for amino acids

A (ala) alanine	K (lys) lysine	T (thr) threonine
C (cys) cysteine	L (leu) leucine	V (val) valine
D (asp) aspartic acid	M (met) methionine	W (trp) tryptophan
E (glu) glutamic acid	N (asn) asparagines	Y (tyr) tyrosine
F (phe) phenylalanine	P (pro) proline	
G (gly) glycine	Q (gln) glutamine	
H (his) histidine	R (arg) arginine	
I (lle) isoleucine	S (ser) serine	

CHAPTER 1

CHAPTER 1: INTRODUCTION

1.1 LIPOPROTEIN METABOLISM

1.1.1 Overview

The most common lipids in the plasma are cholesterol (free and esterified), triglycerides (TGs) and phospholipids (PL). A wide variety of tissues make demands on the lipid pool. Cholesterol and phospholipids are important in the maintenance of the cell plasma membrane, while cholesterol is also a precursor to hormones and bile acids (Shepherd *et al* , 1999). TGs and fatty acids are a vital energy source for cardiac and skeletal muscle. Once absorbed in the intestine, the hydrophobic cholesterol and TGs are packaged into water soluble lipoprotein particles (Lewis, 1997), thus facilitating the transport of these insoluble compounds in the blood plasma to cells in a direct and regulated manner. Lipoproteins consist of a hydrophobic TG and cholesterol ester (CE) core surrounded by a hydrophilic shell of free cholesterol (FC), phospholipids and apolipoproteins (Rosseneu, 1992).

1.1.2 Classification and metabolism of lipoproteins

The various plasma lipoproteins are divided into five classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Rosseneu, 1992). Each lipoprotein class differ in density, size, electrophoretic mobility and composition (table 1.1). Lipoproteins cannot be synthesized and secreted from the liver or intestine without the appropriate apolipoproteins which are pivotal to lipoprotein structure. Apolipoproteins perform key functions in the metabolism of lipoproteins, some for

instance activate enzymes that are important in the covalent modification of lipids or act as receptor ligands. A summary of the various apolipoproteins involved in lipid metabolism are listed in table 1.2.

	Exogenous lipoproteins	Endogenous lipoproteins			
	CM	VLDL	IDL	LDL	HDL
Density (g/ml)	<0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	22-28	5-12
Electrophoretic mobility	0	Pre-β	"broad β"	β	α, Pre-β
Composition (%)					
TG	86	55	23	6	5
PL	7	18	19	22	33
CE	4	12	29	42	17
FC	2	7	9	8	5
Protein	2	8	19	22	40-55
Main apolipoproteins	AI, A-IV B-48,C,E,H	B-100,H C,E, A-V	B-100, C,E	B-100	A-I, A-II, H C,E, A-V
Source	Intestine	Liver	Lipolysis of VLDL	Lipolysis of VLDL, via IDL	Liver, intestine; Lipolysis of CM & VLDL

Table 1.1: Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins (Durrington , 1989; Feher and Richmond , 1997; van der Vliet *et al*, 2001).

Protein	Chromosome	MW (kDa)	Plasma concentration (mg/dl)	Function
ApoA-I	11	28.4	60-160	Activator of LCAT
ApoA-II	1	17.4 (dimer)	25-55	Activator of LCAT
ApoA-IV	11	46.0	15-37	Activator of LCAT
ApoA-V	11	40.9	0.1	Involved in TG metabolism, function undetermined
ApoB100	2	512.0	60-160	Ligand for LDLR
ApoB48	2	245.0	0-2	Receptor ligand
ApoC-I	19	6.6	3-10	Prevent receptor-mediated uptake, inhibitor of CETP and activator of LCAT
ApoC-II	19	9.0	1-6	Activator of LPL, can prevent apoE receptor-mediated binding of VLDL to LDLR, acts as an inhibitor of LCAT
ApoC-III	11	8.8	4-20	Inhibitor of LPL and HL
ApoC-IV	19	14	0.1	TG metabolism (exact role undetermined)
ApoE	19	35.0	2.5-5	Receptor ligand for Chylomicron remnants, inhibitor of LPL and activator of both LCAT and CETP
ApoH	17	50	20	TG clearance and blood coagulation
ApoJ	8	75	10-5	Cellular senescence, aging and cancer

Table 1.2: Summary of various human apolipoproteins involved in lipid metabolism (Jong *et al*, 1999; Li *et al*, 1988; Polz and Kostner, 1979; Pownall , 2004; Trougakos and Gonos, 2002; Weinberg *et al*, 2003).

1.1.3 Apolipoproteins

1.1.3.1 The amphipathic helix, a general feature of exchangeable apolipoproteins

The right handed α helix is formed by repetitive hydrogen bonds between CO and NH groups located 4 residues apart, creating a helix with a pitch of 3.6 residues per turn (varying between 3.5 and 3.7 on average) and a rise of 1.5Å and a radial rotation of 100° between residues (figure 1.1A). This helix tends to be right handed due to the presence of L amino acid residues (Creighton, 1996).

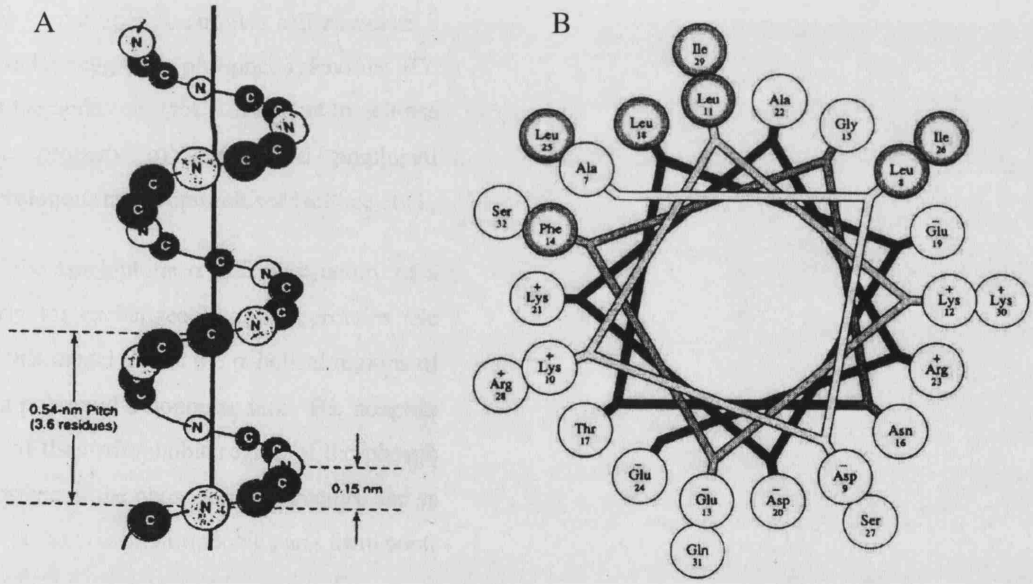


Figure 1.1 A: The classical right handed α-helix (Voet D and Voet JG , 1990). B: Schiffer-Edmundson helical wheel diagram of a well-defined class A amphipathic helix in apoC-I (Segrest *et al*, 1992).

An amphipathic α helix is a unique α helix containing opposing polar and non-polar faces oriented along the long axis of the helix, with hydrophobic and hydrophilic residues alternating every 3 or 4 residues (figure 1.1B) (Segrest *et al*, 1992). It is a versatile structure also found in toxins (Ladokhin and White, 1999) and hormones (Motta *et al*, 1998) not just apolipoproteins. Furthermore amphipathic α helices have been reported to be involved in inter and intra protein-protein interactions, playing essential roles as calmodulin binding sites (Leto *et al*, 1989) and in adheren junctions (Rudiger, 1998).

When exchangeable apolipoproteins are mixed with particular phospholipids, both spontaneously form lipid complexes; analysis by circular dichroism indicates a simultaneous increase of α helicity in apolipoproteins upon binding (Tall *et al*, 1975). The induction of amphipathic α helix formation by apolipoproteins has been postulated to be the mechanism by which they interact with phoshoplipid vesicles and other amphipathic surfaces. The charge distribution of the amphipathic α helice is important in initiating the association of apolipoprotein with the phospholipid surface. Amphipathic helices in apolipoproteins generally consist of a polar face comprising of negatively charged residues at the centre and positively charged residues at the periphery. Such a charge distribution allows the helices to 'snorkel' between the phospholipid head groups, permitting the nonpolar face to interact with the hydrophobic fatty acyl chains within phospholipids, so orientating the hydrophilic faces of the helix towards the aqueous solution (Kaiser and Kezdy, 1984; Segrest *et al*, 1994a). However there is some controversy of the mode by which amphipathic helices bind to lipid. The picket fence model (Phillips *et al*, 1997) proposes that short helical segments align themselves parallel

to the lipid acyl chains. But the widely regarded belt model (Segrest *et al*, 1999) of interaction suggests extended helices, as opposed to short helical segments form a continuous protective strip around the HDL disc and has been substantiated by crystallographic studies (Borhani *et al*, 1997).

The above properties of amphipathic helices facilitate the rapid reversible binding of apolipoproteins to lipid/water interfaces. This enables exchangeable apolipoproteins to effectively switch lipoprotein particles, allowing adjustments required for lipoprotein interactions with enzymes or receptors, thus aiding apolipoproteins to proficiently modulate lipoprotein metabolism.

1.1.3.2 Other features of the apolipoproteins

Apolipoproteins are lipid binding proteins involved in the transport of lipoprotein particles in the plasma. Defects in apolipoprotein structure or synthesis may result in disorders of lipid metabolism and the development of coronary artery disease (CAD) (Herbert *et al* , 1982). Apolipoproteins are synthesised in the rough endoplasmic reticulum and like other secreted proteins, they contain hydrophobic leader sequences that are cleaved post-translationally. These short prosequences present in the amino terminals of secreted apolipoproteins, A-I, A-II and CII contain the crucial elements found generally in numerous leader sequences, including an amino terminal methionine residue, a carboxyl terminal region and a central region rich in hydrophobic amino acids (Rosseneu, 1992). However, the physiological roles of these sequences that are cleaved in the plasma compartment have yet to be discovered.

Based upon their shared properties in solution, structural homology and sequence identity, apolipoproteins can be subdivided into 2 general groups, exchangeable and non-exchangeable (Segrest *et al*, 1994a).

1.1.3.3 Exchangeable apolipoproteins

To date the main human exchangeable apolipoprotein genes *APOE, APOA1, APOA2, APOA4, APOA5, APOC1, APOC2* and *APOC3* have been fully sequenced and cloned. Analysis of these genes has led to the speculation that they evolved via the sequential duplication of an ancestor of the apoCI gene, for they share similar gene organisation with many being situated in clusters together (Li *et al*, 1988; Luo *et al*, 1986). Except for *APOA4* which lacks the first intron, the other exchangeable apolipoprotein genes consist of four exons and three introns (Karathanasis *et al*, 1986). The first intron interrupts the 5' untranslated region, intron 2 interrupts the 3' untranslated region coding for the signal peptide, while intron 3 is within the main sequence coding for the mature protein (Rosseneu, 1992). A key feature of exchangeable apolipoproteins is the presence of 22-mer repeats (tandem array of two 11 mers) coding for amphipathic α helices. In apolipoproteins, these repeating units are often punctuated by helix breakers such as proline or glycine and connected by short peptide segments (hinges) (Fitch, 1977). One of the most noticeable properties of exchangeable apolipoproteins recently described is that in the lipid free form they self associate, forming twisted ribbon like fibrils structurally similar to amyloid fibrils (Bergstrom *et al*, 2001; Hatters and Howlett, 2002).

1.1.3.4 Non-exchangeable apolipoproteins

In contrast non-exchangeable apolipoproteins for example apolipoprotein B (apoB) do not possess such helices with regular proline repeats, but instead the structure of apoB is predominantly comprised of β-sheets (Schumaker *et al*, 1994). The β-sheets of apoB-100 are orientated parallel to the phospholipid monolayer of LDL, with amphipathic β strands contributing to the high affinity of apoB-100 for VLDL and LDL surfaces (Goormaghtigh *et al*, 1989; Segrest *et al*, 1994b). B apolipoproteins are exceptionally insoluble and therefore remain with the lipoprotein particle throughout its metabolism (Kane, 1983). In normal subjects apoB appears in two forms apoB-48 and apoB-100, containing 48% and 100% of the expression product of the normal gene respectively (Davidson and Shelness, 2000). ApoB-100 is exclusively synthesized in the liver and has an indispensable role in the hepatic assembly, secretion and clearance of triglyceride rich lipoproteins (TGRL) (Young, 1990). In fact apoB-100 is the major protein component of LDL and contains the domain required for the interaction with the LDL receptor (Young, 1990). ApoB-48 (amino-terminal 48% of apoB) is synthesised in the liver as well as the small intestine and circulates with and is essential for the assembly of chylomicrons (Hussain *et al*, 1996).

1.1.3.5 Lipoprotein metabolism overview

There are 3 major inter-linked pathways involved in lipoprotein metabolism, the endogenous (transport of hepatic fat), exogenous (transport of dietary fat) and reverse cholesterol transport pathway (Shepherd *et al* , 1999). These pathways are inter-

dependent and defects in one will affect the products and function of the others (Kwiterovich, 2000), the relationship of these pathways is depicted in figure 1.2.

1.1.3.6 The transport of exogenous (dietary) lipid

Dietary lipids are packaged into nascent chylomicrons within the intestinal epithelium via a process requiring apolipoprotein B48 (apoB48) and are secreted by enterocytes (Bisgaier and Glickman, 1983). As the chylomicrons travel through the lymph, apoA-IV and apoA-I are displaced by apoCs and apoE which are essential for chylomicron metabolism (Eisenberg, 1984). The triglyceride core of the chylomicrons are hydrolysed via lipoprotein lipase present on the endothelial surface of peripheral capillaries with apoC-II as a cofactor, yielding smaller chylomicron remnants. During the production of remnants any excess surface material (phospholipids, free cholesterol, apoAI and most of the apoCs) are transferred to HDL via the phospholipids transfer protein (PLTP) (van Tol, 2002), and cholesterol esters (CE) are exchanged for triglyceride with mature HDL by the cholesteryl ester transfer protein (CETP) (Tall, 1993). The products of triglyceride hydrolysis (free fatty acids and glycerol) are either acquired by adipose tissue for storage or delivered to muscle and liver tissue to fulfil metabolic requirements. Although both contain apoB-48 and apoE in comparison to nascent chylomicrons, chylomicron remnants are depleted in TGs and enriched with CE, and are removed primarily by the LDL receptor related protein (LRP) present on hepatic parenchymal cells with apoE as the preferential ligand (Shepherd *et al* , 1999). Thus the CEs, fat-soluble vitamins along with phospholipids and TGs are absorbed by the liver.

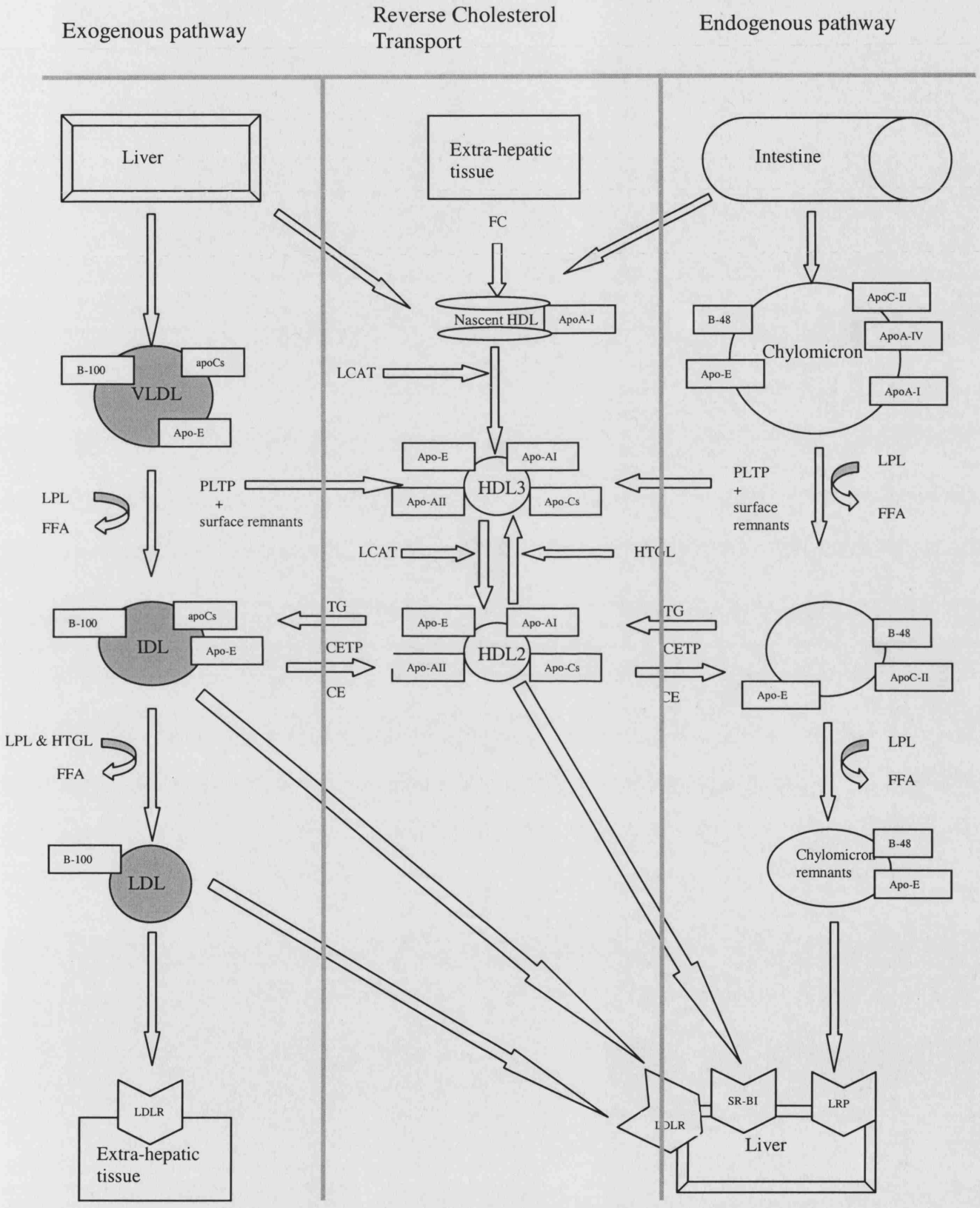


Figure 1.2: Diagram representing the three major inter-linked pathways of lipid metabolism.

1.1.3.7 The transport of endogenous (hepatic) lipid

Excess fatty acid surplus to oxidative requirements in the liver are esterified to produce TGs and are packaged together with cholesterol, CEs, phospholipids, apoB-100, apoCs and apoE into nascent VLDLs which are secreted into the blood. Once in the plasma the TG within VLDL is hydrolysed by LPL to form the cholesterol enriched intermediate density lipoprotein (IDL), resulting in the apoCs, most of the phospholipids and free cholesterol being transferred to HDL. IDL particles can be cleared by LDL receptors (LDLR) and LRP in the liver or subjected to further hydrolysis by hepatic lipase (HL), yielding LDL with the loss of apoE (Eisenberg and Levy, 1975; Feher and Richmond , 1997; Kwiterovich, 2000). CE enriched LDL are the main cholesterol carrying lipoproteins and are normally removed through the interaction of apoB-100 with LDLR present on the liver (Brown and Goldstein, 1986). The endogenous pathway is therefore a means by which fatty acids can be transported out of the liver to peripheral tissues for storage, where it can then be mobilised for use by critically dependent tissues, for example the heart during times of energy shortage (Lewis, 1997).

1.1.3.8 Reverse cholesterol transport (RCT)

RCT is the process enabling free cholesterol to be transported from extra-hepatic cells and tissues to the liver for secretion. The initial process of RCT involves the transfer of excess cholesterol to HDL (Fielding and Fielding, 1995) which can proceed via two mechanisms. Firstly via an aqueous diffusion mechanism, nascent discoidal HDL complexed with apoA-I accepts free cholesterol from the surface membranes of extra-hepatic cells and free cholesterol produced from the LPL-hydrolysis of chylomicrons and VLDL. Secondly, by the energy dependent transfer of cholesterol to lipid deficient

apolipoproteins via the ATP-binding cassette transporter (ABC)A1 or the cyclic adenosine monophosphate (cAMP) induced efflux of cholesterol to apolipoprotein acceptors (Fournier *et al*, 2000; Remaley *et al*, 2001; Smith *et al*, 1996). The cholesterol within the discoidal preβ HDL is then esterified by lecithin cholesterol acyl transferase (LCAT) to produce αHDL spheres (Nichols *et al*, 1985). CE from αHDLs can be transported to the liver directly by transferring the CE to IDL and LDL in exchange for TGs by CETP (Tall, 1993), these CE enriched lipoproteins can then be subsequently removed by hepatic receptors via LDLR. Alternatively the CE in HDL can also be selectively taken up by the scavenger receptor B1 (SR-B1) on the liver (Varban *et al*, 1998).

1.2 ATHEROSCLEROSIS

Coronary heart disease (CHD) is the major source of morbidity and mortality in most Western societies (Braunwald, 1997; Breslow, 1997). Atherosclerosis is the disease of large and medium sized arteries and is the pathological process underlying most of heart attacks and strokes. Atherosclerosis is principally caused by the narrowing of the coronary arteries, with lesions present mainly in large and medium sized elastic and muscular arteries. Atherosclerotic lesions may be present throughout a person's lifetime, with early 'fatty streak' lesions being present in infants (Napoli *et al*, 1997).

The most prominent hypothesis explaining the initiation and progression of atherosclerosis is the'response to injury hypothesis' (Ross, 1979), describing repeated cycles of vascular endothelial injury followed by repair promoting atherogenesis. The

intial step in atherogenesis according to this hypothesis is the physical disruption and denudation of the endothelial layer. This can be accelerated by factors including sheer stress, smoking and oxidative stress, all of which can induce a number of compensatory responses by the endothelium altering the normal homeostatic properties of the endothelial layer (Ross and Glomset, 1976), (Ross, 1986; Ross, 1999). More recently modified LDL, principally oxidised LDL (OXLDL) has been considered to be integral to the atherogenic process. OXLDL can upregulate adhesion molecules and chemotactic protein expression within the endothelium, resulting in increasing amounts of monocytes and lymphocytes entering the vessel wall (Berliner *et al*, 1990), (Cushing *et al*, 1990; Cybulsky and Gimbrone, 1991). The recruited monocytes within the intimal layer differentiate into activated macrophages, secreting growth factors and cytokines. OXLDLs are recognised and internalised via the scavenger receptors SR-A and CD36 by macrophages, leading to foam cell formation (Cushing *et al*, 1990; Febbraio *et al*, 2000; Suzuki *et al*, 1997). The continued accumulation and formation of foam cells within the intima results in fatty streak formation, the first lesion of atherosclerosis.

Atherosclerosis is also considered to be an inflammatory process (Ross, 1999). Cytokines and growth factors secreted by macrophages and T-cells promote smooth muscle cell (SMC) migration, proliferation and extracellular matrix production to form an intermediate lesion (Lusis, 2000). The continued recruitment of foam cells leads to the thickening of the arterial wall which then compensates by gradual dilation, thus up to a point the lumen dimensions remain unaltered, a phenomenon known as remodelling (Glagov *et al*, 1987). Further inflammation results in the continued influx of macrophages

and lymphocytes. Activation of these cells results in the release of proteolytic enzymes, cytokines, chemokines, and growth factors causing additional damage and necrosis (Lusis, 2000). The repeated accumulation of mononuclear cells and the migration and proliferation of smooth muscle cells, results in enlargement and restructuring of the lesion. In turn an advanced lesion is created consisting of a core of lipid and necrotic tissue covered by a fibrous cap (Thompson *et al*, 1995). By this time the bulk of the atheroma becomes too large for the artery to compensate and ultimately encroaches on lumen blood flow (Libby, 1995). If the lesion grows too large and restricts blood flow, the patient may experience coronary ischemia. Advanced plaques can also become unstable, consequently leading to rupture due to endothelial erosion or plaque disruption, this can be followed either by plaque repair or clot formation, generating a new fibrous cap with atheroma enlargement. Once the atherosclerotic plaque reaches a critical size it often becomes occlusive, resulting in a heart attack or stroke depending on the location of the plaque (Davies, 1995; Davies, 2000).

1.2.1 The traditional risk factors for CHD

CHD is a multifactorial disorder and the concept of cardiovascular risk factor originates from the Framingham prospective study (Kannel *et al*, 1961). Using a prospective epidemiological approach the Framingham study revealed consistent, significant associations of risk factors in healthy individuals with the subsequent manifestations of CHD in the same people. Risk factors were subsequently defined as measurable traits that would predict the future development of disease. In particular Framingham demonstrated that a clustering of risk factors for instance smoking, obesity, elevated lipid levels and

blood pressure predisposes an individual to a high risk of developing CHD. Risk factors can be divided into firstly, those which are modifiable such as cholesterol, LDLC, decreased HDLC, smoking, hypertension and obesity. Secondly, into those that are non-modifiable which include family history, age and male gender.

1.2.1.1 Non-modifiable risk factors

1.2.1.1.1 Family history

Family history over the years has been described to be a significant independent risk factor for CHD, even when all known risk factors are controlled for (Goldbourt and Neufeld, 1986). Family history reflects a combination of both shared genetic and environmental factors. Genetic factors are particularly important in the aetiology of early onset CHD; most early CHD events (below the age of 40) appear mainly in families with a positive hereditary history of CHD in first degree relatives below the age of 65 (Pohjola-Sintonen *et al*, 1998). Furthermore analysis of data from the family-based Health Family Tree Study has revealed that 72% of early onset CHD patients and 86% of early stroke sufferers had a positive family history (Koski *et al*, 2000; Williams *et al*, 2001).

1.2.1.1.2 Age

The risk of CHD has been demonstrated to increase with age, regardless of sex (Stamler *et al*, 1999). A reason for this relationship could be due to the increasing number of risk factors acquired and the length of time exposed to them overtime.

1.2.1.1.3 Gender

Below the age of 60, men develop CHD at more than twice the rate compared to women (Nathan and Chaudhuri, 1997). Hormonal factors seem to play an important role. Various epidemiological studies and clinical trials have demonstrated that the gender difference in CHD risk is strikingly reduced after menopause with oestrogen replacement therapy reducing CHD risk by approximately 50% (Barrett-Connor, 1995; Beard *et al*, 1989; Kafonek, 1994).

1.2.1.2 Modifiable risk factors

1.2.1.2.1 Smoking

Cigarette smoking is a major independent CHD risk factor and is known to double the life-time risk of CHD (Doll and Hill, 1966). Middle aged smokers have up to a 10 fold increase in the risk of sudden cardiac death and approximately a 3.6 fold increased risk of myocardial infarction (MI) (Kannel and McGee, 1984). Nonetheless the risk of CHD can be markedly reduced once smoking is stopped (Kuller *et al*, 1991), with excess risk of MI falling by 50% within the first 2 years (Rosenberg *et al*, 1985), (Cook *et al*, 1986). Smokers also have raised plasma fibrinogen and plasminogen levels, resulting in a procoagulant state with an increased risk of arterial thrombosis (Meade *et al*, 1986). There are many mechanisms explaining how smoking could modulate CHD risk. Smoking induced oxidative stress on plasma lipoproteins is thought to cause endothelial damage, smokers additionally have reduced antioxidants and a concomitant increased level of OXLDL (Heitzer *et al*, 1996a; Heitzer *et al*, 1996b) and F2-isoprostanes

(Morrow *et al*, 1995). Smoking is also associated with abnormal lipid profiles consisting of increased LDL and triglyceride levels and lower HDL levels (Craig *et al*, 1989; Cuesta *et al*, 1989; Dullaart *et al*, 1994).

Smoking can also modulate the effects of certain genetic factors influencing the risk of CHD, for example the risk of CHD is increased three fold in male smokers carrying the E4 allele of *APOE* compared to non-smokers (Humphries *et al*, 2001).

1.2.1.2.2 Diet

The process of atherogenesis can be exaggerated by the intake of excess cholesterol and TGs (Jackson *et al*, 1984; Kritchevsky, 1999; Schonfeld *et al*, 1982). Saturated fatty acids are the most important determinants of increased LDLC levels, while in contrast monounsaturated and polyunsaturated fats lower LDLC (Grundy, 1986).

1.2.1.2.3 Obesity

The risk of CHD in obese men and women is approximately three times greater (Seidell *et al*, 1996), with subjects having greater central obesity at particularly high risk (Nielsen and Jensen, 1997) due to the association with raised lipid levels, hypertension and an increased rate of non-insulin dependent diabetes mellitus (NIDDM) (Garrison *et al*, 1996). These aforementioned risk factors could explain why obesity increases the rate of CHD (Schulte *et al*, 1999), despite data from the Nurses Health Study, the Framingham

Heart Study provides evidence that obesity is in fact an independent risk factor for CHD (Hubert *et al*, 1983; Rexrode *et al*, 1996).

1.2.1.2.4 Plasma lipid levels

Epidemiological studies have consistently demonstrated a link between raised total cholesterol (TC) levels in the blood and atherosclerosis. The Multiple Risk Factor Intervention Trial (MRFIT) demonstrated a continuous positive relationship between serum cholesterol and mortality rates due to CHD. In MRFIT participants with TC levels $\geq$ 240mg/dl were found to have a relative risk for CHD death of 3.4 compared to those with TC < 182 mg/dL (Stamler *et al*, 1986). Despite these consistent associations, elevated serum TC alone is not actually an adequate predictor of coronary risk. The TC distribution curves for CHD and disease free patients shows considerable overlap between levels of 150mg/dL and 300mg/dL, producing a 'grey area' of risk (Castelli, 1988). Hence additional factors influence the development of CHD, with numerous CHD patients having TC within the normal range. Gofman *et al* were the first to actually separate serum lipoproteins and notice raised levels of particular lipoproteins to be atherogenic (Gofman and Lindgren, 1950).

Over the years it has been established that LDL-C is the most atherogenic lipoprotein, and that raised levels of LDL-C is associated with increased risk of CHD. Aggressive LDL-C reduction using statins have been successful in the primary and secondary prevention of CHD, significantly benefiting mortality and morbidity (LIPID Study Group, 1998; Sacks *et al*, 1996; Shepherd *et al*, 1995; The Scandinavian Simvastatin

Survival Study (4S), 1994). Nonetheless, risk assessment limited to LDL-C levels fails to identify a substantial proportion of individuals at risk of CHD. Analysis of data from the Prospective Cardiovascular Münster (PROCAM) study indicates that TG and HDL-C levels are important determinants of risk, irrespective of LDL-C levels (Assmann, 2001). A ratio of TC:HDL-C is also considered to be a more accurate indicator of CHD risk (Haq *et al*, 1999). It is now known that the atherogenicity of LDL in most individuals is not due to its cholesterol content, but due to its small size, increased density and particle number (Austin *et al*, 1990; Griffin *et al*, 1994). Small dense LDL compared to its larger counterpart is more readily oxidised (see chapter 6). Furthermore, because of its reduced size it can penetrate the arterial wall more readily and due to an enhanced affinity for proteoglycans has a prolonged residence time in the subendothelial space (Anber *et al*, 1996).

TG levels have now emerged as an independent risk factor for CHD due to the extensive meta-analysis by Hokanson and Austin. Based on data from 17 prospective population-based studies, elevated TG was associated with approximately a 30% increase in CHD risk in men and a 75% increase in women (Hokanson and Austin, 1996). This effect remained statistically significant, although adjustments for HDL and other risk factors attenuated these risks.

Moderately raised TG is associated with an atherogenic lipoprotein phenotype, reduced levels of HDL and a greater proportion of small dense LDL (Austin *et al*, 1990). The inverse relation between HDL-C levels and CHD has been consistently confirmed in numerous studies (Assmann and Schulte, 1992; Gordon *et al*, 1977). HDL possesses many anti-atherogenic properties, for instance HDL down-regulates the expression of adhesive molecules on the surface of vascular endothelium and has anti-oxidant properties. In contrast triglyceride rich lipoproteins (TRLs) including VLDLs and chylomicron remnants are atherogenic, because they are able to penetrate into and become trapped within the arterial wall where they are taken up by macrophages (Hodis, 1999; Ooi and Ooi, 1998).

In summary; an abnormal lipid profile is commonly indicative of increased CHD risk, hence treatment of lipid disorders is useful in reducing atherosclerosis. The World Health Organization system (WHO) have characterised the common hyperlipidaemias by lipoprotein profile based upon the Fredrickson classification (Fredrickson *et al*, 1967), summarised in table 1.3.

Phenotyping of Hyperlipoproteinaemias		
WHO phenotype	Dyslipidaemia	Diagnosis
I: raised chylomicrons	TGs > 10.0mM	Familial LPL deficiency or heterozygous LPL mutations (Soutar, 1998).
IIa: raised LDL.	Mainly hypercholesterolaemia	Familial defective apoB (Myant, 1993), familial and polygenic hypercholesterolaemia (due to defective LDL-R) (Soutar, 1998).
IIb: raised VLDL and LDL.	Hypercholesterolaemia + hypertriglyceridaemia: TGs 2.0-10.0 mM	Combined hyperlipidaemia, genetic factors such as variants in the *LPL*, *CETP*, *APOC1* and *C3* have been implied (Durrington, 2003).
III: raised chylomicrons remnants and IDL.	Hypercholesterolaemia + hypertriglyceridaemia: TGs 5.0-20.0 mM (cholesterol 7.0-12.0mM)	Type III hyperlipoproteinaemia: apoE ε2 homozygosity present in 90% of cases (Durrington, 2003).
IV: raised VLDL	TGs > 1.5mM are unhealthy	Familial or sporadic hypertriglyceridaemia; generally due to mutation in the LPL gene (McDonnell *et al*, 2003) but occasionally due to *APOC2* deficiency (Breckenridge *et al*, 1978).
V: raised chylomicrons and VLDL	TGs > 10.0mM	Familial LPL deficiency or heterozygous LPL mutations (Soutar, 1998).

Table 1.3: The various phenotypes of Hyperlipoproteinaemias.

1.2.1.3 Diabetes Mellitus and CHD

Diabetes mellitus (DM) is a major risk factor for the development of CHD, as documented by several epidemiological and pathological studies (Castelli *et al*, 1986; Wilson, 1998; Wilson *et al*, 1998). There are two classifications of DM, type 1 DM (insulin dependent) and type 2 DM (non-insulin dependent). Approximately 90% of the global DM population is comprised of type 2 diabetics (Quinn, 2002), patients with diabetes have a two to four fold risk of developing CHD and stroke compared to non-diabetics. Furthermore, diabetics have a risk of myocardial infarction (MI) and death from CHD identical to that of a non-diabetic person with a previous MI (Davis *et al*, 1999; Haffner *et al*, 1998).

Diabetes itself has been suggested to be a risk factor for cardiovascular disease (CVD) (The National Cholesterol Education Program, 2001). In type 2 DM and other insulin resistant states, dyslipidemia is the most significant CVD risk factor. Diabetic dyslipidemia is an intricate constellation of abnormalities; the main characteristics of diabetic dylipidaemia are hypertriglyceridaemia (increased VLDL-TG) and reduced HDL-C concentrations (Taskinen, 2002). Type 2 diabetes has been associated with a higher prevalence of small dense LDL in both men and women (Feingold *et al*, 1992; Selby *et al*, 1993) although LDL-C levels are not usually significantly increased in diabetic patients (Mykkanen *et al*, 1991; Taskinen, 2002). Small dense LDL is considered to be a marker for CVD and has been associated with endothelial dysfunction (particularly in DM patients) (Tan *et al*, 1999), in addition to being an independent determinant of intima-media thickness (Skoglund-Andersson *et al*, 1999) and an

independent predictor of CHD in men (Lamarche *et al*, 1997). Normal lipid concentrations are therefore more atherogenic in DM patients because of changes in the composition within lipid particles.

Small dense LDL is mainly produced in DM by the following mechanism: as a result of increased triglyceride rich lipoproteins (TGRL), both chylomicrons and VLDL have increased residence times within the circulation. The consequence of this is that the net increase transfer of CE via CETP results in TG rich LDL which can be hydrolysed by HL, leading to the formation of small dense LDL (Griffin, 1997).

Small dense LDL is particularly atherogenic because of a variety of biochemical mechanisms. The rate of infiltration of serum lipoproteins across the endothelium and into the subendothelial space is directly proportional to particle size (Nordestgaard and Nielsen, 1994), therefore small dense LDL have a greater propensity for uptake by arterial tissue compared to other LDL particles (Bjornheden *et al*, 1996). Futhermore, small dense LDL particles also have reduced affinity to LDLR and increased retention within the subendothelial space due to the preferential binding to proteoglycans (Anber *et al*, 1996; Camejo *et al*, 1985; Galeano *et al*, 1994). According to the response-to-retention hypothesis (Williams and Tabas, 1995) lipoprotein retention is the inciting event of atherosclerosis with the subendothelial retention of lipoproteins provoking a proinflammatory cascade resulting in lesion development. However lipoprotein retention only occurs at specific sites within the arterial tree characterised by the presence of apoB-retentive molecules like proteoglycans (Proctor *et al*, 2002). Small dense LDL is also

more susceptible to oxidation (Tribble *et al*, 1992), a process implicated in the formation of fatty streaks within the arterial intima. In addition to higher small dense LDL, diabetes is also associated with increased oxidative stress resulting in increased lipid peroxidation and hence atherosclerosis according to the oxidative modification hypothesis (section 1.2.1.4) (Amos *et al*, 1997; Davi *et al*, 1999).

The primary causal factor resulting in the pathophysiological alterations in the diabetic vasculature is the chronic exposure to high levels of glucose (Baynes and Thorpe, 2000). Experimental models of diabetes have demonstrated that exposure to cells in the vasculature to high glucose levels results in enhanced oxidant enzyme (NADPH oxidase) activity and superoxide production (Matsumoto *et al*, 2003). Both free and protein bound glucose are capable of undergoing modification (enzymatic and non-enzymatic) resulting in the formation of low molecular mass aldehydes such as glycoaldehyde (Wolff *et al*, 1991). These aldehydes can form adducts with free lysine or arginine residues producing advanced glycation end products (AGEs) (Knott *et al*, 2003) capable of initiating oxidative reactions promoting the formation of OXLDL (Knott *et al*, 2003). Similar glycation reactions can also occur both on the apoB and on phospholipid components of LDL, producing LDL with reduced affinity for the LDL receptor but increased affinity for non-specific scavenger receptors on macrophages encouraging foam cell formation (Bucala *et al*, 1995).

1.2.1.4 The oxidative modification hypothesis of atherosclerosis

The oxidative modification hypothesis of atherosclerosis is established on the idea that LDL in its native state is not atherogenic. However once oxidised, the subsequent OXLDL produced can contribute to atherogenesis via a whole range of biological mechanisms. Minimally modified LDL (MM-LDL) induces local vascular cells to produce chemokines, including monocyte chemotactic protein 1 (MCP-1) that stimulates monocytes recruitment and differentiation in arterial walls (Parhami *et al*, 1993a). The accumulating monocytes and leukocytes further promote lipid peroxidation of LDL, these inflammatory cells contain intracellular host defence enzymes, for instance myleoperoxidase producing diffusible radical species like hypochlorous acid (HOCL) which under certain circumstances can lead to oxidative stress and lipoprotein oxidation (Podrez *et al*, 2000). OXLDL can directly enhance the progression of atherosclerotic lesions. Firstly, OXLDL can chemotactically attract monocytes and stimulate the binding of moncytes to the endothelium (Frostegard *et al*, 1991; Quinn *et al*, 1988). Secondly, OXLDL is cytotoxic to cells in the vasculature promoting the release of lipids and lysosomal enzymes into the intimal space (Frostegard *et al*, 1991; Quinn *et al*, 1988). Evidence in support for the oxidative modification hypothesis indicates that OXLDL occurs *in vivo* and contributes to the development of atherosclerosis. OXLDL has been isolated in atherosclerostic lesions (Yla-Herttuala *et al*, 1989b), products of lipid oxidation have been detected in lesions (Heinecke, 1998) and plasma concentrations of immunoreactive OXLDL are higher in subjects with acute MI than in normal subjects (Holvoet *et al*, 1995). For further details on the oxidation of LDL please turn to chapter 6.

1.2.1.4.1 Oxidative stress

The state of oxidative stress corresponds to an imbalance between the rate of reactive oxygen species (ROS) production and endogenous antioxidant protection or ROS degradation. Over the years oxidative stress has been extensively studied resulting in suggestions that free radical-mediated damage induces a variety of pathologies, including atherosclerosis, cancer, neurodegenerative diseases and a whole host of other diseases (Sorg, 2004). Free radicals are molecules containing at least one unpaired electron, so are highly reactive and can promote electron transfers (oxidation and reduction). ROS has been postulated to enhance the process of atherogenesis via a plethora of mechanisms. Exposure of endothelial cells to ROS such as O_2^- and H_2O_2 induces apoptosis (Dimmeler and Zeiher, 2000), which according to the response to injury theory initiates atherogenesis (Ross and Glomset, 1973). ROS has been shown to induce the expression of adhesion molecules for example vascular cell adhesion molecule-1 (VCAM-1), leading to monocyte adhesion and atherosclerotic lesion development (Marui *et al*, 1993). Elevated ROS and subsequent lipid peroxidation results in the generation of a number of potentially atherogenic products, including reactive aldehydes for instance 3-formyl-3,4-dehydropiperidino (FDP) lysine generated during the oxidation of LDL (Uchida, 2000). These aldehydes can interact with OXLDL apoB, leading to the recognition and the internalisation of OXLDL particles by the scavenger receptors of macrophages and the subsequent formation of foam cells (Steinberg, 1995; Uchida *et al*, 1998).

1.3 APOLIPOPROTEIN A-IV

Little is known about the functions of apoA-IV. The main aim of my thesis was to further investigate this.

1.3.1 *APOA4* gene

The *APOA4* gene has three exons, encoding 396 amino acids which are 162, 127 and 1180 nucleotides in length separated by two introns (beginning with the nucleotides GT and ending with AG) of 357 and 777 nucleotides (Karathanasis *et al*, 1986). In humans the *APOA4* gene has been mapped to the long arm of chromosome 11 (11q23-q24) within the *A1/C3/A4/A5* cluster (Karathanasis, 1985; Pennacchio *et al*, 2001). The *APOA4* gene is transcribed in the same direction as *APOA1/A5* and opposite to *APOC3*, as illustrated in figure 1.3.

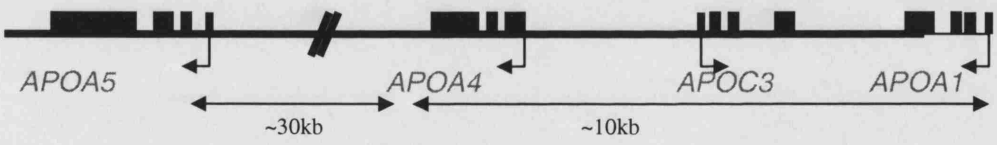


Figure 1.3: The *APOA-I/C3/A4/A5* cluster, the arrows represent the direction of transcription.

1.3.2 ApoA-IV protein

ApoA-IV is a 46kDa plasma glycoprotein and the largest member of the exchangeable apolipoprotein family (Luo *et al*, 1986; Weinberg and Scanu, 1983). In humans apoA-IV is primarily synthesised in intestinal enterocytes during lipid absorption, following co-translational processing the protein is secreted in association with chylomicrons (Hayashi *et al*, 1990). When apoA-IV enters the circulatory system, its association, distribution,

function, and metabolism can be influenced by other apolipoproteins, enzymes and lipid transport reactions (Sun *et al*, 2001). The majority of researchers have reported apoA-IV concentrations to be between 14 and 37 mg/dl, essentially depending upon the nutritional state of the subjects examined (Beisiegel and Utermann, 1979). Humans chronically consuming high fat diets develop significantly elevated plasma apoA-IV concentrations disappearing over time (Weinberg *et al*, 1990a). Due to the various methods of measurement and antibodies used, absolute values for apoA-IV concentrations vary between different studies (Bisgaier *et al*, 1985; Gordon *et al*, 1984; Utermann and Beisiegel, 1979; Weinberg and Scanu, 1983). Reports regarding the distribution of apoA-IV in human plasma are inconsistent. A wide variety of apoA-IV distributions have been observed ranging from apoA-IV existing entirely on HDL (Lagrost *et al*, 1989a) to being unassociated with major lipoprotein fractions (Beisiegel and Utermann, 1979; Bisgaier *et al*, 1985; Dieplinger *et al*, 1992; Duverger *et al*, 1993; Green *et al*, 1980; Malmendier *et al*, 1991; von Eckardstein *et al*, 1995). These discrepancies are a result of the different procedures used to isolate apoA-IV containing lipoproteins. The use of harsh techniques like sequential ultracentrifugation may remove substantial amounts of apoA-IV from HDL, misleadingly increasing the observed amounts of lipoprotein-unbound apoA-IV. The most recently developed non-disruptive technique to separate apoA-IV from human plasma implies that apoA-IV is present in three fractions; lipid free apoA-IV (4%), apoA-IV associated with apoA-I (12%) and in a lipoprotein fraction containing only apoA-IV (84%) (Ezeh *et al*, 2003). Human apoA-IV has a half life of 18-27.5 hours (Green *et al*, 1980) and enters the plasma within chylomicrons where it is rapidly displaced by apoC and apoE (Green *et al*, 1979). The dissociated apoA-IV is either transferred to HDL or

contributes to the lipid 'free' fraction. Within the HDL subfraction, apoA-IV is carried by particles smaller than standard HDL_3 that are metabolically separate from the lipid free fraction (LFF) apoA-IV (Ghiselli *et al*, 1986), (Ohta *et al*, 1984). The ultimate fate of apoA-IV in both these fractions is final catabolism (Ghiselli *et al*, 1986). In humans, fat feeding significantly increases the intestinal content of apoA-IV and its secretion on chylomicrons, results in a 31% elevation of plasma apoA-IV concentration (Bisgaier *et al*, 1985; Green *et al*, 1979; Green *et al*, 1980; Green *et al*, 1982). The postprandial state can also alter the distribution of apoA-IV. Gel filtration of fasted samples reveals apoA-IV elutes in two peaks corresponding to HDL (24%) and LFF (76%). In contrast within postprandial samples apoA-IV elutes in 3 peaks, a minor peak (3%) associated with TGRL, a peak co-eluting with HDL (26%) and a LFF peak (71%) (Bisgaier *et al*, 1985), indicating that during the postprandial state apoA-IV could perhaps recirculate between TGRL and HDL.

1.3.2.1 The structure of apoA-IV

Analysis of the primary sequence of apoA-IV reveals a high content of 22 amino acid repeats punctuated by proline residues, predicted to form 13 amphipathic α helices responsible for the ability of apoA-IV to bind lipid (Boguski *et al*, 1985; Segrest *et al*, 1992; Weinberg and Spector, 1985a). However, the majority of these amphipathic α helices are significantly more hydrophilic compared to those present in other exchangeable apolipoproteins including apoA-I or apoE, making apoA-IV the most hydrophilic of the human exchangeable apolipoproteins (Weinberg, 1987). ApoA-IV also contains many atypical class A domains (A_4 domains) with less well defined class A

snorkel motifs (Segrest *et al*, 1992) prohibiting deep penetration into condensed lipid monolayers (Weinberg and Jordan, 1990). Consequently apoA-IV has a very labile affinity for lipid surfaces and can be displaced by other soluble apolipoproteins, a signature characteristic of apoA-IV (Segrest *et al*, 1992; Weinberg *et al*, 1992; Weinberg and Jordan, 1990; Weinberg and Spector, 1985a). Furthermore, apoA-IV readily undergoes self-association both *in vitro* and *in vivo* forming dimers even at low protein concentrations (Weinberg and Spector, 1985b).

1.3.2.2 Biophysical analysis of apoA-IV

Circular dichroism spectroscopy (CD) has been used to investigate the effect of phospholipid binding on the secondary structure of apoA-IV. The addition of phospholipid to 1μM of apoA-IV resulted in an increase in α-helicity from 43% to 83% at saturation, as measured by a progressive increase in mean residue ellipticity at 222 nm (Θ_{222}) (Weinberg and Jordan, 1990). The induction of α-helical structure is thought to provide the thermodynamic driving force stabilizing the association of apolipoproteins with lipid surfaces (Atkinson and Small, 1986; Morrisett *et al*, 1977; Osborne and Brewer, 1980). Nonetheless the binding of apoA-IV to lipid produced only a very small blue shift (3nm) in comparison to other apolipoproteins signifying a slight decrease in the accessibility of tryptophan, implying that apoA-IV is not deeply buried in the phospholipid surface monolayer (Weinberg and Jordan, 1990).

Spectropic studies have revealed the structural and lipid binding properties of apoA-IV are particularly sensitive to environmental changes. The transition mid point where a 50% reduction of apoA-IV mean ellipticity at 222nm occurs could be induced at 0.4M Guanidinium Chloride (GdmCl), whereas a GdmCl denaturation mid point is induced at much higher concentrations in other human apolipoproteins (Weinberg and Spector, 1985a). In accordance, apoA-IV was also found to be pH sensitive, displaying a substantial decrease in mean ellipticity at 222nm between pH 7.0 and pH 8.0 (Weinberg and Spector, 1985a). The binding of apoA-IV to TGRL particles was significantly reduced in the presence of GdmCl and at pH conditions higher than pH 6.0 (Weinberg and Spector, 1985a), implying that the forces preserving the secondary structure of apoA-IV in aqueous solution are weak. The sensitivity of apoA-IV to denaturation together with the fact that the hydrophilic, atypical class A amphipathic α-helices of apoA-IV prohibit deep penetration into condensed lipid monolayers (Segrest *et al*, 1994a; Weinberg, 1987; Weinberg *et al*, 1990b; Weinberg and Jordan, 1990; Weinberg and Spector, 1985a), indicates that a considerable portion of apoA-IV α-helices could be excluded from the interface at high pressure (Weinberg *et al*, 1992; Weinberg and Scanu, 1983).

ApoA-IV does not possess the typical general structural organisation shared by other exchangeable apolipoproteins such as apoA-I and apoE , with the amino terminal and central regions forming an organised helix bundle and the carboxy-terminal α-helices forming a separate less organised structure (Pearson *et al*, 2004; Saito *et al*, 2003). Instead apoA-IV lacks the C-terminal lipid binding and cholesterol efflux promoting

domains present in apoA-I or apoE, with the amphipathic α-helices of apoA-IV forming a single large domain similar to the N-terminal helical bundle domains of apoA-I and apoE (Pearson *et al*, 2004). In fact the C-terminus of apoA-IV actually hinders the ability of apoA-IV to bind to lipid and promote cholesterol efflux (Pearson *et al*, 2004).

1.3.3 ApoA-IV: postulated physiological functions

Numerous physiological functions for apoA-IV have been postulated, including a role in the absorption of dietary fat and fat soluble vitamins (Kalogeris *et al*, 1997; Ordovas *et al*, 1989). Ordovas *et al* reported plasma levels of essential fatty acids and vitamin A and E to be significantly lower in a patient with apoAI/CIII/AIV deficiency, compared to subjects deficient only in apoA-I and apoC-III (Ordovas *et al*, 1989).

ApoA-IV has been linked to TG metabolism, modulating the postprandial intravascular clearance of TGRL. Based on its interfacial properties, apoA-IV has been speculated to modulate the growth and size of chylomicrons (Weinberg *et al*, 2000; Weinberg, 2002). Elevated plasma apoA-IV levels have been found in hypertriglyceridemic patients (Lagrost *et al*, 1989b; Verges *et al*, 2001) and is a marker of abnormal postprandial lipemia (Verges *et al*, 2001). Recently apoA-IV has been suggested to modulate TGRL assembly and secretion, by perhaps physically interacting with apoB in the secretory pathway (Gallagher *et al*, 2004).

Other proposed roles for apoA-IV include involvement in the metabolism of HDL. *In vitro* studies have shown that it may function as a co-factor for LCAT, modulate the activity of LPL in the presence of apoC-II, enhance the activity of cholesterol ester transfer protein (CETP) and possibly play a role in the conversion of HDL subclasses (Barter *et al*, 1988; Goldberg *et al*, 1990; Main *et al*, 1996; Steinmetz and Utermann, 1985). A number of studies have indicated that apoA-IV could be involved in the efflux of cholesterol from extrahepatic tissues, due to the fact that "lipid free" apoA-IV is abundant in interstitial compartments (Fournier *et al*, 2000; Steinmetz *et al*, 1990). Furthermore apoA-IV has been implicated to function as a satiety factor in rats, but this has never been confirmed in human subjects (Fujimoto *et al*, 1992). Most of these actions are not unique and the precise role or roles of apoA-IV has yet to be determined.

1.3.4 ApoA-IV and atherosclerosis

In vivo studies carried out in transgenic mice propose an anti-atherogenic role for apoA-IV. Transgenic mice over expressing either human or mouse *APOA4* were protected against the formation of diet induced aortic lesions (Cohen *et al*, 1997; Duverger *et al*, 1996). Atherosclerosis was even impeded by the expression of human *APOA4* in *apoe -/-* mice that are hyperlipidemic and spontaneously develop atherosclerosis. Additional analysis revealed that the HDL particles in these animals were more efficient at promoting cholesterol efflux from hepatoma cells (Duverger *et al*, 1996), indicating apoA-IV could perhaps influence the anti-atherogenic properties of HDL.

There has been much research into the actual anti-atherogenic mechanism by which apoA-IV acts. *In vitro*, apoA-IV binds to peripheral cells such as macrophages and promotes cholesterol efflux with the formation of small HDL particles through its participation in reverse cholesterol transport (Fournier *et al*, 2000; Stein *et al*, 1986). These studies coincide with the observation by Kronenberg *et al* that low plasma apoA-IV levels are associated with CHD in men (Kronenberg *et al*, 2000), indicating that apoA-IV could be an anti-atherogenic factor in humans as well.

1.3.4.1 ApoA-IV an anti-oxidant

Human apoA-IV at a concentration below physiological conditions has been shown *in vitro* to inhibit transition metal and free radical mediated lipid peroxidation of very low density lipoprotein (VLDL) (Ferretti *et al*, 2002). The anti-oxidant properties of apoA-IV have been verified *in vivo* using *APOA4/apoe -/-* mice. These transgenic mice compared to *apoe -/-* littermates have reduced oxidative markers including reduced anti-OXLDL antibodies, with apoA-IV accumulating in damaged arterial walls demonstrating that apoA-IV could perhaps act as an *in situ* antioxidant (Ostos *et al*, 2001).

1.3.4.2 ApoA-IV an anti-inflammatory protein

The excess production of cytokines can induce lesion progression from an initial lesion (type I) to a complicated lesion (type VI) (Ross, 1999). Recently based upon a number of animal experiments, anti-inflammatory properties have been assigned to apoA-IV. Expression of human *APOA4* in *apoe-/-* mice significantly reduces Lipopolysaccharide (LPS) induced secretion of cytokines (TNF-α) by macrophages. Furthermore a

diminished cytokine production within atheroscerlotic plaques was observed, indicating a reduction in the secretion of cytokines from macrophages and lymphocytes within the intima (Recalde *et al*, 2004). Similarly the exogenous administration of human apoA-IV has also been found to significantly ameliorate dextran sulphate sodium (DSS) induced inflammatory disease in mice. This anti-inflammatory effect of apoA-IV was assigned to its ability to inhibit leukocyte rolling and adhesion on endothelial cells, by inhibiting P-selectin upregulation (Vowinkel *et al*, 2004).

1.3.5 *APOA4* genetic variation

Genetic polymorphisms are common variants which occur at a frequency of greater than 1% and can include insertions/deletions and single nucleotide polymorphisms (SNPs) (Talmud and Humphries, 2001). Although many common genetic polymorphisms of *APOA4* have been found among Caucasians (table 1.4 and figure 1.4), the wild type apoA-IV1 allele is by far the most common with a frequency in whites ranging between 0.88 to 0.95 and so is considered to be the parent allele (Lohse *et al*, 1990).

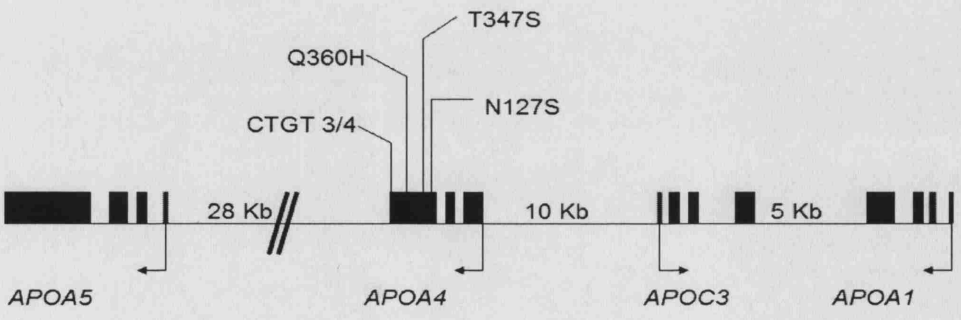


Figure 1.4: Map of the *APOAI/C3/A4/A5* gene cluster and the location of the common *APOA4* variants amongst Caucasians.

The initial analysis of apoA-IV using isoelectric focusing techniques revealed a common apoA-IV2 isoform, one charge unit more basic than the parent isoform (Menzel *et al*, 1982). Further investigation uncovered a guanine to thymine substitution within codon 360 causing a glutamine (CAG) to histidine (CAT) amino acid change at residue 360 (Q360H). This H360 allele has reported frequencies ranging from 0.03 to 0.10 in humans (Hixson and Powers, 1991; von Eckardstein *et al*, 1992; Zaiou *et al*, 1994).

Sequence analysis of *APOA4* has uncovered a range of common *APOA4* variants undetectable by isoelectric focusing techniques. These include an adenine to thymine substitution at codon 347, with observed frequencies of 0.15 to 0.22 (Fisher *et al*, 1999), producing a threonine to serine amino acid substitution at residue 347 (T347S) (Zaiou *et al*, 1992). Other variants found by sequencing include a polymorphism at nucleotide 1687 due to an adenine to guanine substitution, resulting in an asparagine to serine substitution at codon 127 (N127S). The frequency of the S127 allele has only been reported so far in healthy Finns at a frequency of 0.212 (Tenkanen *et al*, 1992). Another common *APOA4* gene variant characterised is the variable number of tandem repeat (VNTR) polymorphism in the non-coding region of exon three, due to a 4 base pair ($CTGT_{3/4}$) deletion/insertion with a reported rare allele frequency of 0.39 in Caucasians (von Eckardstein *et al*, 1992).

ApoA-IV allele	Mutation	Amino acid change	Allele frequency
1 (wt)	-	-	0.88 -0.95 (Lohse *et al*, 1990)
1A	ACT to TCT	Thr^{347} to Ser	0.12 (Saha *et al*, 1997)- 0.35 (Wang *et al*, 1997)
2	CAG to CAT	Gln^{360} to His	0.01-0.12 (Weinberg, 1999)
1B	AAC to AGC	Asp^{127} to Ser	0.212 (Tenkanen *et al*, 1992)
-	$CTGT_{3/4}$ VNTR	-	0.39 (von Eckardstein *et al*, 1992)

Table 1.4: Summarising the common *APOA4* variants in Caucasians

1.3.5.1 The association of *APOA4* polymorphisms with plasma lipid levels and CHD

The reported effects of the *APOA4* Q360H variant have been controversial. Some population studies have observed that carriers of the H360 allele have a favourable lipoprotein profile: increased HDL cholesterol (Menzel *et al*, 1988; Menzel *et al*, 1990), lower LDL cholesterol (Ehnholm *et al*, 1994; Ganan *et al*, 2004), reduced fasting TGs (Eichner *et al*, 1989; Hanis *et al*, 1991; Menzel *et al*, 1990), lower lipoprotein(a) (von Eckardstein *et al*, 1992; von Eckardstein *et al*, 1993) and increased apoA-I concentration (Hanis *et al*, 1991), while other studies have reported no significant associations at all (De Knijff *et al*, 1988; Hanis *et al*, 1991). These differences could be a result of the interactions between the apoA-IV H360 isoform and dietary factors within each of the diverse populations studied. Biophysical studies of the apoA-IV H360 isoform using techniques including circular dichroism and tryptophan florescence have revealed that compared to the parent isoform, apoA-IV H360 has a more compact and α-helical structure and perhaps greater lipid affinity (Weinberg *et al*, 1990b). These physical

properties could account for the increased residence time of apoA-IV H360 in the plasma (Rader *et al*, 1993).

The S347 allele is in strong negative linkage disequilibrium with the Q360 allele (Kamboh *et al*, 1992). A number of population studies have found the S347 allele to be associated with reduced total cholesterol, LDL/apoB levels (noted only in homozygotes) (Saha *et al*, 1997; von Eckardstein *et al*, 1992) and lower lipoprotein(a) levels. But similarly to the H360 allele some studies have reported no influence of the S347 allele on lipid or lipoprotein plasma levels (Larson *et al*, 2002; Miltiadous *et al*, 2002). Although biophysical studies have yet to be performed on the S347 isoform, computer predictions indicate the S347 isoform to have reduced lipid affinity (Lohse *et al*, 1991).

The N127S variant and its associations with lipid and lipoprotein levels have not been examined in any populations other than in a Finnish cohort. The polymorphism was found not to influence lipid or lipoprotein levels and the S127 allele distribution in hyperlipidemic men was found to be similar to that observed in the normal population (Tenkanen *et al*, 1992). Likewise with the VNTR polymorphism ($CTGT_{3/4}$), only a limited number of studies have been performed and no significant associations with lipid and lipoprotein levels have been reported (Groenendijk *et al*, 2001b; von Eckardstein *et al*, 1992).

Before carrying out the investigations for my thesis, despite the H360 allele being associated with a more atherogenic lipoprotein profile amongst Costa Ricans with an urban lifestyle (Campos *et al*, 1997), no associations of the common apoA-IV polymorphisms with CHD risk or MI had been published.

1.4 APPROACHES TO STUDYING THE GENETICS OF CORONARY HEART DISEASE

The main aims of genetic research into CHD include identifying disease causing mutations, hopefully leading to the development of DNA-based tests to identify subjects at risk and to gain further understanding into the pathological processes involved in CHD (Talmud and Humphries, 2002).

The two most common methods developed to identify disease causing mutations are the candidate gene approach and the genome wide indirect test of association method. The candidate gene approach is based upon using the phenotype of interest and the relevant biological pathway to choose various potential genes to be screened for polymorphisms. These variants are then tested for statistically significant associations with CHD. The genome wide indirect test of association method uses genotype data from densely packed markers that are evenly distributed across the genome to detect significant linkage disequilibrium with the disease locus. The gene(s) of interest can then be mapped and further analysed, the main limitation of this approach is that a large number of markers (~ 300,000) need to be genotyped (Kruglyak, 1999). The approach in the Humphries

laboratory over the last 20 years is the candidate gene approach and I have undertaken to examine *APOA4* as a candidate in CHD risk.

CHD is a complex mulifactorial disease involving a wide spectrum of pathophysiological pathways, comprising of many common genetic variants of modest effect interacting with other genes and environmental factors regularly making analysis complicated. The genetics of CHD susceptibility therefore does not conform to straightforward Mendelian monogenic inheritance, and so does not exhibit classical Mendelian recessive or dominant inheritance attributable to a single gene locus (Lander and Schork, 1994). From the many relevant pathophysiological pathways involved in CHD development, lipid metabolism was chosen and *APOA4* the candidate gene, based upon previous data proposing apoA-IV to be an anti-atherogenic protein (section 1.3.4). The association studies in this thesis were carried out in Caucasian cohorts with a greater prevalence of CHD.

Association studies assess the statistical correlation between DNA variants and disease on a population scale, investigating whether genetic variation within or close to the gene of interest is associated with inter-individual differences in the disease phenotype or increased risk of disease. To assess whether a disease associated gene variant is causal or is actually in linkage disequilibrium with another mutation else where, functional studies and haplotype analysis must be performed.

Case-control studies are commonly used, comparing the frequencies of polymorphic alleles of suitable candidate genes in unrelated affected and unaffected individuals from a population. Case-control studies are relatively cheap to perform with recruitment of subjects being straight forward, allowing for the precise characterisation of outcomes. However case-control studies are cross-sectional and are carried out at a single time point, therefore knowledge of events prior to the study is often limited. Because of this one can only state that there is a statistical association between the factor of interest (gene polymorphism) and disease, and not that the factor is likely to cause the disease. Due to the fact that these studies are cross-sectional it is also not possible to estimate the incidence of disease or trends over time. Another option is the use of prospective cohort studies which are longitudinal, following a sample of individuals over time, these studies are useful in studying gene-environment interactions because they measure exposure before disease onset. In addition using prospective studies one can identify genes that influence risk and so detect individuals at high risk whom can be targeted for therapy. Both a case-control and prospective strategy were used in this thesis.

The essential problem that case-control and prospective studies face is the lack of reproducibility. Early association studies tend to overestimate the effect of the genetic polymorphisms on disease with many associations failing to be reproduced at all (Ioannidis *et al*, 2001). This has been because many of these studies in the past have failed to have adequate numbers of cases to be sufficiently powered to examine disease genetics. Similarly to environmental risk, relative risks arising from genetic variation may be small, therefore performing association studies with modest sample sizes could lead to

the overestimation of the size of genetic effects. Furthermore, small association studies are prone to random error often producing significant conclusions with wide confidence intervals (Cardon and Bell, 2001). Failure of replication can also occur if the polymorphism analysed is not the causal variant but is rather in linkage disequilibrium (LD) with the causal variant. LD is dependent upon population history and on the genetic make-up of the founders of that population, hence it is possible that a polymorphism could be in LD with a nearby disease allele in one population but not another (Hirschhorn *et al*, 2002). An additional reason for inconsistent findings could be that gene-gene or gene-environment interactions differ between populations, variants that only manifest their effects in populations with a particular genetic or environment background can only be identified in populations or subgroups with the appropriate genetic or environmental characteristics (Redden and Allison, 2003).

To overcome these discrepancies it has been proposed that statistically significant associations should be reproduced in three independent samples (Humphries and Donati, 2002). Another useful strategy is the use of meta-analysis to evaluate results from a multitude of studies performed, producing a quantitative estimate of the genetic risk factor and disease (Ioannidis *et al*, 2001).

An alternative method to study complex diseases is linkage analysis, where the whole genome is scanned in a number of families with a history of the disease identifying chromosomal regions linked to the disease. Linkage analysis involves the construction of a transmission model explaining the inheritance pattern of the disease, based upon the comparison of the observed segregation of gene markers and trait in affected pedigrees. Linkage is an ideal method to study simple monogenic Mendelian traits because the allowable models are few and easily tested, nevertheless the application of linkage techniques to analyse complex mulitfactorial conditions such as CHD is problematic. Genetic analysis can be confounded by factors such as incomplete penetrance, phenocopy, genetic heterogeneity, polygenic inheritance and by the fact that large multi-generational pedigrees are difficult to find, with misinheritances also possible (Lander and Schork, 1994). Linkage analysis has the ability to identify major gene effects but can be inefficient at detecting genes of modest effect. Nonetheless linkage analysis using 513 western families has located the risk of MI to a single region on chromosome 14 (Broeckel *et al*, 2002) although the exact gene (s) have yet to be determined. Another methodology to study complex diseases is the allele-sharing approach, involving the study of affected relatives (or siblings) in a pedigree to analyse how often identical copies of a chromosomal region are inherited from a common ancestor (Kurtz and Spence, 1993), one then tries to investigate whether the inheritance pattern of this chromosomal region is consistent with random Mendelian segregation. But this is again can be confounded by problems similar to all pedigree based studies.

1.5 HYPOTHESIS

Based upon previous studies proposing that apoA-IV is an anti-atherogenic protein, apoA-IV could be an important determinant of CHD risk. Therefore it is hypothesised that genetic variation in the *APOA4* gene may directly influence the risk of CHD, due to the different anti-atherogenic potentials of the various apoA-IV isoforms.

1.6 AIMS AND OBJECTIVES

1.6.1 Genetic studies to deduce the possible association of *APOA4* with the risk of CHD

To ascertain whether *APOA4* gene variants influence CHD risk, the initial aim of my thesis was to first screen the *APOA4* promoter for genetic variation and this was achieved by single strand conformation polymorphism (SSCP) analysis. Once completed my further task was to genotype any novel and published common variants of *APOA4* detected by PCR and restriction enzyme digestion or heteroduplex generation in the Kronenberg, European Atherosclerosis Research Study (EARS) and the University College Diabetes and Cardiovascular Study (UDACS) case-control studies, containing valuable measurements including apoA-IV plasma levels, lipid parameters and oxidative stress measurements. Consequently these variants were also analysed using the prospective Northwick Park Heart Study II (NPHSII) to calculate the risk of CHD directly and perform survival analysis based upon *APOA4* genotype.

1.6.2 Functional studies to deduce the mechanisms by which any *APOA4* variants could directly influence CHD risk

Due to the strong linkage disequilibrium (LD) across the *APOAI/C3/A4/A5* gene cluster, it is vital to determine whether *APOA4* has functional variants, or whether any associations with *APOA4* polymorphisms and the risk of CHD merely reflects significant LD with functional polymorphisms in the other apolipoprotein genes. To gain further insight into apoA-IV and the possible underlying mechanisms behind any associations of the *APOA4* polymorphisms with the risk of CHD, functional studies were performed based upon the anti-oxidative properties of apoA-IV previously described (Ferretti *et al*, 2002; Ostos *et al*, 2001). The naturally occurring common apoA-IV isoforms were produced as recombinant apolipoproteins in *E.coli* and their ability to inhibit the copper mediated oxidation of LDL *in vitro* examined.

1.6.3 Structural/functional studies to further delineate the mechanism by which apoA-IV activates LCAT and therefore possibly promotes RCT

LCAT is postulated to be the engine for the anti-atherogenic RCT pathway (Jonas, 2000). ApoA-IV has been demonstrated to be a potent activator of LCAT and promote cholesterol efflux *in vitro* (Steinmetz et al, 1990; Fournier et al, 2000). To gain a further understanding of the mechanism by which apoA-IV achieves this, a number of predicted key residues within the LCAT activation of apoA-IV, mimicking the three arginine LCAT activation motif of apoA-I (Roosbeek et al, 2001) were altered and apoA-IV variants synthesised. Biophysical studies were first performed to assess the importance of

these residues on apoA-IV secondary structure and thermal stability, before their ability to activate LCAT *in vitro* was assessed.

CHAPTER 2

CHAPTER 2: MATERIALS AND METHODS

2.1 LABORATORY REAGENTS AND STOCKS

2.1.1 General reagents

All chemicals unless stated were analar grade and deionised water (dH_2O) was used throughout.

Table 2.1: General laboratory reagents

Name	Company of purchase
γ methacryloxy-propyl-trimethoxy-silane	Sigma
30% acrylamide/bisacrylamide 19:1	National diagnostics
2'-deoxyadenosine 5'-triphosphate dATP	Pharmacia
2'-deoxythymidine 5'-triphosphate dTTP	Pharmacia
2'-deoxyguanosine 5'-triphosphate dGTP	Pharmacia
2'-Deoxycytidine 5'-triphosphate dCTP	Pharmacia
3-[N-morpholino]propanesulphonic acid	Sigma
30% acrylamide/bisacrylamide 37.5:1	National diagnostics
Acetic acid (glacial)	Sigma
Agarose	Helena Biosciences
Ampicillin	Sigma
Ammonium hydrogen carbonate	BDH
Ammonium persulphate	Sigma
Bacto-tryptone	Sigma
Bacto-yeast	Sigma
Bacto-agar	Sigma
Beta-hydroxytoluene (BHT)	Sigma
Beta-Mercaptoethanol	Sigma
Biotinylated thrombin	Novagen
Boric acid	Sigma
Bovine serum albumin	GibcoBRL
Bromophenol blue	Sigma
BSA (Fatty acid free)	Sigma
Chelex-100®	Sigma
Chloroform	BDH
Cholesterol	Sigma
[3H]Unesterified cholesterol	Amersham Pharmacia
Coomassie brilliant blue R-250	GibcoBRL

Digitonin	Sigma
Dipotassium hydrogen orthophosphate	Fisher
Disodium hydrogen orthophosphate	Fisher
DMPC (1.2-dimyristoyl-sn-glycero-3-phosphatidylcholine)	Sigma
Ecoscint™ A	National diagnostics
Ethanol 99.7% (v/v)	BDH
Ethidium bromide	Sigma
Ethylenediamine tetraacetic acid disodium salt (EDTA)	BDH
Formamide	BDH
Gelatine	Sigma
Glycerol	BDH
Glycine	BDH
Guanidium Chloride	Fisher
Hydrochloric acid	BDH
Hydrogen peroxide	BDH
Imidazol	Fisher
IPTG (Isopropyl β-D-thiogalactoside)	Sigma
Isopopanol	BDH
Magnesium chloride	Fisher
Magnesium Sulphate	Fisher
Methanol	Fisher
N,N,N',N'-tetraethylethylenediamine (TEMED)	Sigma
Paraffin oil	BDH
Phenylmethylsulfonyl fluoride	Sigma
Phosphate buffered saline (PBS)	GibcoBRL
PLPC (1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine)	Avanti polar lipids
POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine)	Avanti polar lipids
Potassium chloride	Fisher
Potassium dihydrogen orthophosphate	Fisher
Potassium hydroxide	Fisher
Propanol	Fisher
Protogel®	National diagnostics
Sequagel XR	National diagnostics
Sodium acetate	BDH
Sodium azide	Sigma
Sodium chloride	BDH
Sodium Cholate	Sigma
Sodium dihydrogen orthophosphate	Fisher
Sodium dodecyl sulphate	BDH
Sodium hydrogen carbonate	BDH
Sodium hydroxide	Fisher
Sodium phosphate (monobasic, dehydrate)	Fisher
Streptavidin agarose	Novagen

Sucrose	Sigma
Tris	BDH
Triton X-100	BDH
Xylene cyanol	Sigma

2.2 EQUIPMENTS AND REAGENTS

All glass, plasticware and solutions were sterilised by autoclaving.

Table 2.2: Commonly used kits and consumables

Name	Company of purchase
1Kb ladder	Gibco
96 deep well Beckman arrays	Advanced Biotechnologies
96 well digest plate	Bibby Sterlin Ltd
96 well PCR plate	Costar
ABI Prism Bigdye™ terminator V3.1 sequencing kit (BD 3.1)	Applera
Alamo 4/40 PAA gels	VWR international
Amicon ultrapure MWCO 10kDa centrifugal filter	Millipore
BamHI restriction enzyme	NEB
BCA (bicinchoninic acid) protein assay kit	Perbio Science Ltd
Bio-Rad power pac 300	Bio-Rad
BL21 *E.coli*	Novagen
Consort E122 power pack	Consort
DH5α™ *E.coli*	Invitrogen
Dialysis tubing MWCO 12-14 Kda	Medicell international

Dialysis tubing, MWCO 100kDa (Spectra/por Biotech)	NBS biologicals
Econo-Pac™ column	Bio-Rad
Filter paper	Bio-Rad
Gel drying kit	Promega
GenElute™ high performance (HP)	Sigma
HincII restriction enzyme	NEB
HinfI restriction enzyme	NEB
High molecular weight markers	Amersham Biosciences
His-tag® Western blotting alkaline phosphatase (AP) reagents	Novagen
HiTrap™ desalting column (5x5ml)	Amersham
Hybond-C® extra (nitrocellulose) membrane	Amersham
LPE-SL18 18 well applicator	VWR
Microplate sealer	Greiner
Mini-transblott® cell	Bio-Rad
NdeI restriction enzyme	NEB
Ni-NTA His-bind™ resin	Novagen
Non-denaturing pre-cast 4-20% linear gradient Tris-HCL gel	Bio-Rad
Oligonucleotide primers	Invitrogen
Oligonucleotide primers (Hex and Fam)	Operon
Paragon Lipogels	Beckman
pET-14b expression vector	Novagen
Platinum® *PFX DNA* polymersase	Invitrogen

Prestained SDS-PAGE standards (broad range)	Bio-Rad
QuikChange™ site-directed mutagenesis kit	Stratagene
SE 250 mighty small tank	Hoefer scientific
T4 *DNA ligase*	Roche
Taq polymerase	Gibco
Thrombin cleavage capture kit.	Novagen
VGT1 mini-vertical gel unit	BDH
Vivaspin 2ml concentrators MWCO 5 kDa	Vivascience
Wako Phopsholipid B assay	Alpha laboratories Ltd
Wako free cholesterol C assay	Alpha laboratories Ltd
Wizard PCR prep Kit	Promega
XL1-Blue *E.coli*	Novagen

Table 2.3: Addresses of selected companies stated

Company	Address
ABI (applied biosystems)	Applied biosystems, Foster City, CA 94404, U.S.A www.appliedbiosystems.com/
Advanced Biotechnologies	Advanced Biotechnologies Ltd, Surrey, UK www.abionline.com
Alpha Laboratories	Alpha Laboratories Ltd, Hampshire, UK www.alphalabs.co.uk/
Amersham	Amersham Biosciences UK Ltd., Bucks., UK www.amershambiosciences.com
Applera	Applera Corporation, Norwalk, CT 06856-534 U.S.A www.applera.com
Avanti polar lipids	Avanti polar lipids, Alabaster, AL 35007, U.S.A www.avantilipids.com
BDH	BDH Chemicals Ltd., Poole, UK www.bdh.com
Beckman	Beckman Coulter,Buckinghamshire,UK www.beckman.com
Bibby Sterlin	Bibby Sterlin Ltd Staffordshire, UK www.bibby-sterilin.co.uk
Bio-Rad	Bio-Rad Laboratories Ltd., Hertfordshire, UK www.discover.bio-rad.com
Calbiochem	Merck Biosciences, Nottingham, UK www.merckbiosciences.com
Consort	Consort, Turnhout, Belgium www.scie-plas.com
Costar	Costar, New York, USA
Eppendorf	Fisher Scientific UK Ltd., Loughborough, UK www.eppendorf.com/uk/

Fisher	Fisher Scientific UK Ltd., Loughborough, UK www.fisher.co.uk
Greiner Bio-One	Greiner Bio-One Ltd., Gloucestershire, UK www.gbo.com
GibcoBRL	Life Technologies, Paisley, UK www.lifetech.com
Helena biosciences	Helena Biosciences Sunderland, Tyne and Wear, UK) www.helena.co.uk/
Hoefer scientific	Hoefer scientific, San Francisco, USA www.hoeferinc.com/
Invitrogen	Invitrogen Ltd., Paisley, UK www.invitrogen.com
Medicell International	Medicell International Ltd, London, UK www.medicell.co.uk
Millipore	Millipore Ltd, Gloucestershire, UK. www.millipore.com
MJ Research	MJ Reasearch, GRI, Essex, UK. www.gmi-inc.com/BioTechLab/mjresearchptc100.htm
National Diagnostics	National Diagnostics UK Ltd., Yorks, UK www.nationaldiagnostics.com
New England Biolabs	New England Biolabs Ltd, Herts, UK www.neb.com
New Brunswick Scientific (NBS)	NBS Biologicals Ltd, Huntingdon, U.K www.nbsbio.co.uk
Novagen	Novagen, Nottingham. UK www.novagen.com
Operon	Operon, California, USA www.operon.com
Perbio Science	Perbio Science Ltd, Northumberland, UK www.perbio.com

Qiagen	QIAGEN, West Sussex, U.K www1.qiagen.com/
Pharmacia Biosystems Ltd	Pfizer Ltd, New york, U.S.A www.pharmacia.com
Promega	Promega UK. Southampton, UK www.promega.com
Roche	Roche diagnostics ltd, East sussex, U.K www.roche-applied-science.com
Schleicher and Schuell	Dassek, Germany www.schleicher-schuell.com
Severn Biotech	Severn Biotech Ltd, Worcestershire, UK www.severnbiotech.com
Sigma	Sigma-Aldrich Company Ltd., Poole, Dorset, UK www.sigma-aldrich.com
Sorvall	Kendro Laboratory Products Plc., Herts, UK www.sorvall.com
Stratagene	Stratagene, Amsterdam, Netherlands www.stratagene.com
Thermohybaid	Thermohybaid, Hampshire, UK www.thermo.com
Vivascience	Vivascience, Hannover, Germany www.vivascience.com
VWR international	VWR international LTD, Poole, UK www.vwr.com

2.2.2 Commonly used stocks

4% Stacking gel mix: 6ml dH_2O, 1ml 30% w/v acrylamide:bisacrylamide (37.5:1), 1ml 1.87M Tris PH 6.6, 80μl 10% SDS, 10 μl TEMED and 40μl 10% (APS).

10% Protein gel mix: 7.2ml dH_2O, 5.6 mls 30% w/v acrylamide:bisacrylamide (37.5:1), 3.3ml 1.87M Tris pH6.6, 200μl 10% SDS, 15μl TEMED, 100μl 10% ammonium persulphate (APS).

Bind/wash buffer: 10mM imidazole, 6M guanidinium chloride, 50mM sodium phosphate buffer (PH 8.0) and 300mM NaCl.

Bromophenol blue, 1.25ml 1M Tris pH7.5 (1.25mM) and 5.25mls dH_2O.

Coomassie Blue: 0.1g Coomassie Blue R250, 50mls acetic acid, 125mls isopropanol and 325ml dH_2O.

Destain: 100ml methanol, 50ml ascetic acid and 850ml dH_2O.

Elution Buffer: 50mM imidazole, 6M guanidinium chloride (PH 8.0), 300mM NaCl, 50mM Sodium phosphate buffer.

Formamide loading dye: 95% formamide; 10mM EDTA; 0.025% bromophenol blue; 0.025% xlyenol cynol.

Gel drying Solution: 40% methanol, 10% glycerol, 7.5% acetic acid.

Loading buffer: 0.015% bromophenol blue; 0.015% xylene cyanol; 10% glycerol; 10mM EDTA.

L-Agar: 100ml of dH_2O, 10g Bacto-tryptone; 5g Bacto-yeast, 10g NaCl: 20g Bacto-agar.

L-Broth: 100ml of dH_2O, 10g Bacto-tryptone; 5g Bacto-yeast, 10g NaCl.

Lipostain: Sudan Black B in ethanol/water (45/55, v/v) as provided by Paragon.

LOOH assay colour reagent: 163mM potassium dihydrogen orthophosphate (KH_2PO_4), 120mM KI, 37mM dipotassium hydrogen orthophosphate (K_2HPO_4), Triton X-100 (2g/L), 150μM sodium azide (NaN_3), alkylbenzyldimethylammonium chloride (0.1g/L), 10μM ammonium molbdate, pH adjusted to 6.0-6.2. Prepared in fume cupboard and stored at 4°C in the dark.

Lysis buffer: 0.1M sodium phosphate buffer pH8, 1Mm phenylmethylsulfonyl fluoride, 6M guanidinium chloride).

MOPS buffer (Chelex-100®-treated): 10mM 3-[N-morpholino]propanesulphonic acid (MOPS), 150mM NaCl, pH 7.4. Chelex-100® (washed in distilled water to remove transition metal contamination) added at 1g/L, mixed overnight and removed by filtration prior to pH adjustment.

Nuclei lysis buffer: 10mM/L Tris-HCL pH 8.2, 400mM/L NaCl, 2mM/L EDTA, 1% SDS.

Polmix buffer: 500mM KCL; 100mM Tris-HCL PH8.3; 0.01% gelatine; 2mM dATP; 2mM dTTP; 2mM dGTP; 2mM dCTP.

Protein loading buffer: 2ml Glycerol, 1ml SDS (20%), 500μl β-mercaptoethanol, 0.01g bromophenol blue, 1.25ml 1M Tris pH7.5 and 5.25 dH_2O.

Running Buffer: 28.8g glycine, 6.2g Tris, 100ml 10% SDS and 1.9 L dH_2O.

Sequencing loading dye: 25ml 0.5M EDTA; 25g Ficoll; 0.0625g bromophenol blue and 175ml dH_2O.

SOB: 20g Tryptone, 5g yeast, 0.5g NACL, 10ml 1M MgCl2, 10ml MgSO4, 20ml glucose (20%) and 960mls dH_2O.

Sticky Silane: 0.5% v/v glacial acetic acid; 0.5% v/v γ methacryloxy-propyl-trimethoxy-silane.

Sucrose lysis buffer: 0.32M sucrose, 10mM/L Tris-HCL pH 7.5, 5mM/L $MgCl_2$, 1% (v/v) Triton X-100.

Sucrose Solution loading buffer: 0.25% Bromophenol blue, 0.25% Xylene cyanol and 40% sucrose.

TBE buffer: 0.04M Tris-borate; 1mM EDTA pH7.6.

TE buffer: 0.01M Tris-HCL; 1mM EDTA pH 7.6.

Transfer buffer: 12mM Tris-HCL pH 8.3, 96mM glycine, 20% (v/v) methanol.

Tris Buffer Saline (TBS) pH7.4: 6.005 g tris, 43.83g NaCl, 0.3g NaN_3, 0.25g EDTA-Na_2 and 5L dH_2O.

2.3 HUMAN GENETIC STUDIES

2.3.1 DNA extraction from blood, the 'salting out' method

Genomic DNA was isolated from potassium EDTA-anti-coagulated whole blood (stored at -20°C till use) via the salting out method by Miller *et al* (Miller *et al,* 1995). The salting out protocol involves cellular lysis using a sucrose lysis buffer and nuclear lysis with a nuclei lysis buffer containing 10% (w/v) sodium dodecyl sulphate (SDS). The DNA is finally ethanol precipitated out following deproteinisation.

Blood was thawed (at room temperature) and transferred to a 30ml centrifuge tube filled with 12ml cold (4°C) sucrose lysis buffer and mixed several times by inversion. After centrifuging the tubes at 1300g for 10 minutes at 4°C (Sorvall RC5C centrifuge using rotor SA-600), the supernatant was discarded and the pellet re-suspended in 12ml cold (4°C) sucrose lysis buffer and the sample was centrifuged again at 1300g.

The supernatant was discarded, 2ml nuclei buffer was added and the pellet mixed thoroughly using a pipette. 5M sodium perchlorate (1ml) was applied and the tube mixed by inversion followed by the addition of 2ml of cold (20°C) chloroform, the sample was again mixed by inversion and then centrifuged at 1300g for 5 minutes at room temperature. The upper aqueous phase was transferred to a clean 30ml centrifuge tube without disturbing the organic phase and 10ml cold (20°C) 100% ethanol was gently applied to the aqueous phase. The precipitated DNA on the interface appeared as a fine woolly network and was spooled off with a Pasteur pipette, washed in 70% ethanol and transferred to a sterile microtube containing 1ml TE buffer. To fully dissolve the DNA, it was incubated at 37 °C overnight.

To assess the DNA concentration, 20μl of the DNA solution was diluted into 1ml TE buffer and the absorbance was measured at 260nm (one optical density unit is equal to 50μg/ml of double stranded DNA). The stock DNA was diluted in dH_2O to a concentration of 20μg/ml, aliquoted into 96 deep well Beckman arrays and stored at 4°C.

2.3.2 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in a final volume of 20μl using 30ng of DNA. Each 20μl reaction contained 1X Polmix buffer, $MgCl_2$ (concentration differed per set of primers), 8 pmol of each oligonucleotide primer and 0.2U *Taq* polymerase. The reactions were performed in 96 well PCR plates, overlaid with 20μl paraffin oil and sealed with a microplate sealer to prevent evaporation.

1.5-3 μl (~10-20 ng/μl) of genomic DNA was transferred from a deep well Beckman arrays to 96 well PCR plates using a Finnpippette munltichannel (Life Sciences). The DNA was centrifuged briefly (185g) to ensure DNA was at the bottom of the well, and then dried at 80°C for 8 minutes using the PTC/225 Peltier thermocycler (MJ research UK). The PCR reaction mix was distributed evenly to all the wells via an automatic biohit dispenser (Alpha laboratories). Parrafin was subsequently added in the same way, before the PCR plate was again briefly spun (185g) and sealed with a microplate sealer.

2.3.3 Restriction enzyme digestion

The DNA samples were digested with restriction enzymes in the appropriate supplied buffers according to the manufacturer's protocol. The digestions were carried out in an incubator at the recommended temperature for the specific enzyme.

2.3.4 Agarose gel electrophoresis

Agarose was melted in a microwave oven to the appropriate concentration (1-2%, depending upon the PCR fragment size) in 1x TBE. Once the agarose had cooled to approximately 50°C, it was poured into a gel tray (10 x 14cm) with the suitable comb inserted and allowed to set at room temperature. DNA was run in the appropriate amount of loading buffer in 1x TBE at 100-150V. All DNA samples were analysed by staining with ethidium bromide (EtBr) (0.3mgs/L) post electrophoresis and visualised in a transilluminator box and photographed using the UVP Gel Documentation System.

2.3.5 Microplate array diagonal gel electrophoresis (MADGE) theory

MADGE adheres to the traditional methods of electrophoretic separation of DNA, based upon fragment size performed either in an agarose or polyacrylamide gel matrix. However compared to standard gels containing well forming combs it is a compact horizontal polyacrylamide gel (figure 2.1).

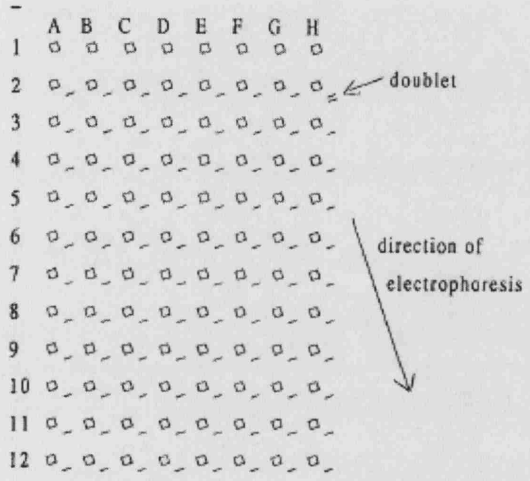


Figure 2.1: Schematic of a MADGE gel image. The squares represent wells and the lines represent bands in the lanes, adapted from Day *et al* (Day *et al*, 1999).

MADGE consists of an open faced "swimming-pool" (100x150mm), 2mm deep with 8x12 wells. Each well is at an angle of 71.6° to the row axis of the array, but perpendicular to the long axis of the perspex former (Day *et al*, 1999). The maximum track length is 26.5mm sufficient for most post-PCR analysis.

Using a multichannel DNA samples were directly transferred from a 96 PCR plate, stained with formamide loading dye and loaded into a MADGE GEL. Using ethidium bromide imaging in ultraviolet light, the DNA bands were illuminated. The protocol followed for the preparation of MADGE gel is summarised below.

1.) A glass plate (160 x 100 x 2mm) is cleaned with detergent followed by ethanol and dried.

2.) One side of the glass was glazed with "sticky silane" containing: glacial acetic acid (0.5% v/v), γ methacryloxy-propyl-trimethoxy-silane (0.5% v/v). This is imperative to enable the gel to adhere to the glass plate.

3.) A 6-7.5% MADGE gel solution (250ml) was then prepared from a 30% polyacrylamide stock solution (19:1 acrylamide:bis-acrylamide) containing 1X TBE buffer and distilled water. To initiate polymerization 150 μl of both NNN'N'-Tetramethylethylendediamine (TEMED) and 25% ammonium persulphate (APS) was added.

4.) The gel components were thoroughly mixed and poured into the centre of the gel former. The glass plate was placed gently on the top, coated side down, and a weight placed on top to maintain pressure between glass and the former. Once polymerisation was completed, the glass plate containing the attached gel was prised from the former. The gels on glass were then stored in 1 X TBE buffer at 4°C.

5.) Formamide loading dye (3 µl) was mixed with 10 µl PCR product and 8.5 µl of the mix was loaded directly onto the gel in columns of eight using a multichannel pipette. Gels were typically run at 100 V for 1-2 hours, depending on the difference in size of

the products to be resolved, and the DNA visualised by staining with EtBr either pre- or post-electrophoresis.

All MADGE gel eletrophoresis was carried out using the Consort E122 power pack.

2.3.6 Genotype analysis: PCR amplification of the *APOA4* gene

PCR primer pairs were designed from the published sequence of human *APOA4*, available from Genbank (accession n.o: J02758) and used to amplify regions of the *APOA4* gene. The primer sequence, annealing temperature, cycle number and $MgCl_2$ concentration for the various amplifications of exon 3 are reviewed in table 2.4. All reactions were carried out using the high throughput PCR protocol described in section 2.3.2.

2.3.7 Detection of polymorphisms, genotyping

Genotypes of the *APOA4* gene variants were determined by PCR amplification and restriction enzyme digestion. Alleles were assigned as 11 or 22 signifying the presence or absence of the cutting sites respectively. Table 2.4 summarises the restriction endonucleases used to digest PCR products.

Polymorphism	Forward (F) and Reverse (R) primer nucleotide sequence.	Annealing temp (Cycles), buffer, [$MgCl_2$]	Restriction enzyme	Fragment Size
T347S	(F) GGTCAACTCCTTCTTCAGCAC (R) AGGGGCAGCTCAGCTCTCCAA	69°C (30), Polmix, [1.5mM]	*Hinf I*	151bp
$CTGT_{(3/4)}$	(F) TGCACTGGCCCCACCCTCGT (R) CCAACTGGACATGTGTCCTCAAGTTCATA	60°C (29), Polmix, [1.5mM]	-	99/103bp (3/4 repeats)
N127S	(F) GGACAACCTGCGAGAGCTTCA (R) ATTCGTCAGCGTAGGGCGTAA	60°C (29), Polmix, [1.5mM]	*Hinc II*	248bp

Table 2.4: Primers and PCR conditions used to genotype *APOA4* for the T347S, $CTGT_{(3/4)}$ and N127S polymorphisms.

2.3.7.1 *HinfI* digestion of the T347S PCR product

The T347S PCR product was subjected to restriction enzyme analysis by digestion with 3 units of *HinfI* per 8 μl of PCR sample at 37 °C, overnight in the buffer recommended by the manufacturer. The products of restriction digestion were separated by MADGE, section 2.3.5.

2.3.7.2 *HincII* digestion of the N127S PCR product

The N127S PCR product was subjected to restriction enzyme analysis by digestion with 3 units of *Hinc II* per 8 μl of PCR sample at 37 °C, overnight in the buffer recommended by the manufacturer. The products were then analysed by MADGE, section 2.3.5.

2.3.7.3 Detection of the $CTGT_{(3/4)}$ polymorphism

The CTGT PCR products was separated and genotyped using polyacrylamide gel electrophoresis. Two plates were used to pour the gel, one larger plate (33 x 42 cm) and one smaller plate (33 x 39 cm). Before use, each plate was thoroughly cleaned using detergent, rinsed in dH_2O and 100% ethanol before being dried. The plates were placed together and separated by two 0.4mm spacers. The following gel mix (50ml) was made consisting of 17.5 % bisacrylamide:acrylamide (37.5:1) and 1 x TBE buffer. For polymerisation 115μl of both 25% APS and TEMED was added to the gel mix and thoroughly stirred before pouring. The gel mix was poured between the plates using a 50 ml syringe and left for two hours to ensure complete polymerisation before use.

2.3.7.4 Heterodulpex generation to determine simultaneously the *APOA4* T347S and Q360H genotypes

The heteroduplex method involves the PCR amplification of the region containing the two polymorphic sites (T347S and Q360H) within apoA-IV, which is then hybridised to a synthetic molecule called an induced heterodulplex generator (IHG). This results in the formation of heteroduplexes that can be separated on a non-denaturing polyacylamide gel and visualised using EtBr (Bolla *et al*, 1999; Li and Humphries, 2002).

The sequence of the primers used to PCR amplify the T347S and Q360H sites of the *APOA4* gene are summarised in table 2.5. The IHG was supplied by Nigel Wood (University of Bristol) and is homologous to the sequence being investigated, apart from two and three adenine insertions downstream of codon 347 (nucleotide 1150) and codon 360 (nucleotide 1191) respectively. The sequence of the IHG and its relation to primers used to PCR the relevant portion of the *APOA4* gene containing the polymorphic sites (T347S and Q360H) are illustrated in figure 2.2 adapted from Li, L.K *et al* (Li and Humphries, 2002).

Polymorphism	Forward (F) and Reverse (R) primer nucleotide sequence.	Annealing Temp (Cycles), buffer, [$MgCl_2$]	Fragment size
T347S and Q360H	(F) GGTCAACTCCTTCTTCAGCAC (R) AGGGGCAGCTCAGCTCTCCAA	51°C (35), Polmix, [1.5mM]	151bp

Table 2.5: The oligonucleotides and PCR conditions used to amplify the *APOA4* region containing both the T347S and Q360H polymorphic sites.

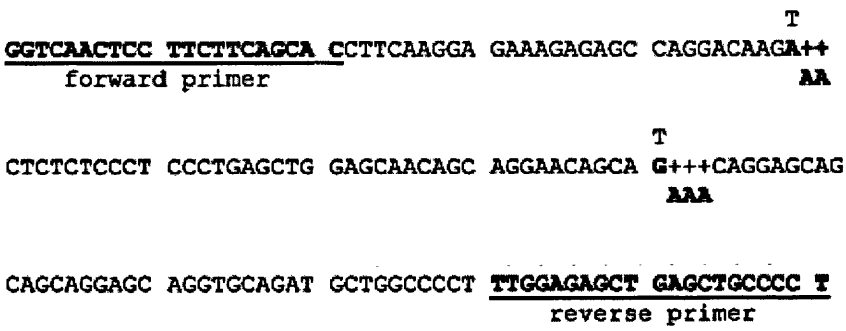


Figure 2.2: Illustrates the 5'to 3' nucleotide sequence of the 156bp *APOA4* IHG used with the PCR primers underlined. The two and three adenine insertions at nucleotides 1150 and 1191, downstream of codons 347 (A to T) and 360 (G to T) respectively within the IHG are represented by + symbols (Li and Humphries, 2002).

Each PCR reaction was carried out on 2.5μl of genomic DNA pipetted from a deep well array into a sterile 96 well PCR plate using a multi-channel pipette (Finnpipette; life Sciences, Basingstoke, UK). The PCR template was placed on a PCR block for 10 mins at 75 °C and to each dried DNA template a 20μl PCR mix was added. Each reaction consisted of 2μl 10 X polmix, .0.6μl 50mM $MgCl_2$, 0.8μl of each PCR primer, 16.12μl sterile distilled water and 0.12μl Taq DNA polymerase (5U/ml). Every well was overlaid with 20μl of paraffin oil, sealed with a microplate sealer and centrifuged for 1 minute at 1600g (Sorvall T60000B; DuPont, Newtown, CT). The IHG was amplified using 1μl of the 10^{-5} dilution of the IHG (figure 2.2) using the identical PCR reaction mixture listed above. Both the IHG and the genomic DNA were amplified separately in a MJ Tetrad peltier thermal cycler using the cycling conditions; 95 °C for 5 mins (1 cycle), 95 °C for 1 min, 51°C for 1 min, 72 °C for 1 min (35 cycles) and 72 °C for 5 minutes (1 cycle). Both PCR products were then run on a 2% agarose gel with a 1kB ladder.

Heteroduplexes were generated by mixing 5μl of IHG, 8μl of PCR product and 2μl of sucrose solution loading buffer in a clean microtitre plate, sealed with a microplate sealer and denatured on a PCR block at 94 °C for 10 mins. The samples were then allowed to cool for 15 mins, allowing renaturation and the formation of heteroduplexes. Each denatured sample (15μl) was separated on a non-denaturing 15% polyacrylamide gel (bisacrylamide:acrylamide (37.5:1) and 1 X TBE) poured in between 2 glass plates (33 x 42 cm and 33 x 39 cm in size) separated by two 0.4mm spacers via the same protocol used to produce the gels for $CTGT_{(3/4)}$ polymorphism detection (section 2.3.7.3). The gels were then run in 1 X TBE buffer at 130V for 3 hours at approximately 14 °C. Staining post-electrophoresis was carried out with 0.5μg/ml EtBr.

Following hybridisation of the IHG with the amplified *APOA4* fragment, a number of discriminatory DNA heteroduplex patterns for the T347S and Q360H *APOA4* alleles in homozygous and heterozygous combinations were generated. Each allele generates two different DNA heteroduplexes corresponding to the coding and non-coding DNA strands of the allele, with homozygotes generating two and heterozygotes four heteroduplex bands/patterns. These bands are clearly separated on a non-denaturing 15% polyacrylamide gel and visualised with 0.5μg/ml ethidium bromide (figure 2.3).

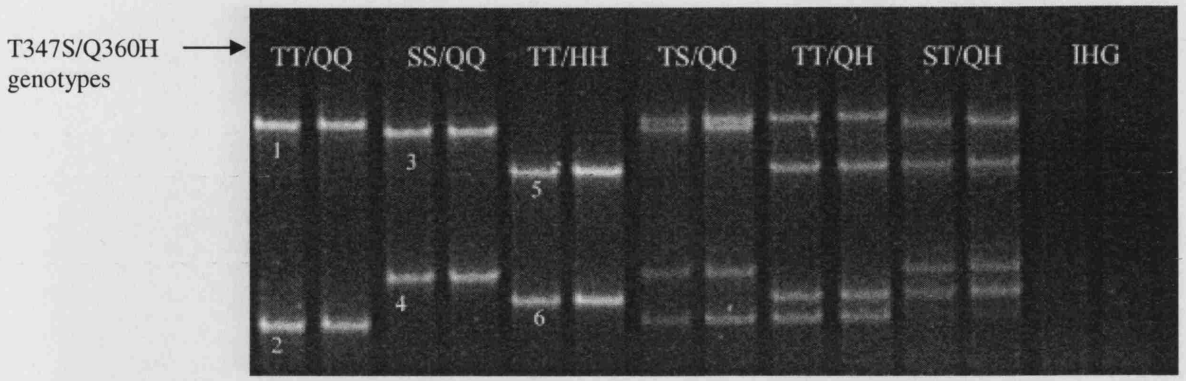


Figure 2.3: A 15% polyacrylamide gel showing all six possible combinations of the T347S and Q360H alleles, the unique bands are labelled 1-6 with each sample run twice including the IHG control. Adapted from Li, L.K *et al* (Li and Humphries, 2002).

2.3.8 Screening the *APOA4* promoter: Single Strand Conformation Polymorphism (SSCP)

Single stranded DNA has the ability to fold up and form intricate structures held together by hydrogen bonds. The electrophoretic mobilities of single strands can therefore vary notably not only due to strand length but also due to conformation which is largely determined by the DNA sequence. Under non-denaturing conditions individual DNA strands will form a secondary structure exclusive to that particular sequence, which will migrate differentially through a polyacrylamide gel. SSCP is a simple method for detecting genetic variation in a single strand of DNA up to 300-500bp long, although it does not reveal the nature or precise location of any changes detected (Mogensen *et al*, 2003).

SSCP was performed using the Amersham MegaBace capillary sequencer with a non-denaturing long read matrix and LPA buffer supplied by Amersham. Table 2.6 summarises the instrumental parameters used.

Instrumental parameter	Setting	Instrumental parameter	Setting
Matrix/ high pressure time	200 mins	Run temp	28^{O}C
Matrix fill/ relaxation time	1 min	Sleep temp	25 ^{O}C
Pre-run time	5 mins	Matrix flush time	22secs
Pre-run voltage	10 mins	Low pressure time	240 secs
Pre-injection voltage	10V	Pre-injection time	10 secs
PMT1 Volt	10 secs	PMT2 voltage	780V

Table 2.6: The parameters used for SSCP analysis of the *APOA4* promoter on the MegaBace.

Previously ~4000bp of the 6.6kb intergenic sequence between *APOC3* and *APOA4* had been analysed by SSCP, thus only the remaining ~2000bp from *APOA4* (-1550 to + 244bps, from the start of translation) of this intergenic region was screened. SSCP was performed by designing overlapping primers covering the region of interest (table 2.7) and analysing the resultant PCR products on a non-denaturing matrix. All forward primers were FAM labelled and reverse primers HEX labelled at their 5' ends respectively, all primers were designed from the published *APOC3/APOA4* sequence

(accession number X78901). The PCR products were heated at 95°C for 5 min and then immediately placed on wet ice before being analysed on the MegaBace.

Region amplified	Sequence 5' to 3' forward (F) and reverse (R).	Annealing temp (^{O}c) cycle number	[$MgCl_2$] mM	PCR product length
NT -1358 to -1026	(F) CATGGGAGGATTGCTTGAGGC (R) CGGAAGTGCTGTGTGAATGTT	58 (30 cycles)	2.25	332 bp
NT -1158 to -835	(F)CCAGCCTGGGTGACAGAGTAA (R)CATTGCTTGCGAAGTGGGTAGT	66 (30 cycles)	2.25	323 bp
NT -910 to -536	(F)CTGCAGAGGGTCCTGGAATCA (R) ATCCGCCACTAATGCTGACTCC	65 (30 cycles)	2.25	374 bp
NT -636 to -364	(F)TGTCTGCAGTCCTGGATGGTC (R)AAGGGAGGAGGGGAACGGAAA	65 (30 cycles)	2.25	272 bp
NT -463 to -94	(F)ACAAACCCAGAGCCGCCAGCA (R)CCTGCGCTGCAGTGGGAACTG	60 (30 cycles)	2.25	369 bp
NT -106 to +244	(F)GGGTGGCGATAGGGAGAGAGT (R) TAGCACCAATGCCAATGGGCC	64 (30 cycles)	1.5	410 bp
NT -1551 to -1296	(F) GTGAGTGGCTCATGCCTGTAA (R) CGGGGTTTCACCATGTTGCTCA	64 (30 cycles)	1.5	255 bp

Table 2.7: Oligonucleotide primers used for SSCP analysis of the *APOA4* promoter.

2.4 BACTERIAL WORK

2.4.1 DNA extraction from bacterial cells

The Sigma GenElute™ high performance (HP) plasmid maxiprep kit was used for plasmid DNA preparation according to the manufacturer's instructions. Briefly, 150ml of overnight SOB culture was centrifuged at 5,000g for 10 minutes and the supernatant discarded, yielding a bacterial pellet. The cells were then resuspended in a buffer containing RNase solution before being subjected to alkaline lysis and neutralised. The lysate was then poured into a column containing an anion-exchange resin binding the plasmid DNA under appropriate low salt and pH conditions. Ribonucleic acid (RNA), proteins, dyes and low molecular weight impurities were removed by a medium salt wash. The DNA was then eluted under high salt conditions and concentrated by ethanol precipitation as follows: to the plasmid DNA, 3M sodium acetate (1/10 volume) and 4 °C 100% ethanol (2x volume) were added before the mixture was incubated at -70 °C for 1 hour. The samples were then centrifuged at 12,000 rpm for 30 minutes, the supernatant discarded and the pellet washed in 1ml 70% ethanol. After a final centrifugation at 12,000 rpm, the supernatant was removed and the pellet allowed to air dry before resuspension in 400μl of 10mM Tris HCL (pH 8). The absorbance measured at 260nm was used to estimate the concentration of DNA.

2.4.2 Cloning

2.4.2.1 PCR amplification of *APOA4* cDNA from the pTYB11 vector

Fragments to be cloned were generated by PCR reactions and inserted into the pET14b expression vector (figure 2.4). The *APOA4* cDNA inserted within the pTYB11 vector (a kind gift from Richard B. Weinberg [Wake forest University School of Medicine, North Carolina, U.S.A]) was amplified, excluding the signal peptide (21bp) with *NdeI* and *BamHI* restriction enzyme cleavage site overhangs. (The respective restriction enzyme recognition sites are underlined and the cleavage position marked, *NdeI* forward: 5'ATTATC▼ATATGGAGGTCAGTGCTGACCAGGTGGCCACAGTG3', *BamHI* reverse: 5'TACAG▼GATCCTCAGCTCTCCAAAGGGGCCAGCAT3'.) The PCR was carried out in a 500μl final volume, containing 1X polmix, 10 pmol of each primer, 1.5mM $MgCl_2$, 0.2U proof-reading *taq polymerase* (Platinum® *PFX DNA polymersase*). The PCR reaction was performed using a MJ Tetrad peltier thermal cycler with the cycling conditions; 95 °C for 5 mins (1 cycle), 95 °C for 45 sec, 65°C for 1 min, 72 °C for 1 min (30 cycles) and 72 °C for 10 minutes (1 cycle). The 1.2 kB PCR product was analysed by 2% agarose gel electrophoresis including a Gibco 1kB ladder.

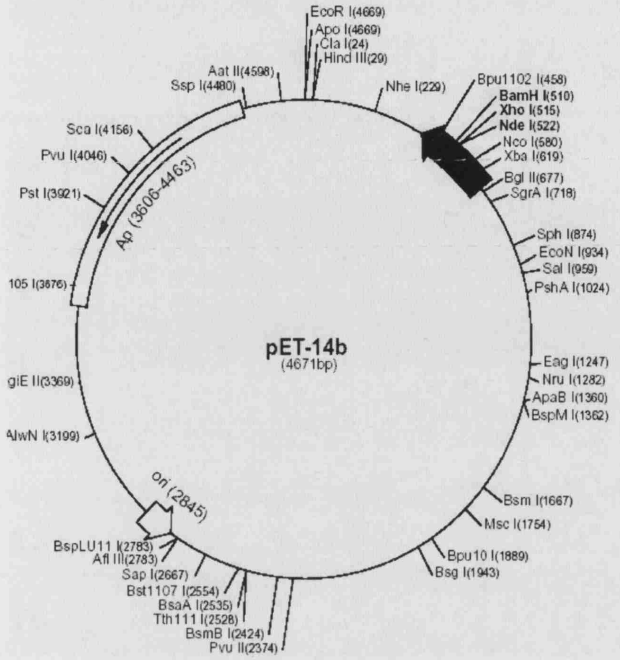


Figure 2.4: A schematic representation of the pET-14b expression vector that was used to express *APOA4*.

2.4.2.2 Purification of PCR products from agarose gels

The PCR products were separated by electrophoresis using 2% agarose and excised from the gel under UV illumination. To purify the PCR product, the Wizard PCR prep kit was used according to the manufacturer's instructions. The gel slice was placed in a pre-weighed eppendorf tube and incubated at 65 ^{o}C for 5 minutes with 1ml of DNA binding resin. Using a 2ml syringe, the resin-gel mixture was applied to a spin column and washed with 80% isopropanol. In order to remove excess isopropanol the spin column was spun (1000 rpm, 10 seconds) in a micro centrifuge and the column allowed to air dry before the addition of 50μl dH_2O. Finally the DNA was eluted by spinning the column into a fresh eppendorf tube at 1000 rpm for 20 seconds.

2.4.2.3 Ligation

The insert DNA and vector were digested with 20 units of the appropriate restriction enzymes (*NdeI* and *BamHI*) for 1 hour at 37 °C with the supplied buffer and purified on an agarose gel using the Wizard PCR prep kit. The ligation reaction was carried out at 14 °C overnight; in a 3:1 molar ratio of insert to vector DNA (100ng), using 10 units of T4 ligase in a final volume of 20μl with the addition of the manufacturer's supplied buffer.

2.4.2.4 Transformation

The ligation mixture (10μl) was mixed with 50μl of competent cells and allowed to equilibrate on ice for 30 minutes. The cells were then heat shocked at 42 °C for 45 seconds and allowed to recover in 1ml of SOB at 37 °C for 1 hour. Following this the cell mixture was spread evenly between two LB agar plates containing the appropriate antibiotic and incubated at 37 °C for 16 hours. Colonies were picked and checked if they contained the correct insert by maxi prep and sequencing (sections 2.4.1 and 2.4.4).

2.4.3 Mutagenesis

All the apoA-IV variants were created using the QuikChange™ site-directed mutagenesis kit. Plasmid DNA of the wild type apoA-IV was used as a template for PCR using synthetic primers containing the desired mutation, complementary to opposite strands of the vector. All PCR reactions were carried out using PfuTurbo *DNA polymerase*, according to the manufacturer's guidelines (supplied in the Quikchange™ kit). PCR was performed with an initial denaturation step at 95 °C for 30 seconds, followed by 16 cycles of 95 °C for 30 sec, 55 °C for 1 minute and 68 °C for 10 minutes. After removing the dam-methylated parental template by *DpnI* digestion, the synthesised mutated DNA was transformed into *E.coli* XL1 Blue competent cells. Candidate clones were screened by sequencing the entire *APOA4* insert to ensure the presence of the desired base change and that no unwanted changes were created. All primers used in the site-directed mutagenesis of *APOA4* are listed in table 2.8, base changes created are in bold. Every mutation generated was confirmed by sequencing (section 2.4.4).

Mutation produced	Forward primer sequence	Reverse primer sequence
R123Q	5'CGCGGACCAGCTGC**AG**ACCC AGGTCAACACG3'	5'CGTGTTGACCTGGGT**CT**G CAGCTGGTCCGCG3'
R134Q	5'GGCCGAGCAGCTGC**A**GCGCC AGCTGACCC3'	5'GGGTCAGCTGGCGC**T**GCA GCTGCTCGGCC3'
N127R	5'GCGCACCCAGGTC**CG**CACGC AGGCCGAGC3'	5'GCTCGGCCTGCGTG**CG**GA CCTGGGTGCG3'
R123Q+R134Q	Combination of R123Q and R134Q on a wild type template.	
R123Q+N127R	Combination of R123Q and N127R on a wild type template	
N127R+R134Q	Combination of N127R and R134Q on a wild type template	
H1 K.O	Combination of N127R, R134Q and R123Q on a wild type template	
R149Q	5'GGAGAGAGTGCTGC**A**GGAGA ACGCCGACAGC3'	5'GCTGTCGGCGTTCTCC**T**GC AGCACTCTCTCC3'
R160Q	5'CCTGCAGGCCTCGCTG**CA**GCC CCACGCCGACG3'	5'CGTCGGCGTGGGGC**TG**CA GCGAGGCCTGCAGG3'
Q156R	5'CCCAGGTCAACACGC**G**GGCC GAGCAGCTGCG3'	5'CGCAGCTGCTCGGCC**C**GC GTGTTGACCTGGG3'
R149Q+R160Q	Combination of R149Q and R160Q on a wild type template	
R149Q+Q156R	Combination of R149Q and Q156R on a wild type template	
R160Q+Q156R	Combination of R160Q and Q156R on a wild type template	
H2 K.O	Combination of R160Q,Q156R and R149Q on a wild type template	
Q360H	5'GCAGGAACAGCA**T**CAGGA GCAGCAGC3'	5'GCTGCTGCTCCTG**A**TGC TGTTCCTGC3'
T347S	5'GCCAGGACAAG**T**CTCTCTC CCTCCC3'	5'GGGAGGGAGAGAG**A**CT TGTCCTGGC3'
Q360H+T347S	Combination of Q360H and T347S on a wild type template	
N127S	5'GCGCACCCAGGTCA**G**CACGC AGGCCGAGC3'	5'GCTCGGCCTGCGTG**C**TGA CCTGGGAGCGC3'
L166Q	5'CCCACGCCGACGAGC**AG**AAG GCCAAGATCGACC3'	5'GGTCGATCTTGGCCTT**CT**G CTCGTCGGCGTGGG3'

Table 2.8: Primers used for the site directed mutagenesis of *APOA4*, mutations are in bold.

2.4.4 DNA sequencing

The ABI Prism Bigdye™ terminator V3.1 sequencing kit (BD 3.1) was used to perform all sequencing reactions based upon the chain termination sequencing method (Sanger and Coulson, 1975). Plasmid inserts to be sequenced were amplified by PCR in a 10μl reaction consisting of Maxi prep purified plasmid DNA (~800ng), 2μl BD 3.1 mix and 2μl 5X sequencing buffer. The reaction mixture was overlaid with 10μl of paraffin oil before being PCR amplified. The conditions of the cycle sequencing were 96°C for 1 minute x 1 cycle followed by (96°C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes) x 25 cycles, all sequencing primers used are summarised in table 2.9. Following the sequencing reaction, the samples were ethanol precipitated. 10μl of dH_2O, 2 μl 125mM EDTA, 2μl 3M sodium acetate and 50μl of 100% ethanol (4 °C) was added to each sample and left at room temperature for 15 minutes. All samples were then centrifuged at 3000g for 30 minutes, the supernatent removed by inversion and the pellet washed with 70 μl of 70% ethanol and centrifuged at 3000g for 15 minutes. The supernatant was removed by inverting the PCR plate and spinning the sample at 50 rpm for 30 seconds, the pellet was resuspended in 4μl of loading dye (5:1, formamide: blue dextran).

5% acrylamide gels (20ml ultra pure Sequagel XR and 5ml Sequagel complete buffer from National diagnostics) were polymerised by the addition of 200μl fresh 10% APS and 35μl of TEMED and pre-warmed at 65W (1600V, 80A) in 1X TBE for 30 minutes. The resuspended sequencing products were denatured at 95 °C for 5 minutes and placed straight on to ice to prevent re-annealing. The mixture (2μl) was loaded on to the

polyacrylamide gel and run in 1 x TBE buffer at 1600V for 9 hours on an ABI 377 prism DNA sequencer. Sequence analysis was performed using the Sequence analysis 2.0 software (PE-ABI, UK).

Primer name	Forward	Reverse
pET-14b Seq	5'TAATACGACTCACTATAGG3'	5'CTAGTTATTGCTCAGCGG3'
Seq 641-364	5'TCCCACTGCAGCGCAGG3'	5'GGGCTCCACCCGGCGTC3'
Seq 481-842	5'AGATCGGGGACAACCTG3'	5'CGTTCTTCTTCATCTGG3'
Seq 451-725	5'CGTAGGGCGTAAGGCGT3'	5'CAACACGCAGGCCGAGC3'

Table 2.9: Primers used to sequence the *APOA4* insert within the pET-14b vector.

2.5 APOA-IV PROTEIN WORK

2.5.1 Structural analysis of apoA-IV

2.5.1.1 Secondary structure prediction

The consensus secondary structure prediction programme available from the Network Protein Sequence Analysis website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) was used to assess the possible secondary structure of apoA-IV. The consensus sequence was generated using various predictive methods: SOPMA (Geourjon and Deleage, 1995), PHD (Rost and Sander, 1993), (Rost and Sander, 1994), Predator (Frishman and Argos, 1996), GORIV (Garnier *et al*, 1996), DPM (Deleage and Roux, 1987), DSC (King and Sternberg, 1996), SIMPA96 (Levin *et al*, 1986; Levin, 1997) and HNNC (Guermeur *et al*, 1999).

2.5.1.2 Predicting the possible LCAT activation domain within apoA-IV

Based upon the study carried out by Emmanuel *et al* (Emmanuel *et al*, 1994) the LCAT activation domain of apoA-IV has been reported to reside between residues 117-160 of the mature protein. Therefore we decided to perform various alignments using Clustal W (http://www.ebi.ac.uk/clustalw/) with this section of apoA-IV to search for regions of homology with the LCAT activation domain of apolipoprotein A-I (apoA-I) as published by Roosbeek *et al* (Roosbeek *et al*, 2001), paying close attention to the three arginine residue (R149, R153 and R160) critical for apoA-I LCAT activation.

Residues 117-160 of apoA-IV are predicted by the consensus secondary structure prediction programme from NPS to be in a region of helical content. Hence once appropriate potential apoA-IV LCAT activating sequences were found, through alignments of the human apoA-IV amino acid sequence with the apoA-IV sequences of other species and human apoA-I, WinPep 3.01 (Hennig, 1999) was used to plot Edmundson wheel representations of the regions to analyse their location within possible amphipathic helices.

2.5.2 The production of recombinant apoA-IV from *E.coli* cells

The appropriate pET14b expression vector constructs were transformed into competent BL21 (DE3) *E.coli* as previously described section 2.4.2.4. A single colony was picked and incubated at 37°C in 20ml of Luria broth medium, supplemented with ampicillin (100μg/ml). Five millilitres of this overnight culture was used to inoculate a 1 litre flask of the same medium. These 1 litre production cultures were grown at 37°C till an

absorbance between 0.6 and 1 OD unit at 540nm was reached. Isopropyl β-D-thiogalactoside (IPTG) was added at a final concentration of 1mM, the cultures were further fermented at 37°C for 2 hours to induce T7 polymerase expression and the production of recombinant apoA-IV. The cells were harvested by centrifugation and frozen at -80 °C.

To extract the recombinant apoA-IV proteins, the frozen pellets were resuspended in lysis buffer overnight at 4 °C. The cells were disrupted by 3 cycles of 5 minute sonications using a Soniprep 150 (Sanyo) sonicator. Cell debris were removed by centrifugation at 12,000 rpm for 30 mins in a Sorvall RC 5C plus centrifuge at 4 °C and the supernatent was kept for protein extraction.

In order to purify the recombinant apoA-IV proteins from the supernatant, Ni-NTA His-bind™ nickel resin was equilibrated with bind buffer for 20 minutes, following this to a 5ml bed volume of resin up to 20 mls of supernatant was added. The mixture of resin and cell lysate was mixed for 1 hour at room temperature and the resin washed thoroughly twice with 20 volumes of bind buffer for 10 minutes to remove all unbound proteins and centrifuged at 3000 rpm, allowing the bind buffer to be easily removed. The resin was transferred and settled in an Econo-Pac™ column before being finally eluted with 15mls (in 1ml aliquots) of elution buffer at pH8. The various elution aliquots were analysed at 280 nm, using a Cecil CE 2041 spectrophotometer, those with the highest reading were pooled and tested for the presence of apoA-IV by SDS-PAGE (section 2.5.2.2).

2.5.2.1 Detection of free protein concentration

The concentration of apoA-IV was measured using the bicinchoninic acid (BCA) protein assay kit. Throughout the standard microwell plate protocol was followed using standards ranging from 25μg/ml to 2000μg/ml according to the manufacturer's instructions. The molar concentration of apoA-IV was also estimated using a spectrophotometer (Cecil CE 2041), by dividing the absorbance at 280nm by the molar extinction co-efficient of the mature recombinant apoA-IV and multiplying this value by the dilution factor. The molar extinction of the recombinant apoA-IV was taken to be 0.348 as calculated by the ProtParam program available at http://us.expasy.org/tools/protparam.html.

2.5.2.2 The analysis of proteins by Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and non-denaturing pre-cast 4-20% linear gradient Tris-HCL gels

All recombinant apoA-IV proteins were analysed on a 10% SDS-PAGE, consisting of a 10% protein gel mix and a 4% stacking gel mix using prestained SDS-PAGE standards ranging from 7.2-203kDa. To further analyse the recombinant apoA-IV proteins, all samples were also run on a non-denaturing pre-cast 4-20% linear gradient Tris-HCL gel (according to the manufacturer's instructions). Both SDS-PAGE and non-denaturing pre-cast 4-20% linear gradient gel analysis was carried out at 50V for approximately 4 hours in a Mini protean® II cell (Bio-Rad).

2.5.2.2.1 Coomassie blue staining of polyacrylamide gels

Coomassie blue staining was carried out according to Maniatis *et al* (Maniatis T , 1989). All gels were submerged in 5 volumes of Coomassie blue stain for at least 3 hours and then immersed in 5 volumes of destain for 3 hours or until the appearance of bands.

2.5.2.2.2 Drying of SDS or agarose gels

The drying of gels was performed using the gel drying kit from Promega following the manufacturer's protocol. After destaining, the gels were soaked in gel drying solution for 10 minutes and gel drying films also thoroughly moistened with gel drying solution. The gels were then placed between two moistened gel films and air bubbles removed prior to the film being placed carefully into the frame provided and stretched. The assembled frame with the gel was then allowed to air dry for 16 hours (overnight) at room temperature.

2.5.3 Removal of N-terminal 6 X histidine tag from wild type apoA-IV

The wild type N-terminal 6 X histidine tag (his-tag) was removed using the thrombin cleavage capture kit according to manufacturer's instructions. An optimisation assay was first performed with 10μg of apoAIV being cut with biotinylated thrombin at a series of dilutions containing 0.04, 0.02, 0.01 and 0.005 U/μl. The presence of his-tag in each sample was checked using his-tag mouse monoclonal antibodies via the western blot protocol (section 2.5.4). The optimal condition found to completely remove the his-tag from 0.5 mgs of apoA-IV was 0.25 U/μl of biotinylated thrombin, in 1X thrombin cleavage/capture buffer (20mM tris-HCL pH8.4, 0.15 M NaCl, 2.5mM $CaCl_2$). The his-

tag cleaved apoA-IV was then analysed on a SDS PAGE (section 2.5.2.2) to ensure that the protein was intact. To remove the biotinylated thrombin from the samples, they were incubated with streptavidin agarose for one and a half hours at room temperature using 16 μl of resin per unit of enzyme. The successful removal of the biotinylated thrombin was examined by SDS PAGE.

The his-tag cleaved apoA-IV protein, with the biotinylated thrombin removed was desalted into PBS using a HiTrap™ desalting column (5 x 5 ml) placed within an Amersham AKTA FPLC machine. Protein concentration was then assessed using the BCA protein assay kit.

2.5.4 Western Blotting

Western Blotting was used to confirm that the recombinant 46kDa protein produced was apo-A-IV and to check the efficient removal of the 6X histidine tag from recombinant apoA-IV. Approximately 10μg of recombinant apoA-IV separated by SDS-PAGE or non-denaturing pre-cast 4-20% linear gradient Tris-HCL gels was electrophoretically transferred to a Hybond-C® extra (nitrocellulose) membrane using the Bio-Rad mini-transblott® cell. After SDS-PAGE, a gel sandwich was prepared inside a transfer cage in this order: Cathode (-), fibrous pad, filter paper, gel, Hybond-C® extra nitrocellulose membrane, filter paper, fibrous pad, anode (+). The transfer cage once inside the transfer electrode was placed into the transfer tank filled with cold transfer buffer. Transfer was carried out at a constant current of 220mA for 2.5 hours, using the Bio-Rad power pac® 300.

Visualisation of all Western Blotts was carried out using His•Tag® Western and LumiBlot™ reagents supplied by Novagen. The membrane were washed twice for 10 minutes each time using 15ml 1X TBS detergent (supplied in the His•Tag® Western and LumiBlot™) and blocked overnight with blocking solution consisting of 3% BSA in 1xTBS. The membrane was washed twice for 10 minutes each time using 20ml 1X TBSTT (0.5M NaCl, 20mM Tris-HCL, 0.2% Triton® X-100, 0.005% v/v Tween®-20, pH 7.5) and then once with 15ml 1X TBS for 10 minutes before being incubated with the primary antibody (either rabbit IgG apoAIV, a kind gift from M. Jauhiainen [department of Biochemistry, National Public Health Institute, Helsinki, Finland] or His•Tag mouse monoclonal IgG) diluted 1:1000 in blocking solution. After blocking was complete, the membrane was washed with 1X TBSTT and 1X TBS as before and incubated with the alkaline phosphotase (AP) conjugated secondary antibody (either 1:3000 diluted rat anti-rabbit antibody, a kind gift from T.Segal, [department of medicine, University College London, UK] or 1:5000 diluted goat anti-mouse IgG) for 1 hour. The blot was washed 5 times with 20ml 1X TBSTT before being visualised with developing solution containing: 60μl Nitro-Blue Tetrazolium Chloride solution, 60μl BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) in 15ml 1X AP buffer (trade marked). Once a strong purple signal appeared, the reaction was stopped by washing the blot in dH_2O and the blot was then allowed to air dry.

2.6 FUNCTIONAL STUDIES PERFORMED ON RECOMBINANT APOA-IV

2.6.1 Lipid binding properties of apoA-IV

2.6.1.1 Preparation of the 1.2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) multilamellar vesicles (MLV)

DMPC was dissolved in choloform and dried under nitrogen to produce a film and placed in a vacuum for 2 hours to remove any residual chloroform. The DMPC film was re-dissolved in 5mM NH_4HCO_3 (pH 8.0) to produce a concentration of DMPC of 2mg/ml by vortexing the sample at room temperature until the emulsion was homogeneous.

2.6.1.2 Analysing the lipid binding properties of recombinant apoA-IV

The lipid binding properties of all apoA-IV variants produced was assessed by monitoring the decrease in turbidity of DMPC MLV at 325nm, after being mixed with recombinant apoA-IV protein as a function of temperature from 16 °C-36 °C. All turbidity measurement were carried out as follows; 40μg of apoA-IV was mixed with 80μg DMPC MLV in 5 mM NH_4HCO_3 (pH 8.0) buffer and the turbidity of DMPC was measured in an Uvikon 931 spectrophotometer.

2.6.2 The generation of apoA-IV: 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) complexes, the cholate dialysis method

The recombinant apoA-IV (as produced according to section 2.5.2) at a concentration of 10mg/ml was placed into dialysis tubing with a molecular weight cut off (MWCO) of 12-14 kDa and dialysed against 1X TBS over 5 days. Meanwhile a 10mg/ml unesterfied cholesterol (UC) solution was prepared in a 2:1 chloroform/methanol solution on ice using UC supplied by Sigma. The second solution prepared was a 30mg/ml sodium cholate solution in 1X TBS (pH7.4), while the POPC purchased from Avanti polar lipids was supplied in a 10 mg/ml choloform mix.

All apoA-IV:POPC complexes were made to the molar ratio of 180M POPC: 10M UC: 1M apoA-IV: 100M sodium cholate. The correct molar ratio of POPC and UC dissolved in a 2:1 chloroform/methanol solution was aliquoted into fresh test tubes and dried using N_2, producing a thin film. These lipid films were dried for 2 hours at ~40 °C under N_2 before being placed on ice. After this about 200μl of sodium cholate (30mg/ml) and 300μl of 1X TBS was added to the films and the mixture vortexed every 15 minutes until the solution became optically clear, this procedure was carried out on ice at 4 °C. Once optically clear the appropriate moles of recombinant apoA-IV were added and the mixture was allowed to stand on ice. The sodium cholate was eliminated by dialysing the sample in 1X TBS over 5 days, using Spectra/Por Biotech dialysis tubing with a MWCO of 100kDa to aid the removal of all uncomplexed recombinant apoA-IV protein. Radioactive complexes were made with the addition of [3H] unesterified cholesterol (0.55MBq).

2.6.3 Characterisation of the apoA-IV:POPC complexes

2.6.3.1 Calculating the molar apoA-IV:POPC:UC ratio of the discs

The phospholipid and cholesterol content of the complexes were measured using the Wako phospholipids B (cat n.o 999-54006) and free cholesterol C (cat n.o 279-47106) kits respectively. Throughout the use of these kits the manufacturer's guidelines were followed.

The Phospholipid B kit is based upon the enzymatic hydrolysis of phospholipids to free choline by phospholipase D. In turn the free choline is subjected to oxidation by choline oxidase producing betaine and H_2O_2. The H_2O_2 produced quantitatively, oxidatively combines 4-aminoantipyrine and phenol, yielding a chromogen with a maximal absorption at 505nm. Thus the concentration of phospholipid can be deduced by measuring the absorbance of the chromogen at 505nm, via a set of standards of known phospholipid concentration. Similarly the free cholesterol C kit is based upon the enzymatic oxidation of cholesterol by cholesterol oxidase to cholestenone, simultaneously producing H_2O_2 causing phenol and 4-aminoantipyrine to undergo quantitatively an oxidative condensation in the presence of peroxidase, producing a red colour. The concentration of free cholesterol in the test sample can therefore be measured by determining the absorbance of the red colour at 505nm, via a set of standards of known cholesterol concentration.

The concentration of apoA-IV was measured using the BCA assay (described in section 2.5.2.1), using the relative molecular weights of apoA-IV, POPC and UC as 43374.5g, 760.09 and 386.66g respectively, the ideal molar ratio with apoA-IV set as 1 was calculated.

2.6.3.2 Determining the Stoke's diameter of the complexes

All apoA-IV:POPC complexes were analysed on a 4-30% non-denaturing alamo polyacrylamide gradient gel and the Stoke's diameter determined by running the samples adjacent to a set of high molecular weight markers of known diameter. All 4-30% non-denaturing alamo polyacrylamide gradient gels were run on the VGT1 mini-vertical gel unit. Once the gels were pre-run at 120V for 1 hour, samples were loaded using an LPE-SL18, 18 well applicator with loading buffer. Following this, the gels were subjected to electrophoresis in the following sequence: 15V for 15min, 70V for 20 min, 125V for 24 hours (3,000 Volt hours) and stained with coomassie blue stain, visualised with destain solution and the bands scanned using an ImageScanner® and LabScan® 5 software (Amersham). Quantification of the major and minor bands was performed using Image J software (http://rsb.info.nih.gov/ij/).

2.6.3.3 Circular Dichroism (CD) measurements

CD spectra for both the lipid free recombinant apoA-IV and the apoA-IV:POPC complexes in 1X TBS buffer were measured in an AVIV instrument, Model 202SF, stopped flow CD spectrometer between 200 and 260nm at room temperature. All measurements were performed using a 0.1cm path length quartz cuvette containing

500μg/ml of sample in 1 X TBS buffer. Spectra data from 200 to 260nm were collected at 0.5nm intervals with a scanning speed of 20nm/minute and at a time constant of 2 seconds. Five spectra were collected and averaged for each sample. The increase in α-helicity was calculated by directly comparing the increase in 208nm and 222nm minima between the lipid free apo-AIV and apoA-IV complex CD spectra. Deconvolution of the CD spectra was performed using the CDPro software bundle (available at http:/lamar.colostate.edu/~sreeram/CDPro) containing the popular CONTIL/LL and SELCON3 software (Sreerama and Woody, 2000).

2.6.3.4 Thermal denaturation of apoA-IV

Denaturation was executed by placing 500μg/ml of each apoA-IV recombinant protein (diluted in 1 X TBS) in a 0.1cm path length quartz cuvette heated within a AVIV instrument, Model 202SF, stopped flow CD spectrometer at a rate of 1 °C/min from 15 °C to 95 °C. Electronic recordings of the CD change monitored temperature induced unfolding at 222 nm. The temperature of midtransition (T_m), heat capacity (ΔCp) and folding enthalpy (ΔH) of each recombinant apoA-IV protein in the lipid free state was calculated by fitting the CD curve data to the equation below:

$$f_U(T) = \frac{1}{1 + \exp\left(\frac{-1}{R}\left(\Delta H_{fold}(T_M)\left(\frac{1}{T} - \frac{1}{T_M}\right) + \Delta C_{p,fold}\left(1 - \frac{T_M}{T} - \ln\left(\frac{T}{T_M}\right)\right)\right)\right)}$$

deduced by Cliff *et al* (Cliff *et al*, 2005) using mathematica® notebook (Wolfram research Inc, USA), errors were generated using montecarlo simulations of 100 data sets using the method of Cliff *et al* (Cliff *et al*, 2005).

2.6.4 Lecithin cholesterol acyl transferase (LCAT) assay

The LCAT assay was performed on all the recombinant apoA-IV:POPC complexes (generated according to section 2.6.2) made with radioactive trititum UC (^{3}H UC), using a method adapted from Piran *et al* (Piran and Morin, 1979). This rapid radioassay for LCAT activity relies on radiolabelled UC as a substrate for LCAT. After incubation of LCAT with the radiolabelled UC substrate (radioactive apoA-IV:POPC complex), an ethanolic digitonin solution is added directly to the mixture to extract the lipids, excess cholesterol is added to the incubation mixture and the labelled cholesterol-digitonide and denature proteins are sedimented by centrifugation at 1500g for 10 mins, leaving the radiolabelled esterified cholesterol in solution which can be measured in an aqueous scintillation mixture. LCAT activity was assessed by measuring the amount of radiolabelled esterified cholesterol present. A variety of control incubation mixtures were also set up, the first containing no LCAT to obtain the background supernatant radioactivity, the second control consisted of an incubation mixture with 95% ethanol lacking digitonin to determine the total cholesterol radioactivity in the mixture, while the final mixture was a positive control consisting of lipoprotein deficient serum (LPDS) and radioactive apoA-IV:POPC complex without LCAT. All experiments were performed in triplicate.

The following stock solutions were made; 10mg/ml FAF (fatty acid free) BSA in 1x TBS buffer, a β-mercaptoethanol solution diluted 1:10 with 1 x TBS buffer, a 1 % solution of digitonin in 95% ethanol, a 5mg/ml solution of UC in ethanol and a 1/50 solution of LCAT enzyme. Subsequently 45 tubes with their contents summarised in table 2.10 were

set up, including the 3 control mixtures and the radioactive apoA-IV complexes in a series of dilutions from 0.5 to 100μmol/ml of UC.

	Tube	Substrate (AIV /PLPC/Chol complex)	BSA (μl)	ß-ME (μl)	TBS (μl)	Final vol (μl)
Total counts	1,2,3	use tube to give final conc. 100μmol/L	50	5	40	135
Blank	4,5,6,	add 40μl	50	5	40	135
LCAT	7,8,9		50	5	40	135
Total counts	10,11,12	use tube to give final conc. 50μmol/L	50	5	40	135
LCAT	13,14,15	add 40μl	50	5	40	135
Total counts	16,17,18	use tube to give final conc. 20μmol/L	50	5	40	135
LCAT	19,20,21	add 40μl	50	5	40	135
Total counts	22,23,24,	use tube to give final conc. 10μmol/L	50	5	40	135
LCAT	25,26,27	add 40μl	50	5	40	135
Total counts	28,29,30	use tube to give final conc. 2μmol/L	50	5	40	135
LCAT	31,32,33	add 40μl	50	5	40	135
Total counts	34,35,36	use tube to give final conc. 0.5μmol/L	50	5	40	135
Blank	37,38,39	add 40μl	50	5	40	135
LCAT	40,41,42		50	5	40	135
Pos. control	43,44,45	use tube to give final conc. 2μmol/L add 40μl	50	5	40	135

Table 2.10: Summarising the contents of the 45 tubes set up for each LCAT assay performed.

All 45 incubation mixtures were equilibrated at 37 °C under nitrogen for 20 minutes. To the tubes marked as total counts or blank, 20μl of TBS were added, to those marked positive control and LCAT, 20μl of LPDS and LCAT were added respectively. Subsequently all tubes were then incubated at 37 °C for an hour. After this incubation, total count tubes had 500μl of 95% ethanol added, while all other tubes had 500μl of digitonin added instead. Both sets of mixtures were vortexed for 15 seconds, before adding 25μl of UC followed by another 15 seconds of vortexing. Finally all tubes were centrifuged at 1500g for 10 minutes sedimenting all proteins and UC. The supernatant containing the radiolabelled cholesterol ester products of LCAT activity were measured by placing 200μl of the supernatant in 5mls of scintillation fluid (Ecoscint™ A). All radioactivity was measured using a Tri-Carb liquid scintillation analyzer (Packard Biosciences, San Diego, USA).

The kinetic parameters K_m(app) and V_{max} were determined by nonlinear regression using GraphPad Prism 4.0 computer software (GraphPad Software Inc.) with the help of Professor Kerry Ann Rye (Lipid Research Laboratory, Royal Adelaide Hospital, Australia). The data was fitted directly to the Michaelis-Menten equation: $V = (V_{max} * [S])/(K_m + [S])$, where V is the velocity of LCAT esterification and [S] is the concentration of reconstituted HDL (rHDL). Lineweaver-Burk linear transformations are for display purposes only.

2.6.5 Measurement of LDL oxidation

2.6.5.1 The continuous monitoring of copper-mediated LDL oxidation by measuring conjugated diene accumulation

A method adapted from Esterbauer *et al* (Esterbauer *et al*, 1989) was used, in which the extent of LDL oxidation is monitored by following the formation of conjugated dienes that absorb at 234nm. Oxidised LDL (OXLDL) with the general structure of –CH=CH-CH=CH-CHOOH are fully soluble in PBS and so the generation of these conjugated dienes can directly be monitored in a reproducible manner. The kinetics of conjugated diene formation can be subdivided into 3 phases (figure 2.5). The first is the lag phase in which the LDL becomes depleted of its anti-oxidants with minimal lipid peroxidation occurring at an approximate rate of one molecule of conjugated lipid hydroperoxide in each LDL particle every 6 minutes (Esterbauer *et al*, 1992). Once depleted of all its antioxidants, LDL is subjected to the autocatalytic reactions of lipid peroxidation accelerating lipid peroxidation in an exponential manner, signalling the transition from the initial lag to the propagation phase of LDL oxidation. All the conjugated dienes produced during the propagation phase are intermediary products. Such products of lipid peroxidation thus rise to a maximum concentration and thereafter subsequent decomposition reactions predominate resulting in conjugated diene breakdown, leading to the decomposition phase (Rice-Evans *et al*, 1996).

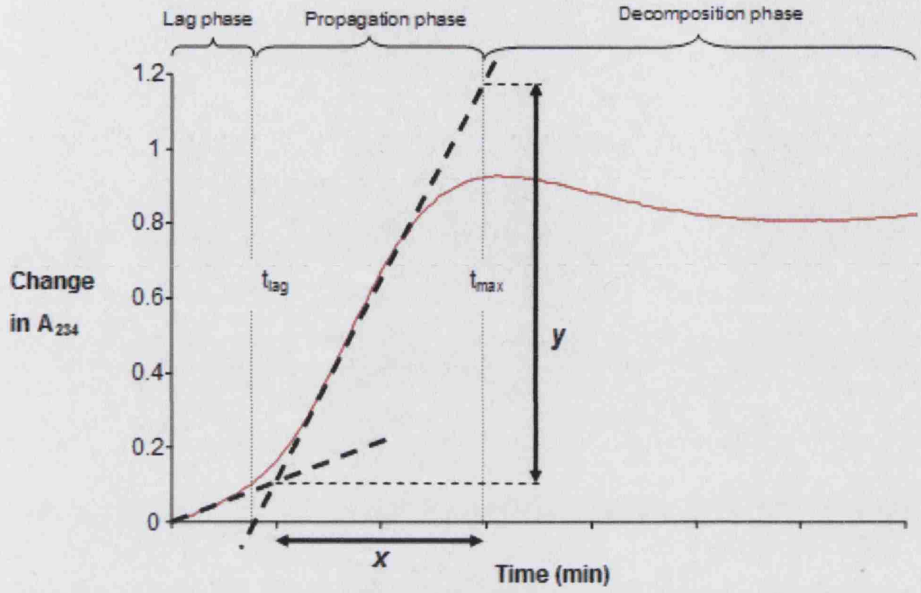


Figure 2.5: The continuous measurement of copper mediated LDL oxidation by conjugated diene absorption at 234nm (A_{234}), adapted from Esterbauer *et al* (Esterbauer *et al*, 1992). The lag (t_{lag}), propagation and decomposition are represented, with t_{max} referring to the time at which peak A_{234} is reached.

2.6.5.2 The conjugated diene assay used to assess the anti-oxidant abilities of the common apoA-IV isoforms (Wild type, H360, S347, S127)

Native LDL (nLDL) [50μg/ml] from a healthy donor stored in MOPS buffer, a kind gift from David S Leake (University of Reading, School of Animal and Microbial Sciences, UK) was oxidised at 37 °C in the presence of 5μM $CuSO_4$ in capped quartz cuvettes. The formation of conjugated dienes was monitored at one minute intervals at 234nm in either a Lambda2 6-cell or a Lambda Bio 40 8-cell spectrophotometer with UV Winlab software (Perkin Elmer, UK) against reference cuvettes containing no LDL. To assess

the anti-oxidant abilities of the common apoA-IV isoforms (Wild type, H360, S347, S127), 100μg/ml of each isoform was incubated with 50μg/ml nLDL in the presence of 5μM $CuSO_4$, along with both a positive (50μg/ml LDL + 5μM $CuSO_4$) and negative control (50μg/ml LDL + 5μM $CuSO_4$ + 100μg/ml BSA) and the formation of conjugated dienes measured. The antioxidant ability of each isoform was assessed by comparing the time for 50% conjugated diene formation ($T_{1/2}$) of each apoA-IV isoform to that of the wild type isoform.

2.6.5.3 Measurement of LDL oxidation by measuring the concentration of lipid hydroperoxides iodometrically

The total amount of lipid hydroperoxides (LOOH) were quantified via an assay adapted from El-Saadani *et al* (El Saadani *et al*, 1989). In principle, this assay relies on the ability of lipid peroxides to oxidise iodide ions ($2I^-$) into molecular iodine (I_2) which subsequently combines with iodide (I^-) to form the tr-iodide chromophore (I_3^-) that can be measured photometrically at 365 nm. The LOOH content of LDL can then be estimated from calibration curves produced using known concentrations of hydrogen peroxide (H_2O_2), expressed as nmol H_2O_2 equivalents per mg LDL protein (figure 2.6).

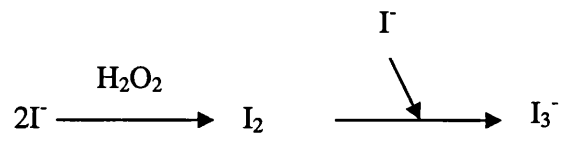


Figure 2.6: Illustrating the oxidation of iodide ions ($2I^-$) by H_2O_2 to molecular iodine (I_2), which in combination with iodide (I^-) produces the chromophore tri-iodide (I_3^-).

All LOOH measurements were performed in triplicate, to 250μl LDL samples containing 100μg protein/ml, 1ml of colour reagent (CHOD-iodide, Merck, Darmstadt, FRG) was added. H_2O_2 standards were freshly prepared at a concentration up to 25nmol per assay tube and were treated identically to samples. After vortexing, both standard and sample tubes were analysed at 365nm using a Unicam Heliosβ spectrophotometer. The LOOH content of each LDL sample was determined by comparison with the H_2O_2 standard plot.

2.6.5.4 The tri-iodide assay used to assess the anti-oxidant abilities of the common apoA-IV isoforms (Wild type, H360, S347, S127)

At various time points throughout the conjugated diene assay, 250μl of each reaction mixture (50μg/ml LDL + 5μM $CuSO_4$ + apoA-IV isoform) including both positive (50μg/ml LDL + 5μM $CuSO_4$) and negative controls (50μg/ml LDL + 5μM $CuSO_4$ + 100μg/ml BSA) were removed and 20mM of BHT and 1mM EDTA added to halt the oxidation of LDL and any further LOOH generation. Once both the sample and standard tubes had 1ml of colour reagent (CHOD-iodide, Merck, Darmstadt, FRG) added they were vortexed and incubated for 1 hour at room temperature in the dark. The LOOH content of the samples were determined by comparison with a H_2O_2 standard plot and expressed as nmol H_2O_2 equivalents per mg LDL protein.

2.6.5.5 Measurement of Plasma total antioxidant status (TAOS)

Plasma TAOS was measured by Dr Jeffrey Stephens using a photometric microassay previously described by Sampson *et al.*(Sampson *et al*, 2002). The TAOS of plasma was determined by its capacity to inhibit the peroxidase-mediated formation of the 2,2-azino-

bis-3-ethylbensthiazoline-6-sulfonic acid ($ABTS^+$) radical. In the assay, the relative inhibition of $ABTS^+$ formation in the presence of plasma is proportional to the antioxidant capacity of the sample. Therefore, there are two arms to the assay, a control arm and test arm. In the control arm, phosphate-buffered saline is used instead of plasma. The difference in absorbance at 405nm (control absorbance minus test absorbance) divided by the control absorbance (expressed as a percentage) was used to represent the percentage inhibition of the reaction.

2.6.5.6 Electrophoresis of LDL

As LDL becomes gradually oxidised it acquires a net negative charge, partly due to the aldehydes (a by-product of lipid peroxidation) binding and neutralising the positively charged lysyl residues of apoB-100. Thus the extent of LDL oxidation can be assessed by the net negative charge of LDL, measured by agarose gel electrophoresis with LDL of greater net negative charge migrating further towards the anode. Native or OXLDL (3μl, at 100μg/ml) were loaded on to a pre-cast 0.5% (w/v) agarose gel (Lipogels®; Beckman Coulter, Fullerton, CA, USA) and allowed to diffuse into the gel for 5 minutes, before being subjected to electrophoresis at 100V for 40 minutes using a Beckman Paragon electrophoresis system in the barbitone buffer (pH 8.6) supplied. Following this, the gels were placed in a fixative solution supplied and allowed to dry for a few hours. Once dry the gels were stained for 10 minutes with a lipid stain (lipostain®) consisting of Sudan Black B in 55% (v/v) ethanol, destained in 45% (v/v) ethanol for a few minutes, rinsed in dH_2O and dried. The electrophoretic mobility of each oxidised LDL species can then be calculated as the distance migrated by the oxidised LDL from the origin in comparison to the distance migrated from the origin by the native LDL.

CHAPTER 3

CHAPTER 3: *APOA4* POLYMORPHISMS AND INTERMEDIATE PHENOTYPES OF CHD RISK

3.1 INTRODUCTION

APOA4 has been mapped to the long arm of chromosome 11 (11q23-q24) within the *APOA1/C3/A4/A5* cluster (Karathanasis, 1985; Pennacchio *et al*, 2001). At present little is known about the regulation of *APOA5* expression, the fact that it is not under the influence of the *APOC3* enhancer (-780/-580) which contains regulatory elements and tissue specific enhancers influencing the expression of the *APOAI/C3/A4* genes, suggests that its expression is independently regulated (Baroukh *et al*, 2004; Bisaha *et al*, 1995; Groenendijk *et al*, 2001a). *APOA4* expression is limited to intestinal and hepatic cells, implying a tissue specific transcriptional regulation of the gene. Analysis of the *APOA4* promoter (-300 to +10) promoter using *DNaseI* footprinting, revealed 4 protected sites: AIVA (-32 to –22), AIVB (-84 to –42), AIVC (-148 to –92) and AIVD (-274 to –250) (Ktistaki *et al*, 1994). Element AIVC was required for maximal promoter activity, which can be activated by hepatocyte nuclear factor 4 (HNF-4) and repressed by Arp-1 and Ear-3 (Ktistaki *et al*, 1994). *In vitro* experiments have revealed that the (–700 to +10) *APOA4* promoter region is incapable of driving optimal transcription *in vivo*, requiring the upstream *APOC3* enhancing region for maximal expression (Ktistaki *et al*, 1994). More recent studies have elucidated that the distal region of the *APOA4* promoter (-700/-310) contains a hormone response element essential for the correct, restricted, endogenous *APOA4* expression pattern within the intestine, with the ratio of HNF-4α and HNF-4β controlling the spatial expression of *APOA4* along the crypt to villus axis *in vivo* (Le

Beyec *et al*, 1999; Sauvaget *et al*, 2002). Various studies have revealed the intestinal synthesis of apoA-IV is increased with lipid absorption (Kalogeris *et al*, 1994; Stan *et al*, 2003), and that apoA-IV synthesis is linked to chylomicron assembly after lipid supply (Hayashi *et al*, 1990). Recent evidence implies that lipid supply in the basal pole of the enterocytes increases *APOA4* transcription and mRNA stabilization, while an apical supply of lipid induces *APOA4* transcription through a HNF-4 dependent mechanism (Carriere *et al*, 2005).

The association of the common *APOA4* variants with lipid levels and CHD risk have been investigated, yielding controversial results. Some population studies have observed that carriers of the H360 allele (of the Q360H polymorphism) have a favourable lipoprotein profile: increased HDL cholesterol (Menzel *et al*, 1988; Menzel *et al*, 1990), lower LDL cholesterol (Ehnholm *et al*, 1994; Ganan *et al*, 2004), reduced fasting TGs (Eichner *et al*, 1989; Hanis *et al*, 1991; Menzel *et al*, 1990), lower lipoprotein(a) (von Eckardstein *et al*, 1992; von Eckardstein *et al*, 1993) and increased apoA-I concentration (Hanis *et al*, 1991). However other studies have reported no significant associations at all (De Knijff *et al*, 1988; Hanis *et al*, 1991). These differences could be a result of the interactions between the apoA-IV H360 isoform and dietary factors within each of the populations studied, therefore reflecting population heterogeneity. A number of population studies have found the S347 allele (of the T347S polymorphism) to be associated with reduced total cholesterol, LDL levels with lower apoB levels (noted only in homozygotes) (Saha *et al*, 1997; von Eckardstein *et al*, 1992)and lower lipoprotein(a) levels. But similarly to the H360 allele, some studies have reported the S347 allele to

have no influence on lipid or lipoprotein plasma levels (Larson *et al*, 2002; Miltiadous *et al*, 2002). The N127S variant and its associations with lipid and lipoprotein levels has yet to be examined in any population other than in a Finnish cohort. The N127S polymorphism was found not to influence lipid or lipoprotein levels and the S127 allele distribution in hyperlipidemic men was discovered to be similar to that observed in the normal population (Tenkanen *et al*, 1992). Likewise with the VNTR polymorphism ($CTGT_{3/4}$), only a limited number of studies have been performed and no significant associations with lipid and lipoprotein levels are reported (Groenendijk *et al*, 2001b; von Eckardstein *et al*, 1992). Before carrying out the investigations for my thesis, despite the H360 allele being associated with a more atherogenic lipoprotein profile amongst Costa Ricans with an urban lifestyle (Campos *et al*, 1997), no associations of the common apoA-IV polymorphisms with CHD risk or MI had been published.

3.2 METHODS

3.2.1 Study groups

3.2.1.1 The Kronenberg study

The Kronenberg study is a cross sectional study comprising of 114 Caucasian male cases (from Austria), whom underwent coronary angiography with coronary stenosis of $\geq 50\%$ of the luminal diameter in at least one coronary artery (Kronenberg *et al*, 2000). The mean age of Caucasian case subjects was 60 ± 10 years and these were compared with 114 male controls (mean age 53 ± 8 years) recruited from the Project of Munster Heart

Study (PROCAM) from Germany (Assmann *et al*, 1996). In addition the Kronenberg study also included 68 male patients from India and 68 age matched Indian male controls (mean age 52 ± 8 years), recruited using the same criteria as the Caucasian males. Plasma apoA-IV levels were measured using an ELISA assay (Kronenberg *et al*, 1994; Rosseneu *et al*, 1988).

3.2.1.2 European Atherosclerosis Research Study (EARS)

Male and female students aged 18 to 26 years were recruited from 14 universities from 11 European countries (Austria, Belgium, Denmark, Finland, France, Germany, Italy, Spain, Sweden, Switzerland, and the United Kingdom) (The EARS group 1994, 1994), "cases" were defined as those whose fathers had documented acute myocardial infarction (MI) before the age of 55 years. Two age- and sex-matched controls were recruited by random selection from the same university population. Detailed descriptions of lifestyle and quantitative trait measurements as well as additional protocols have been previously described (The EARS group 1994, 1994). Serum apoAIV concentrations were measured by sandwich enzyme-linked immunosorbent assay (ELISA) in Bruges (Rosseneu *et al*, 1988).

3.2.1.3 Northwick Park Heart Study II (NPHSII)

NPHSII is a large prospective study of healthy middle-aged (50 to 61years) men drawn from 9 general medical practices (family doctors) throughout the UK. Of the initial cohort of 3052 men, 2808 DNA samples were available. The study has been ongoing for 9 years, and men have been followed-up annually for lipid and clotting factor measures.

Full details of anthropometric and biochemical measurements and other aspects of the study are well documented elsewhere (Miller *et al*, 1995; Miller *et al*, 1996). Lipid measures were taken nonfasting, the exact instruction for clinic attendance and the possible implications of nonfasting measures are presented elsewhere (Talmud *et al*, 2002). CHD events taken as end-points were fatal (sudden or not) and nonfatal MI (n=134) based on WHO criteria (World Health Organization Regional Offfice for Europe and Myocardial Infarction Community Register , 1976), plus coronary artery surgery (n=33) and silent MI on the follow-up ECG (n=20) (in which case the time to event was assumed to have been midway between the baseline and follow-up records). Ethical approval was obtained from the USA National Institutes of Health, who partly funded the study and from the local ethical committee in the UK.

3.2.1.4 The University College Diabetes and Cardiovascular Study (UDACS)

The University College Diabetes and Cardiovascular Study (UDACS), is a cross-sectional study designed to analyse the association between common variants in inflammatory/metabolic genes and biochemical risk factors implicated in CVD in patients with diabetes (Stephens *et al*, 2004). Briefly, UDACS comprises of 1,011 consecutive subjects recruited from the diabetes clinic at University College London Hospitals National Health Service Trust (UCLH) between the years 2001 and 2002. All patients had diabetes according to World Health Organization criteria (Alberti and Zimmet, 1998). Analysis was confined to Caucasian subjects (780, of whom 731 were successfully genotyped for the T347S gene variant) to avoid the confounding effect of ethnicity. Subjects were categorized by the presence/absence of clinically manifested CVD. CVD

was recorded if a patient had one or more of CHD, peripheral vascular disease (PVD), or cerebrovascular disease (CbVD). The presence of CHD was recorded if any patient had positive coronary angiography or angioplasty, coronary artery bypass, a positive cardiac thallium scan or exercise tolerance test, or documented evidence of myocardial infarction or symptomatic/treated angina. The presence of PVD was recorded in any patient with absent peripheral pulses and abnormal lower-limb doppler pressures or an abnormal lower-limb angiogram, previous angioplasty, or limb bypass graft. CbVD was recorded if a patient had been investigated for symptoms or signs consistent with a cerebrovascular accident and had a brain computed tomography scan showing any evidence of infarction (diffuse/localized) or hemorrhage. Subjects who were asymptomatic for CHD/CbVD/PVD or who had negative investigations were categorized as having no CVD.

3.2.2 SSCP protocol

DNA from 96 healthy UK Caucasian men from the Northwick Park Heart Study (NPHS II) was screened for variation in the final 1.8 Kb region of the *APOA4* promoter (from -1550 to +244, with nucleotides numbered from the translation start site). The SSCP protocol, including the sequence of all primers used and parameter settings for the MegaBace capillary sequencer are listed in section 2.3.8.

3.2.3 DNA sample preparation and genotyping

DNA from the participants of the Kronenberg study was extracted as previously mentioned in section 2.3.1 via the 'salting out method'. The primers, PCR conditions and restriction enzyme analysis used for T347S, N127S and CTGT polymorphism detection are described in section 2.3.5-2.3.7. While the heteroduplex method to simultaneously genotype the T347S and Q360H *APOA4* variants are described in section 2.3.7.4.

3.2.4 Statistical analysis

3.2.4.1 General statistics

Analysis was performed using SPSS (version 11.5, SPSS Inc.,Chicago). Deviations from Hardy-Weinberg equilibrium were considered using chi-squared tests. Allele frequencies are shown with the 95% confidence intervals. Results for quantitative traits are presented as mean ± standard deviation or median (interquartile range). To assess the differences between CVD and CVD-free patients, two-sided T-tests were performed on normally distributed data or after appropriate transformation (log or square root). In all cases a P-value of less than 0.05 was considered statistically significant.

3.2.4.2 Kronenberg/EARS statistical analysis

The association of *APOA4* T347S and apoA-IV levels was analysed by analysis of variance (ANOVA) adjusted for case-control status, age, sex, region, BMI, physical activity, TG and contraception (only in EARS).

3.2.4.3 NPHSII statistical analysis

Survival analysis with respect to T347S, N127S and CTGT genotypes was performed using the Cox's proportional hazard ratio. Ninety five percent confidence intervals (CI) for the estimates were calculated from the standard error assuming a normal distribution.

3.2.4.4 UDACS statistical analysis

The interaction between T347S genotype and CVD in determining plasma total anti-oxidant status (TAOS) levels was performed using linear regression including an interaction term for genotype and CVD status. ANOVA was used to assess the association between genotype and plasma TAOS in subjects with and without CVD, *a priori* combining the 347TT and TS groups due to the reported recessive effect on apoA-IV levels of the S347 allele was performed (Wong *et al*, 2003). The relationships between baseline parameters and plasma TAOS were tested by the Spearman rank correlation co-efficient.

3.2.5 Haplotype analysis method

The NPHSII haplotype construction was carried out by Emma Hawe our statistician, using the PHASE programme (Stephens *et al*, 2001), parameters and algorithms that were used are fully explained in Talmud *et al* (Talmud *et al*, 2002).

3.3 RESULTS

3.3.1 Screening the *APOA4* promoter for variation, SSCP results

ApoA-IV has been postulated to play a role in the anti-atherogenic reverse cholesterol transport pathway. This is based upon *in vitro* experiments indicating that apoA-IV acts as a cofactor for Lecithin Cholesterol Acyltransferase (LCAT), modulates LPL activity in the presence of apoC-II and enhances the activity of cholesterol ester transfer protein (CETP) (Barter *et al*, 1988; Goldberg *et al*, 1990; Main *et al*, 1996; Steinmetz and Utermann, 1985). *In vivo* studies performed using transgenic mice propose an anti-atherogenic role for apoA-IV (Cohen *et al*, 1997; Duverger *et al*, 1996), furthermore the association of low plasma apoA-IV levels with CHD in men (Kronenberg *et al*, 2000) implies apoA-IV could be an anti-atherogenic factor in humans as well. Hence variation in the *APOA4* gene could affect an individuals risk of CHD and this was investigated in various cohorts (section 3.2.1). Whilst known *APOA4* variants were analysed in this thesis, the remaining sequence of the apoA-IV promoter (-1550 to +244 bps, from the start of translation) yet to be screened was also analysed by SSCP (section 2.3.8).

Approximately 1.8 Kb of the promoter region of *APOA4* was successfully screened by SSCP (figure 3.1). Using overlapping PCR pairs on the MegaBace no polymorphisms detectable by SSCP within the *APOA4* promoter were found. A typical MegaBace readout for two invariant PCR products is shown in figure 3.2.

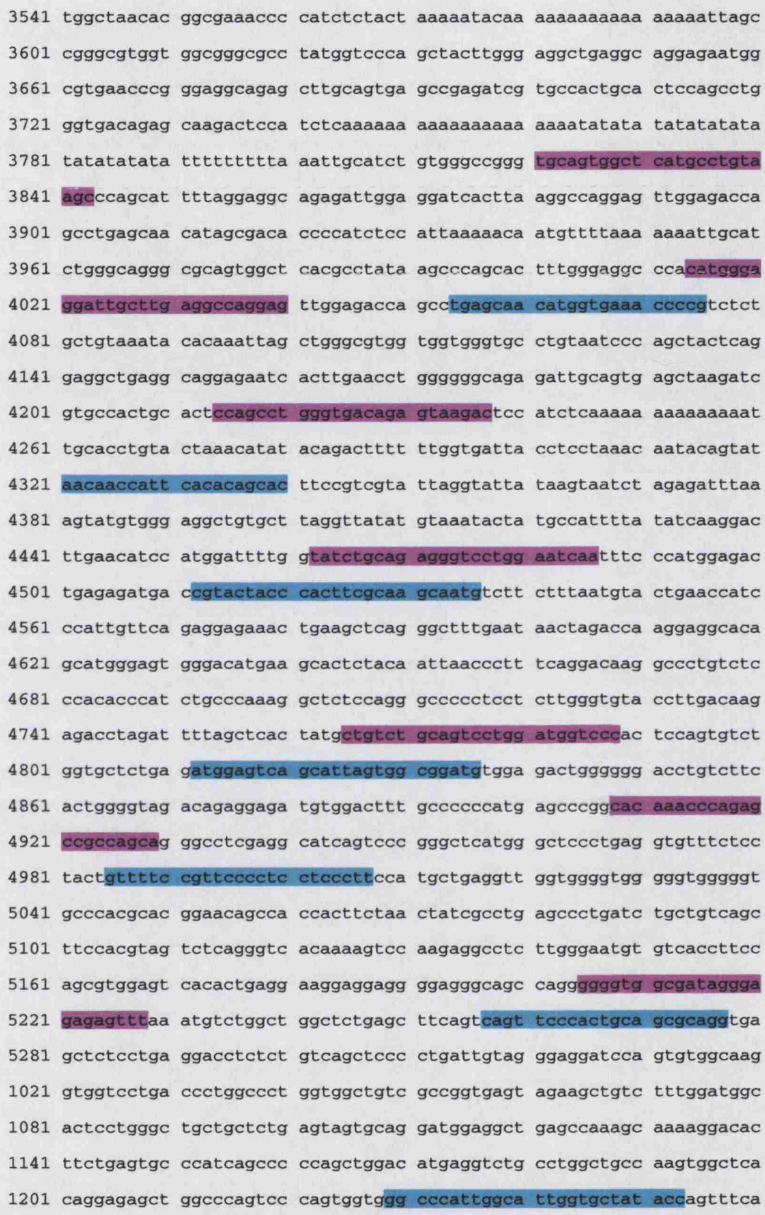


Figure 3.1: Illustrating the overlapping oligos designed for SSCP analysis of the *APOC3/A4* intergenic region (ac n.o X78901). The forward oligos are highlighted in pink and the reverse in blue.

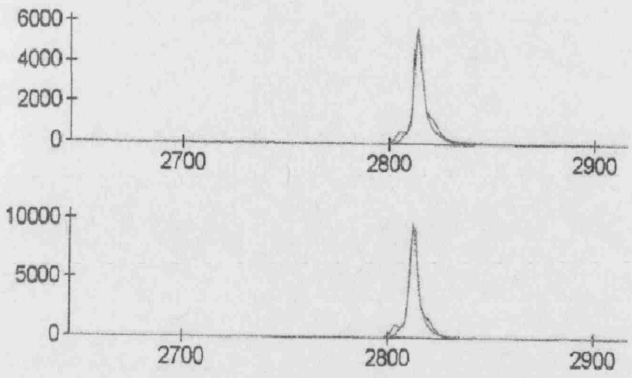


Figure 3.2: A characteristic MegaBace readout of two invariant PCR products.

3.3.2 Variation in the *APOA4* gene and the risk of CHD

3.3.2.1 The *APOA4* variants T347S and Q360H demonstrate no association with apoA-IV plasma levels in the Kronenberg study

The *APOA4* T347S and Q360H variants were genotyped only in the Caucasian case samples provided. Both the T347S and Q360H genotype distribution were found to be in Hardy Weinberg equilibrium with a rare allele frequency of 0.182 and 0.1 respectively. No significant associations were found with any parameters measured in the Kronenberg study (table 3.1 and 3.2) and there were no significant differences in apoA-IV levels in males or females. Finally the lack of association between either T347S or Q360H genotype on apoA-IV levels remained once apoA-IV plasma levels were adjusted for age, sex, urea and creatine measurements (P=0.160 and P=0.438 respectively).

Trait	T347S Genotype				
	TT	TS	SS	Probability	Probability
	N=90	N =39	N = 5	3 way	TT/TS v SS
Age (years)	62.1 (13.7)	60.16 (11.25)	63.6 (8.23)	0.634	0.687
Sex (F/M)	25/66 (27.2%/71.7%)	9/5 (64.2%/35.7%)	3/2 (0.6%/0.4%)	0.523	0.488
Plasma apoA-IV levels (mg/dl)	10.57 (3.90)	9.77 (3.24)	9.72 (6.22)	0.504	0.754
Urea (mmol/l)	36.74 (9.54)	37.16 (12.74)	38.15 (8.32)	0.634	0.841
Creatinine (mmol/l)	1.13 (0.17)	1.12 (0.17)	1.23 (0.16)	0.671	0.272
Body mass index (kg/m^2)*	25.9 (24.81-32.05)	25.41 (22.7-34.14)	26.73 (24-27.8)	0.405	0.635
Height (m)	1.70 (8.21)	1.68 (6.67)	1.69 (7.98)	0.711	0.907

Table 3.1: Baseline characteristics by *APOA4* T347S genotype in the Kronenberg study .Mean (SD) or Median (Interquartile range) shown, *Log transformed. Analysis was performed by two-sided T-tests after appropriate transformation.

Trait	Q360H Genotype				
	QQ	QH	HH	Probability	Probability
	N=113	N =26	N = 1	3 way	QQ/QH v HH
Age (years)	61.63 (10.72)	62.19 (9.93)	63	0.964	0.905
Sex (F/M)	27/86 (23.9%/76.1%)	8/18 (30.8%/69.2%)	Female	0.183	0.089
Plasma apoA-IV levels (mg/dl)	10.22 (3.87)	10.52 (3.58)	11.27	0.903	0.795
Urea (mmol/l)	37.24 (10.74)	35.93 (9.86)	26.7	0.539	0.333
Creatinine (mmol/l)	1.13 (0.18)	1.10 (0.139)	1.01	0.568	0.485
Body mass index (kg/m^2)*	25.95 (24.10-33.10)	27 (24.86-32.05)	25.8	0.897	0.886
Height (m)	1.69 (7.74)	1.69 (7.76)	1.61	0.501	0.265

Table 3.2: Baseline characteristics by *APOA4* Q360H genotype in the Kronenberg study .Mean (SD) or Median (Interquartile range) shown, *Log transformed. Analysis was performed by two-sided T-tests after appropriate transformation.

3.3.2.2 The *APOA4* T347S is associated with reduced apoA-IV plasma levels in the EARS study

Previously the *APOA4* Q360H variant was genotyped in the EARS study, no association with apoA-IV plasma concentration or strong genetic differences between plasma lipid concentrations in 'cases' and 'controls' were found (Ehnholm *et al*, 1994). Based on three case-control studies demonstrating apoA-IV levels to be significantly lower in CHD cases compared to controls (Kronenberg *et al*, 2000; Warner *et al*, 2001), we decided to determine the relationship between the *APOA4* S347 variant and apoA-IV levels in the EARS study (apoA-IV levels were not availible in NPHSII).

The frequencies of the *APOA4* T347S variant within the different regions are shown in table 3.3. All genotype distributions were in Hardy Weinberg equilibrium. The successful genotyping of the T347S variants is illustrated in figure 3.3. There was significant evidence for allele frequency heterogeneity among regions (P=0.04) with the "middle" region having the highest S347 allele frequency. No significant heterogeneity of T347S frequency between cases and controls (P=0.14) was found, thus for all subsequent analyses cases and controls were considered together. Considering the effect of genotype on apoAIV levels, because there was no significant heterogeneity of the genotype effect between cases and controls, across regions, or between sexes, the effect of T347S on apoAIV levels is presented in the sample as a whole (figure 3.4). After correcting for age, gender, case-control status, BMI, physical activity, TG, HDL, and contraception, individuals homozygous for the S347 had significantly lower apoAIV plasma levels (13.68±0.59 mg/dL) than those carrying the T347 allele (14.90±0.12 mg/dL) (P=0.035,

figure 3.4). There was no statistically significant effect of this genotype on any lipid variable, BMI, or waist hip ratio (WHR).

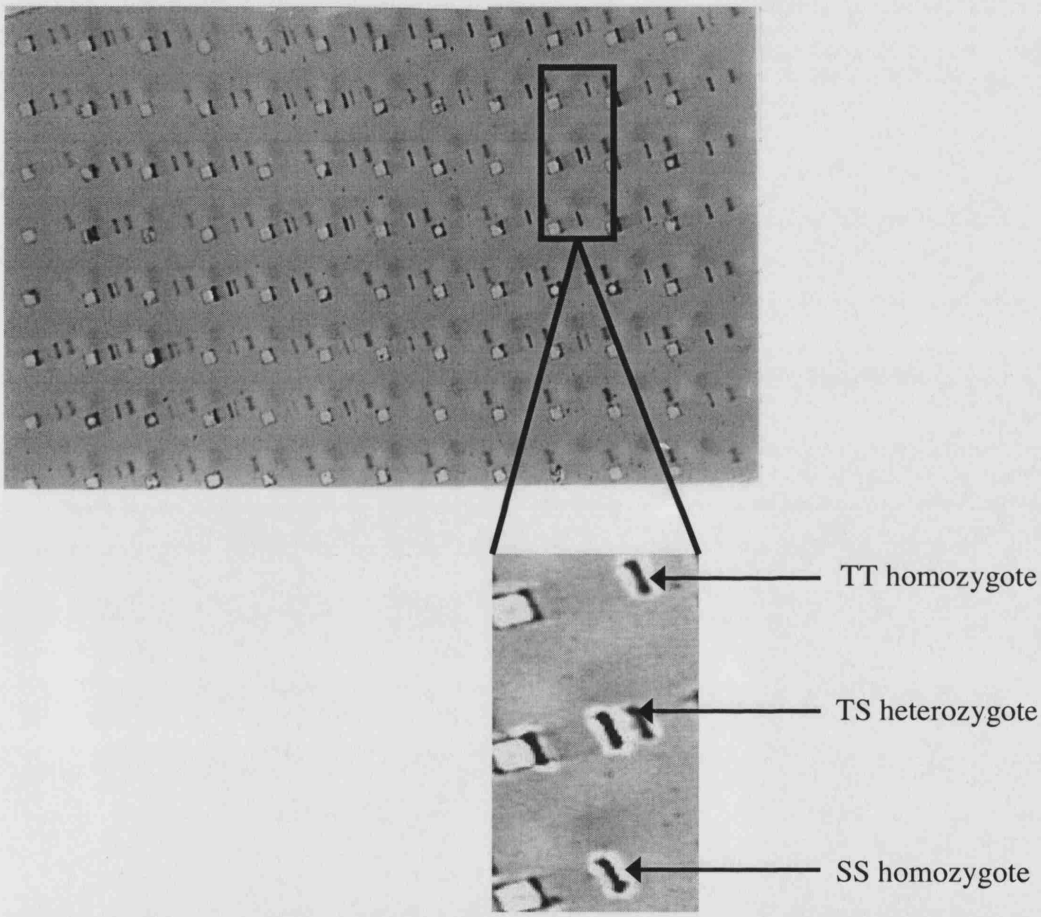


Figure 3.3: *Hinf I* digestion of the T347S PCR product analysed by MADGE.

	T347S	
Region	Number Genotyped	S347 Allele Frequency
Finland		
Case	84	0.137
Control	183	0.169
Britain		
Case	57	0.140
Control	107	0.164
North		
Case	152	0.184
Control	303	0.200
Middle		
Case	129	0.209
Control	214	0.224
South		
Case	139	0.169
Middle	250	0.194
All regions		
Case	561	0.175
Middle	1057	0.194

Table 3.3: Distribution of the *APOA4* T347S in the cases and controls in the 5 regions of Europe participating in the in EARS study. There was no significant departure from Hardy-Weinberg equilibrium. Case-control differences in allele frequency, adjusted for region: P=0.14. Difference between regions, adjusted for status: P=0.04

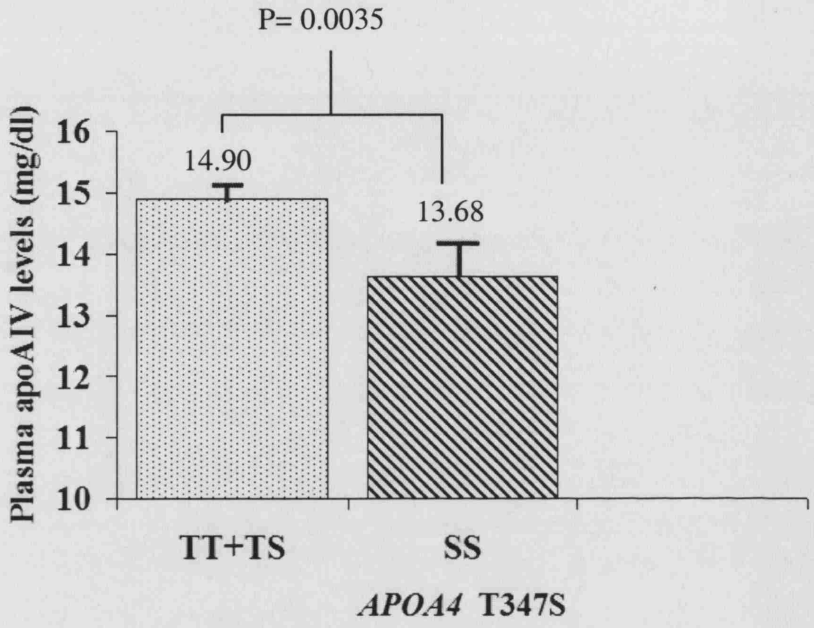


Figure 3.4: Mean apoA-IV plasma levels according to *APOA4* T347S genotype in EARS subjects, adjusting for case-control status, age, sex and region, BMI, physical activity, TG, HDL and contraception. Mean values and SEM are given for TT+SS (n=1559) and SS (n=52).

3.3.2.3 The *APOA4* gene variant S347 is associated with increased risk of CHD in NPHSII, independent of any lipid parameters

Analysis of the baseline characteristics of NPHSII men revealed that compared to men whom remained free of CHD (n=2621), men who had an event (n=187) were significantly older, had a greater BMI and higher systolic and diastolic blood pressures, were more likely to be smokers, had higher; triglyceride (TG), total and low-density lipoprotein density lipoprotein (LDL)–cholesterol and apoB levels, in addition to lower high-density lipoprotein (HDL)-C and apoAI levels (table 3.4).

Trait	No CHD Event (n=2621)	CHD Event (n=187)	*P*
Age, y	56.01 (3.42)	56.67 (3.62)	0.01
Body mass index, kg/m²*	26.19 (3.37)	26.96 (3.42)	<0.0005
Current smoking	27.43%	38.50%	0.001
Diastolic blood pressure, mmHg*	83.58 (11.21)	86.69 (11.95)	<0.0005
Systolic blood pressure, mmHg*	136.64 (18.60)	142.65 (20.16)	<0.00005
Cholesterol, mmol/L	5.71 (1.01)	6.10 (1.04)	<0.00005
Triglyceride, mmol/L†	1.78 (0.93)	2.11 (1.14)	<0.00005
ApoB, mg/dL†	0.86 (0.24)	0.92 (0.23)	0.0007
ApoAI, mg/dL	1.64 (0.32)	1.58 (0.27)	0.02
LDL-C, mmol/L‡	3.06 (1.01)	3.41 (0.97)	<0.00005
HDL-C, mmol/L‡	1.71 (0.61)	1.53 (0.56)	<0.0005

Table 3.4: Mean baseline characteristics (and SD) of the men in NPHSII considering all those genotyped for the variants in the *APOC3-A4-A5* gene cluster who had a CHD event or not. *Geometric means presented with approximate standard deviations. †Given are the anti-log of the log-transformed mean, and standard deviations are approximated. ‡Calculated according to Walldius *et al* (Walldius *et al*, 2001).

Genotype distributions were found to be in Hardy Weinberg with a rare allele frequency for the S347 variant being 0.18. There was borderline significant differences in allele frequency between carriers of the T allele and SS homozygotes in subjects with or without CHD (SS vs T allele; P=0.053). The risk of a coronary event was assessed using hazard ratio (HR) via Cox regression analysis with the TT homozygotes set as 1. Homozygosity for the 347S allele showed a significant increase effect on risk [HR 2.04 (95%CI 1.02-4.05)] after adjustment for age, general clinical practice and TGs. Initial analysis also revealed that T347S genotype was associated with differences in TG levels, with SS homozygotes having 20.5% lower TG concentrations than carriers of the T allele (SS vs T allele; P= 0.001). T347S genotype was not associated with other lipid or CHD risk traits (table 3.5). Given that the relationship between raised plasma TGs and increased CHD risk has been confirmed by meta-analysis as an independent CHD risk factor (Hokanson and Austin, 1996), this SS allele association in NPHSII with increased CHD risk yet reduced TG levels was hard to rationalize.

Trait	T347S Genotype			
	TT	TS	SS	Probability
	N=1496	N =679	N = 77	3 way
Age (years)	55.89 (3.29)	55.97 (3.24)	55.65 (3.44)	0.683
Body mass index (kg/m^2)*	26.04 (24.04-28.22)	26.31 (24.04-28.5)	26.10 (24.05-28.2)	0.184
Current smoking	28.7%	27.9%	26%	0.835
Diastolic blood pressure (mmHg)	84.41 (3.37)	84.76 (11.19)	83.86 (10.9)	0.702
Systolic blood pressure (mmHg)	138.28 (19.4)	138.8 (19.5)	138.42 (17.88)	0.846
Cholesterol (mM)	5.77 (1.01)	5.77 (1.03)	5.77 (1.02)	0.244
Triglycerides (mM)*	1.75 (1.25-2.65)	1.68 (1.23-2.47)	1.47 (2.85)	0.003
ApoB (mg/dL) *	0.87 (0.74-1.03)	0.86 (0.73-1.03)	0.82 (0.67-1.01)	0.381
ApoAI (mg/dL)	1.63 (0.31)	1.63 (0.35)	1.63 (0.32)	0.846
LDL-C (mM)	3.12 (1.00)	3.13 (1.02)	3.02 (1.03)	0.706
HDL-C (mM)*	0.81 (0.66-0.99)	0.82 (0.66-1.00)	0.81 (0.67-1.02)	0.964

Table 3.5: Baseline characteristics by *APOA4* T347S genotype in NPHSII. Mean (SD) or Median (Interquartile range) shown, *Log transformed. Analysis performed by two-sided T-tests after appropriate transformation. $\chi 2$ tests used for categorical data.

3.3.2.4 Haplotype analysis of the *APOC3-A4-A5* gene cluster to deduce the *APOA4* S347 association with increased risk and low TGs

In humans the *APOA4* gene has been mapped to the long arm of chromosome 11 (11q23-q24) within the *APOA1/C3/A4/A5* cluster (Karathanasis, 1985; Pennacchio *et al*, 2001). The location of the *APOA4* T347S variant, including the 8 previously genotyped single nucleotide polymorphisms (SNPs) in NPHSII are illustrated in figure 3.5. Each SNP was examined separately for an association with CHD risk and the hazard ratio (HR) values are presented in table 3.6. Of the *APOC3* variants, 1100CT and TT showed a significant (protective) effect on risk [HR 0.65 (95%CI 0.56 to 0.92) and 0.28 (95%CI 0.09 to 0.87), respectively]. Neither *APOA5* 1131T>C nor the S19W variants had a significant impact on risk, whereas the *APOA4-A5* intergenic T>C showed a borderline statistically significant effect on risk, with CC men having a HR of 1.59 (95%CI 0.99 to 2.56). Using stepwise regression analysis examining all those SNPs with a significant effect on risk, after adjustment for age, cholesterol, TG, and stratification by general medical practice, *APOA4* T347S alone remained in the model. Compared with TT homozygotes, TS heterozygotes had a HR of 1.2 (95%CI 0.93 to 1.79) and SS homozygotes had a HR of 2.07 (95%CI 1.04 to 4.12), demonstrating a codominant effect on CHD risk independent of TG levels.

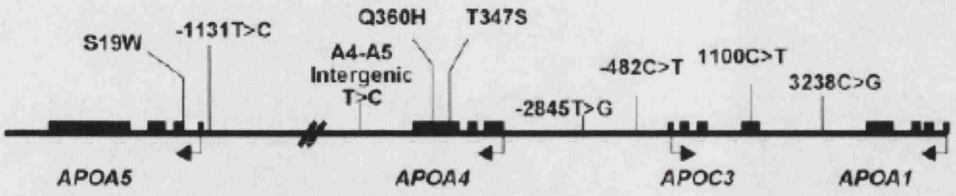


Figure 3.5: Map of the *APOC3-A4-A5* gene cluster, illustrating the position of the apolipoprotein genes, direction of transcription and location of variants studied.

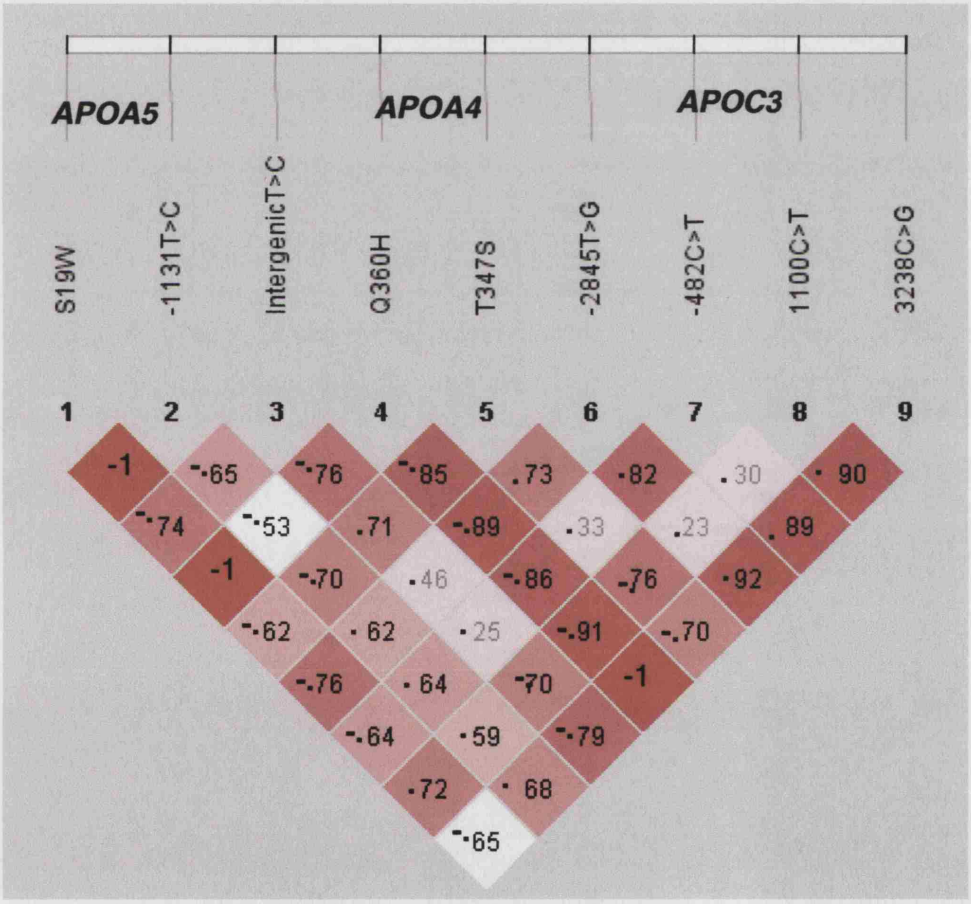


Figure 3.6: Haploview analysis showing the strong linkage (D') between the nine SNPs genotyped in NPHSII across the *APOC3-A4-A5* gene cluster. Software available at www.haploview.com.

	Hazard Ratio (95% CI)*	Hazard Ratio (95% CI)†
APOA5 −1131T>C		
TC	0.90 (0.54, 1.48)	0.91 (0.55, 1.51)
CC	1.10 (0.15, 7.83)	1.43 (0.20, 10.3)
Intergenic *APOA4-A5* T>C		
TT	1.37 (0.98, 1.91)	1.36 (0.97, 1.90)
CC	1.60 (1.00, 2.58)	1.59 (0.99, 2.56)
APOA5 S19W		
SW	0.79 (0.45, 1.36)	0.78 (0.45, 1.35)
WW	1.27 (0.18, 9.06)	1.47 (0.20, 10.56)
APOA4 T347S		
TS	1.32 (0.95, 1.82)	1.31 (0.95, 1.82)
SS	1.79 (0.91, 3.55)	2.04 (1.02, 4.05)
APOA4 Q360H		
QH	0.95 (0.62, 1.46)	0.91 (0.59, 1.41)
HH	3.31 (0.46, 24.02)	3.27 (0.45, 23.69)
APOC3 1100C>T		
CT	0.66 (0.47, 0.93)	0.65 (0.56, 0.92)
TT	0.29 (0.09, 0.92)	0.28 (0.09, 0.87)
APOC3 3238C>G		
CG+GG	0.72 (0.45, 1.15)	0.70 (0.44, 1.12)
APOC3 −482C>T		
CT	0.84 (0.60, 1.18)	0.85 (0.60, 1.19)
TT	0.97 (0.51, 1.86)	0.93 (0.48, 1.79)
APOC3 −2854 T>G		
TG	0.91 (0.66, 1.26)	0.90 (0.65, 1.24)
GG	1.27 (0.81, 1.98)	1.25 (0.80, 1.96)

Table 3.6: Univariate hazard ratios (187 coronary events out of 2808 individuals) for the nine *APOC3-A4-A5* variants. All compared to common allele homozygotes with a hazard ratio set at 1 (unless otherwise stated). *Adjusted for age and practice. †Adjusted for age and practice and triglyceride levels.

Due to the strong linkage disequilibrium (LD) existent across the *APOC3-A4-A5* gene cluster (figure 3.6) (Talmud *et al*, 2002) to further deduce the association of the *APOA4* T347S SNP with increased CHD risk yet reduced TG levels, haplotype analysis was performed using the 9 genotyped SNPs located across the *APOC3-A4-A5* gene cluster. Nineteen haplotypes that occurred in more than 10 individuals with at least 1 recorded CHD event, representing 88% of the sample were studied. The proportion of CHD events for each haplotype was calculated and ranked according to the proportion of risk (figure 3.7A). A comparison was made with the TG associated haplotypes ranked in the same order (figure 3.7B). 6 of the 5 high-risk haplotypes, haplotypes 1, 3, and 5 (representing 17.5% of the sample) all carried the *APOA4* S347 in combination with the intergenic *APOA4-A5* C and/or *APOC3* -2845G and/or *APOC3* -482T alleles. Haplotypes 2 and 4 (only found in 0.7% and 0.8% of the sample respectively) were defined by *APOC3* -2845G and *APOA5*W19, respectively, on the wild-type background.

It is clear that the ranking by proportion of events and by TG did not correspond (figures 3.7A and 3.7B). Haplotypes 1, 3, and 5 were associated with TGs below or around the sample mean of 1.80 mmol/L (1.71, 1.79, and 1.82 mmol/L, respectively), whereas haplotypes 2 and 4, defined by *APOC3* -2854G and *APOA5* W19, respectively, were associated with TG levels of 1.67 and 2.16 mmol/L, respectively (figure 3.7B). The common haplotype (haplotype 6), representing 36% of the sample was associated with an event rate of 8.1% significantly higher than the mean event rate of 6.7% (P=0.04) (figure 3.7A), yet men who carried this haplotype had mean TG levels of 1.75 mmol/L, ie, below the sample mean.

Because each individual will have two haplotypes, if one was the common haplotype, the second could be risk-raising, risk lowering, or risk-neutral. The overall risk-effect associated with the common haplotype would therefore depend on the haplotype frequencies of these other haplotypes. To analyze this in more detail, we estimated the risk associated with the 2 common risk-raising haplotypes 1 and 3, characterized by *APOA4* S347, intergenic *APOA4/A5* C, and/or *APOC3* -2845G, considering only those men who had in addition the common haplotype (haplotype 6), and compared this to the risk of all other haplotypes combined with the common haplotype (table 3.7). The proportion of events for men carrying haplotypes 1/6 and 3/6 was 14.3% compared with 7.2% for the haplotype 6/all other haplotypes pooled (P=0.02).

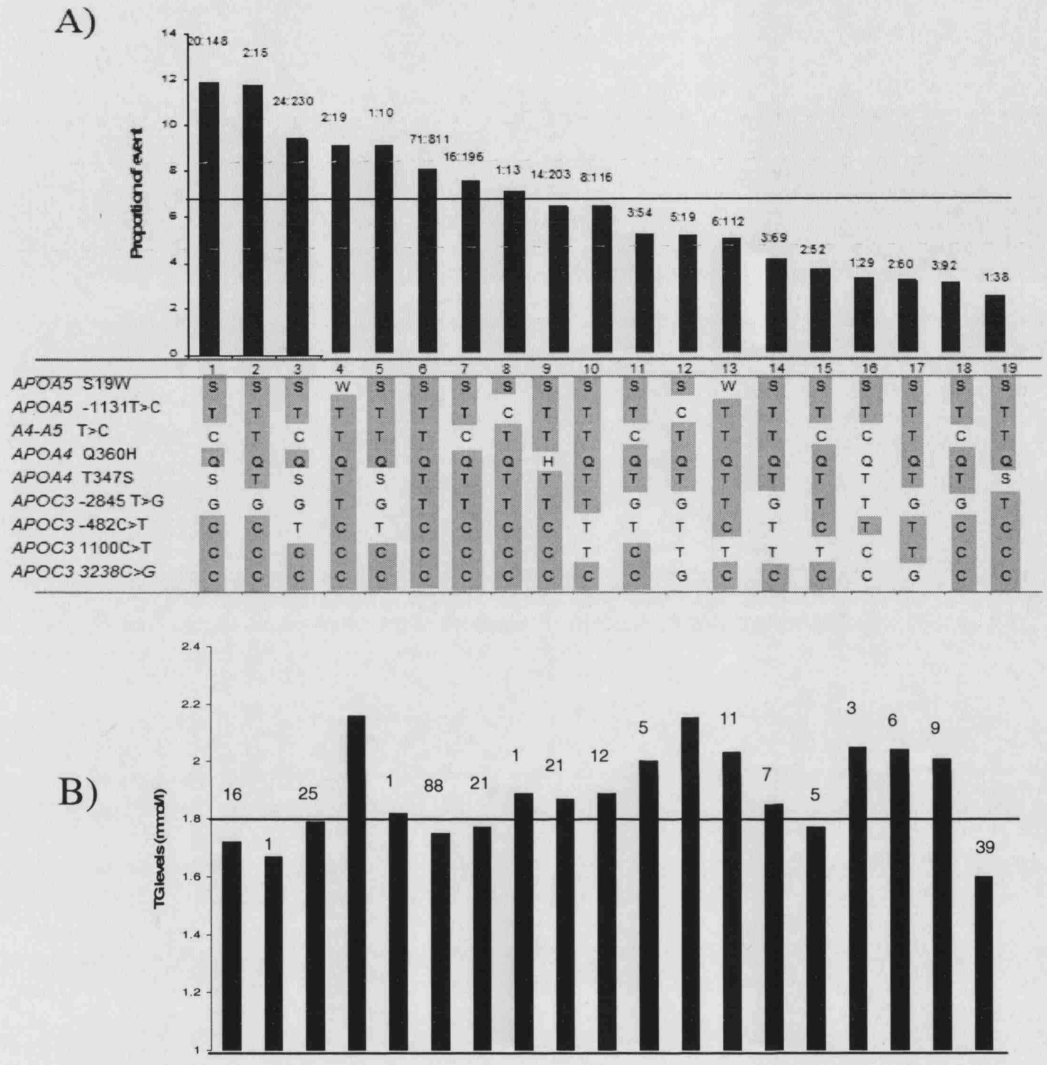


Figure 3.7A: Ranking of the haplotypes derived from the 9 SNPs according to the proportion of events given. Number of events to nonevents appears above each bar. Mean proportion of events for the sample is represented by a horizontal line. Common alleles are shaded. Figure 3.7B: Triglyceride levels associated with the haplotypes derived from the 9 SNPs, ranked in the same order as the proportion of events. Number of men with each haplotype is given above. Mean TG level for the sample is represented by a horizontal line.

Haplotypes	No CHD Event	CHD Event	Event Rate
1+3	126	18	14.3%
All others	555	40	7.2%

Table 3.7: Proportion of events associated with haplotypes in those individuals who carried the wild type haplotype. Comparing haplotypes 1 and 3 (defined by a combination of the rare alleles of *APOA4* S347, *APOA4-A5* intergenic C, and *APOC3* -2845G) to all other haplotypes was significant (P=0.02).

3.3.2.5 *APOA4* S347 carriers have reduced survival rates

Considering the HRs, Q360H showed no statistically significant effect on risk although men homozygous for the H360 allele had a HR of 3.27 (95%CI 0.45, 23.69). Because there is strong negative allelic association (D=-0. 91; P=0.0005) between the two *APOA4* variants (Talmud *et al*, 2002), we determined whether the survival rate of T347S was independent of Q360H by examining only those men homozygous for the Q360 allele. The Kaplan Meier survival curves produced (figure 3.8) clearly shows the lower survival rate in men homozygous for the S347 allele [HR 2.08 (95%CI 1.04, 4.18)] compared with men homozygous for T347, with heterozygous men showing intermediate survival [HR 1.40 (95%CI 0.98, 2.02)] (overall P=0.05).

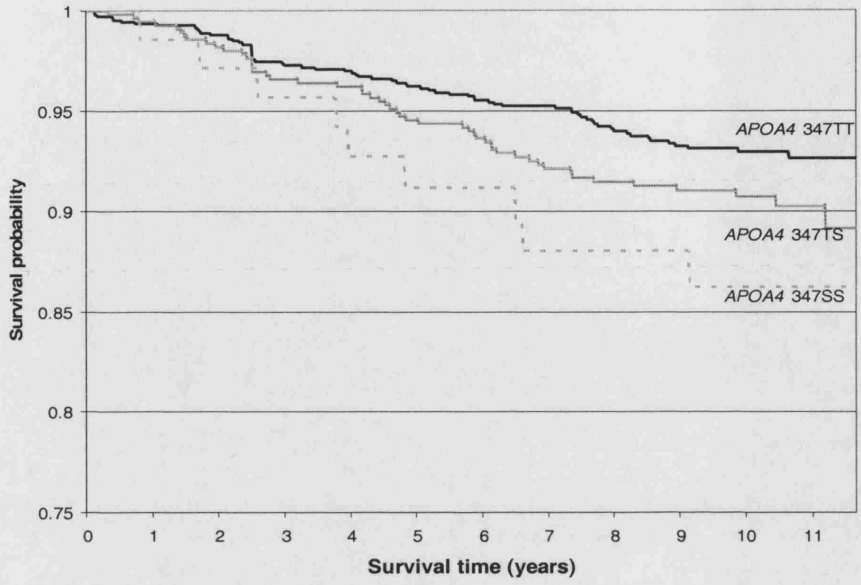


Figure 3.8: Survival functions of CHD events in NPHSII by *APOA4* T347S genotype. Graph of the estimated survivor functions from the Cox proportional hazard model stratified by T347S genotype in those men who were homozygous for the Q360 allele.

3.3.2.6 The *APOA4* T347S variant is associated with reduced plasma total antioxidant status in subjects with diabetes mellitus and cardiovascular disease

One possible pathological mechanism to explain the reported inverse association between apoA-IV levels and CHD (Kronenberg *et al*, 2000) is the ability of apoA-IV to act as an effective inhibitor of lipid peroxidation, a key process implicated in CHD progression (Chisolm and Steinberg, 2000; Navab *et al*, 2002). Based on the potential anti-oxidant properties of apoA-IV and our previous finding that the *APOA4* S347 variant is associated with increased CHD risk (Wong *et al*, 2003), the association of the *APOA4* S347 variant with plasma total anti-oxidant status (TAOS), which is inversely related to

oxidative stress, in a sample of subjects with diabetes mellitus and therefore at high risk of cardiovascular disease (CVD) was investigated.

3.3.2.6.1 Initial analysis of the *APOA4* S347 variant in the UDAC cohort

In total 734 (94.0%) Caucasian patients within the UDAC study were successfully genotyped for the *APOA4* T347S variant. Genotype distributions were found to be in Hardy Weinberg equilibrium with a rare allele frequency of 0.21 (95% CI 0.19-0.23). There was no significant differences in allele frequencies between subjects with or without CVD (P=0.4). CVD was present in 202 individuals, whose mean age, plasma creatine and C-reactive protein were significantly higher than those without CVD. A significantly higher proportion of patients with CVD were taking ACE inhibitors (ACEI), aspirin, insulin and statins (table 3.8), explaining the lower LDL-C levels and diastolic blood pressure in subjects with CVD compared to those without. Plasma TAOS was positively correlated with HDL-C plasma levels and negatively correlated with triglycerides, glucose and HBA1c (correlation coefficient r=0.11, -0.14, -0.12, -0.07, respectively all $p<0.05$). T347S had no effect on any baseline characteristics (table 3.9).

3.3.2.6.2 *APOA4* S347 and plasma TAOS

There was no heterogeneity of effects due to gender with respect to TAOS, thus analysis was performed on men and women together. In the sample there was no significant difference in the mean plasma TAOS by CVD status (Table 3.8), nevertheless a significantly higher percentage of subjects with CVD were found to be present in the lowest quartile of TAOS compared to the upper quartiles (P=0.04, figure 3.9). In the

group as a whole there was no association of T347S genotype with TAOS levels (P=0.63). However when the patients were stratified on the basis of CVD status, in those patients with CVD, homozygotes for the *APOA4* S347 allele had significantly lower plasma TAOS compared to carriers of the T347 allele (42.5% vs 31.2%, respectively, P= 0.0024, figure 3.10). This association remained significant after adjustment for age, triglycerides, HDL-C, glucose and HbA_{1c} (SS vs T allele, P=0.019). No such differences were observed in those subjects without CVD (P=0.32, figure 3.10). The interaction between CVD and genotype on plasma TAOS was statistically significant (P=0.014).

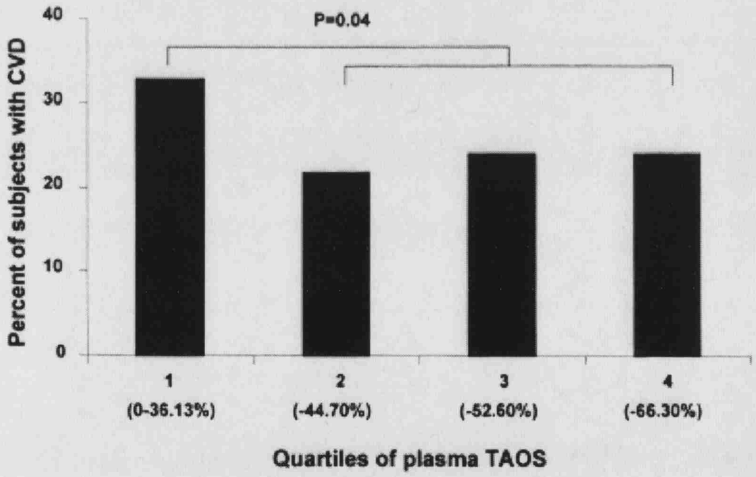


Figure 3.9: A bar chart representing the percentage of patients with cardiovascular disease by quartiles of total antioxidant status (TAOS).

Trait	No CVD (n=529)	CVD (n=202)	P Value
Age (years)	60.2 (14.1)	68.8 (10.1)	<0.001
Sex (F/M)	(43.2/56.8%)	(29.4/70.6%)	0.001
Duration (years)+	11 (5-11)	11 (6-18)	0.804
Systolic BP (mmHg)*	138 (127-150)	141 (128-153)	0.657
Diastolic BP (mmHg)*	81 (74-88)	76 (70-84)	<0.001
Body mass index (kg/m^2)*	28.1 (24.9-31.6)	29.0 (26.1-32.9)	0.277
HbA_{1c} (%)*	7.8 (6.8-9.0)	7.6 (6.6-8.8)	0.156
Glucose (mmol/l)*	10.1 (7.0-14.4)	9.6 (7.0-13.3)	0.735
Creatinine (mmol/l)*	87 (76-100)	98 (84-121)	<0.001
CRP (mg/l)*;	1.61(0.91-2.90)	1.86 (1.03-3.45)	0.050
LDL-C (mmol/l)	2.9 (0.9)	2.6 (0.9)	<0.001
TAOS %	44.8 (12.8)	44.2 (13.10)	0.510
ACEI (No/Yes)	(59.4/40.6%)	(43.1/56.9%)	<0.001
Aspirin (No/Yes)	(65.7/34.3%)	(26.0/74.0%)	<0.001
Insulin (No/Yes)	(52.7/47.3%)	(62.9/37.1%)	0.013
Statin (No/Yes)	(83.1/16.9%)	(45.2/54.8%)	<0.001
APOA4 T347S distribution	TT TS SS	TT TS SS	
N=	321 193 15	126 69 7	0.786
Rare allele freq	0.21 [0.15-0.27]	0.21 [0.15-0.26]	

Table 3.8: The baseline characteristics of the 731 Caucasian subjects genotyped by CVD status in UDACS. Mean (SD) or Median (Interquartile range) shown, *Log transformed, +Square root transformed. Analysis performed by two-sided T-tests after appropriate transformation. χ^2 test were used to compare groups.

Trait	T347S Genotype				
	TT	TS	SS	Probability	Probability
	N=447	N =262	N = 22	3 way	TT/TS v SS
Age (years)	62.1 (13.7)	63.2 (13.6)	64.2 (15.4)	0.50	0.57
Sex (F/M)	182/265 (40.8%/59.2%)	103/159 (39.4%/60.6%)	4/18 (18.2%/81.8%)	0.12	0.04
Duration (years)+	10.5 (5.0-10.0)	11.0 (5.0-20.0)	10.5 (4.0-22.0)	0.55	0.69
Systolic blood pressure (mmHg)*	138 (126-150)	140 (128-151)	136 (114-143)	0.14	0.10
Diastolic blood pressure (mmHg)*	80 (73-87)	80 (73-87)	80 (71-84)	0.16	0.07
Body mass index (kg/m^2)*	28.5 (25.2-31.7)	28.4 (25.4-31.6)	30.5 (24.3-32.7)	0.70	0.40
HbA_{1c} (%)*	7.7 (6.6-8.9)	7.9 (6.8-9.1)	7.5 (7.0-8.2)	0.15	0.64
Glucose (mmol/l)*	9.8 (7.0-14.0)	10.3 (7.5-14.7)	8.1 (5.0-13.3)	0.06	0.07
Creatinine (mmol/l)*	90 (77-107)	89 (78-104)	95 (79-113)	0.98	0.94
CRP (mg/l)*;	1.66 (0.92-2.83)	1.68 (0.93-3.32)	1.64 (0.98-2.70)	0.92	0.90
LDL-C (mmol/l)	2.8 (1.0)	2.9 (0.9)	2.9 (1.3)	0.51	0.64
Plasma TAOS (%)	42.40 (13.17)	43.18 (12.67)	41.35 (10.60)	0.67	0.64
CVD (%)	321/126 (71.8%/28.2%)	193/69 (73.7%/26.3%)	15/7 (68.2%/31.8%)	0.79	0.66
ACEI (No/Yes)	236/211 (52.8%/47.2%)	154/108 (58.8%/41.2%)	11/11 (50.0%/50.0%)	0.27	0.64
Aspirin (No/Yes)	246/201 (55.1%/44.9%)	146/116 (55.6%/44.4%)	8/14 (38.1%/61.9%)	0.30	0.12
Insulin (No/Yes)	261/186 (58.3%/41.7%)	134/128 (51.1%/48.9%)	11/11 (50.0%/50.0%)	0.16	0.60
Statin (No/Yes)	324/123 (72.4%/27.6%)	192/70 (73.3%/26.7%)	16/6 (72.7%/27.3%)	0.97	1.00

Table 3.9: Baseline characteristics by *APOA4* T347S genotype in UDACS. Mean (SD) or Median (Interquartile range) shown, *Log transformed, +Square root transformed. Analysis performed by two-sided T-tests after appropriate transformation. $\chi 2$ tests used for categorical data.

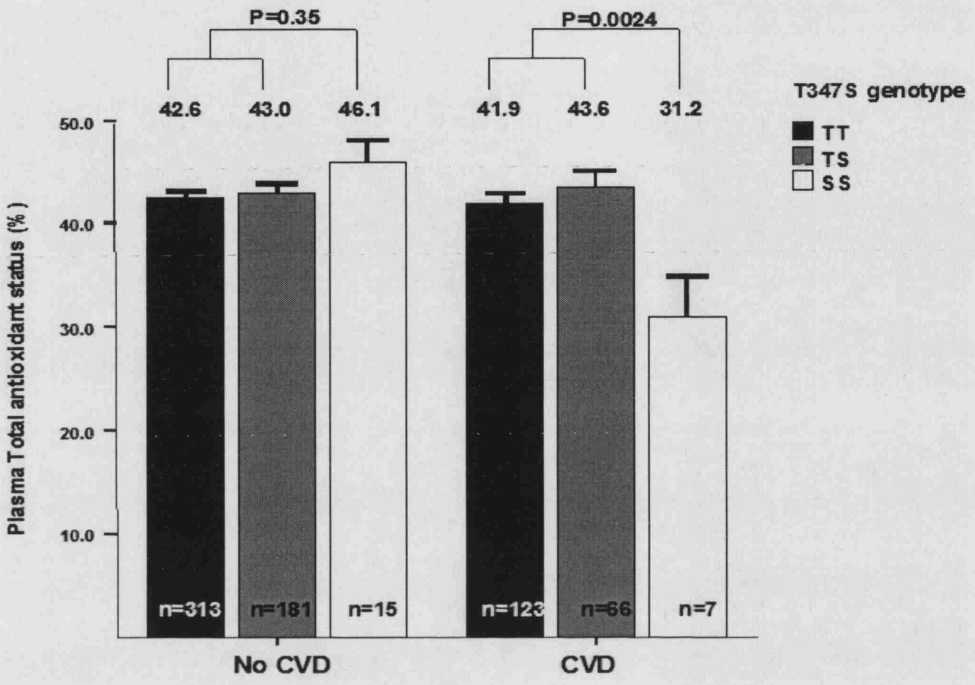


Figure 3.10: Mean plasma TAOS (%, ± SE) in relation to the *APOA4* T347S genotype in the UDAC cohort. Numbers of subjects are shown at the base of each column.

3.3.2.7 Analysing the *APOA4* N127S and CTGT $_{3/4}$ variants in NPHSII

Two additional *APOA4* SNPs were also examined in this thesis. The first additional polymorphism analysed was at nucleotide 1687 due to an adenine to guanine substitution resulting in an asparagine to serine substitution at codon 127 (N127S). The second polymorphism assessed was a CTGT variable number of tandem repeat (VNTR) polymorphism in the non-coding region of exon three, due to a 3 or 4 base pair insertion/deletion ($CTGT_{3/4}$). Both these additional *APOA4* variants have previously only been examined in a limited number of studies with no clear associations found. Given the previous association of the *APOA4* T347S variant with CHD risk, it was of interest to examine these additional polymorphisms and their possible association with CHD risk.

The genotype distributions of both the N127S and CTGT variants were found to be in Hardy Weinberg equilibrium with rare allele frequencies of 0.11 and 0.44 respectively in NPHSII men. The successful genotyping of the two polymorphisms is shown in figures 3.11 and 3.12. Baseline clinical and biochemical characteristics by N127S and CTGT are presented in tables 3.11 and 3.12 respectively. Both apoA-IV variants demonstrated no association between plasma total cholesterol, total cholesterol, triglyceride, apoA-I or apoB concentrations. As shown in table 3.10 there was no significant difference in the number of CHD events by genotype.

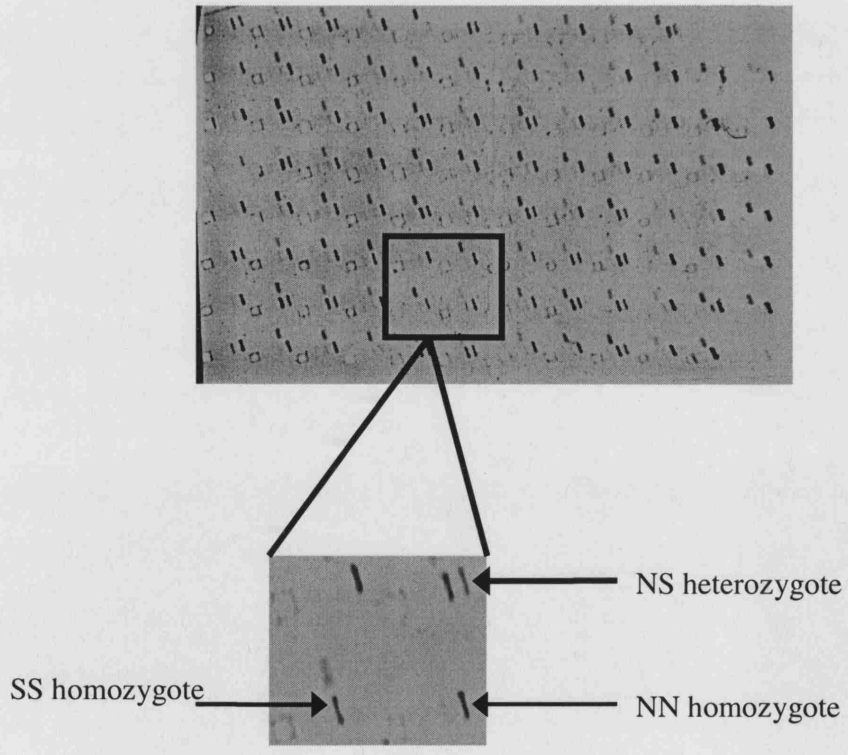


Figure 3.11: *Hinc II* digestion of the N127S PCR product analysed by MADGE.

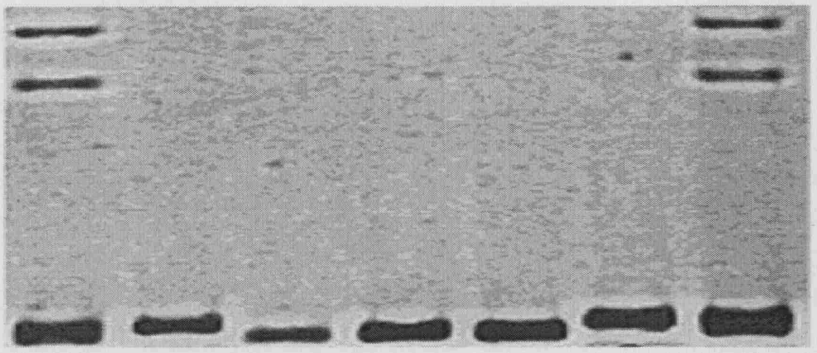


Figure 3.12: The 105bp CTGT PCR product run on a 17% polyacrylamide gel at 120V for 5 hours, with the corresponding CTGT genotypes (number of repeats listed below each band).

Polymorphism	Event	Genotype			P (3 way)
		11	12	22	
N127S	No CHD	176	458	24	0.341
	CHD	154	487	2	
CTGT	No CHD	765	1201	465	0.735
	CHD	62	91	41	

Table 3.10: Overall number of CHD events by N127S and CTGT genotype.

Trait	N127S Genotype			
	NN	NS	SS	P value from
	N=1943	N =487	N = 26	ANOVA
Age (years)	56 (3.44)	56.14 (3.51)	56.58 (3.75)	0.498
Cholesterol (mM)	5.72 (1.01)	5.74 (1.02)	5.53 (1.18)	0.547
ApoB*	0.85 (0.71 – 1.21)	0.86 (0.73-1.01)	0.78 (0.69-1.11)	0.511
HDL* (mM)	0.81 (0.66-0.99)	0.78 (0.61-1.04)	0.86 (0.61-1.05)	0.145
Body mass index (kg/m^2)*	26.04 (24-28.5)	26.31 (24.3-28.5)	25.8 (23.1-28.5)	0.72
LDL (mM)*	3.06 (2.38-3.79)	3.03 (2.38-3.74)	2.92 (2.33-3.56)	0.467
TG (mM)*	1.71 (1.23-2.56)	1.75 (1.30-2.58)	1.58 (1.26-3.06)	0.278
Smoking (never/former/current) (%)	29.4/38.4/27.2	31.1/36.9/24.5	17.2/44.8/27.6	0.133

Table 3.11: Baseline characteristics by N127S genotype in NPHSII. Mean (SD) or median (interquartile range) shown. Analysis was performed by two-sided t-tests after appropriate transformation. *log transformed.

Trait	CTGT Genotype			
	33	34	44	P value from
	N=827	N =1292	N = 506	ANOVA
Age (years)	56 (3.46)	56.09 (3.45)	56 (3.41)	0.874
Cholesterol (mM)	5.72 (0.99)	5.72 (1.07)	5.72 (1.04)	0.998
ApoB*	0.86 (0.73-1.02)	0.85 (0.72-1.01)	0.86 (0.72-1.01)	0.314
HDL* (mM)	0.82 (0.67-1.02)	0.78 (0.64-0.96)	0.82 (0.68-0.98)	0.073
Body mass index (kg/m^2)*	25.7 (24-28.2)	26.04 (24.2-28.5)	26.3 (24.05-28.8)	0.06
LDL (mM)*	3.15 (2.34-3.81)	2.00 (2.40-3.90)	3.06 (2.41-3.73)	0.885
TG (mM)*	1.71 (1.23-2.50)	1.77 (1.26-2.61)	1.65 (1.21-2.49)	0.164
Smoking (never/former/current) (%)	31.1/38.8/30.1	31.4/40.4/28.1	32/42.5/25.5	0.6

Table 3.12: Baseline characteristics by CTGT genotype in NPHSII. Mean (SD) or median (interquartile range) shown. Analysis was performed by two-sided t-tests after appropriate transformation. *log transformed.

3.3.2.7.1 The *APOA4* CTGT and N127S association with risk of CHD in NPHSII

3.3.2.7.1.1 *APOA4* CTGT genotype and CHD risk with smoking

Although in the overall study there was no significant evidence of a difference in CHD event rate according to CTGT genotype, there was significant evidence for a heterogeneity of effect between smokers and non-smokers (P=0.02). The risk (hazard ratio; HR) of CHD in non smokers did not vary significantly with CTGT genotype, although initial analysis did reveal that whilst smokers homozygous for 3 repeats ($CTGT_{3/3}$) had a 2.89 fold (95% CI 1.67-5.01; P>0.005) increased risk of CHD, heterozygotes ($CTGT_{3/4}$) had a reduced 1.80 fold (95% CI 1.09-2.98; P=0.024) increased risk of CHD. Although not statistically significant smokers homozygous for 4 repeats ($CTGT_{4/4}$) seemed to be protected, with a reduced risk of 1.56 (95% CI 0.90-2.76; P<0.05). In NPHS II men, age, BMI, baseline concentrations of plasma cholesterol, triglyceride and systolic blood pressure have been found to be statistically independent determinants of CHD risk (Miller *et al*, 1995). Adjustment for these parameters did not significantly alter the observed HR for $CTGT_{3/3}$ or $CTGT_{4/4}$ individuals, but did result in the loss of statistical significance for the protective effect seen in $CTGT_{3/4}$ subjects (HR; 1.64; 95% CI 0.93-2.90; P=0.09). These results as summarised in figure 3.13 A and table 3.13.

3.3.2.7.1.2 *APOA4* N127S genotype and CHD risk with smoking

Similarly although there was no significant evidence of a difference in CHD event rate according to N127S genotype, there was significant evidence for a heterogeneity of effect between smokers and non-smokers (P=0.03). The risk (hazard ratio; HR) of CHD in non smokers did not vary significantly with N127S genotype, while smokers homozygous for the N127 allele had a 1.90 fold (95% CI 1.38-2.62; p>0.005) increased risk of CHD, reflecting the well known effect of smoking on CHD (Doll and Hill, 1966) (illustrated in figure 3.13 B). Adjustment for age, BMI, baseline concentrations of plasma cholesterol, triglyceride and systolic blood pressure did not statistically alter the HR for N127S genotype (table 3.14).

Genotype	Hazard ratio (95% CI)*	Adjusted hazard ratio (95%CI) †
Non-Smokers		
CTGT $_{3/3}$	1.00	1.00
CTGT $_{3/4}$	1.27 (0.82-1.98)	1.37 (0.84-2.21)
CTGT $_{4/4}$	1.62 (0.98-2.69)	1.58 (0.90-2.76)
Smokers		
CTGT $_{3/3}$	2.83 (1.72-4.67)	2.89 (1.67-5.01)
CTGT $_{3/4}$	1.80 (1.09-2.98)	1.64 (0.93-2.90)
CTGT $_{4/4}$	1.56 (0.90-2.76)	1.34 (0.60-3.00)

Table 3.13: Hazard ratios used to estimate the risk of CHD events in men according to CTGT genotype.* Adjusted for age † adjusted for age, practice, BMI, baseline concentrations of plasma cholesterol, triglyceride and systolic blood pressure.

Genotype	Hazard ratio (95% CI)*	Adjusted hazard ratio (95%CI)†
Non-Smokers		
N127	1.00	1.00
SS+NS 127	1.02 (0.66-1.60)	1.09 (0.68-1.77)
Smokers		
N127	1.90 (1.38-2.62)	1.80 (1.26-2.57)
SS+NS 127	0.65 (0.28-1.60)	0.71 (0.31-1.66)

Table 3.14: Hazard ratios used to estimate the risk of CHD events in men according to N127S genotype. .* Adjusted for age † adjusted for age, practice, BMI, baseline concentrations of plasma cholesterol, triglyceride and systolic blood pressure.

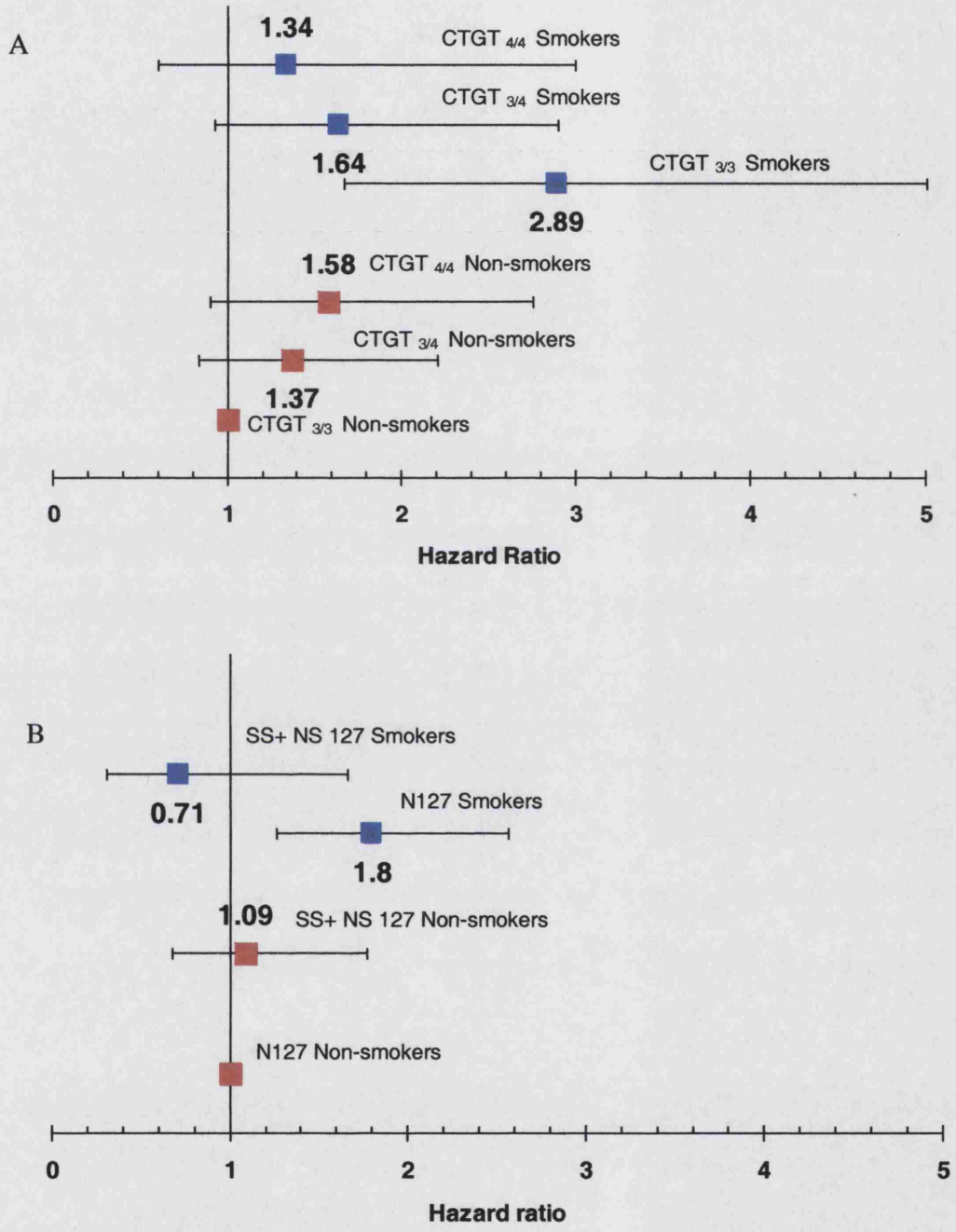


Figure 3.13: Chart illustrating the heterogeneity of effect between smokers and non-smokers according to A.) CTGT and B.) N127S genotype, adjusted for age, BMI, baseline concentrations of plasma cholesterol, triglyceride and systolic blood pressure.

3.4 DISCUSSION

3.4.1 Analysis of the *APOA4* promoter by SSCP

The first aim of these studies was to scan the promoter region of *APOA4* in search for new polymorphisms to be investigated along with published genetic variants of the *APOA4* gene for their involvement in CHD risk. Using SSCP no variants within the *APOA4* promoter were discovered, indicating that the *APOA4* promoter region investigated is invariate. SSCP unlike direct sequencing is a technique which is not 100% effective at detecting SNPs (Mogensen *et al*, 2003). Nonetheless, the sensitivity of SSCP can be improved by altering experimental conditions which impact on DNA conformation, these include temperature, ionic strength and pH (Jordanova *et al*, 1997). Greater sensitivity could perhaps also be achieved by varying the size of the DNA fragment with some studies implying that the optimal PCR fragment size for SSCP to be 150bp (Sheffield *et al*, 1993). Thus it is most likely due to the unpredictable electrophoretic nature of the mutant strands that limits the resolution of SSCP. This can be overcome to some extent by conducting SSCP under a variety of different conditions, which could be done in the future.

3.4.2 Variation in the *APOA4* gene and the risk of CHD

3.4.2.1 Investigating the possible association of the *APOA4* T347S and Q360H variants with apoA-IV plasma levels in the Kronenberg case-control study

Based upon the fact that low plasma apoA-IV levels have been associated with CHD in men (Kronenberg *et al*, 2000), we decided to investigate the possible association of the two common *APOA4* variants (T347S and Q360H) with apoA-IV plasma levels available in the Kronenberg study. No associations with the T347S or Q360H polymorphism were found in the Kronenberg study, despite a trend with SS homozygotes showing reduced apoA-IV levels.

3.4.2.2 The *APOA4* S347 variant is associated with reduced apoA-IV plasma levels in the EARS study

In order to investigate whether the apoA-IV T347S association with CHD risk (independent of lipid parameters) (Wong *et al*, 2003) was related to genotypic effects on plasma apoA-IV levels, we additionally examined the association of the T347S variant with apoA-IV plasma levels in the more powerful EARS study, where apoA-IV concentrations were available. Unlike previous studies showing significantly lower apoA-IV levels in cases compared to controls, in the EARS study no significant case-control difference in apoA-IV levels were observed. This could be because EARS is an offspring study comprised of healthy young subjects; any risk effect in the parents would have been diluted in the offspring (Tiret *et al*, 1993). However further analysis revealed that S347 homozygosity was associated with significantly lower plasma apoA-IV levels compared

with T347 carriers. Although none of the two previous studies examining this relationship found a significant association (Larson *et al*, 2002; Zaiou *et al*, 1994), one would anticipate that this genotypic effect would be similar in all Caucasians. In part this inconsistency could be explained by previous studies having insufficient power to detect such an association, for example the study by Larson *et al* (Larson *et al*, 2002), in 743 men and women had only 23% power at P= 0.05 to detect an association based on the genotypic levels reported in EARS. This could also account for the lack of association detected in the Kronenberg study with either the T347S or Q360H polymorphisms.

3.4.2.3 Haplotype analysis of the *APOC3-A4-A5* cluster in NPHSII

Analysis of nine SNPs within the *APOC3-A4-A5* cluster demonstrated that the *APOA4* T347S polymorphism alone was associated with a significant effect on the risk of CHD in healthy UK men. Men homozygous for the S347 had a 2 fold increased risk of CHD compared with T347 homozygotes independent of lipid parameters. Further haplotype analysis as published by Talmud *et al*, reveals that the TG lowering effect of *APOA4* S347 initially found might merely reflect the strong negative linkage disequilibrium with the common alleles of the major TG raising alleles *APOA5* W19 and *APOC3* -482T (Talmud *et al*, 2002). Although in univariate analysis there was significant evidence that the *APOC3* 1100C>T was risk-protective, considering the simultaneous effects of all the variants, *APOA4* T347S alone remained statistically significant and independent of established risk factors such as BMI, smoking, blood pressure, age, cholesterol, TG and apoAI levels. It is now becoming clear that when examining the association of SNPs where there is strong LD across the gene or cluster, it is potentially misleading to use

single SNPs, and using haplotypes is the best analytical approach. Our results strongly suggest that the apparent protective effect of the *APOC3* 1100C>T merely reflects the strong LD across the cluster and does not suggest a protective role for *APOC3* per se.

It is of no surprise that haplotype analysis revealed that the *APOA5* and *APOC3* variants were both associated with plasma TG levels. The clear and contrasting phenotypes of transgenic and knock out mouse models of *APOA5* and *APOC3* implies that both these apolipoproteins play an important role in TG metabolism. The over expression of human *APOA5* in mice results in hypotriglyceridemia, while *APOA5* deficiency increases TG levels (Pennacchio *et al*, 2001). In contrast, the over expression of human *APOC3* in mice causes hypertriglyceridemia (Ito *et al*, 1990) whereas *APOC3* deficiency decreases TG levels (Maeda *et al*, 1994). The primary function of apoC-III is the inhibition of lipoprotein lipase (LPL) -mediated hydrolysis of TGs by displacement of apoC-II (the activator of LPL) from lipoproteins (Wang *et al*, 1985). Similarly apoC-III has been proposed to affect VLDL and chylomicron TG metabolism by displacing apoE, inhibiting the hepatic uptake of chylomicrons and VLDL (Quarfordt *et al*, 1982; Windler and Havel, 1985). ApoA-V is the latest member of the plasma apolipoprotein family and its exact physiological function is unknown. Because the *APOA5* gene is expressed exclusively in the liver and its plasma concentrations are so low (van der Vliet *et al*, 2001), it has been postulated that apoA-V could affect plasma TG levels intracelluarly by modulating hepatic VLDL assembly and secretion (Weinberg *et al*, 2003). In fact the adenovirus mediated expression of murine *apoa5* in mice was found to reduce the hepatic VLDL-TG production rate by impairing the lipidation of apoB (Schaap *et al*, 2004).

Alternatively, there is also evidence to explain the hypotriglyceridemic effect of apoA-V based upon the apoA-V mediated activation of LPL *in vitro* and *in vivo* in both transgenic *APOA5* mice and those injected with *apoa5* via adenovirus mediated gene transfer (Merkel *et al*, 2005; Schaap *et al*, 2004). In both studies the increase in apoA-V levels in mice resulted in the faster catabolism of VLDL and chylomicron due to faster LPL hydrolysis of plasma TGs. This could result in the greater conversion of VLDL and chylomicrons into their respective remnants allowing for a more efficient clearance of remnants by the liver.

Recent evidence also demonstrates that *APOC3* and *APOA5* in fact independently influence plasma TG levels in an opposing manner, with both 'double transgenic' and 'double knockout' mice displaying normal TG concentrations (Baroukh *et al*, 2004). In addition compared to *APOA1/C3/A4* trangenic mice (Vergnes *et al*, 2000), *APOA1/C3/A4/A5* transgenics due to the addition of *APOA5* have dramatically reduced TG levels within normal physiological range (Baroukh *et al*, 2004). This is an important finding due to the close proximity of these two genes (*APOA5* and *APOC3* are only separated by ~ 35kb) in determining whether both genes in fact have functional variants, or whether polymorphisms in one of the genes are in significant linkage disequilibrium with functional polymorphisms in the other gene.

There is good evidence that both the *APOA5* S19W and *APOC3* -482 C>T SNPs could both functionally affect plasma TG levels. The *APOC3* -482C>T is situated within the *APOC3* insulin response element, and the C>T change has been demonstrated to abolish

insulin repression of *APOC3* resulting in elevated apoC-III levels and so higher TG levels (Li *et al*, 1995). While the apoA-V S19W change resulting in the substitution of a hydrophilic residue for a hydrophobic residue within the apoA-V signal peptide could potentially be of functional consequence. The S19W substitution has been proposed to affect the translocation of growing apoA-V peptide and therefore the amount of mature apoA-V secreted across the endoplasmic reticulum where it will be associated with nascent apoB containing particles (Talmud *et al*, 2005). Such alteration in apoA-V metabolism could result in the hepatic production of TG enriched VLDL, coupled with reduced LPL activity and so increasing TG levels.

Comparing the triglyceride levels associated with the haplotypes derived from the same nine SNPS ranked in the same order as the proportion of events, it is clear that that those haplotypes associated with the greatest CHD risk were not those associated with the highest TG levels. Three haplotypes representing 17% of the study sample, associated with among the highest proportion of events (11.9%, 9.5%, and 9.1%, respectively) were defined by *APOA4* S347 in combination with flanking markers *APOC3* -2854G in the intergenic region between *APOA4* -*APOC3* and/or the intergenic *APOA4-APOA5*C. These SNPs are in tight positive LD (Talmud *et al*, 2002), implicating *APOA4* as a gene involved in CHD risk determination. Very few studies have examined the association of *APOA4* T347S with CHD risk. A small case-control study from Finland (Tenkanen *et al*, 1992) found no difference in frequency of the T347S in the 180 cases compared with 73 controls, but was underpowered to do so, whereas in a Japanese study the T347S rare allele frequency was too low to give a risk estimate (Bai *et al*, 1996). Taken together the

association of S347 with increased risk and lower apoA-IV levels (Wong *et al*, 2003), indicates that variation in *APOA4* could be directly affecting risk.

3.4.2.4 Association of the *APOA4* S347 variant with reduced plasma TAOS in the UDACS cohort

One possible pathological mechanism to explain the inverse association of apoA-IV levels and CHD is the ability of apoA-IV to act as an effective inhibitor of lipid peroxidation (Ferretti *et al*, 2002), (Ostos *et al*, 2001), a key process implicated in the CHD progression (Chisolm and Steinberg, 2000; Navab *et al*, 2002). On the basis of this I decided to investigate the association of the *APOA4* T347S variant with plasma TAOS which is inversely related to oxidative stress in the UDACS study. Homozygosity for the *APOA4* S347 allele was found to be associated with reduced TAOS and therefore increased oxidative stress in diabetic patients with CVD. The association remained significant after adjustment for any confounding effects, including all lipid or lipoprotein measurements. Nonetheless the association of the T347S genotype with reduced TAOS was not observed in subjects free of CVD. A significantly greater percentage of subjects with CVD were found to be present in the lowest quartiles of TAOS (P=0.04), indicating greater oxidative stress in patients with CVD. Despite TAOS not being a highly specific measure of plasma oxidative stress, there is evidence supporting the use of TAOS as a marker of oxidative stress with a previous study observing a highly significant correlation (r= -0.65; P= 0.003) between plasma TAOS and esterified F_2-isoprostanes (Dhamrait *et al*, 2004). This data implies that individuals homozygous for the S347 allele have reduced protection against free radical attack by reactive oxygen species (ROS). An

elevated concentration in ROS could result in subsequent lipid peroxidation leading to the generation of a variety of potentially atherogenic products. These include the formation of reactive aldehydes such as FDP (3-formyl-3,4-dehydropiperidino) lysine generated during the oxidation of LDL (Uchida, 2000). Such aldehydes can react with oxidized LDL (OX-LDL) apoB, resulting in the internalization of OX-LDL particles by the scavenger receptors of macrophages and the subsequent formation of foam cells (Steinberg, 1995; Uchida *et al*, 1998), hence contributing to the progression of atherosclerosis.

This association data does not differentiate whether the increased risk associated with S347 homozygosity is a) a result of inherent lower apoA-IV levels and therefore lower antioxidant potential, b) whether the apoA-IV S347 protein itself has poorer antioxidant activity compared to apoA-IV T347, and therefore S347 homozygotes have lower antioxidant potential or c) whether by accelerating post-prandial lipid clearance, the 347S variant causes changes in lipid metabolism (e.g., increased hepatic oxidation of dietary polyunsaturated fats) that are themselves associated with an increased oxidation status. To gain further insight into the association of S347 homozygosity and reduced TAOS, S347 apoA-IV protein was produced and its antioxidant capabilities compared to wild type apoA-IV was examined in this thesis (see chapter 6).

It is worth noting that *APOA4* S347 is in strong linkage disequilibrium (LD) with flanking SNPs, and that the effect of the *APOA4* S347 variant on apoA-IV plasma levels could be directly modulated by the *APOA4-APOC3* intergenic –2854T>G variant in

strong LD with it (D'=0.76, p<0.0005) (Talmud *et al*, 2002). The –2854T>G is located in close proximity to a hepatic nuclear factor 4 (HNF4) responsive element and the two variants, S347 and –2854T together define a haplotype associated with increased CHD risk (Wong *et al*, 2003). Enhancers which mapped within the *APOA4-APOC3* intergenic region are known to co-regulate the expression of the entire *APOA4-C3-A1* cluster and not just *APOA4* (Vergnes *et al*, 1997). Hence altered enhancer activity due to the –2845T>C change could directly impact on apoA-IV levels as well possibly apoCIII and apoA-I.

We compared the frequency of the T347S variant in UDACS with that previously reported in the 2192 UK Caucasian men in the prospective Northwick Park Heart Study II (excluding those with a diagnosis of diabetes, n =71). The frequency of the S347 allele in the UDAC cohort (0.208 [0.19, 0.23]) was significantly higher than in the NPHSII study (0.184 [0.17, 0.20] P=0.04), supporting the concept of the clustering of CVD risk genes in patients with diabetes and the increased progression of CVD in those patients (Barakat and Hitman, 2001). Diabetes is a major risk factor for CVD and is associated with enhanced oxidative stress and subsequently increased lipid peroxidation (Amos *et al*, 1997; Davi *et al*, 1999). In such high-risk patients, apoA-IV may play a particularly important protective role, and our results suggest that the mechanism of this might be protection against lipid peroxidation and, thus, inhibition of the progression of atherosclerosis.

3.4.2.5 The analysis of the *APOA4* N127S and CTGT variants in NPHSII

Although both variants displayed a significant interaction with smoking and the risk of CHD, a significant effect with smoking causing a 1.8-2.8 fold increase in risk was only seen in the common wild type homozygotes. The S127 carriers and $CTGT_{4/4}$ homozygotes demonstrated a non-statistically significant protection against smoking and CHD, with $CTGT_{3/4}$ heterozygotes showing intermediate protection. Smoking is well known to increase the risk of CHD and induce oxidative stress (Doll and Hill, 1966; Heitzer *et al*, 1996a; Heitzer *et al*, 1996b). Any possible association could be a result of the different antioxidant capacity of the S127 apoA-IV isoform compared to wild type apoA-IV and functional studies were carried out in this thesis to test this (chapter 6).

3' UTR sequences are well known to contain regulatory elements that play important roles in the post-transcriptional control of gene expression (Chiaromonte *et al*, 2003), (Hesketh, 2004). Various regulatory motifs have been discovered in different mRNAs and 3'UTRs have been reported to be involved in mRNA stability (Chen and Shyu, 1995), localization and translation (Hesketh, 2004). These sequences within the 3' UTR can influence mRNA stability by either promoting endonucleolytic cleavage of the mRNA transcript, or by promoting the loss of the poly (A) tail and so increasing the vulnerability of the mRNA transcript to exonucleolytic attack via its free 3' end (Latchman, 1998). The length of 3' UTRs in mammalian mRNA varies considerably from 60-80 nucleotides (nt) to approximately 4000 nt, with humans on average having a 3' UTR of 740nt (Pesole *et al*, 1997). It is generally not possible to predict expression levels by just examining gene sequences, hence implying a potential effect of the CTGTn

polymorphism located in the 3' UTR of *APOA4* on *APOA4* expression levels is challenging without functional work. However using a bioinformatics approach examining only the minimal housekeeping transcriptome (MHKT), Chiaromonte *et al* have calculated that on a genome wide scale highly expressed genes produce significantly shorter mRNAs with shorter 3'UTRs (Chiaromonte *et al*, 2003). This indicates that an additional 4 nt repeat ($CTGT_{4/4}$) within the 3'UTR of *APOA4* could perhaps reduce apoA-IV plasma levels. Due to the strong LD existent across the *APOA5/A4/C3/AI* gene cluster, haplotype analysis should be carried out along with additional functional work (see chapter 7) to fully deduce the likely importance of the $CTGT_n$ polymorphism on *APOA4* mRNA stability and its possible association with CHD risk and smoking. Unfortunately no plasma apoA-IV level data was available in NPHSII and I did not have time to genotype or analyse either the N127S or CTGT variants in EARS, therefore it is unknown whether these variants are associated with apoA-IV plasma levels.

CHAPTER 4

CHAPTER 4: THE PRODUCTION AND CHARACTERISATION OF RECOMBINANT WILD TYPE AND SITE DIRECTED MUTATION OF APOA-IV: LIPID BINDING, SECONDARY STRUTURE ANALYSIS AND APOA-IV PHOSPHOLIPID COMPLEX SYNTHESIS

4.1 INTRODUCTION

4.1.1 Protein modelling to further isolate the LCAT activation domain within apoA-IV

Protein modelling is a useful tool to predict protein structure from amino acid sequence and can be used to gain further knowledge of protein function. In this thesis protein modelling was used to identify important domains in apoA-IV in order to probe the protein through point mutation and biophysical studies to obtain further insight into the structure/function relationship of apoA-IV. The initial modelling was performed with the help of Frank Peelman (University of Ghent, Belgium), all additional modelling including alignments and secondary structure prediction shown here was done by myself.

4.1.2 Expression of apoA-IV protein in *E.coli*

E.coli bacteria are the most commonly used production host enabling the cost efficient production of large amounts of protein. But being a prokaryotic organism, *E.coli* expression systems are incapable of post translational modification processes including glycosylation, which is a part of native apoA-IV expression. The pET-14b vector was used containing the insert of interest (*APOA4* cDNA) under the control of the T7 promoter, which is only transcribed by the T7 RNA polymerase used in a strain of *E.coli*

(BL21) carrying a T7 RNA polymerase gene under the tight control of a *lac* promoter (see section 2.4.2 for *APOA4* construct production). Strict regulation of *APOA4* expression is important to ensure that early overproduction of the heterologous protein does not impair cell growth. Once a moderate cell density was reached and before unfavourable changes in the growth medium occured, the production of apoA-IV was induced by isopropyl β-D-thiogalactopyranoside (IPTG). The apoA-IV protein produced contained a molecularly encoded N terminal 6X histidine tag (his-tag), making it ideal for metal ion affinity purification using nickel resin (see section 2.5.2).

4.1.3 The physiochemical characteristics of the lipid free and lipid bound apoA-IV variants

To further the understanding of the molecular basis for the conformational adaptability of the exchangeable apolipoprotein apoA-IV to both aqueous and lipid environments, structural analyses were carried out on lipid free and POPC apoA-IV complexes. Due to time constraints thermodynamic analysis was confined to lipid free apoA-IV samples only.

4.2 METHODS

All methods used for protein modelling are summarised in section 2.5.1, while the methods used for recombinant apoA-IV production and the subsequent characterisation of these proteins are described in section 2.5.2 and section 2.6 respectively.

4.3 RESULTS

4.3.1 The isolation of 2 atypical class A amphipathic helices possibly involved in the activation of LCAT

Given that apoA-IV and the extensively studied LCAT activator apoA-I are derived from a common evolutionary ancestor (Karathanasis *et al*, 1986), a multiple alignment using Clustal W (Thompson *et al*, 1994) was performed between the two proteins (figure 4.1). The LCAT activation domain within apoA-IV is located between residues 117-160 of the mature protein (Emmanuel *et al*, 1994). Alignment of this region with the apoA-I LCAT activation domain was performed, paying close attention to the 3 strictly conserved arginine residues (R149, R153 and R160) critical for apoA-I LCAT activation (Roosbeek *et al*, 2001). However, only one LCAT activating arginine residue (R153) seemed to be conserved between apoA-I and apoA-IV. The consensus secondary structure prediction of apoA-IV revealed that residues 117-160 are in fact within a region of helical structure (figure 4.2), any interestingly conserved residues between apoA-IV and apoA-I were therefore subjected to helical wheel analysis.

```
ApoA-IV_X13629__      MFLKAVVLTLALVAVAGARA----------EVSADQVATVMWDYFSQLSNNAKEAVEHLQ
ApoAI_P02647__        --MKAAVLTLAVLFLTGSQARHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFE
                        :**.*****:: ::*::*          : . *:*  :   *.. *.:..:: *.:::

ApoA-IV_X13629__      KSELTQQLNALFQDKLGEVNTYAGDLQKKLVPFATELHERLAKDSEKLKEEIGKELEELR
ApoAI_P02647__        GSALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVK
                       * * :***  : *: ..*.:  ..*:::* *.: *: :.* *::* *::*:.*:***::

ApoA-IV_X13629__      ARLLPHANEVSQKIGDNLRELQQRLEPYADQLRTQVNTQAEQLRRQLTPYAQRMERVLRE
ApoAI_P02647__        AKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARA
                      *:: *: ::..:*  :::.  :*::**   :*:  .. : .:*:.:*:* .:.*.   *

ApoA-IV_X13629__      NADSLQASLRPHADELKAKIDQNVEELKGRLTPYADEFKVKIDQTVEELRRSLAPYAQDT
ApoAI_P02647__        HVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDL
                      :.*:*:: * *::***: ::  .:* ** .  .   *::.*  : :. * ..  *  :*

ApoA-IV_X13629__      QEKLNHQLEGLTFQMKKNAEELKARISASAEELRQRLAPLAEDVRGNLRGNTEGLQKSLA
ApoAI_P02647__        RQGLLPVLESFKVSFLSALEEYTKKLNTQ-----------------------------
                      :: *   **.:...: .  ** . ::.:.

ApoA-IV_X13629__      ELGGHLDQQVEEFRRRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDK
ApoAI_P02647__        ------------------------------------------------------------


ApoA-IV_X13629__      VNSFFSTFKEKESQDKTLSLPELEQQQEQQQEQQQEQVQMLAPLES
ApoAI_P02647__        -----------------------------------------------
```

Figure 4.1: Clustal W alignment of human apoA-IV (accession number X13629) with human apoA-I (accession number P02647) with the arginine residues critical for LCAT activation (R149 □, R153□ and R160□) highlighted.

```
          10        20        30        40        50        60        70
                    |         |         |         |         |         |         |
apoA-IV   MFLKAVVLTLALVAVAGARAEVSADQVATVMWDYFSQLSNNAKEAVEHLQKSELTQQLNALFQDKLGEVN
DPM       cchhheeeehhhhhhhhhhhhhhhheheeeehehhhhtcchhhhhhhhhhhhhhhhhhhhhhhhhhchht
DSC       ccchhhhhhhhhhhhhcccccchhhhhhhhhhhhhhhhhcchhhhhhhhhhhhhhhhhhhhhhhcccccc
GOR4      ccceeeehhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccccc
HNNC      cchhhhhhhhhhhhhhcccchcchhhhhhhhhhhhhhhhchchhhhhhhhhhhhhhhhhhhhhhhhccchh
PHD       chhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
Predator  ccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcchhhhhhhhhhhhhhhhhhhhhhhhhccccc
SIMPA96   chhhhhhhhhhhhhhhcchhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcchh
SOPM      hhhhhhhhhhhhhhhhtchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
Sec.Cons. cchhhhhhhhhhhhhhh?hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh?cchh

                    80        90        100       110       120       130       140
                    |         |         |         |         |         |         |
apoA-IV   TYAGDLQKKLVPFATELHERLAKDSEKLKEEIGKELEELRARLLPHANEVSQKIGDNLRELQQRLEPYAD
DPM       cchcchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhtcchhhhhhhhhhhhhh
DSC       hhhhhhhhhccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhhhhhhhccchhh
GOR4      cccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccchhhhhhchhhhhhhhhhchhhh
HNNC      hhhhchhhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhccccchhhhhhhhhhhhhhhhhccchh
PHD       hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
Predator  cchhhhhhhcccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcchhhhhhcchhhhhhhhhhhhhhhh
SIMPA96   hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
SOPM      hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh
Sec.Cons. hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh?hhhhhhhhhhhhhhhhhhhhhhhh
```

	150 160 170 180 190 200 210
apoA-IV	QLRTQVNTQAEQLRRQLTPYAQRMERVLRENADSLQASLRPHADELKAKIDQNVEELKGRLTPYADEFKV
DPM	hhhhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhchhcchhhhhhh
DSC	hhhhhcchhhhhhhhhcchhhhhhhhhhhhhhhhhhhccchhhhhhhhhhhhhhhhhcccccccccccee
GOR4	hhhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccchhhhhh
HNNC	hhhhhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccccchhhhhhh
PHD	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccchhhhhhhh
Predator	hhhhhhhhhhhhhhhhhhcchhhhhhhhhhcchhhhhhchhhhhhhhhhhhhhhhhhhcccccccchhhh
SIMPA96	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhh
SOPM	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhh
Sec.Cons.	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccc?hhhhh

	220 230 240 250 260 270 280
apoA-IV	KIDQTVEELRRSLAPYAQDTQEKLNHQLEGLTFQMKKNAEELKARISASAEELRQRLAPLAEDVRGNLRG
DPM	hhhhhhhhhhhhhhchhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccttc
DSC	cchhhhhhhhhcchhhhhhhhhhhhcchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcchhhhhhccccc
GOR4	hhhhhhhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccccc
HNNC	hhhhhhhhhhhhhcccchchhhhhhhhhcccceehhcchhhhhhhhhcchhhhhhhhhhchhhhhcccccc
PHD	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcc
Predator	hhhhhhhhhhhhcccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccccccc
SIMPA96	hhhhhhhhhhhhhhhhchhhhhhhhcchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccccc
SOPM	hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccc
Sec.Cons.	hhhhhhhhhhhhh?hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhccccc

```
                290       300       310       320       330       340       350
                 |         |         |         |         |         |         |
apoA-IV    NTEGLQKSLAELGGHLDQQVEEFRRRVEPYGENFNKALVQQMEQLRQKLGPHAGDVEGHLSFLEKDLRDK
DPM        tcccchhhhhhhhcchhhhhhhhhhhhhhhtctcthchhhhhhhhhhhhhhhtctccthhchhhhhhhhhhhh
DSC        ccchhhhhhhhhhcchhhhhhhhhhhcccccchhhhhhhhhhhhhhhhhhcccccccccccchhhhhhhhccc
GOR4       cchhhhhhhhhhhcchhhhhhhhhhhhcccccchhhhhhhhhhhhhhhhhcccccccccccchhhhhhhhhhh
HNNC       chhhhhhhhhhhcccchhhhhhhhhhhhhcccchhhhhhhhhhhhhhhhhhcccccccchhhhhhhhhhhh
PHD        cchhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhhhhhhhcccccchhhhhhhhhhhhh
Predator   ccchhhhhhhhhcccchhhhhhhhhhhhcccccchhhhhhhhhhhhhhhhhhcccccccccchhhhhhhccc
SIMPA96    hhhhhhhhhhhhcchhhhhhhhhhhhhhhhcchhhhhhhhhhhhhhhhhhcccccchhhhhhhhhhhhh
SOPM       chhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccchhhhhhhhhhhhh
Sec.Cons.  cchhhhhhhhhh?c?hhhhhhhhhhh?ccchhhhhhhhhhhhhhhhhhhcccccc?chhhhhhhhhhh

                360       370       380       390
                 |         |         |         |
apoA-IV    VNSFFSTFKEKESQDKTLSLPELEQQQEQQQEQQQEQVQMLAPLES
DPM        eteehhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcc
DSC        ccchhhhhcccccccccccchhhhhhhhhhhhhhhhhhhhhcccc
GOR4       hhhhhcccccccchhhhhchhhhhhhhhhhhhhhhhhhhhhceec
HNNC       hhhhhhhcccccccccccccccccccchhhhhhhhhhhcccc
PHD        hhhhhhhhhhcccccccccchhhhhhhhhhhhhhhhhhhcccc
Predator   ccccccccccccccccccchhhhhhhhhhhhhhhhhhhcccccc
SIMPA96    hhhhhhhhcccccccccchhhhhhhhhhhhhhhhhhhhhhhhc
SOPM       hhhhhhhhcccccccccccchhhhhhhhhhhhhhhhhhhhhhcc
Sec.Cons.  hhhhhhhhcccccccccccchhhhhhhhhhhhhhhhhhhh?cccc
```

Figure 4.2: The consensus secondary structure prediction of apoA-IV, h= α helix e= β sheet, c= coil and t= turn. The LCAT activation domain of apoA-IV according to Emmanuel *et al* (Emmanuel *et al*, 1994)(residues 117-160) are highlighted □.

Helical wheel analysis between residues 117-160 yielded 6 interesting amino acid residues (R123, R134Q, N127, Q156, R149, R160) found to be within the polar face of two atypical class A amphipathic helices, helix 1 (residues 117-138) and helix 2 (residues 146-161) as shown in figure 4.3, mimicking the cluster of polar arginine residues (R149, R153, R160) found in the LCAT activation helix of apoA-I (Roosbeek *et al*, 2001) .To investigate the potential importance of these residues they were mutated to residues of opposite charge. Arginine residues (basic, hydrophilic) were altered to glutamine residues (neutral, uncharged) and vica versa, while asparagines residues (neutral, uncharged) were mutated to arginine residues. A Clustal W alignment analysis of human apoA-IV (figure 4.4) revealed that the mutations are only conserved in higher mammals. Each helix was altered by site directed mutagenesis and in total 20 different apoA-IV variants were produced, these are summarised in table 4.1.

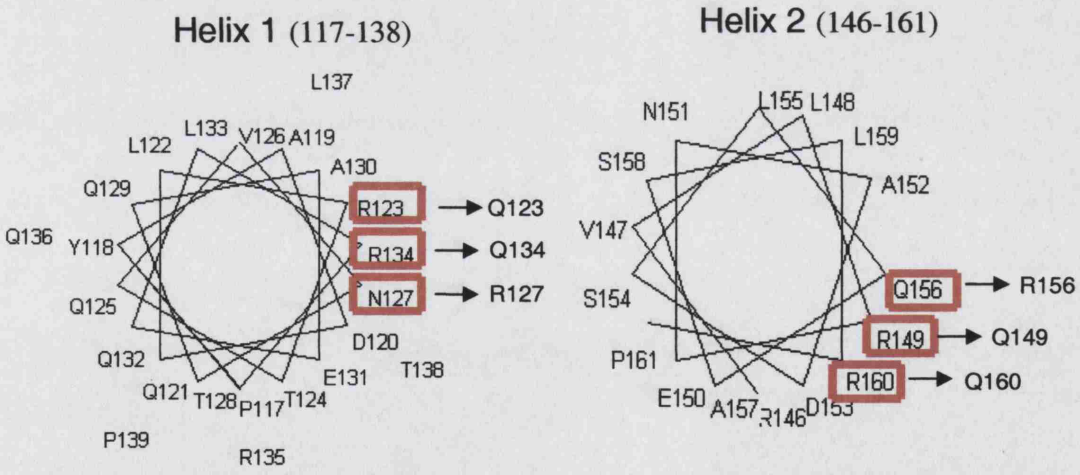


Figure 4.3: Edmundson wheel representations of the two predicted atypical class A amphipathic helices found between residues 117-160 of apoA-IV. The cluster of residues targeted for point mutation are highlighted.

ApoA-IV variants produced
1. Wild type apoA-IV
2. R123Q
3. R134Q
4. N127R
5. R123Q+R134Q
6. R123Q+N127R
7. N127R+R134Q
8. Helix 1 K.O- R123Q+ R134Q+ N127R
9. R149Q
10. R160Q
11. Q156R
12. R149Q+R160Q
13. R149Q+Q156R
14. R160Q+Q156R
15. Helix 2 K.O- R149Q+R160Q + Q156R
16. Q360H
17. T347S
18. Q360H+T347S
19. N127S
20. L166Q

Table 4.1: A listing of all the types of recombinant apoA-IV proteins produced throughout this thesis.

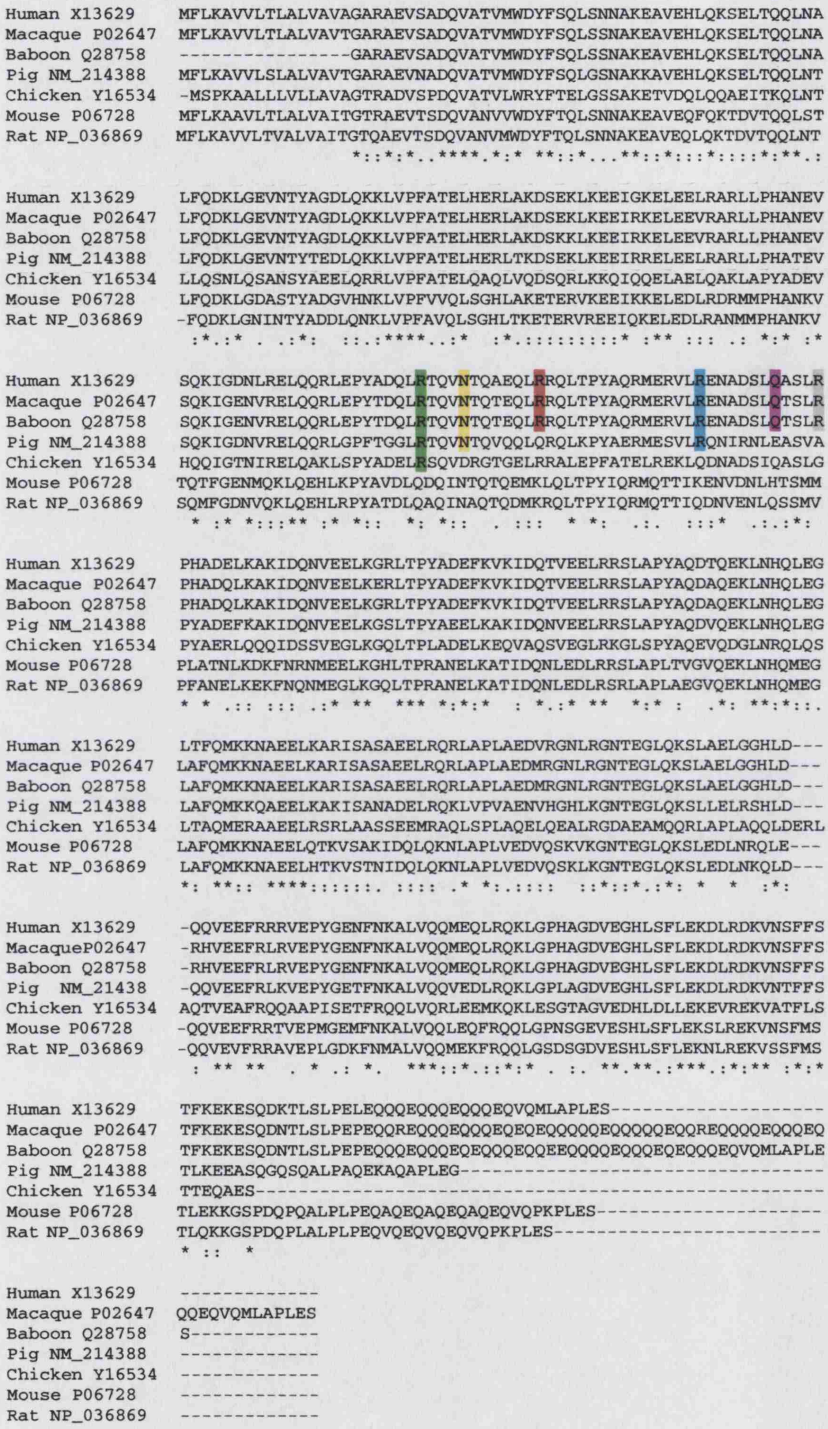


Figure 4.4: Clustal W alignment of apoA-IV from various species compared to human apoA-IV, all appropriate accession numbers are listed next to each alignment (residues targeted for mutation are indicated R123, N127, R134, R149, Q156 and R160). All sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov).

4.3.2 The production of recombinant apoA-IV using *E.coli*

The optimal induction time of the different apoA-IV variants in the BL21 *E.coli* strain was found by inducing the *E.coli* BL21 media (at OD ~ 1) for 30 minutes, 1 , 2 and 3 hours with 1mM IPTG and comparing the amount of apoA-IV protein produced by SDS PAGE (figure 4.5). Most of the variants had an optimal induction time of 2 hours with others working equally well between 1 and 3 hours (figure 4.5). All samples were subjected to SDS PAGE and purity assessed (figure 4.6), before large scale recombinant apoA-IV production was performed. All apoA-IV sample production was performed in bind and elution buffers containing 6M guanidinium chloride to prevent aggregation and the formation of inclusion bodies (section 2.5.2). As illustrated in figure 4.6, small scale metal affinity purification of apoA-IV was successful, with a distinct 46kDA band produced. Next, large scale production of apoA-IV was performed in 1 litre SOB batches according to section 2.5.2 and again analysed by SDS PAGE (figure 4.7).

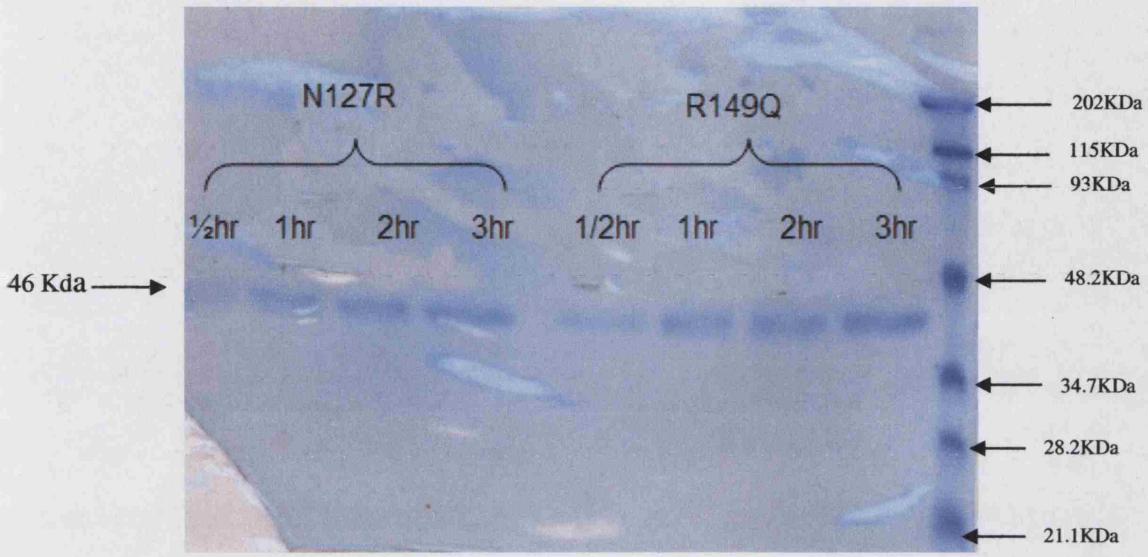
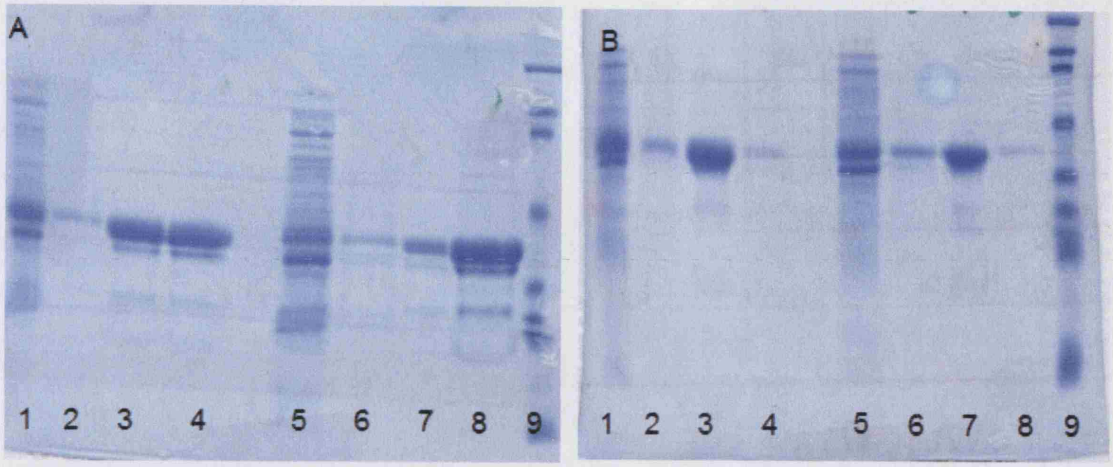


Figure 4.5: Time course of apoA-IV expression, recombinant apoA-IV was expressed in SOB media and induced with 1mM IPTG at 37°C for different induction times. The samples were purified using nickel resin (section 2.5.2) and analysed by SDS PAGE (section 2.5.2.2). All recombinant proteins were optimised this way, only the N127R and R149Q variants are shown here.



Calibrated molecular weights of prestained SDS-PAGE standards (BioRad)

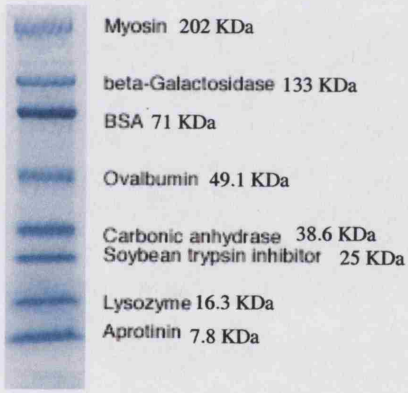
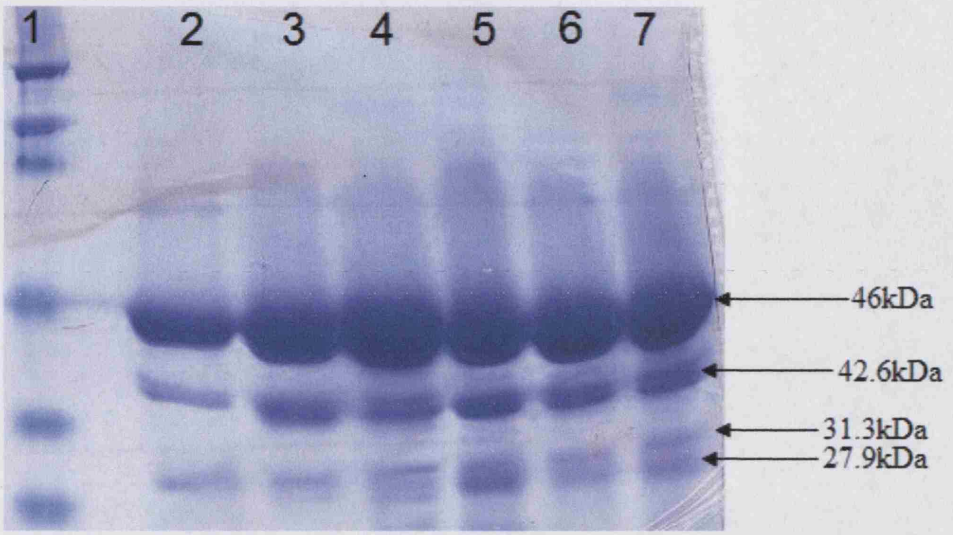


Figure 4.6: Dried SDS PAGE gels, run to check the purity of small scale apoA-IV production, this procedure was carried out for all 20 apoA-IV isoforms expressed of which 4 are shown here. A.) H2 K.O 1.) flowthrough 2.) wash 3.) first elution aliquot 4.) Second elution aliquot. R160Q+Q156R 5.) flowthrough 6.) wash 7.) first elution aliquot 8.) second elution aliquot. B.) R160Q+R149Q 1.) flowthrough 2.) wash 3.) first elution aliquot 4.) second elution aliquot. L166Q 5.) flowthrough 6.) wash 7.) first elution aliquot 8.) second elution aliquot. Lane 9 in both figures consists of the prestained SDS-PAGE standards (broad range).



Calibrated molecular weights of prestained SDS-PAGE standards (BioRad)

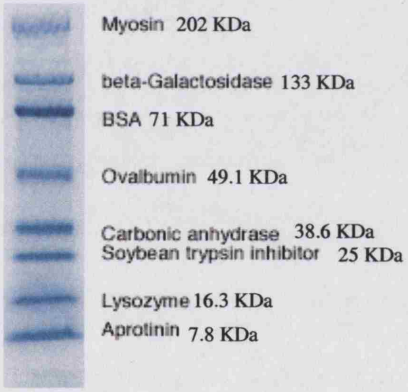
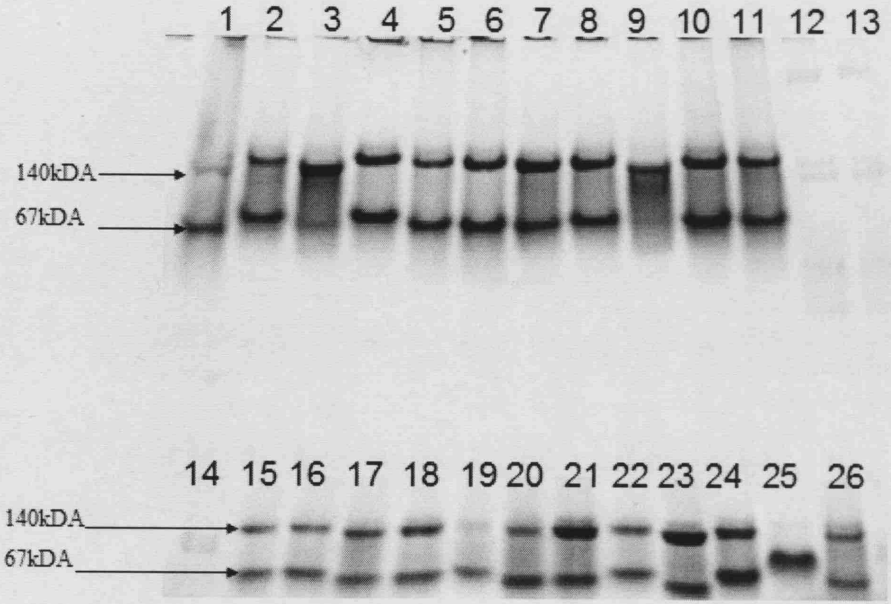
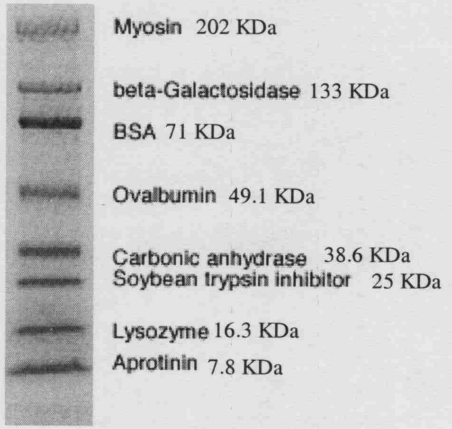


Figure 4.7: Dried SDS PAGE gel of the large scale production of recombinant apoA-IV protein, all 20 variants produced were checked this way of which 6 are shown here. 1= Prestained SDS-PAGE standards, 2= apoA-IV-WT, 3= apoA-IV-S347, 4= apoA-IV-H360, 5= apoA-IV-Q123+Q134, 6=apoA-IV-Q146. The additional 42.6, 31.3, 27.9 kDa bands most likely represent apoA-IV degradation products.

4.3.2.1 Analysis of the apoA-IV recombinant proteins by Non-denaturing polyacrylamide gradient gel electrophoresis

All recombinant apoA-IV proteins produced were subjected to electrophoresis on a non-denaturing pre-cast 4-20% linear gradient Tris-HCL gel (section 2.5.2.2). Similar results were achieved in comparison to Emmanuel *et al* (Emmanuel *et al*, 1994), with the majority of recombinant apoA-IV proteins appearing as two major bands of roughly 67 and 140 kDa (figure 4.8). The same pattern was also found by Weinberg and Spector *et al* (Weinberg and Spector, 1985a), whom attributed the two major bands to be monomeric and dimeric forms of apoA-IV despite their relative molecular masses being higher than 46 and 92 kDa respectively. They hypothesised that the reason for this unexpectedly slow migration was due to the presence of carbohydrates in apoA-IV isolated from human plasma. Yet the recombinant apoA-IV proteins produced in this thesis using *E.coli* were not glycosylated but had equally reduced mobility, consequently it seems likely that the two major bands actually correspond to dimeric and trimeric forms of apoA-IV. This is as expected based upon previous observations that apoA-IV readily undergoes self-association both *in vitro* and *in vivo,* forming dimers even at low protein concentrations (Weinberg and Spector, 1985b).





1 R149Q+Q156R	2 N127R+R134Q	3 R123Q+R134Q	4 R123Q+N127R	5 R123Q	6 T347S
7 N127S	8 N127R	9 H1	10 Q156R	11 WT	12 Markers
13 Markers	14 Markers	15 WT	16Q360H	17 R134Q	18 R160Q
19 R160Q+Q156R	20 H2	21 R149Q	22 Q360H+T347S	23 R149Q+R160Q	24 WT
25 L166Q	26 WT				

Figure 4.8: Analysis of all 20 recombinant apoA-IV proteins using non-denaturing pre-cast 4-20% linear gradient Tris-HCL gels.

4.3.2.2 Western Blot analysis of apoA-IV proteins

The wild type apoA-IV proteins produced with or without 6M GdmCl were subjected to electrophoresis on a non-denaturing pre-cast 4-20% linear gradient Tris-HCL gel and examined by western blotting (section 2.5.4), to clarify that the proteins produced were in fact apoA-IV. The resultant western blot (figure 4.9) clearly demonstrates that when produced in the presence of GdmCl, a greater proportion of the faster migrating (dimeric) apoA-IV is produced.

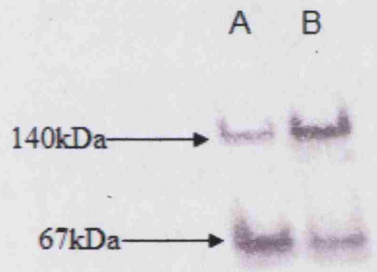


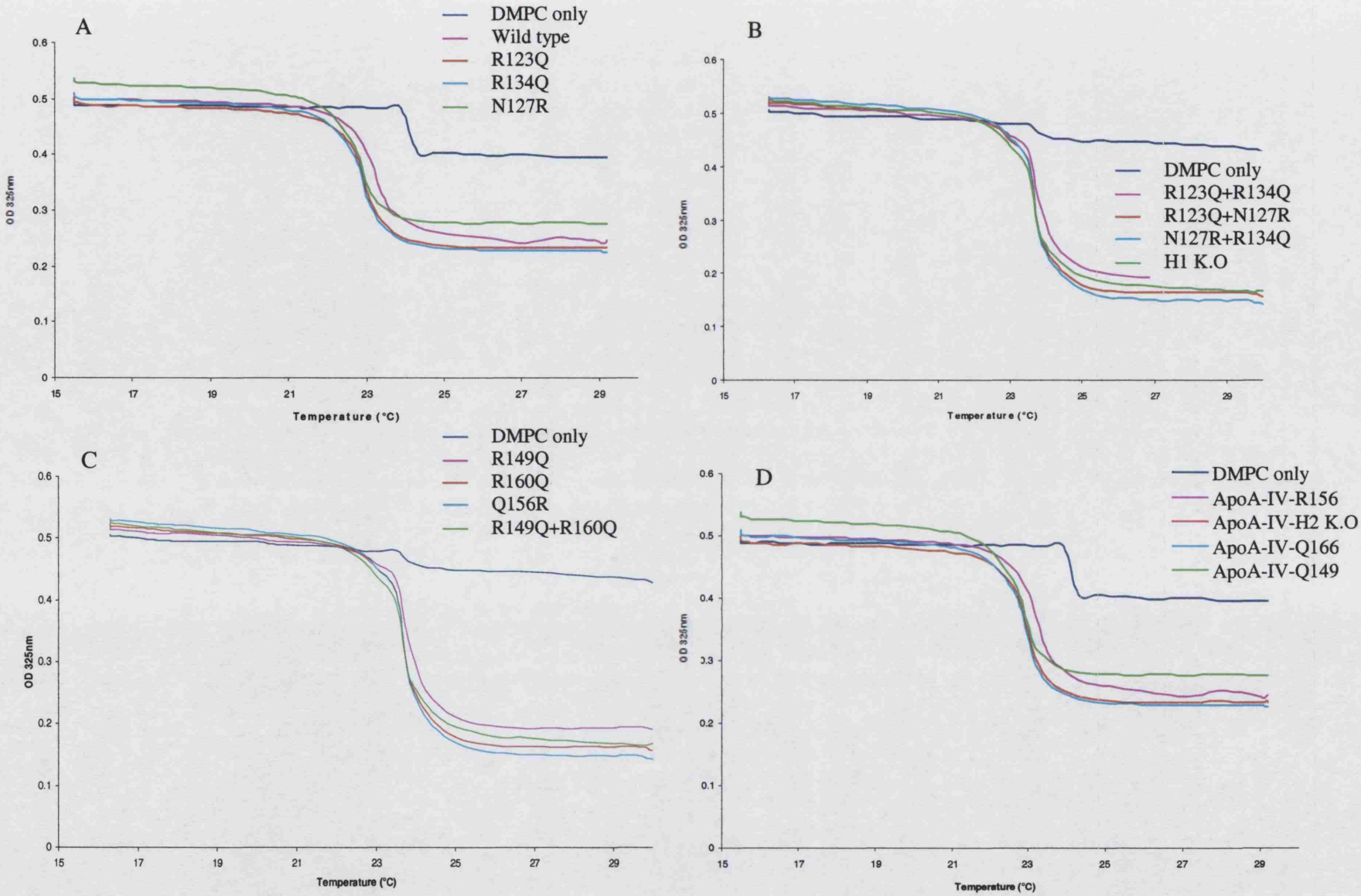
Figure 4.9: Western blot of recombinant wild type apoA-IV proteins produced in the presence of GdmCl (lane A) and without (lane B), prestained broad range markers were used but they did not blot sufficiently. Both samples A and B were separated on a non-denaturing pre-cast 4-20% linear gradient Tris-HCL gel.

4.3.3 Analysis of the physiochemical characteristics of the lipid free and lipid bound apoA-IV variants

4.3.3.1 Lipid binding properties of the apoA-IV variants

The lipid binding properties of all 20 recombinant apoA-IV proteins generated were examined by assessing their interaction with 1.2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC). Briefly, it was carried out by monitoring the turbidity change of DMPC MLVs (multilamellar vesicles) at 325nm after being mixed with recombinant apoA-IV proteins as a function of the temperature, through the transition temperature of DMPC MLVs (23.5°C) from 16 °C to 36 °C (see section 2.6.1). The principle of these experiments is that when the protein binds to DMPC MLVs, the turbidity of the mixture will decrease due to the formation of discoidal complexes between the protein and DMPC.

When all the recombinant apoA-IV proteins were examined, a similar large decrease in the turbidity of DMPC MLV at 325nm was observed that was maximal around the transition temperature of DMPC (25°C) for all wild type and mutant apoA-IV proteins, indicating that all recombinant apoA-IV proteins successfully bound to DMPC with similar lipid affinities (figure 4.10). In addition these results also imply that neither the lack of glycosylation or the presence of an N-terminal his-tag affects the lipid binding properties of apoA-IV.


A
DMPC only
Wild type
R123Q
R134Q
N127R
OD 325nm
Temperature (°C)
B
DMPC only
R123Q+R134Q
R123Q+N127R
N127R+R134Q
H1 K.O
OD 325nm
Temperature (°C)
C
DMPC only
R149Q
R160Q
Q156R
R149Q+R160Q
OD 325nm
Temperature (°C)
D
DMPC only
ApoA-IV-R156
ApoA-IV-H2 K.O
ApoA-IV-Q166
ApoA-IV-Q149
OD 325nm
Temperature (°C)

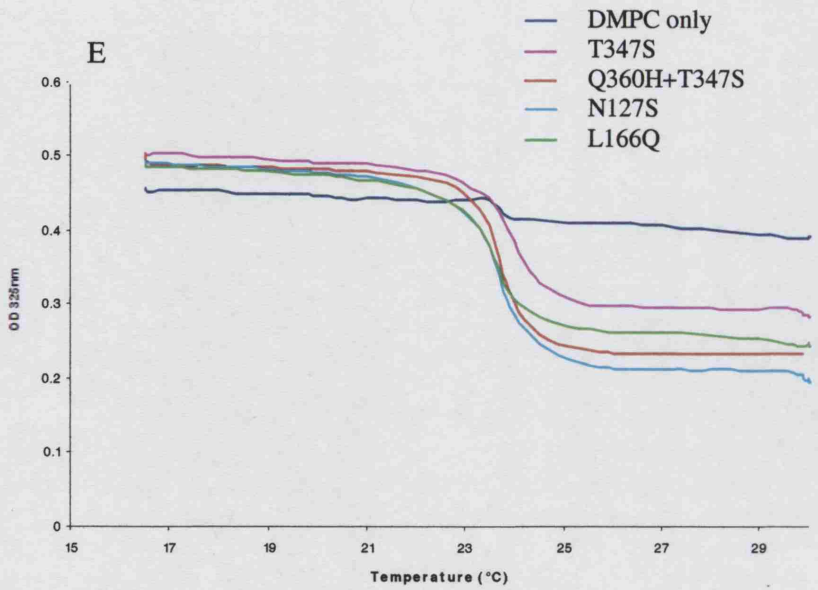


Figure 4.10 A-E Turbidity decrease of DMPC MLVs mixed with the 20 apoA-IV recombinant proteins produced as a function of the temperature. 80μg of DMPC was mixed with 40μg of apoA-IV protein in 5mM NH_4HCO_3 (pH8.0) and the OD at 325 nm was followed.

4.3.3.2 Preparation of apoA-IV: 1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine (PLPC) complexes

At first the protein lipid complexes were made to a molar ratio of apoA-IV 1: PLPC 150: unesterified cholesterol 10, according to section 2.6.2 but using 1X TBS with the addition of 0.3m GdmCl. GdmCl (0.3M) was added to increase the amount of monomeric apoA-IV present, hopefully increasing the chances of complex formation. At first the apoA-IV wild type complexes synthesised were compared to pure recombinant apoA-IV by gel filtration, to ensure a complex was in fact present (figure 4.11). All other complexes formed were then also inspected by gel filtration (a Superdex 200 pg 10/60), as illustrated in figure 4.12 A and B the production of apoA-IV PLPC complexes was successful.

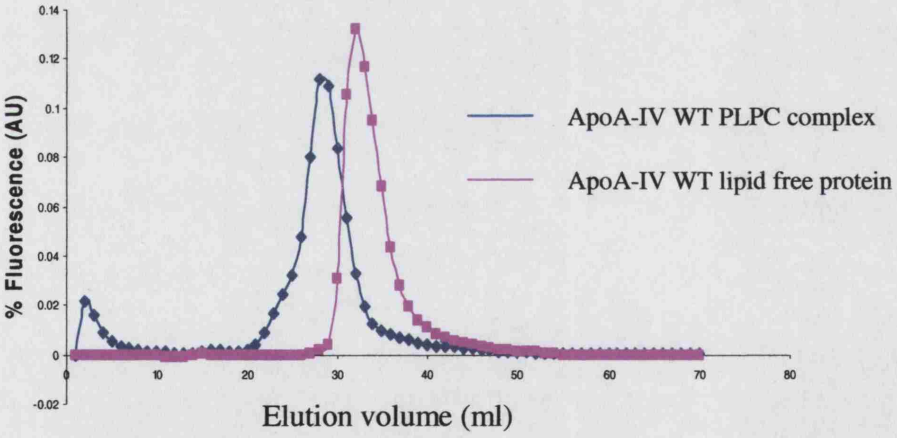
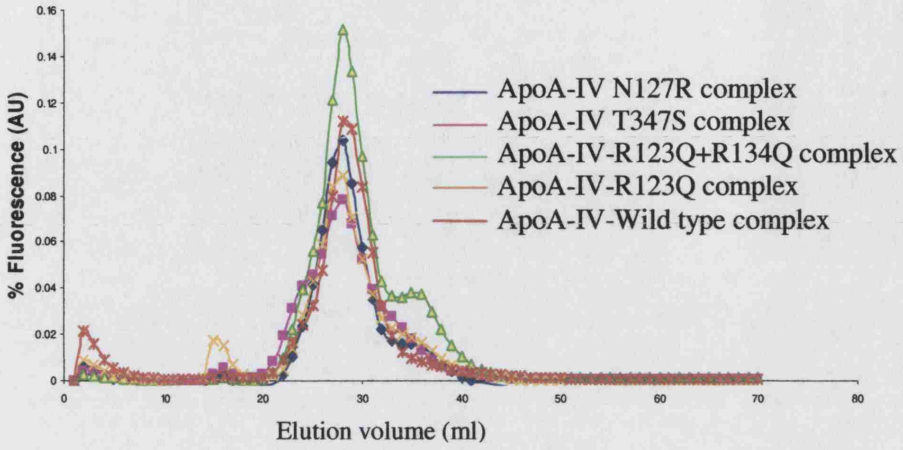


Figure 4.11: The apoA-IV wild type complex was analysed by gel filtration (Superdex 200 PG 10/60) in comparison to the purified lipid free apoA-IV to double check that a complex had formed.

A



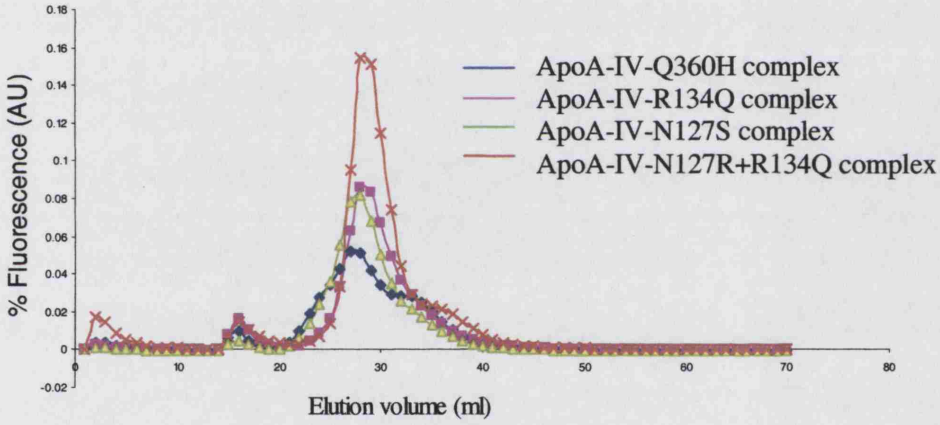


Figure 4.12 A and B: All the apoA-IV PLPC complexes successfully produced were subjected to gel filtration analysis (Superdex 200 PG 10/60).

However despite this success, although being stored at 4°C the complexes were found to be unstable and dissociated in 24 hours. The instability of these complexes was first noticed when the complex mixtures became cloudy, to further investigate a few of the complexes were subjected to gel filtration using a Superdex 200 HR column giving greater resolution/separation than the Superdex 200 PG 10/60 column previously used (figure 4.13). All cloudy samples were found to contain an abundance of lipid free apoA-IV.

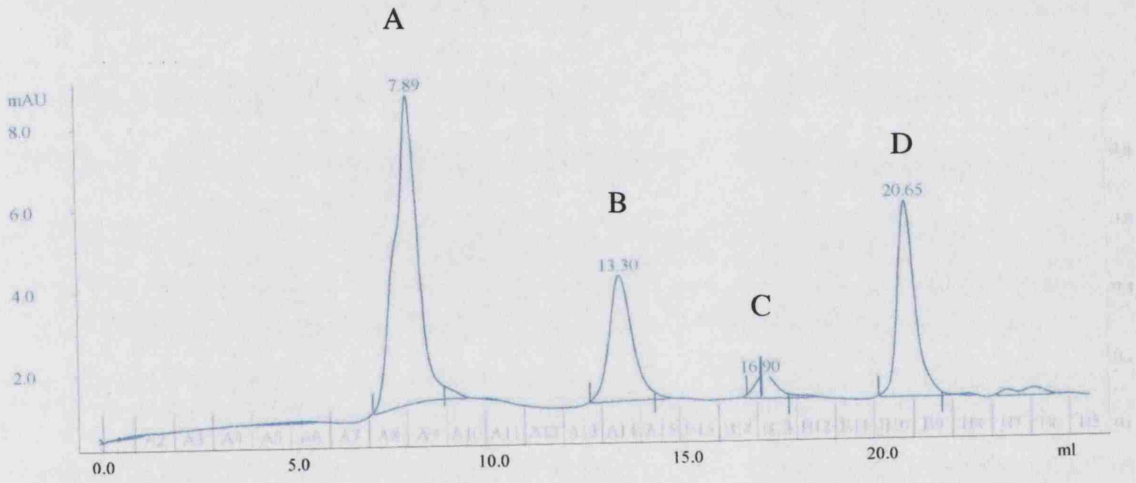
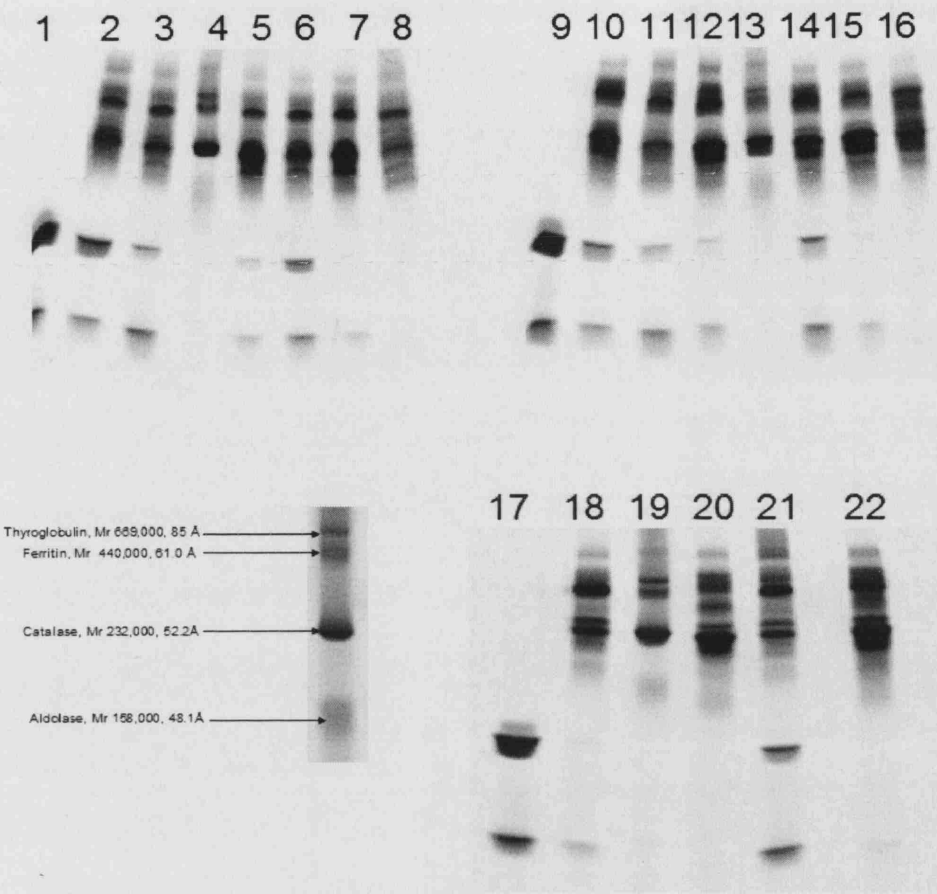


Figure 4.13: The cloudy degraded wild type complex mixture analysed by gel filtration using a Superdex 200 HR column. The corresponding elution fraction of each peak is shown above. Peak A= WT complex, B= trimeric apoA-IV, C= dimeric apoA-IV and D= monomeric apoA-IV.

A possible explanation for the instability of such complexes is that apoA-IV has weak affinity for lipid (Weinberg and Jordan, 1990) and the presence of 0.3M GdmCl although increasing the amount of monomeric apoA-IV could have sufficiently unfolded the protein enabling it only to bind to lipid transiently.

4.3.3.3 Preparation of apoA-IV:POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine) complexes

The complexes were remade this time using POPC which like PLPC is an excellent, widely used substrate for LCAT studies, as this was available already solubilised in choloroform making the production of lipid films easier and more accurate. To improve the stability of the apoA-IV complexes, all apoA-IV samples were dialysed against 1X TBS removing all GdmCl before complex formation. To increase the formation of complexes and to minimise any free unbound apoA-IV being present, the apoA-IV: phospholipid molar ratio was increased to 1:180 (section 2.6.2). The complexes were then checked on a non-denaturing 4-30% gradient polyacrylamide gel (4-30% PAA) and the Stokes radius calculated using HMW markers of known diameter. As shown in figure 4.14 the complexes were successfully made and the samples did not become cloudy after overnight storage at 4°C. Due to time constraints only the apoA-IV helix 1 (117-138) was studied. On average all the apoA-IV proteins were resolved as 2 types of complexes consisting of a major band of 48.6 Å at 351Kda and a minor band of 57.6 Å at 416Kda, most likely due to 2 different conformations of apoA-IV (table 4.2 and 4.3). Although the majority of apoA-IV was incorporated into complex, most of the preparations contained on average 11 % free apoA-IV (table 4.4 and 4.5). Radioactive complexes were made with the addition of [3H] unesterified cholesterol (0.55MBq).



1 WT lipid free	5 R134Q complex	9 WT lipid free	13 HMW	17 WT lipid free	21 N127R+R134Q complex
2 WT complex	6 R123Q+N127R complex	10 WT* (radioactive) complex	14 R134Q* complex	18 N127R+R134Q* complex	22 H.1 K.O complex
3 R123Q complex	7 R123Q+R134Q complex	11 R123Q* complex	15 R123Q+N127R* complex	19 HMW	
4 HMW	8 N127R+R134Q complex	12 N127R* complex	16 R123Q+R134Q* complex	20 H.1 K.O * complex	

Figure 4.14: The analysis of all radioactive (*) and non-radioactive complexes produced using 4-30% non-denaturing polyacrylamide gradient gels.

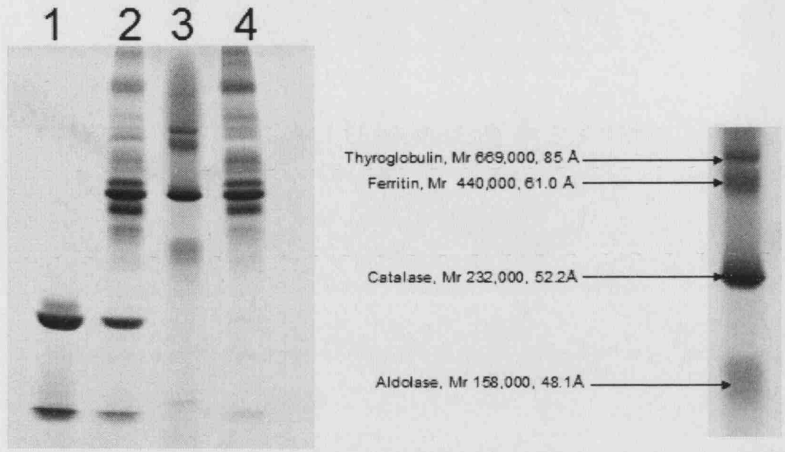


Figure 4.15: Analysis of the N127R complex on 4/30 PAA, 1= lipid free apoA-IV, 2= WT complex, 3=HMW, 4= N127R complex using a 4-30% non-denaturing polyacrylamide gradient gel.

The apolipoprotein A-IV:POPC:UC molar ratio shown in table 4.2 and 4.3 was calculated using the BCA assay kit to estimate protein concentration (as described in section 2.5.2.1), and the Wako phospholipid B and Wako free cholesterol C assay kits (section 2.6.3.1) to calculate the phospholipid and UC concentrations.

ApoA-IV mutant	Diameters of complexes (Å)	ApoA-IV/POPC/Cholesterol *(mol/mol/mol)*
WT	48.6, 57.6	1/240/16
R123Q	48.6, 57.6	1/231/16
N127R	48.6	1/212/11
R134Q	48.6, 57.6	1/212/16
R123Q+N127R	48.6, 57.6	1/275/26
R123Q+R134Q	48.6, 57.6	1/266/16
N127R+R134Q	48.6, 57.6	1/266/13
Helix 1 K.O	48.6, 57.6	1/310/20

Table 4.2: The physical characteristics of the non radioactive apoA-IV POPC complexes produced for circular dichroism analysis.

ApoA-IV mutant	Diameters of radioactive complex (Å)	ApoA-IV/POPC/Cholesterol *(mol/mol/mol)* ratio of radioactive complex.
WT	48.6, 57.6	1/246/11
R123Q	48.6, 57.6	1/235/9
N127R	48.6, 57.6	1/257/17
R134Q	48.6, 57.6	1/230/13
R123Q+N127R	48.6, 57.6	1/271/18
R123Q+R134Q	48.6, 57.6	1/281/12
N127R+R134Q	48.6, 57.6	1/255/12
Helix 1 K.O	48.6, 57.6	1/277/15

Table 4.3: The physical characteristics of the radioactive apoA-IV POPC complexes produced for LCAT studies.

Complex	Major complex (%)	Minor complex (%)	Free protein (%)
Wild type apoA-IV	69	11	20
R123Q	54	23	23
R134Q	64	27	9
N127R	81	16	3
R123Q+R134Q	72	22	6
R123Q+N127R	54	25	22
N127R+R134Q	70	29	1
Helix 1- R123Q+ R134Q+ N127R	54	43	3

Table 4.4: Quantification of the apoA-IV POPC complexes.

Radioactive complexes (*)	Major complex (%)	Minor complex (%)	Free protein (%)
Wild type apoA-IV*	52	28	20
R123Q*	51	28	21
R134Q*	51	27	22
N127R*	59	30	11
R123Q+R134Q*	60	40	0
R123Q+N127R*	65	27	8
N127R+R134Q*	61	32	7
Helix 1- R123Q+ R134Q+ N127R*	71	28	1

Table 4.5: Quantification of the radioactive apoA-IV POPC complexes.

4.3.3.4 Analysis of the secondary structure of the free and complexed recombinant apoA-IV proteins produced

Circular dichroism is a general method used to analyse the secondary structures of proteins (Johnson, 1988). Both the lipid free and complexed apoA-IV variants (500μg/ml) were analysed using a 0.1cm path length quartz cell as described in section 2.6.3.3. The secondary structure of each sample (% helicity) was estimated from the ellipticity curves produced using the method described in section 2.6.3.3. As presented in figure 4.16 all samples clearly demonstrated a characteristic α-helix CD spectra with two minima at 208 and 222nm and that the formation of POPC complexes resulted in increased α-helicity (figure 4.17). On average wild type apoA-IV was more α helical compared to the apoA-IV variants in the lipid free state, but once complexed all recombinant apoA-IV proteins had very similar secondary structures (table 4.6). In order to deconvolute the various CD spectra I used the CD pro software bundle (available at http:/lamar.colostate.edu/~sreeram/CDPro) containing the popular CONTIL/LL and SELCON3 software (Sreerama and Woody, 2000; Sreerama and Woody, 2004), these results are summarised in table 4.6. All deconvolution results were analysed sceptically because none of the reference databases available contained any apolipoproteins. The increase in the 208nm and 222nm minima upon POPC complex formation was most pronounced in the H1 variant with a 135% and 169% increase at 208nm and 222nm respectively, while the R134Q variant only demonstrated a 12% and 16% increase at 208 nm and 222nm (summarised in figure 4.17), demonstrating the different abilities of each apoA-IV variant to fold as an α-helix.

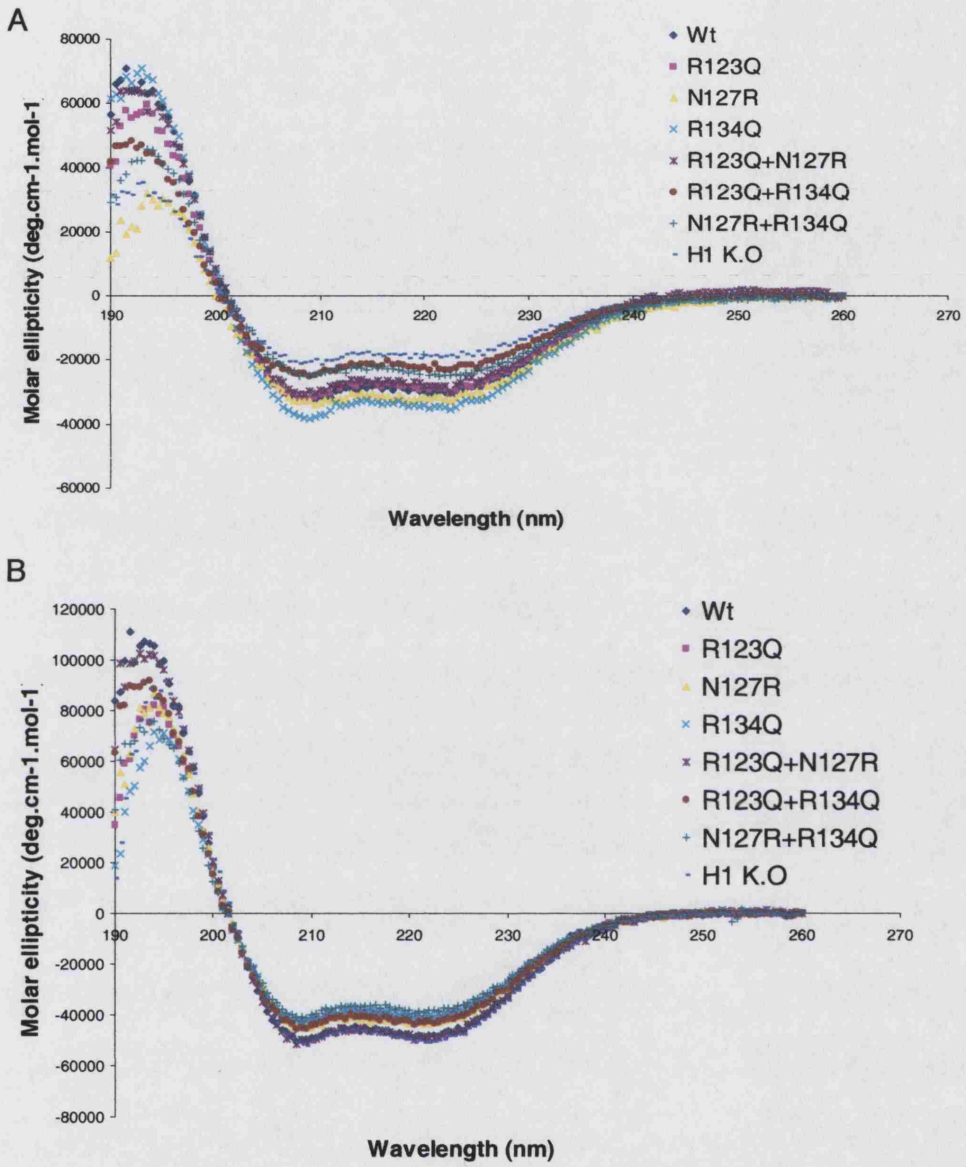
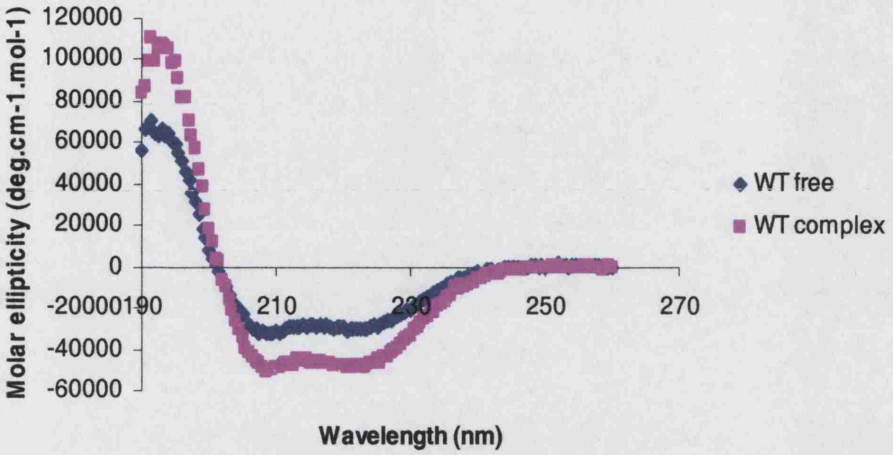


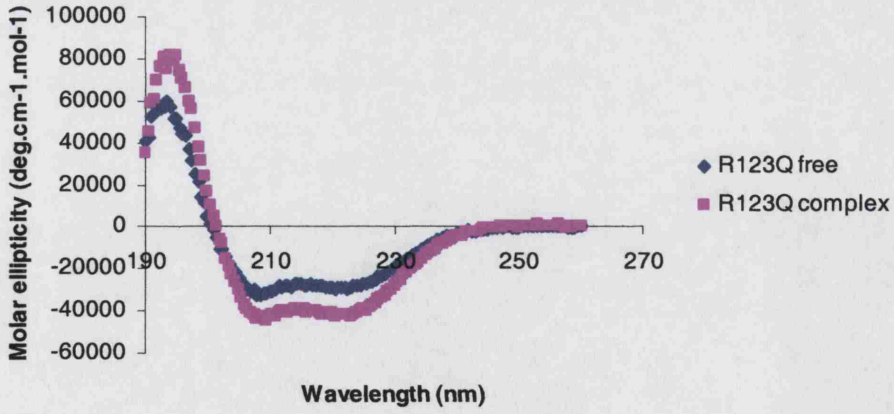
Figure 4.16: The CD spectra of all the recombinant apoA-IV proteins produced, both in the A.) lipid free state and B.) in POPC complexes.

A



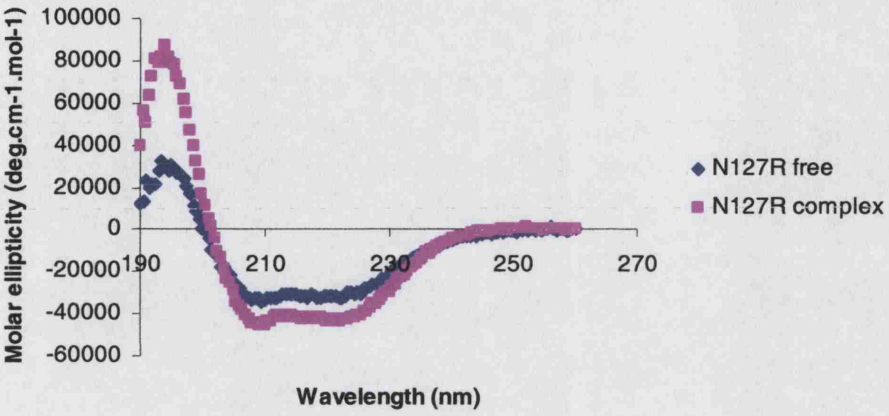
Wavelength (nm)	WT free	WT complex	% Increase
208	-31675.07227	-48329.13	52.58
222	-29941.87109	-48127.34	60.74

B



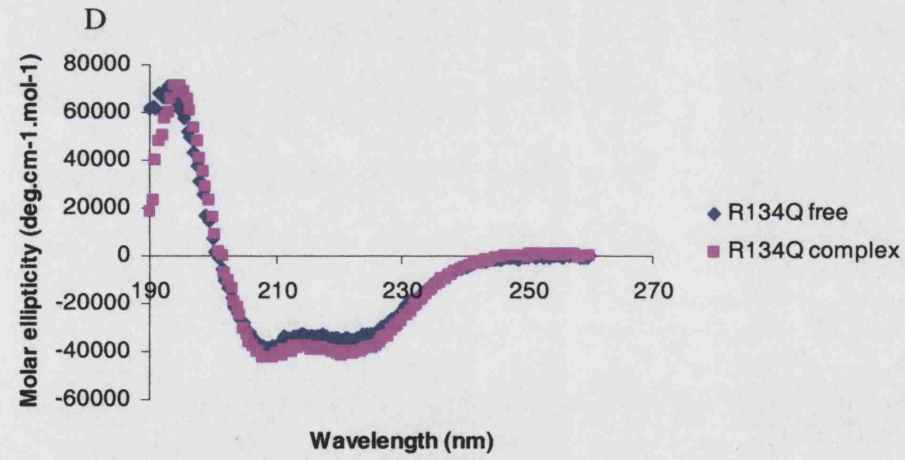
Wavelength (nm)	R123Q free	R123Q complex	% Increase
208	-32408.11	-43454.14	34.08
222	-28916.30	-42118.30	45.66

C



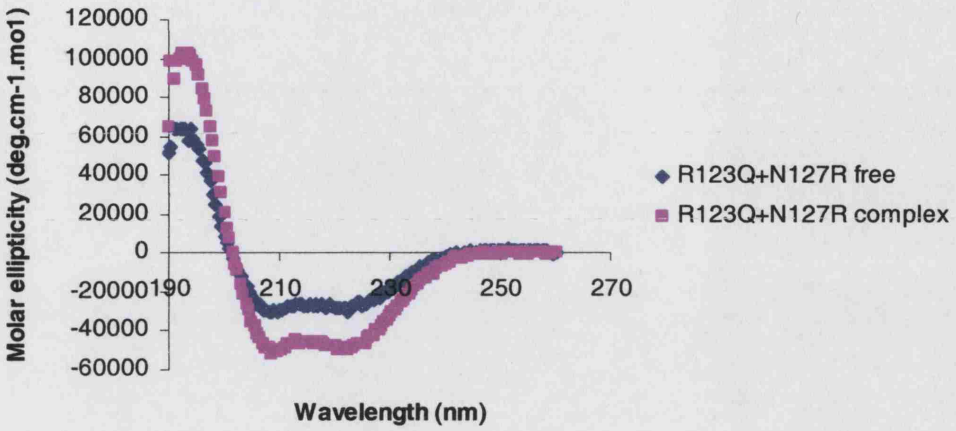
Wavelength (nm)	N127R free	N127R complex	% Increase
208	-33034.47	-44661.10	35.20
222	-32394.17	-43289.99	33.64

D



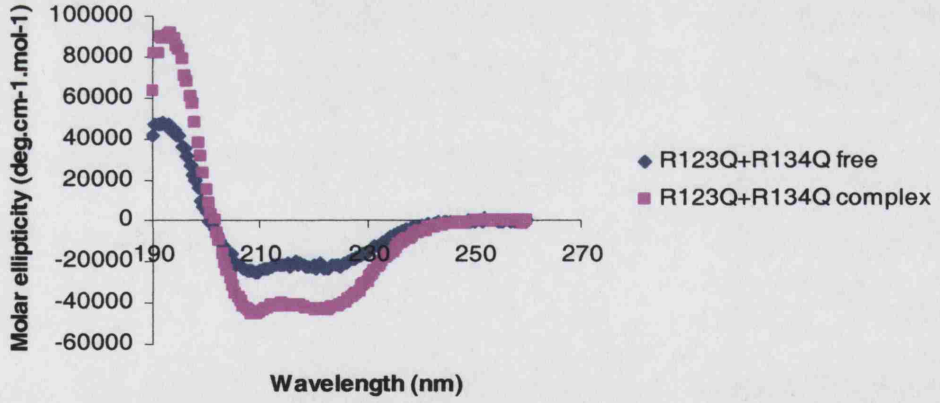
Wavelength (nm)	R134Q free	R134Q complex	% Increase
208	-37538.99	-42058.27	12.04
222	-34929.21	-40482.48	15.90

E



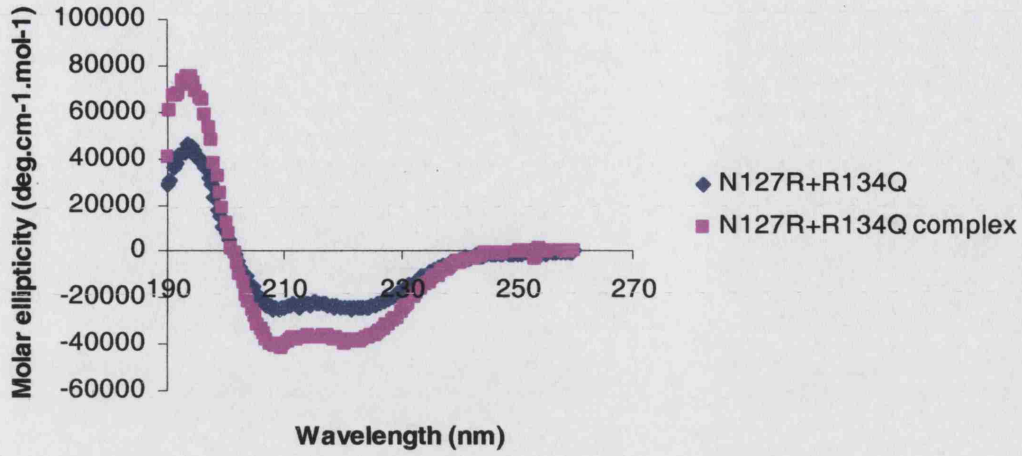
Wavelength (nm)	R123Q+N127R free	R123Q+N127R complex	% Increase
208	-30720.85	-48277.07	57.15
222	-27988.23	-48763.45	74.23

F



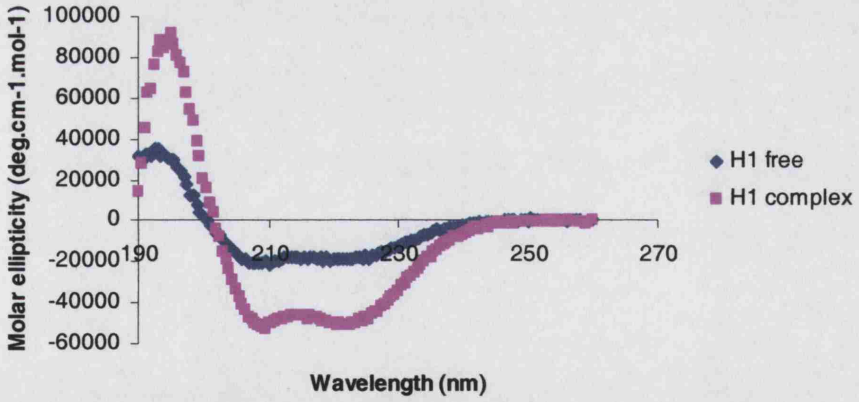
Wavelength (nm)	R123Q+R134Q free	R123Q+R134Q complex	% Increase
208	-24175.69	-43482.95	79.86
222	-23212.36	-42761.91	84.22

G



Wavelength (nm)	N127R+R134Q free	N127R+R134Q complex	% Increase
208	-25232.32	-40171.33	59.21
222	-24730.93	-38479.51	55.59

H



Wavelength (nm)	H1 lipid free	H1 complex	% Increase
208	-21090.09	-49552.61	134.96
222	-18980.28	-51031.18	168.86

Figure 4.17 A-H: Comparing the secondary structure of the individual apoA-IV variants in their lipid free and POPC complexed state.

Sample	Method	α_R	α_D	β_R	β_D	Turn	Unrd
WT	CONTIN/LL	59	30.2	0	3.8	1.4	5.5
	SELCON3	58.1	22.4	0	0	5.4	14.8
WT complex	CONTIN/LL	69.2	29.7	0	1.2	0	0
	SELCON3	58.7	22.9	0	0.1	5.3	14.8
R123Q	CONTIN/LL	57.8	32.9	0	8.7	0	0.7
	SELCON3	58.7	22.7	0	0	8.2	16.3
R123Q complex	CONTIN/LL	60.2	34.6	0.2	2.9	2.1	0
	SELCON3	64	26.1	0	0	5.8	11.4
R134Q	CONTIN/LL	61.5	36.5	0	2	0	0
	SELCON3	58.1	22.3	0	0	5.3	14.6
R134Q complex	CONTIN/LL	56.7	39.9	0.7	1.7	1	0
	SELCON3	62.2	25.7	0	0	6.5	11.7
N127R	CONTIN/LL	37.3	31.7	0	3.6	20	7.4
	SELCON3	26.2	14.9	3.5	0.6	21.9	27.9
N127R complex	CONTIN/LL	60	35.5	2	2.2	2.1	0
	SELCON3	63.9	26.2	0	0	5.3	11.1
R123Q+R134Q	CONTIN/LL	44.9	24.9	0	2.9	9.6	17.8
	SELCON3	42.6	21.5	0	1.8	13.3	22.4
R123Q+R134Q complex	CONTIN/LL	67.2	31.3	0	1.5	0	0
	SELCON3	63.2	22.7	0	0	4.6	14.5
R123Q+N127R	CONTIN/LL	49.9	36.2	0	8.7	5.2	0
	SELCON3	59.2	23.9	0	0.2	6.2	14.4
R123Q+N127R complex	CONTIN/LL	68.4	30.7	0	0	0.4	0
	SELCON3	63.1	23.8	0	0	4.8	13.3
N127R+R134Q	CONTIN/LL	34.6	36.7	0	7	21.7	0
	SELCON3	30.5	23.6	0	3.4	26.9	21.9
N127R+R134Q complex	CONTIN/LL	63.2	34.5	0	1.3	1.1	0
	SELCON3	64	26.5	0	0	6.1	11.5
Helix 1	CONTIN/LL	37.3	21.3	0.3	2.2	14.5	24.3
	SELCON3	36.3	21	1.5	2.5	15	23.4
Helix 1 complex	CONTIN/LL	54	38.6	0.4	2.6	4.4	0
	SELCON3	61.6	24.9	0	0	6.5	12.6

Table 4.6: Deconvolution of the apoA-IV/complex CD spectra using CONTIN/LL and SELCON3; α_R, regular α-helix; α_R, distorted α-helix; β_R, regular β-strand; β_D, distorted β; Turn; beta turn and Unrd; unordered.

4.3.3.5 The thermal stability of the free recombinant apo-AIV proteins

The stability of the lipid free apoA-IV proteins was estimated by following the decrease in their α helical content measured by CD with the increase in temperature from 15 °C to 95 °C. Exactly 500μg/ml of each apoA-IV recombinant protein (diluted in 1 X TBS) was heated from 15 °C to 95 °C and the CD change at 222 nm was recorded and used to monitor the temperature induced denaturation of the protein (figure 4.18) . The temperature of midtransition (T_m) was calculated for each protein and compared to the wild type apoA-IV (please see section 2.6.3.4 for more details). As summarised in table 4.7 the recombinant apoA-IV proteins differed in their susceptibility to thermal denaturation with wild type apoA-IV having the greatest T_m (WT> N127R> R134Q> N127R+R134Q> R123Q+N127R> R123Q> H1>R123Q+R134Q), indicating that on average the α-helices in the mutant apoA-IV variants produced were less stable (figure 4.19).

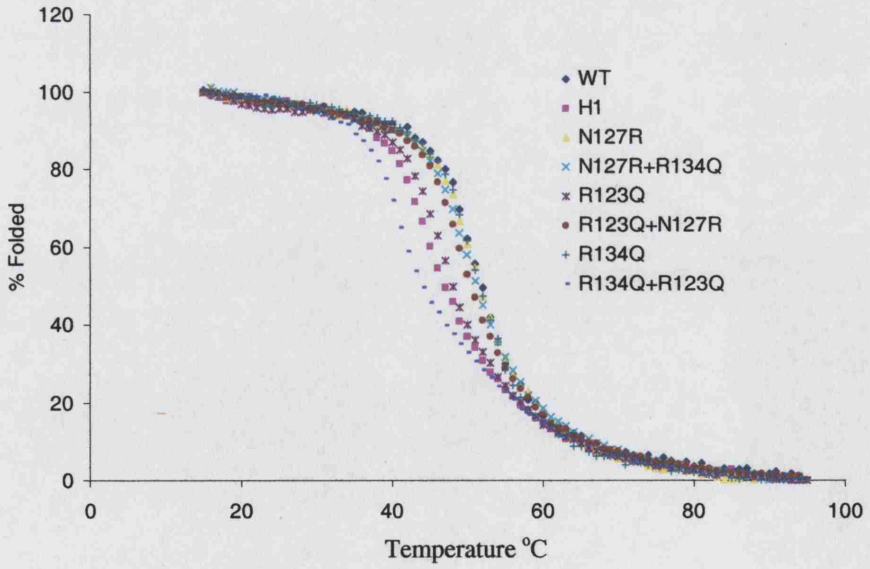
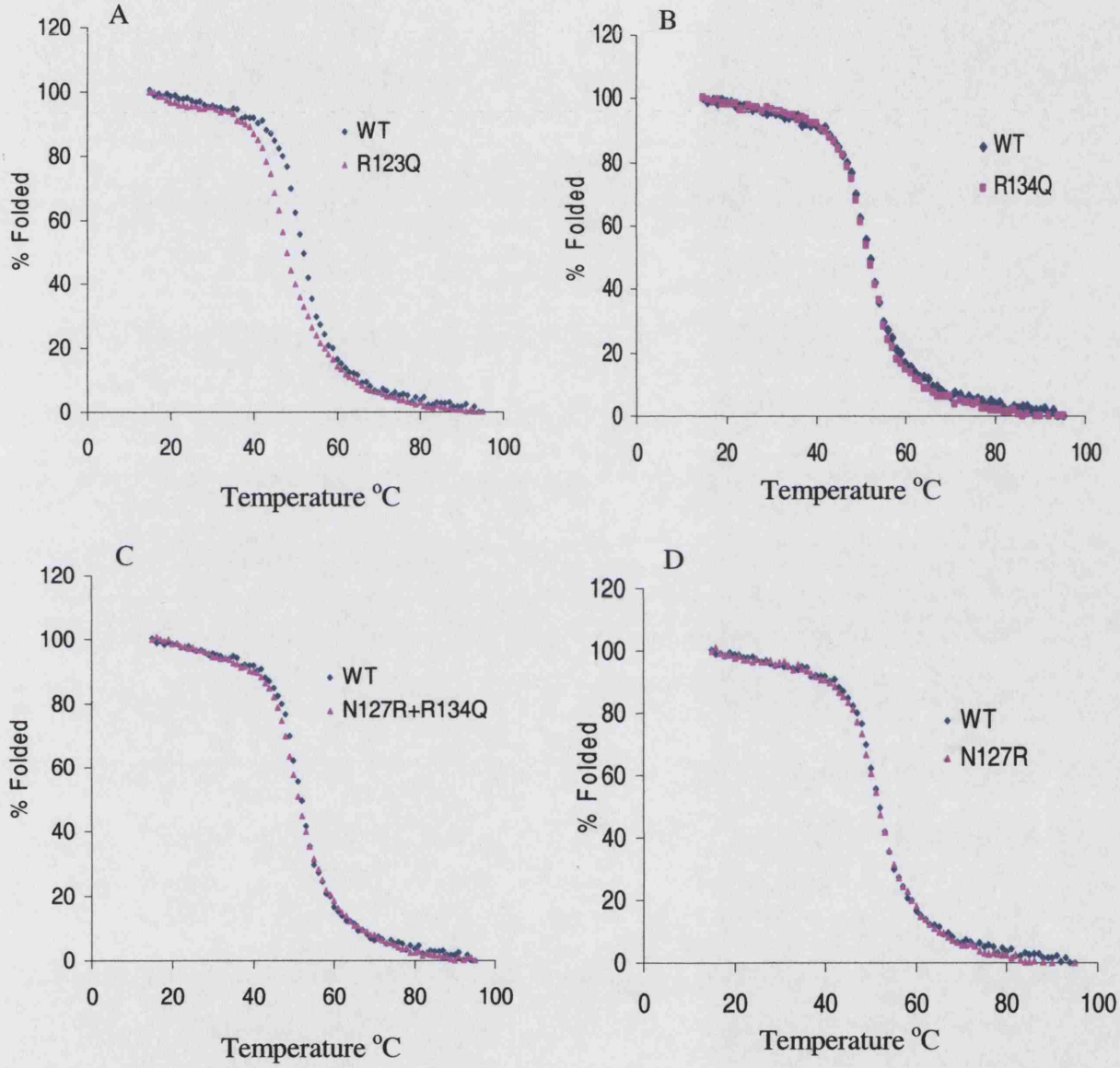


Figure 4.18: The thermal denaturation (15 °C to 95 °C) of the recombinant apoA-IV proteins, monitored at 222nm.

As presented in figure 4.18 all variants produced sigmoidal thermal denaturation plots implying that all apoA-IV proteins underwent similar unfolding behaviour, apart from mutant R123Q+R134Q which underwent thermal denaturation that could not be modelled using the two state hypothesis. All other samples demonstrated a relatively narrow thermal transition (unfolding occurred over a small temperature range) indicating that unfolding proceeded via a two state process (folded and unfolded) with no intermediate formation occurring. Such a steep thermal transition implies that apoA-IV undergoes high cooperative unfolding with a significant heat capacity (ΔCp) increment, supporting the recent finding that apoA-IV exists as a single cooperative domain between residues 40-332 (Pearson *et al*, 2004). Since the majority of the change in heat capacity (ΔCp) arises from water ordering by apolar protein groups unfolding (Makhatadze and Privalov, 1990), the reduced ΔCp inferred from thermal unfolding of the apoA-IV mutants compared to wild type apoA-IV implicates a reduction in the amount of hydrophobic core. This is based on the fact that a positive ΔCp change associated with protein unfolding is due to the exposure of hydrophobic residues to water (Freire, 1995). The R123Q+R134Q variant with the lowest T_m did not fit well to a 2 state unfolding transition, indicating that the thermal denaturation of this variant could have resulted in the formation of one or more intermediate states, highlighted by the inaccurate ΔCp and folding enthalpy with large errors generated (table 4.7). The increase change in folding enthalpy (ΔH fold) reflecting the order of thermal stability of the recombinant apoA-IV samples, corresponds to the heat of unfolding of an average cooperative unit. Therefore the differences in ΔH fold between the wild type protein and mutants reflects the differences in cooperativity of unfolding and the gradient of the thermal transition slope.


A
% Folded
Temperature °C
WT
R123Q
B
% Folded
Temperature °C
WT
R134Q
C
% Folded
Temperature °C
WT
N127R+R134Q
D
% Folded
Temperature °C
WT
N127R

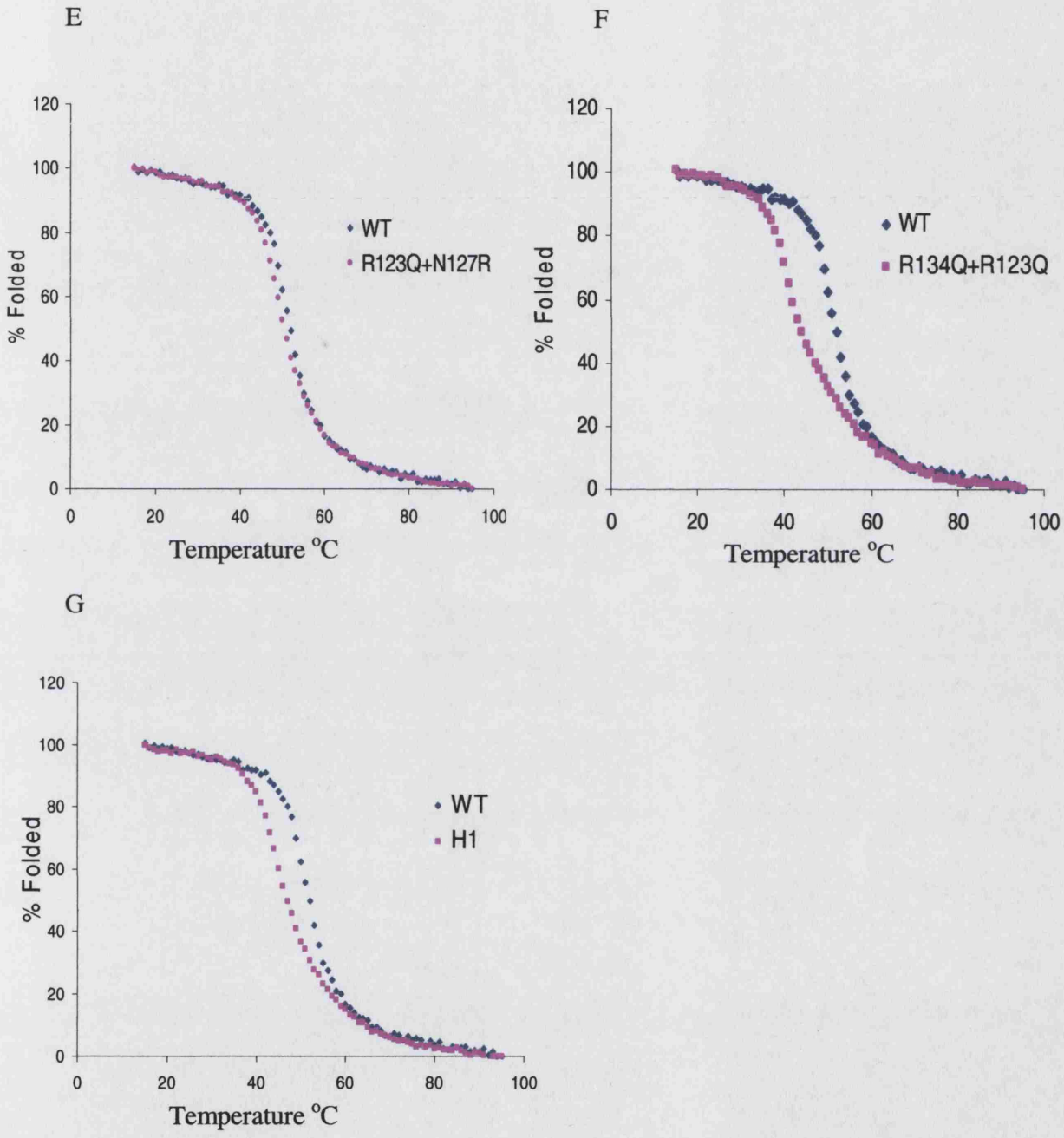


Figure 4.19: Graphs illustrating the thermal stability of each apoA-IV variants compared to wild type apoA-IV.

ApoA-IV (500μg/ml)	T_m (°C)	ΔCp fold (kJ/mol^{-1}/K)	ΔH fold (kJ/mol^{-1})
Wild type	52.82 (±0.09)	11.152 (±0.64)	-302.611 (±6.32)
R123Q	47.98 (±0.11)	8.095 (±0.36)	-233.145 (±6.61)
R134Q	51.77 (±0.08)	10.181 (±1.79)	-287.237 (±6.71)
N127R	51.73 (±0.01)	9.799 (±0.48)	-264.318 (6.70)
R123Q+R134Q	41.96 (±0.64)	-4.054 (±4.36)	-0.015 (±80.26)
R123Q+N127R	50.50 (±0.11)	8.907 (±0.49)	-252.869 (±6.35)
N127R+R134Q	51.25 (±0.13)	9.867 (±0.47)	-259.719 (±8.15)
Helix 1 - R123Q+ R134Q+ N127R	46.53 (±1.95)	6.451 (±1.34)	-201.619 (±48.47)

Table 4.7: Conformation and stability properties of wild type apoA-IV and the various mutants produced. Errors were generated using montecarlo simulations of 100 data sets using the method of Cliff *et al,* (Cliff *et al*, 2005).

4.4 DISCUSSION AND CONCLUSION

Previous experiments have suggested that the key residues for the activation of LCAT by apoA-IV are located between residues 117-160 of the mature protein (Emmanuel *et al*, 1994). After subjecting this region of apoA-IV to helical wheel analysis and alignment with the LCAT activation domain of apoA-I, paying close attention to the apoA-I key residues R134Q, R153Q and R160Q (Roosbeek *et al*, 2001), six interesting residues within the polar face of two atypical class A amphipathic helix of apoA-IV were identified. A Clustal W alignment revealed that the 6 mutated residues within the polar face of helix 1 (R123, R134, N127) and helix 2 (Q156, R149 and R160) were only conserved in higher mammals. Using the pET-14b system in *E.coli* cells, in total 20 apoA-IV recombinant proteins were successfully produced and purified using nickel resin. Analysis of all apoA-IV proteins produced using non denaturing 4-20% gradient gels revealed that the majority of recombinant apoA-IV proteins migrated as two major bands of roughly 67 and 140 kDa, likely corresponding to dimeric and trimeric forms of apoA-IV (Weinberg and Spector, 1985a). A western blot using a monoclonal rabbit apoA-IV antibody further verified that the recombinant proteins produced were indeed apoA-IV.

All 20 recombinant apoA-IV proteins produced successfully bound to DMPC at its transition temperature (23.5°C), indicating that all recombinant apoA-IV proteins had equal lipid binding affinity and that the presence of an N-terminal 6X histidine tag or the lack of glycosylation did not affect the lipid binding properties of apoA-IV. Due to time constraints only helix 1 (residues 117-138) was investigated further with 7 apoA-IV

variants in total being converted into POPC complexes and their physical characteristics analysed. Two sets of complexes were produced, a radioactive (^{3}H) set which was subjected to the LCAT assay (chapter 5) and a non-radioactive set which was analysed by CD. Both sets of complexes had very similar diameters and apoA-IV/POPC/cholesterol molar ratios. All recombinant apoA-IV proteins as expected gave α-helical CD spectra with two minima at 208 and 222nm that increased upon the formation of POPC complexes. Despite some variants (N127R, R123Q+R134Q, H1 and N127R+R134Q) in the lipid free state demonstrating reduced α-helical content compared to wild type apoA-IV, once complexed all apoA-IV proteins produced had limited differences in secondary structure.

To further analyse the energetic structure/function relationship in apoA-IV, all lipid free recombinant apoA-IV proteins were subjected to thermal denaturation. The differences in thermal denaturation behaviour between wild type apoA-IV and mutants suggest differences in the overall stability of the protein folds. However all apoA-IV proteins examined showed similar narrow thermal transition profiles (except the R123Q+R134Q variant that did not fit accurately to a 2 state unfolding transition), with relatively native conformations present up to about 40°C after which rapid denaturation occurred followed by a plateau (indicative of a two state process: folded and unfolded) . Such a narrow thermal transition profile of apoA-IV is expected given the recent finding that apoA-IV exists as a single cooperative domain between residues 40-332 (Pearson *et al*, 2004). This is in contrast to apoE, apoA-II and apoA-I (Gursky and Atkinson, 1996a; Gursky and Atkinson, 1996b; Morrow *et al*, 2002) in which denaturation occurs over a large

temperature interval indicating the induction of a molten globular-like state (a compact folding intermediate with a well defined near native secondary structure and a lax non-specific tertiary structure) and therefore a three state model of denaturation (native/intermediate/unfolded). Indeed the broad temperature induced unfolding transition of apoA-II (25-85°C) can even be compared to the unfolding of isolated α-helices in aqueous solution (20-100°C) (Gursky and Atkinson, 1996a; Scholtz *et al*, 1991), indicating that the α-helices of apoA-II may be only weakly stabilised by tertiary interactions. The lack of specific tertiary contacts could confer greater conformational adaptability to apoA-I, II and E compared to apoA-IV, essential for the action of these exchangeable apolipoproteins and could possibly explain the ease with which apoE can displace apoA-IV on chylomicrons once in the plasma (Eisenberg, 1984).

Despite having a thermal denaturation curve with a greater sigmoidicity and thus a superior cooperativity compared to apoA-I, the T_m for apoA-IV is 52.8°C compared to 60 °C for apoA-I indicating that on average the α-helices in apoA-IV are slightly less stable (Pearson *et al*, 2004). This is further supported by the fact that apoA-IV compared to apoA-I is less stable and is completely denatured in 0.5M guanidine HCL (Weinberg and Spector, 1985a). Nonetheless the data here suggest that the domain structure of apoA-IV is highly cooperative and organised in such a way to sequester hydrophobic residues within the interior of the protein molecule. Such a high level of organisation can be seen in figure 4.5 with monomeric apoA-IV running as a tightly focused band on a SDS PAGE. The seemingly opposed relationship between stability and high structural organisation in apoA-IV could be linked to the fact of all the exchangeable

apolipoproteins, apoA-IV has the least hydrophobic helical repeats (Weinberg, 1987). It is thought in comparison to apoA-I or apoE, apoA-IV is dominated by less hydrophobic forces acting upon sequestered non-polar residues and more hydrophilic interactions among exposed polar residues, these polar interaction on the surface could result in a delicate but defined tertiary structure of apoA-IV (Pearson *et al*, 2004).

In conclusion, alterations in the charge distribution in helix 1 (residues 117-138) or net charge of the apoA-IV molecule affects the secondary structure of the lipid free protein. However, once complexed to lipid, differences in protein secondary structure between the apoA-IV mutants as examined by CD were only minor. Next we investigated the impact of these alterations on apoA-IV function by examining the ability of the various radioactive complexes to activate LCAT.

CHAPTER 5

CHAPTER 5: THE ACTIVATION OF LCAT BY APOA-IV VARIANTS

5.1 INTRODUCTION

5.1.1 The LCAT (Lecithin-Cholesterol Acyltransferase) reaction

LCAT is a pivotal enzyme in maintaining cholesterol homeostasis and regulating cholesterol transport in the blood. The majority of LCAT is synthesised in the liver (Osuga and Portman, 1971) and is associated with HDL, containing the main LCAT apolipoprotein activator apoA-I (Fielding *et al*, 1972). Although predominantly associated with HDL, LCAT has also been shown to be active in LDL subfractions using apoE as an activator (Barter, 1983; Zhao *et al*, 2005). The most important physiological role of LCAT is the transesterification of the sn-2 acyl chain from phosphatidylcholine (PC) to the 3β-hydroxyl group of cholesterol, producing cholesterol ester (CE) and lyso-PC (Glomset, 1968; Glomset , 1972).The above esterification reaction by LCAT results in the accumulation of CE in the core of HDL, converting discoidal HDL into spherical particles, sustaining a chemical gradient for the flux of cholesterol from cell membranes into HDL. LCAT has therefore been postulated to be the engine for reverse cholesterol transport (Jonas, 2000). In addition LCAT also possess two other activities. Firstly, when lyso-PC concentrations are elevated, LCAT can catalyse the reacylation of lyso-PC producing PC on LDL, an enzymatic reaction termed as lysolecithin acyltransferase (LAT) (Subbaiah and Bagdade, 1978). Secondly, LCAT can catalyse CE hydrolysis followed by re-esterification of a second cholesterol molecule, a catalytic reaction termed cholesterol acyltransferase (CAT) (Sorci-Thomas *et al*, 1990; Subbaiah and Bagdade, 1978).

5.1.2 LCAT predicted structure

The complete tertiary structure of LCAT remains to be elucidated, predictions from linear sequences and circular dichroism measurements imply a low α helical content (~22%) and greater β sheet content (Jonas, 2000). Nonetheless a computer predicted structure of LCAT has been produced based upon its homology to other members of the α/ β hydrolase fold family (figure 5.1) (Peelman *et al*, 1998). The LCAT model by Peelman *et al* indicates that the central domain of LCAT is comprised of seven parallel beta strands, connected by four α-helices and separated by loops containing the catalytic triad residues (D345, H377, S181) (Adimoolam *et al*, 1998; Peelman *et al*, 1998). Based upon studies by Jauhiainen and Dolphin, it is predicted that the D345, H377 and S181 residues create a catalytic triad with S181 enabling a fatty-acyl-Serine acyl-enzyme intermediate to form, following lecithin cleavage and prior to the donation of the fatty acid to the sterol acyl-acceptor (cholesterol) (Jauhiainen and Dolphin, 1986). LCAT residues 56-68 have been predicted to form a 'lid domain' important in the interfacial binding of LCAT to HDL and LDL surfaces (Jin *et al*, 1999; Peelman *et al*, 1999). The most prominent helix in LCAT is helix 4-5 (residues 151-174) preceding the S181 residue, with functional studies postulating it to be important in LCAT binding to lipoprotein surfaces or phospholipids substrates (Peelman *et al*, 1997).

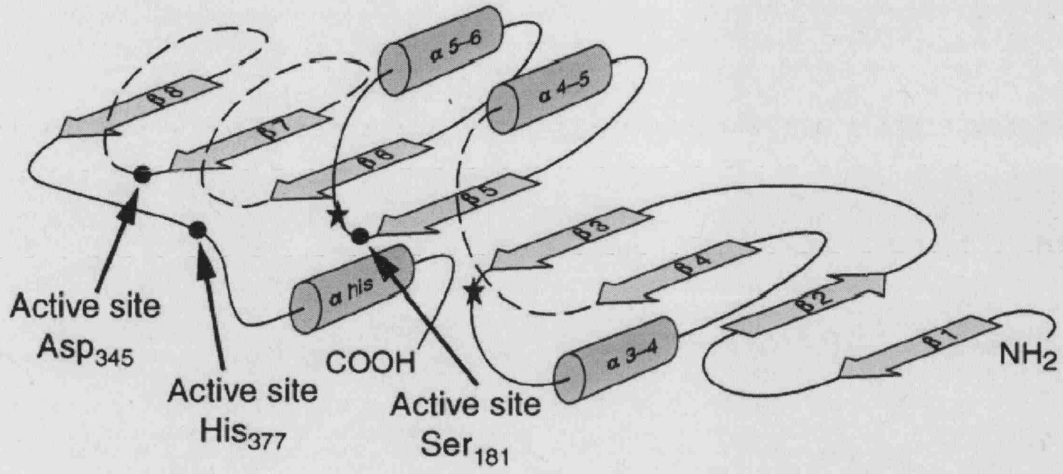


Figure 5.1: The predicted tertiary structure of LCAT, adapted from Jonas *et al* (Jonas, 2000). Catalytic triad residues are represented by dots while the other catalytic residues are denoted as stars.

5.1.3 The catalytic mechanism of LCAT

The LCAT conversion of cholesterol and phophatidylcholine (PC) to cholesterol ester (CE) and lysophosphotidylcholine (lysoPC) occurs in multiple steps on the surface of HDL. These include the interfacial binding, activation, PC binding (the rate limiting step), acyl enzyme formation, release of lyso-PC, cholesterol binding and release of cholesterol ester enzyme (Jonas, 2000; Parks and Gebre, 1997). It is thought that the LCAT reaction proceeds as follows; the interfacial recognition domain (residues 50-74) (Peelman *et al*, 1998) swings out and inserts into the lipid, within HDL particles containing apoA-I in a favourable conformation an interaction between apoA-I (residues 143-186) and a complementary region of LCAT occurs leading to a change of LCAT conformation resulting in an LCAT enzyme optimally active with its lipid substrates (Jonas, 1998).

The initial LCAT cleavage of the sn-2 ester bond of PC produces lyso-PC and a transient oxyester fatty acyl intermediate linked to the serine residue within the LCAT active site. This trans fatty acyl moiety is transesterified to a cysteine residue within the LCAT activation site, producing a thioesterified enzyme capable of donating its fatty acyl group to cholesterol producing cholesterol ester, thus regenerating a non-acylated enzyme (Jauhiainen and Dolphin, 1986).

5.1.4 Regulation of LCAT activity by substrate

The regulation of LCAT activity is not dependent upon processes that control its synthesis and secretion. LCAT activity is regulated by the concentration, composition and subclass distribution of its lipoprotein substrates (Jonas, 1998).

Studies involving the use of reconstituted HDL (rHDL) have indicated that LCAT activity can vary *in vitro* up to 100 fold, depending upon the fatty acyl composition of the rHDL substrate (Jonas *et al*, 1987). Bolin and Jonas using direct binding assays for LCAT and rHDL have described K_m (Michaelis constant) to reflect the interfacial binding affinity, and V_{max} (maximum rate of reaction) to reflect the active site preference of LCAT (Bolin and Jonas, 1994).

Using defined PC species with long chain polyunsaturated fatty acids (PUFA), it has been identified that rHDL with long chain PUFA in the *sn*-2 position of PC have decreased catalytic efficiency. The LCAT reactivity of rHDL was ordered as follows POPC (18:1) = PLPC (18:2) > PAPC (20:4) > PEPC (20:5) > PDPC (22:6) (Huggins *et al*, 1998; Parks

and Gebre, 1997). Various experiments have revealed that PC species containing PUFA are more disordered, hydrated along the fatty acyl chain, hydrophilic and contain a larger free volume (Holte *et al*, 1995), (Ho *et al*, 1995), furthermore that PC containing long chain PUFA (PEPC and PDPC) have a larger overall molecular surface area compared to POPC containing PC (Parks and Thuren, 1993). Such an increase in molecular mass and presence of water in the membrane could weaken the association of apoA-I to PC. A reduced interaction between apoA-I and PC may also result in increased intra-helical interactions between adjacent apoA-I α-helical regions, destabilising the regions of apoA-I not bound to PC (Huggins *et al*, 1998). The diminished stability of apoA-I in such complexes is demonstrated by the reduced amount of guanidine chloride needed to denature half of the apoA-I on rHDL containing long chain PUFA, compared to those containing POPC (1.57-1.70M vs 2.83M). The K_m values were similar among all rHDL investigated, with apparent disparities between PC species due to V_{max} differences (Parks and Gebre, 1997). This indicates that the reduced reactivity of LCAT for long chain PUFA does not reflect a decreased interfacial binding of LCAT to apoA-I rHDL surfaces, but rather a decreased preference of LCAT for long chain PUFA at the enzymes active site. However it was also observed that rHDL containing long chain PUFA, despite having reduced V_{max}, had lower activation energies relative to POPC. The lower activation energy required for the LCAT reaction in these complexes could be an intrinsic property of PUFA, due to the disordered structure of PCs containing long chain PUFA facilitating the access of lipid substrate to LCAT (Huggins *et al*, 1998).

5.1.5 The effect of substrate particle size on LCAT activation

Particle size is also an important parameter; analysis of L-alpha-dipalmitoylphosphotidylcholine (DPPC) apoA-I complexes prepared by cholate dialysis ranging from 103Å to 380Å established that complexes with a diameter of 110 Å had optimal LCAT reaction rates. In contrast when apoA-I complexes were formed with egg phosphotidylcholine (egg-PC) producing a limited spectrum of complexes ranging from 105 Å to 214 Å, all apoA-I complexes had similar LCAT reaction rates (Jonas and McHugh, 1984). The maxima fluorescence measurements in these smaller DPPC complexes indicate an optimal apoA-I conformation at the interface with PC, while all egg-PC complexes produced had uniform spectral properties (Jonas and McHugh, 1984).

5.1.6 Apolipoprotein activation of LCAT

The principal activator of LCAT is apoA-I (Yokoyama *et al*, 1980), while by comparison other apolipoproteins such as apoA-IV, apoE-2 and apoE-3 are inferior activators of LCAT (Chen and Albers, 1985; Steinmetz and Utermann, 1985). It is now known that the helical residues 144-186 of apoA-I play a key role in LCAT activation (Frank *et al*, 1998). ApoA-I helix 144-164 is thought to interact with residues 151-174 of LCAT allowing access to free UC in discoidal rHDL, while apoA-I helices 143-164 and 165-186 have been predicted via electrostatic interactions to facilitate and stabilise the interaction of LCAT with rHDL (Meng *et al*, 1993; Sparks *et al*, 1998). A cluster of three strictly conserved arginine residues (R149, R153 and R160) in apoA-I that can interact with LCAT via salt bridge formation or hydrogen bonds (Roosbeek *et al*, 2001), and which are critical for LCAT activation in all species have been found. The side chains of these

arginine residues are predicted to be orientated towards the surface of rHDL discs enabling them to electrostatically interact with LCAT and induce full enzymatic activity (figure 5.2) (Roosbeek *et al*, 2001).

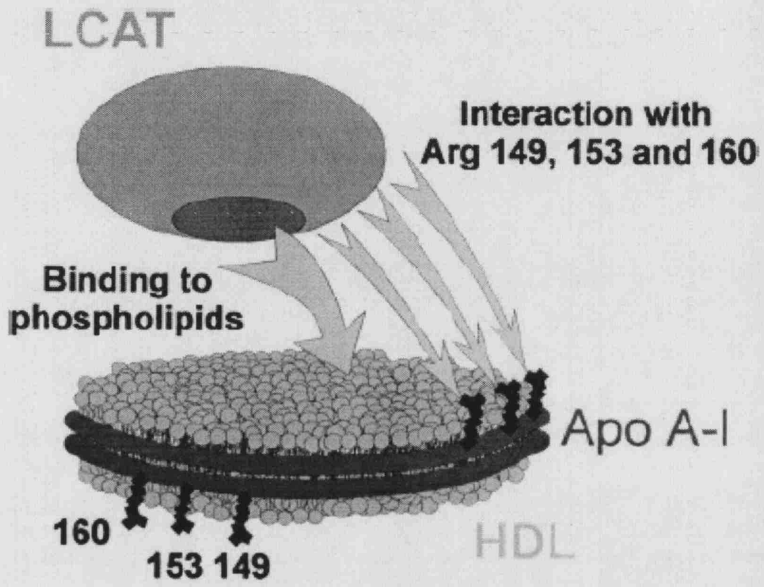


Figure 5.2: Schematic representation of the proposed mode of interaction of LCAT with apoA-I arginine residues 149, 153, and 160, in a discoidal rHDL particle, adapted from Roosbeek *et al* (Roosbeek *et al*, 2001).

The ability of apoA-I to activate LCAT has been ascribed to the propensity of its α-amphipathic helices to insert between the polar head groups of phospholipids, resulting in the *sn*-2 ester linkage of PC being re-orientated rendering it more susceptible for hydrolysis by LCAT (Yokoyama *et al*, 1980). It is thought that the transfer of the olefinic groups present in the unsaturated fatty acid side chains that usually occupy the gaps between the phospholipid head groups into the hydrophobic region of the monolayer by

apoA-I, hence re-orientating the *sn*-2 ester linkage is critical for LCAT activation (Yokoyama *et al*, 1980).

Recently apo-E has been postulated to be the principal activator of LCAT on apo-B containing lipoproteins (Zhao *et al*, 2005). The addition of apoE to VLDL from *apoAI -/- apoE-/-* mice resulted in a 3 fold increase in CE formation, whereas the addition of apoA-I resulted in only a modest increase (Zhao *et al*, 2005). However on rHDL discs apoE is only 17-18% as effective as apoA-I at activating recombinant LCAT (Zhao *et al*, 2005), implying that the secondary structure of apoE, unlike apoA-I on discoidal rHDL may not be optimal for LCAT activation (Wald *et al*, 1990). In contrast on larger lipoprotein particles like LDL the conformation of apoA-I may prohibit LCAT activation, whereas apoE conformation in such an environment may be optimal for LCAT activation.

ApoA-IV has been found *in vitro* to be a more potent activator of LCAT than apoA-I when two saturated fatty acids were used as acyl donors (Steinmetz *et al*, 1990). Complexes containing two saturated fatty acids were even found to act as LCAT substrates without an activator protein. This could be due to such complexes possessing 'gaps' in between the polar head groups of phospholipids, enabling LCAT to interact with the hydrocarbon chain.

5.2 METHODS

To further delineate more precisely the specific region of apoA-IV involved in LCAT activation, the LCAT activation region of apoA-IV (residues 117-248) (Emmanuel *et al*, 1994) was subjected to alignment with apoA-I and helical wheel analysis. Using protein modelling techniques as described previously in section 4.2 two atypical class A amphipathic helices were isolated. Due to time constraints only helix 1 (residues 117-138) was analysed and three interesting residues within the polar face of this helix (R123, R134Q, N127) were isolated, mimicking the cluster of polar arginine residues (R149, R153, R160) critical for apoA-I LCAT activation (Roosbeek *et al*, 2001). To investigate the potential importance of these residues in apoA-IV mediated LCAT activation, they were mutated to residues of opposite charge and several recombinant apoA-IV mutant proteins generated (R123Q, R134Q, N127R, R123Q+R134Q, R123Q+N127R, N127R+R134Q and Helix 1 K.O- R123Q+ R134Q+ N127R). The mutants and wild type apoA-IV, produced as recombinant poly-histidine tagged apolipoproteins in *E.coli* (section 2.5.2) were then assembled with 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) and radioactive unesterified cholesterol (^{3}H UC) to produce apoA-IV reconstituted high density lipoproteins (rHDLs). The ability of each individual recombinant protein to activate LCAT was measured as the amount of labelled (^{3}H) cholesterol ester produced, and thus the cholesterol esterification rate when the individual apoA-IV:POPC: ^{3}H UC complexes at various concentrations were incubated with purified LCAT (section 2.6.4).

5.3 RESULTS

5.3.1 Optimisation of the LCAT assay, initial velocity experiments

To make certain that the LCAT assay was performed correctly allowing for the accurate calculation of V_{max} and K_m, two optimisation assays were performed to ensure that the CE production rate for each of the experiments would remain within the linear range (<30% CE formation) and that precise saturation curves were produced.

5.3.1.1 Finding the correct dilution factor of LCAT to be used, ensuring CE formation remains within the linear range (initial velocity experiment 1)

To keep CE formation within the linear range (<30% CE formation), two optimisation experiments were initially performed adjusting the dilution factor of LCAT but keeping the concentration of WT apoA-IV:POPC: ^{3}H UC complex constant at [UC] 0.5 μM/L. Based upon the results summarised in table 5.1 A and B, an LCAT dilution factor of 1/50 was chosen because it generated a cholesterol esterification rate that was less than 30% . For a detailed description of the LCAT assay please refer to section 2.6.4.

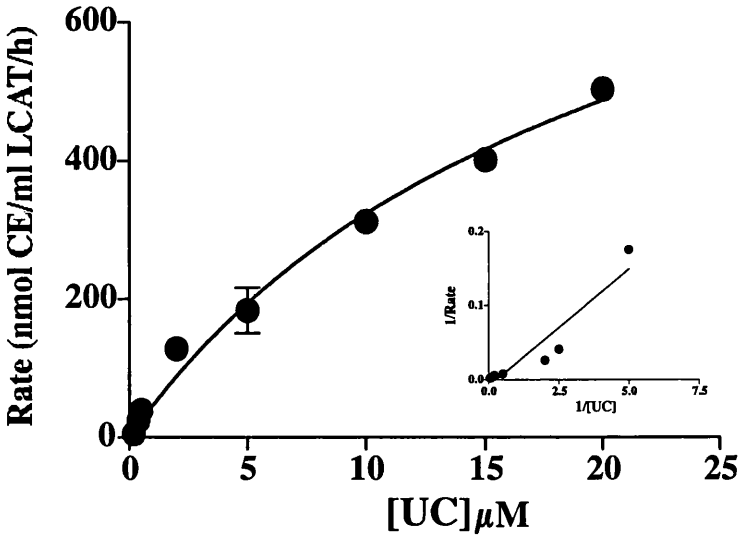
A.) [UC] 0.5 μM/L	Average total cpm	Average total cpm	% Esterified	% Esterified-blank
Total counts	172.3			
Total counts	192.71	174		
Total counts	156.91			
Blank WT	27			
Blank WT	16	23	13	
Blank WT	26.45			
WT rHDL+ 1/20 LCAT	95			
WT rHDL+ 1/20 LCAT	97	93	54	41
WT rHDL+ 1/20 LCAT	88			
WT rHDL+ 1/30 LCAT	88.96			
WT rHDL+ 1/30 LCAT	88.74	87	50	37
WT rHDL+ 1/30 LCAT	83.17			

B.) [UC] 0.5 μM/L	Average total cpm	Average total cpm	% Esterified	% Esterified-blank
Total counts	83			
Total counts	100	92		
Total counts	92			
Blank WT	16			
Blank WT	17	17	18	
WT rHDL+ 1/40 LCAT	45			
WT rHDL+ 1/40 LCAT	46	48	52	34
WT rHDL+ 1/40 LCAT	52			
WT rHDL+ 1/50 LCAT	33			
WT rHDL+ 1/50 LCAT	28	33	36	18
WT rHDL+ 1/50 LCAT	40			

Table 5.1A and 5.1B: Summary of the results from two optimisation experiments to find the ideal dilution of LCAT to obtain a < 30% cholesterol esterification rate.

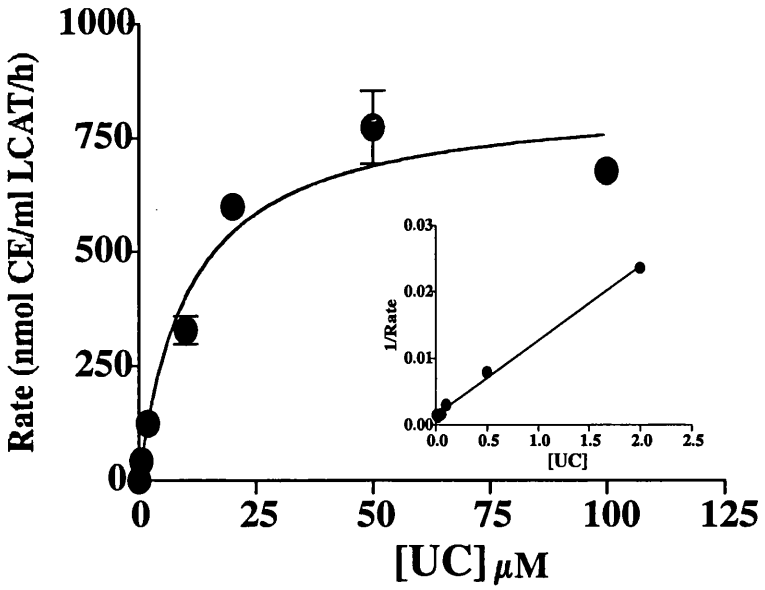
5.3.1.2 Optimisation experiments to find the concentrations of apoA-IV rHDL to produce a saturation curve yielding accurate V_{max} and $K_m$values (initial velocity experiment 2)

Initially the LCAT experiment was performed by incubating the following concentrations of WT apoA-IV rHDL ([UC] μM/L); 0.2, 0.4, 0.5, 2 and 5μM UC with a constant amount of LCAT (1/50). CE formation was determined by measuring the generation of radiolabelled (^{3}H) CE in the incubation as described in section 2.6.4. All kinetic parameters were calculated by nonlinear regression analysis fit to the Michaelis –Menten equation : $V = (V_{max} * [S])/(K_m + [S])$, where V is the velocity of LCAT esterification and [S] is the concentration of rHDL. Lineweaver-Burk linear transformations are for display purposes only. The saturation curve produced using the initial apoA-IV WT rHDL concentrations did not reach a plateau, making the calculation of V_{max} and K_m completely inaccurate (figure 5.3). When the experiment was repeated with the following WT apoA-IV rHDL concentrations ([UC] μM/L); 0.5, 2, 10, 20, 50, and 100 μM, a saturation curve reaching a plateau, enabled the calculation of V_{max} and K_m with small SEMs and narrow confidence intervals (figure 5.4). Ideal rHDL and LCAT dilutions were therefore determined and all LCAT assays were carried out with apoA-IV rHDL ([UC] μM/L) concentrations of 0.5, 2, 10, 20, 50, and 100 μM, while the LCAT dilution was kept constant at 1/50.



Results for optimisation 1	
Apparent V_{max}, nM CE formed/ml of LCAT/h	982.3
Apparent K_m, μM UC	20.28
V_{max} Standard error	131.5
K_m Standard error	4.611
V_{max} 95% confidence intervals	708.7 to 1256
K_m 95% confidence intervals	10.79 to 29.97

Figure 5.3: Kinetic parameters calculated by performing the LCAT assay using the following concentrations of WT apoA-IV rHDL ([UC] μM/L); 0.2, 0.4, 0.5, 2 and 5μM UC with a constant amount of LCAT (1/50). The rate of CE formation as a function of substrate concentration is shown in the main panel, while the minor panel shows a Lineweaver-Burk double reciprocal plot of the kinetic data. All values are means ± SEM of triplicate determinations.



Results for optimisation 2	
Apparent V_{max}, nM CE formed/ml of LCAT/h	839.7
Apparent K_m, μM UC	10.93
V_{max} Standard error	53.98
K_m Standard error	2.56
V_{max} 95% confidence intervals	726.7 to 952.7
K_m 95% confidence intervals	5.6 to 16.28

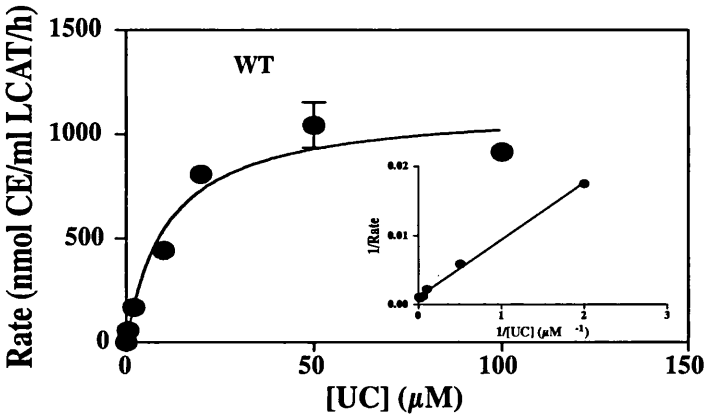
Figure 5.4: Kinetic parameters calculated by performing the LCAT assay using the following concentrations of WT apoA-IV rHDL ([UC] μM/L); 0.5, 2, 10, 20, 50, and 100 μM UC with a constant amount of LCAT (1/50). The rate of CE formation as a function of substrate is shown in the main panel, while the minor panel shows a Lineweaver-Burk double reciprocal plot of the kinetic data. All values are means ± SEM of triplicate determinations.

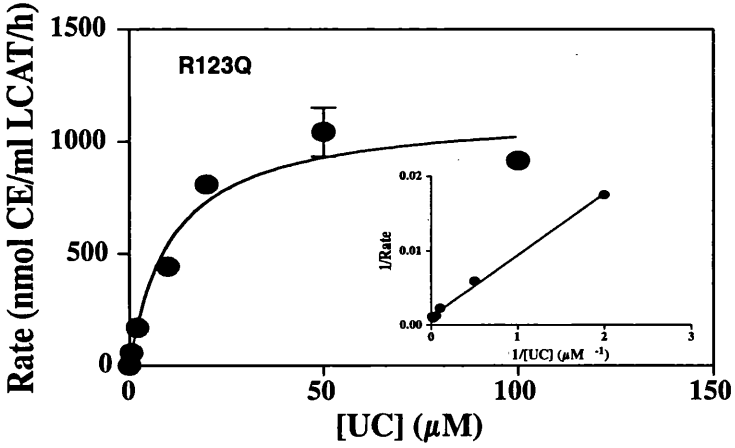
5.3.2 The kinetics of LCAT mediated CE formation in the apoA-IV variant rHDLs

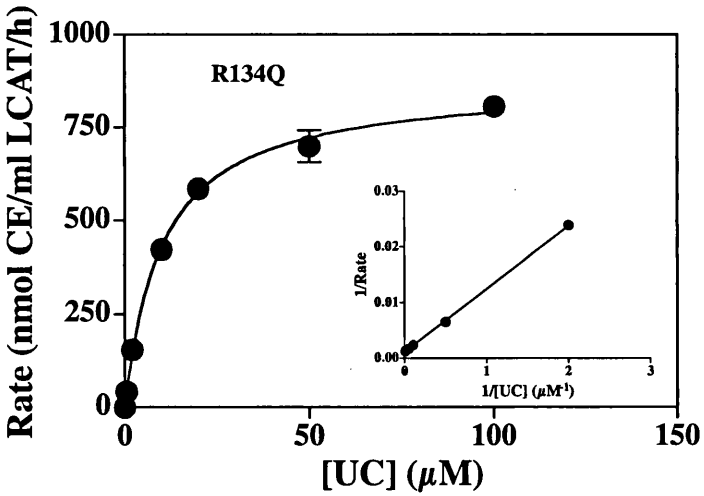
To compare the kinetics of LCAT mediated CE formation, various concentrations of each apoA-IV rHDL produced (final concentration of 0.5-100μM UC) were incubated with a constant amount of LCAT. Substrate saturation curves for CE formation by LCAT with the addition of the various radioactive (^{3}H) rHDLs were plotted (as shown in figure 5.5). Based upon optimisation experiments the dilution of LCAT used was adjusted to keep the CE formation within the linear range (<30% CE formation). As illustrated in figure 5.6 all the variants apart from R123Q + R134Q had a reduced rate of CE formation compared to the wild type isoform. Nonetheless the effect of the apoA-IV mutations on V_{max} and K_m were within a limited range. As summarised in table 5.2 the V_{max} and K_m values were of the same order of magnitude and did not even vary 2 to 3 fold in comparison to the wild type apoA-IV isoform. This is clearly shown by the very similar enzyme efficiencies V_{max}/K_m calculated for each of the mutations (table 5.2). Hence it could be possible that the variations in the rate of CE formation between the mutant and wild type apoA-IV rHDLs could just reflect the minor differences in composition, size and structure between the apoA-IV complexes (table 4.3).

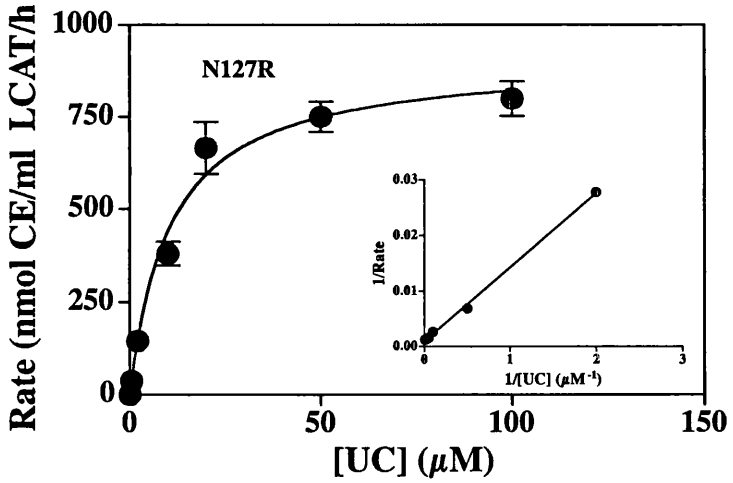
ApoA-IV mutant in rHDL	Apparent V_{max}, nM CE formed/ml of LCAT/h	Apparent K_m, μM UC	Catalytic efficiency, V_{max} / K_m (apparent)
Wild type apoA-IV	1134 ± 72.87	10.93 ± 2.56	104
R123Q	773.2 ± 44.19	7.61 ± 1.80	102
R134Q	871.2 ± 22.12	10.28 ± 1.00	85
N127R	909.1 ± 47.06	10.67 ± 2.03	85
R123Q+R134Q	1285 ± 92.42	16.08 ± 3.69	80
R123Q+N127R	736.3 ± 62.54	7.33 ± 2.59	100
N127R+R134Q	944.1 ± 45.81	11.83 ± 2.03	80
Helix 1 - (R123Q+ R134Q+ N127R)	792.3 ± 35.05	10.13 ± 1.68	78

Table 5.2: Effect of the various apoA-IV rHDLs on the LCAT kinetic constants (app V_{max} and app K_m). Spherical rHDL (0.5-100μM UC) were incubated with a constant amount of LCAT as described in section 2.6.4. CE formation was determined by measuring the generation of radiolabelled (^{3}H) CE in the incubation mixture. The values are the means ± SEM (standard error of the mean) of triplicate determinations.


WT
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)
1/Rate
0.02
0.01
0.00
1/[UC] (μM -1)


R123Q
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)
1/Rate
0.02
0.01
0.00
1/[UC] (μM -1)


R134Q
Rate (nmol CE/ml LCAT/h)
1000
750
500
250
0
0
50
100
150
[UC] (μM)
1/Rate
0.03
0.02
0.01
0.00
1/[UC] (μM-1)


N127R
Rate (nmol CE/ml LCAT/h
1000
750
500
250
0
0
50
100
150
[UC] (μM)
1/Rate
0.03
0.02
0.01
0.00
1/[UC] (μM-1)

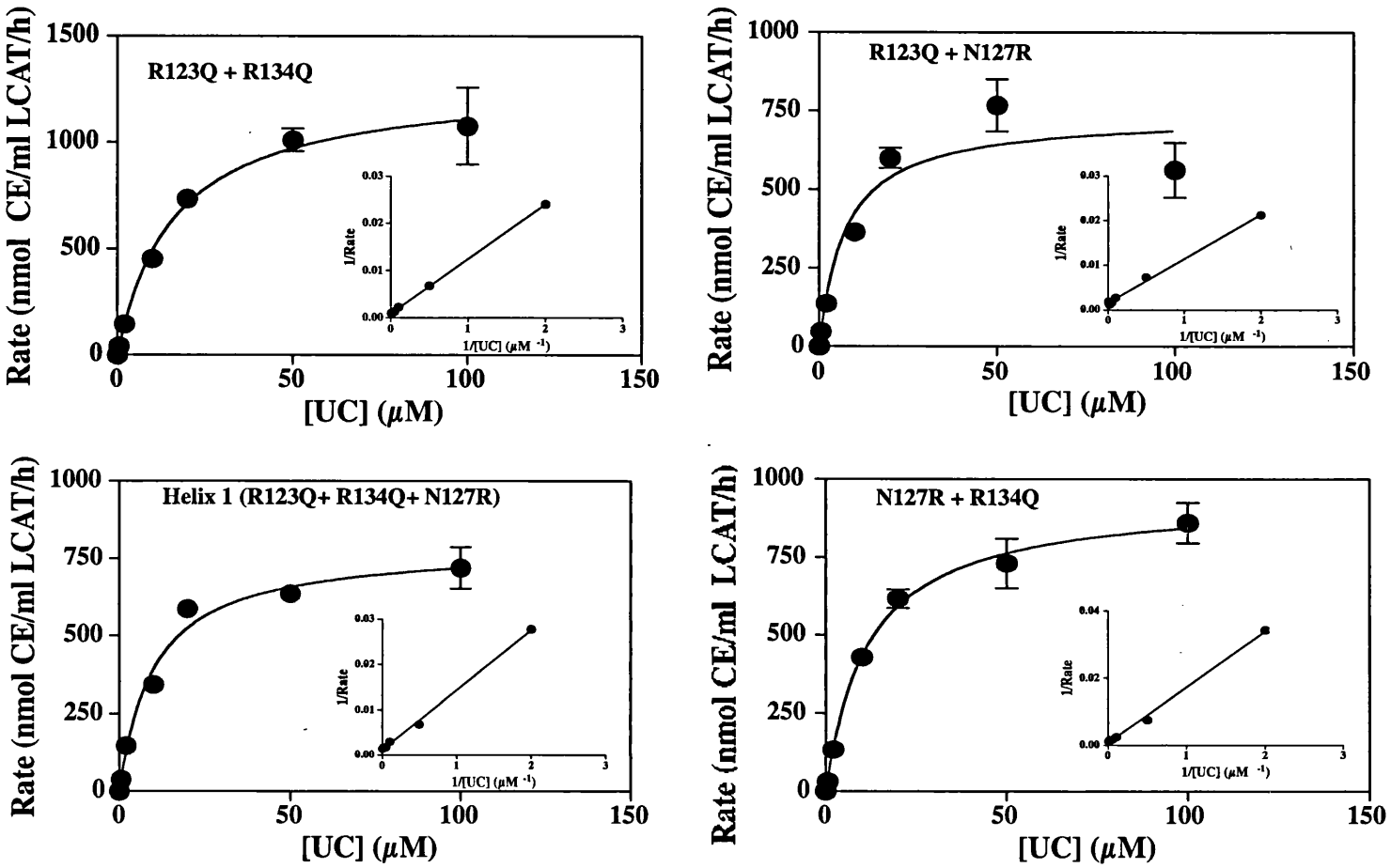
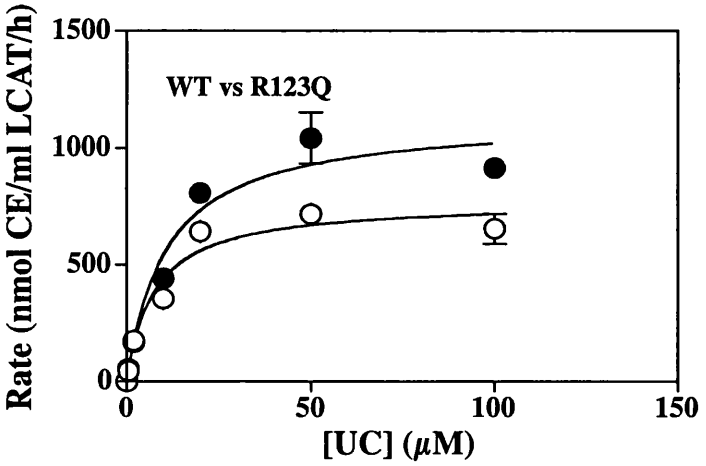
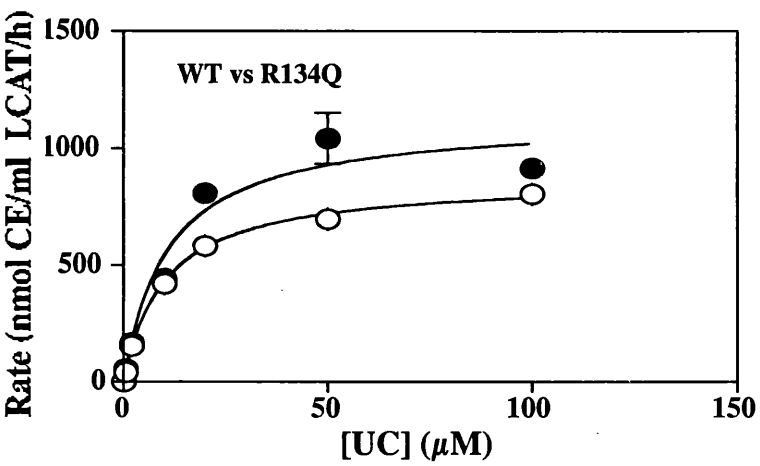
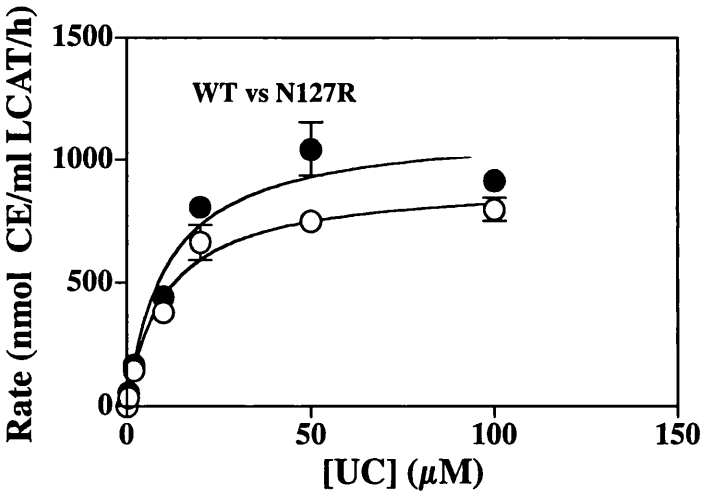
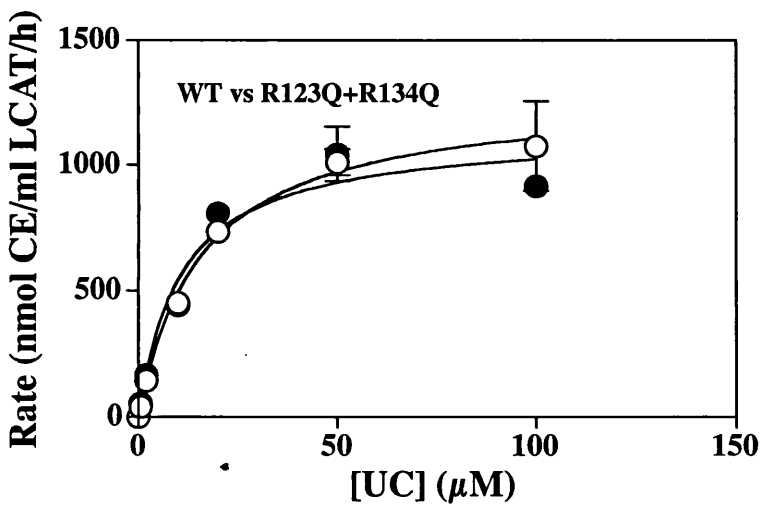


Figure 5.5: The kinetics of cholesterol ester (CE) formation of the various apoA-IV variant rHDLs (0.5-100μM UC) incubated with LCAT for 1 hour at 37°C. The rate of CE formation in the various apoA-IV variant rHDLs as a function of substrate is shown in the main panels, while the minor panels show Lineweaver-Burk double reciprocal plots of the kinetic data. All values are means ± SEM of triplicate determinations.


WT vs R123Q
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)


WT vs R134Q
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)


WT vs N127R
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)


WT vs R123Q+R134Q
Rate (nmol CE/ml LCAT/h)
1500
1000
500
0
0
50
100
150
[UC] (μM)

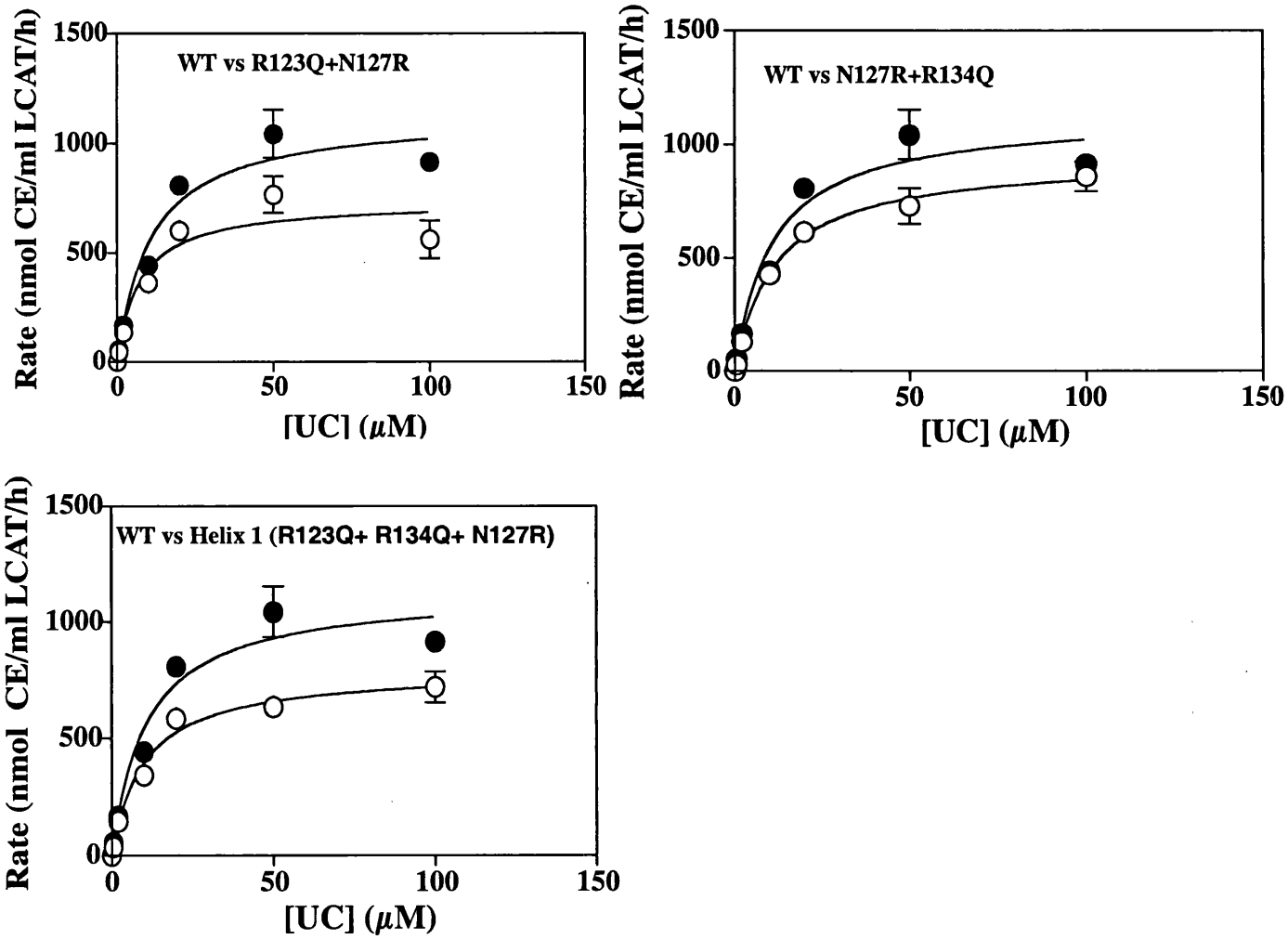


Figure 5.6: Comparison of the kinetics of CE formation using WT apoA-IV rHDL (●) versus the various mutant apoA-IV rHDLs (○). The corresponding ANOVA P values are shown, all values are means ± SEM of triplicate determinations.

5.4 DISCUSSION

Previous experiments have indicated that the key residues for the activation of LCAT by apoA-IV are located between residues 117-160 of the mature protein (Emmanuel *et al*, 1994). After subjecting this region of apoA-IV to helical wheel analysis and alignment with the LCAT activation domain of apoA-I, paying close attention to the apoA-I key residues R149Q, R153Q and R160Q, various interesting residues within the polar face of 2 atypical class A amphipathic helix of apoA-IV were identified. Due to time constraints only helix 1 (117-138) was examined, with 7 apoA-IV variants (in total) made into rHDLs and their ability to activate LCAT compared to wild type apoA-IV investigated. Helix 1 (117-138) was found to contain two arginine residues (R123 and R134), both of which were mutated to neutral, uncharged glutamine residues. The other residue altered was an asparagine residue (N127) which was replaced with a basic, hydrophilic arginine residue producing apoA-IV with a helix 117-138 containing 3 arginine residues (R123, R127, R134), arranged in the appropriate 1-4-11 configuration required for their side chains to be in the right position to interact with a molecule of LCAT bound to the surface of the rHDL discs (based upon the belt model). These mutations were designed to investigate if the mechanism by which apoA-IV activates LCAT is similar to that of apoA-I, which occurs via a three critical arginine residue (R149, R153, R160) LCAT activation motif (Roosbeek *et al*, 2001) (figure 5.2).

Initial velocity experiments (V_0) showed a linear increase of up to 30% cholesterol esterification in the substrate and subsequent measurements were carried out within that range. V_{max} and K_m were determined for wild type and mutant apoA-IV proteins from V_0 experiments at varying ^3H cholesterol concentrations, by fitting the experimental data to the Michaelis Menton equation using GraphPad Prism 4.0. Although comparisons of the rate of CE formation between the majority of apoA-IV variants and wild type apoA-IV were significant, the effects of the mutations on V_{max} and K_m were limited. All V_{max} and K_m values were of the same order of magnitude and did not vary even 2 or 3 fold. Calculation of the catalytic efficiency V_{max}/K_m clearly demonstrates that mutations in at least one of the arginine residues within the polar face of helix 117-138 of apoA-IV did not drastically alter the LCAT activating properties of apoA-IV. Also that the addition of an arginine residue (R127) mimicking the three critical LCAT activating arginine residues (R149, R153 and R160) of apoA-I did not increase apoA-IV LCAT activity as expected, but gave similar results compared to the other apoA-IV variants. Because of the small magnitude of differences in LCAT activity between the apoA-IV variants and wild type apoA-IV, these differences could have been attributable to the minor differences in size, composition and structure of the complexes produced. Additional experiments further examining the structure of apoA-IV in each of the variants could be carried out to investigate this. These include subjecting the complexes to tryptophan fluorescence and thermal melt analysis, to investigate the depth of apoA-IV penetration and the stability of each apoA-IV variant within the individual complexes. In addition the complexes could be further examined by electron microscopy, producing a precise measurement of the distribution of the various complexes and the range of their particle sizes.

The limited effects that the charge distribution alterations in helix 117-138 had upon apoA-IV LCAT activity, does not necessarily imply that helix 117-138 is not involved in apoA-IV LCAT activation. As noted earlier in chapter 4, another atypical class A amphipathic helix was found between residues 146-161 within the LCAT activation domain of apoA-IV (residues 117-160). Hence despite the loss of helix 117-138 apoA-IV LCAT activation could still occur via helix 146-161, especially if apoA-IV LCAT activation occurs through cooperative segments, helix 146-161 could compensate for the alterations in helix 117-138. An experiment to solve this would be to produce apoA-IV mutants with or without helix 117-138 and helix 146-161 and analyse their ability to activate LCAT. The current experiments do nevertheless propose that unlike the three highly conserved arginine residues (R149, R153 and R160) in apoA-I (Roosbeek *et al*, 2001), the two arginine residues (R123 and R134) within helix 117-138 of apoA-IV are not critical for LCAT activation.

Further analysis of the electrostatic potentials of apoA-IV by Frank Peelman (University of Ghent, Belgium) revealed that unlike apoA-I (figure 5.7), the electrostatic potentials around apoA-IV are not predominantly negative and that there are no particular focal points of positive charge (personal communication). This indicates that the two arginine residues R123 and R134 in apoA-IV may not be vital in determining the electrostatic interaction between apoA-IV and LCAT with plenty of other positively charged residues available to stabilise the interaction of LCAT with rHDL (by either forming salt bridges with negative residues of LCAT, or hydrogen bonds with other polar residues of the

enzyme), allowing residues elsewhere to interact with LCAT and induce full enzymatic activity.

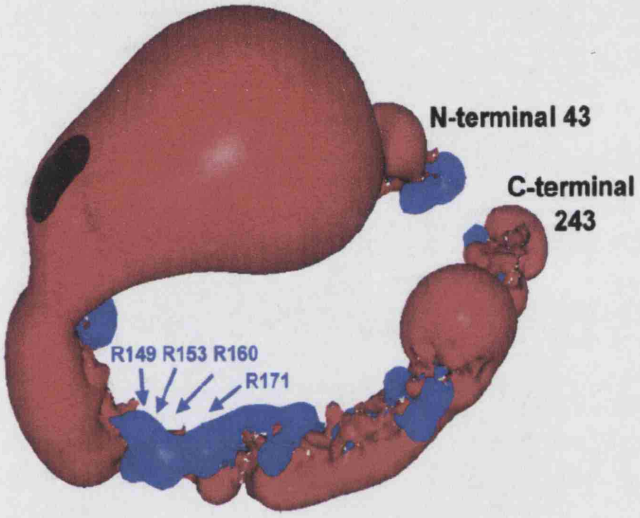


Figure 5.7: The electrostatic potentials around a molecule of apoA-I(□1–43), adapted from Roosbeek *et al* (Roosbeek *et al*, 2001). Red, negative potential at 21 kt/e; blue, positive potential at 10.5 kt/e.

ApoA-IV is an activator of LCAT, and is a better activator than apoA-I when saturated phosphatidylcholine (PC) is used as the fatty acyl donor (Steinmetz and Utermann, 1985). LCAT activity directly influences the distribution of apoA-IV in the human plasma, redistributing apoA-IV from the lipoprotein deficient fraction on to plasma lipoproteins (Bisgaier *et al*, 1987). The mechanism explaining the LCAT mediated redistribution of apoA-IV proposes that LCAT activity could result in the generation of 'gaps' within the lipoprotein surface due to the consumption of substrates and the simultaneous expansion of the lipoprotein core, lipid 'free' apoA-IV may then bind to these 'gaps' where the packing density of the phospholipid head groups is reduced (Bisgaier *et al*, 1987). Although apoA-I has been proposed to be the main physiological activator of LCAT, a unique physiological role for apoA-IV in the LCAT reaction has been postulated. Because unlike other apolipoproteins apoA-IV is largely 'free' (Green *et al*, 1980), it could therefore provide a reservoir of amphipathic proteins to stabilise lipoproteins as they enlarge under the influence of LCAT. By filling in the 'gaps' present on the lipoprotein surface, apoA-IV could also allow the utilisation of residual PC molecules (especially the saturated PC) for which LCAT in the presence of apoA-I has lower activity for. Hence by binding to lipoprotein particles under the influence of LCAT activity, apoA-IV could not only stabilise the lipoproteins as they expand but also facilitate the access of substrates to LCAT, promoting the further depletion of surface PC remnants and so the creation of additional CE (Bisgaier *et al*, 1987).

CHAPTER 6

CHAPTER 6: THE COMMON ISOFORMS OF APOA-IV DIFFER IN THEIR ABILITY TO INHIBIT COPPER MEDIATED LDL OXIDATION

6.1 INTRODUCTION

6.1.1 Structure of human low density lipoprotein (LDL)

Human LDL is defined as a population of lipoproteins which can be isolated by ultracentrifugation within a density range of 1.019 to 1.063 g/mL (Esterbauer *et al*, 1992). LDL particles are spherical lipoproteins, 22-28nm in diameter having a relative molecular mass of $1.8\text{-}2.8\text{x}10^6$. Being a typical lipoprotein LDL consists of a core of cholesterol esters (CE), triacylglycerols (TG) and endogenous antioxidants (e.g. carotenoid) surrounded by a phospholipid (PL) monolayer containing phosphatidylcholine, sphingomyelin, non-esterified cholesterol and small amounts of lysophosphatidylcholine (figure 6.1) (Esterbauer *et al*, 1992). Additionally the outer layer also contains antioxidants such as α-tocopherol and a large apolipoprotein B (apoB) (Rosseneu, 1992). The approximate percentage composition by weight of LDL is 22% protein, 22% PL, 6% TG, 10% non-esterified cholesterol and 42% CE. A single LDL particle contains approximately 1600 molecules of CE, 170 molecules of TG, 700 molecules of PL and 600 molecules of free cholesterol. The fatty acid composition of LDL varies from donor to donor. LDL contains on average 2700 fatty acid molecules of which half are polyunsaturated free fatty acid (PUFA), mainly linoleic acid with minor amounts of arachidonic and docosahexaenoic acid (Esterbauer *et al*, 1992).

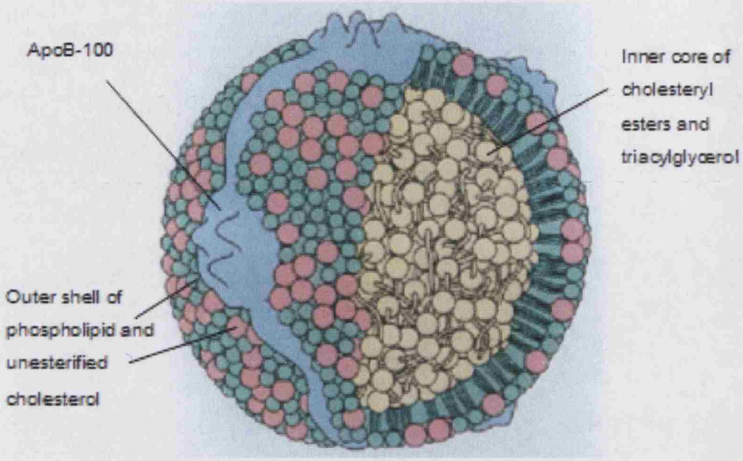


Figure 6.1: A diagram illustrating the structure of low density lipoprotein, adapted from Stryer (Stryer L , 1995)

6.1.2 Lipid peroxidation and the oxidation of LDL

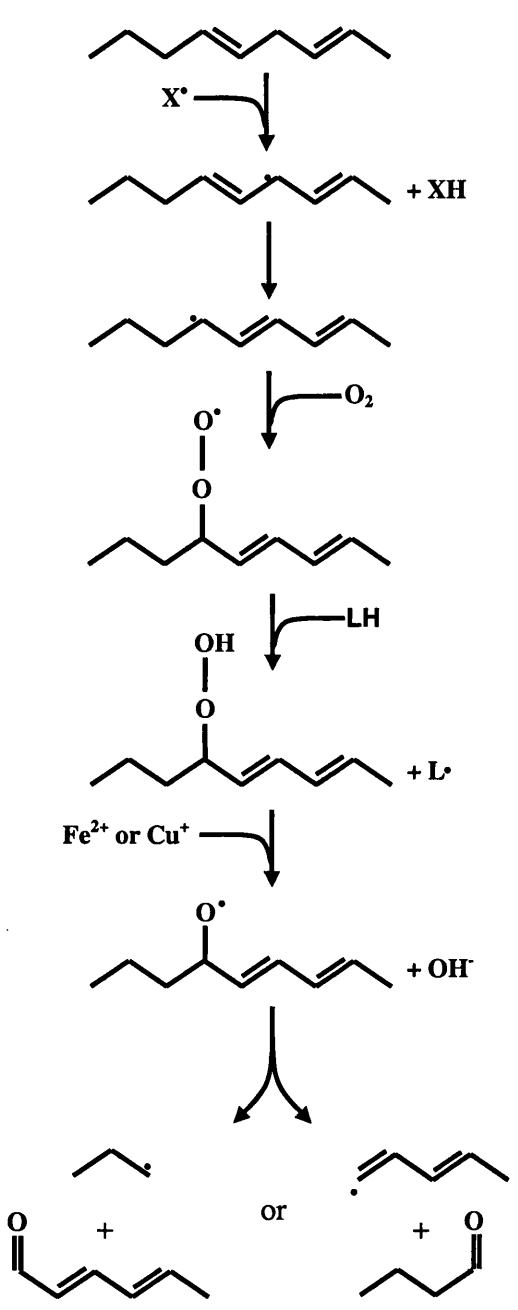
The term oxidised LDL (OXLDL) does not define a well characterised molecular species, as different laboratories are known to have induced LDL oxidation to different extents using a variety of conditions. Even if the conditions to oxidatively modify LDL were strictly adhered to there would still be huge variation due to the heterogeneous nature of native LDL (nLDL) itself, for instance some donors may have a higher concentration of endogenous antioxidants such as vitamin E, resulting in the extent of oxidation varying from one preparation to the next (Chisolm and Steinberg, 2000). The term OXLDL is therefore best used to describe LDL preparations that have acquired new functions as a result of oxidation (Chisolm and Steinberg, 2000).

Lipid peroxidation is defined as the oxidative deterioration of lipids containing carbon-carbon double bonds. The oxidation of LDL is the most extensively studied modification of LDL, primarily due to the oxidative modification hypothesis of atherosclerosis (section 1.2.1.4). Oxidation of LDL results in a wide variety of alterations in LDL particle composition dependent upon the concentration and type of oxidant used, including the time of exposure to the oxidant (Berliner and Heinecke, 1996; Stocker, 1994). The oxidation of LDL is a free radical-chain process which can be divided into 3 stages and is illustrated in figure 6.2.

1. Initiation: The peroxidation of LDL can commence with the attack of a bisallylic carbon atom in a PUFA by reactive oxygen species (ROS) with sufficient reactivity to abstract a hydrogen atom (H) from a methylene group ($-CH_2-$), resulting in the generation of polyunsaturated fatty acid free radicals (lipid alkyl radical). Polyunsaturated fatty acids are primary targets for peroxidation due to the presence of double bonds in the fatty acid chain weakening the C-H bond on the carbon atom adjacent to the double bond, making H removal easier. The resultant lipid alkyl radical undergoes rearrangement to form a stable conjugated diene. Under aerobic conditions conjugated dienes can combine with oxygen (O_2) yielding a peroxyl radical.
2. Propagation: The peroxyl radical is capable of abstracting H from an adjacent fatty acid (a process that can be enhanced by metal ions such as copper) resulting in a autocatalytic chain reaction. At this stage lipid hydroperoxides (LOOH) are generated from the combination of the peroxyl radical with H. The presence of

metal ions can lead to the formation of lipid alkoxyl radicals, eventually resulting in the formation of chain cleavage products such as aldehydes and lipid alkyl radicals.

3. Termination: The formation of hydroperoxides, resulting in termination can be achieved by the reaction of peroxyl radicals with 'chain-breaking molecules' such as α-tocopherol present in cell membranes. In addition, alkyl radicals can react with lipid peroxide to give non-initiating, non-propagating species or two peroxide molecules can combine yielding hydroxylated derivatives. Lastly termination of lipid peroxidation can be achieved by the bonding of lipid peroxides and membrane proteins.



Within PUFA, the C atom in close proximity to C=C double bonds is susceptible to free radical attack leading to H being abstracted

A Lipid alkyl radical is produced

The lipid alkyl rearranges to a more stable form producing a conjugated diene alkyl radical

Under aerobic conditions the addition of molecular oxygen gives a lipid peroxyl radical

H abstraction, such as from a neighbouring PUFA forms a lipid hydroperoxide and another lipid alkyl radical

Reaction with Cu^{+} or Fe^{2+} gives a lipid alkoxyl radical

Cleavage products of lipid alkoxyl radicals include alkyl radicals and aldehydes

Figure 6.2: A schematic representation of the LDL oxidation process, as described in section 6.1.2.

The physical and structural properties of OXLDL vary greatly and are dependent upon the type and extent of oxidation to which a particular LDL sample has been subjected to. Copper induced lipid peroxidation of LDL results in the complete peroxidation of PUFAs, extensive decomposition of lipid hydroperoxide (LOOH) producing aldehydic fragmentation products and the extensive fragmentation of apoB-100 with the formation of oxysterols (Carpenter *et al*, 1994; Fong *et al*, 1987; Lougheed and Steinbrecher, 1996; Steinbrecher *et al*, 1984). In the presence of other oxidants, for example peroxidase, peroxyl radicals or low concentrations of iron results in less peroxide decomposition or damage to apoB-100 (Noguchi *et al*, 1994). While oxidants for instance hypochlorous acid, a product of the myeloperoxidase enzyme (MPO) are capable of altering the protein components of LDL (Hazell and Stocker, 1993) but can only oxidise lipids under certain conditions (for example in the presence of tyrosine) (Hazell *et al*, 1999). The most readily oxidised components of LDL are the polyunsaturated fatty acyl chains of phospholipids, cholesterol esters and triacyglycerols, while cholesterol and mono-unsaturated fatty acids react less vigorously with oxidants (Carpenter *et al*, 1994).

A small amount of cholesterol in LDL is oxidised to oxysterols. Minimally oxidised LDL contains 7β or 7α-hydroperoxycholesterol (7β or 7α-OOH) (Brown *et al*, 1997), while heavily oxidised LDL contains a significant portion of 7-oxygenated sterols, including 7-ketocholesterol (7K), 7β-hydroxycholesterol (7β-OH) (Brown *et al*, 1996) and aldehydes (Brown *et al*, 2000; Karten *et al*, 1999). Oxysterols are 27-carbon products of cholesterol oxidation (figure 6.3) that have been shown to be present in atherosclerotic plaques (Brown *et al*, 1997), and are postulated to play an active role in plaque development.

Oxysterols for instance are cytotoxic to many cell types including smooth muscle (SMC) and endothelial cells (EC) (Ramasamy *et al*, 1992), the death of these cell types can lead to the initiation and progression of atherosclerosis according to the 'response to injury hypothesis' (Ross and Glomset, 1976). OXLDL therefore represents a complex mixture of various components, such as LOOH, oxysterols, lysophophatidylcholine and aldehydes.

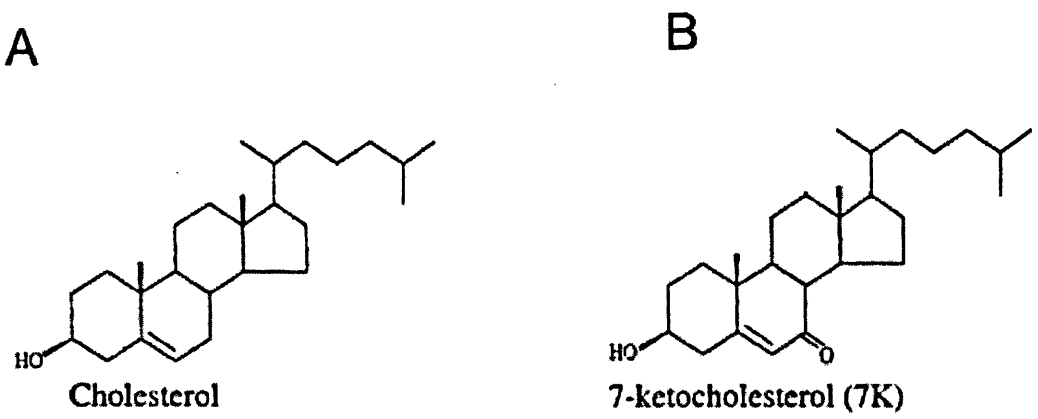


Figure 6.3: A.) The 27-carbon four-fused cyclopentanophenanthrene ring structure of cholesterol (5-cholesten-3β-ol; $C_{27}H_{46}0$). B.) The oxysterol 7-ketocholesterol (7K). Both figures are adapted from Brown *et al* (Brown and Jessup, 1999).

The copper induced oxidation of LDL is the most frequently used method to study the oxidation of LDL. This form of LDL oxidation *in vitro* results in the formation of sterols oxygenated in the 7-position, with 7K being the major oxysterol formed (Brown *et al*, 1996; Brown *et al*, 1997; Carpenter *et al*, 1994; Chang *et al*, 1997). A similar composition of OXLDL is also produced as a result of macrophage, 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) and lipoxygenated mediated oxidation of LDL (Carpenter *et al*, 1994). *In vivo*, the presence of 7K and 7β-OH in fatty streaks indicates that the formation of oxysterols and hence the oxidation of LDL cholesterol frequently occurs at the early stages of atherogenesis (Carpenter *et al*, 1995).

In this thesis the antioxidative qualities of the common apoA-IV isoforms were assessed, by investigating their respective abilities to inhibit the copper induced oxidation of LDL.

6.1.3 The biological effects of OXLDL

OXLDL exerts a multitude of proatherogenic effects *in vitro* and *in vivo* affecting cell growth (Martens *et al*, 1999), fatty streak formation (Henriksen *et al*, 1981), cell death (Quinn *et al*, 1988) and monocyte adhesion (Parhami *et al*, 1993a) to name a few.

As briefly mentioned in section 1.2.1.4 extensively modified LDL (OXLDL) with a loss of apoB-100 lysine residues or malondialdehyde (MDA) treated LDL are no longer recognised by LDLR, but instead by scavenger receptors on macrophages and internalised to form foam cells (Henriksen *et al*, 1981; Requena *et al*, 1997; Steinbrecher, 1987). Macrophages usually export excess cholesterol to HDL, avoiding foam cell

formation (Oram and Vaughan, 2000). However in macrophages engorged with OXLDL such export is inhibited due to the direct inhibitory effects of oxysterols, for example 7K mediated lysosomal sequestration results in sterols sequestered in lysosomes that are resistant to removal by normal receptors, promoting foam cell formation (Brown *et al*, 2000; Gaus *et al*, 2001b; Gaus *et al*, 2001a).

Foam cell formation: has both pro and anti-atherogenic effects, for instance foam cells can sequester potentially bioactive proatherogenic lipids (Berliner and Heinecke, 1996). Nonetheless foam cells are a feature of lesions and data strongly indicates that they contribute to lesion development by a variety of mechanisms, especially with the large cholesterol-rich core of advanced rupture-prone lesions appearing to be substantially derived from the necrosis/apoptosis of foam cells (Tabas, 2000). In addition, foam cells contain and accumulate a significant amount of lipid oxidation products such as oxysterols (7K) (Brown *et al*, 1996) and advanced oxidation products like core aldehydes (Karten *et al*, 1999), both of which are proatherogenic.

Cytotoxicity: OXLDL are cytotoxic to macrophages, smooth muscle cells, endothelial cells and fibroblasts (Bjorkerud and Bjorkerud, 1996; Harada-Shiba *et al*, 1998; Morel *et al*, 1983; Siow *et al*, 1999). Over 60 oxysterols have been discovered, with 7K and 7β-OH able to induce apoptosis even at micromolar concentrations in a variety of normal and tumour cells (Aupeix *et al*, 1997; Lizard *et al*, 1999; O'Callaghan *et al*, 2001). Vascular cell death (apoptosis) has been found to be greater in atherosclerotic plaques compared to normal vessels (Bennett *et al*, 1995), contributing to the necrotic core in

advanced lesions, promoting plaque rapture (Bennett *et al*, 1995) and thrombosis (der Thusen *et al*, 2002).

Cell function: OXLDL promotes the proliferation of macrophages within the intima, which is thought to be an essential feature for the progression of atherogenesis (der Thusen *et al*, 2002). OXLDL can stimulate endothelial cells to produce a concoction of proinflammatory molecules, including adhesion molecules and growth factors like macrophage colony stimulating factor (M-CSF) that enhance monocyte recruitment and differentiation into macrophages within the arterial wall (Lusis, 2000; Parhami *et al*, 1993a). OXLDL is chemotactic for monocytes, promoting the recruitment and retainment of monocyte/macrophages during atherogenesis (Quinn *et al*, 1987; Quinn *et al*, 1988). However, the presence of OXLDL can also diminish the innate immune response, altering the transcriptional response of macrophages to inflammatory stimulus (Mikita *et al*, 2001) and reducing the lipopolysaccharide induced upregulation of cell adhesion molecules in endothelial cells (Parhami *et al*, 1993b).

6.1.4 The mechanisms of LDL oxidation *in vivo*

There is now substantial evidence implying OXLDL to be present within human atherosclerotic lesions, for example within atherosclerotic lesions both the characteristic epitopes of OXLDL and immunoglobulins that recognise OXLDL have been isolated (Palinski *et al*, 1989; Yla-Herttuala *et al*, 1989a). But due to the presence of numerous water and lipid soluble antioxidants within the extracellular fluid (Esterbauer *et al*, 1992) and the fact that OXLDL is taken up by liver Kupffer cells and sinusoidal endothelial

cells (de Rijke and van Berkel, 1994), it is uncertain whether significant oxidation of LDL occurs in the circulation.

A possible location for LDL oxidation to take place is within the intima, where it is within close proximity to EC, SMC, lymphocytes and macrophage cells that can potentially incite LDL oxidation (Gaut and Heinecke, 2001). Macrophages due to the activity of NADPH oxidase and myeloperoxidase (MPO) are a potential source of oxygen and nitrogen radicals (Cathcart, 2004; Hazen and Heinecke, 1997). Mononuclear phagocytes are capable of generating a variety of diffusible radical species including reactive oxygen or nitrogen species and hypochlorous acid as part of their normal host defense functions. However under certain conditions an excess of these oxidising species can overwhelm local anti-oxidant defence mechanisms, resulting in oxidative stress and oxidative tissue injury. Typically activated phagocytes (which are present in atherosclerotic lesions) can undergo abrupt superoxide ($O_2^{\cdot -}$) formation from oxygen, a process called the respiratory burst catalysed by the NADPH oxidase enzyme (Babior, 1999). This is an important mechanism by which activated leukocytes kill pathogenic microorganisms through the generation of reactive oxygen species (ROS). The respiratory burst is accompanied by the secretion of enzymes, such as MPO that are capable of using hydrogen peroxide (H_2O_2), the by-product of $O_2^{\cdot -}$ generation, to produce a wide variety of diffusible radical species including tyrosyl radicals that have the capability to initiate oxidation of lipid in the plasma (Podrez *et al*, 2000). In addition the production of ROS inside the cells can also induce apoptosis (Kasahara *et al*, 1997) with membrane vesicles from apoptotic cells containing biologically active oxidised

phospholipids, further perpetuating atherogenesis (Huber *et al*, 2002). Evidence for the involvement of enzymes such as MPO in atherogenesis include the enrichment of products formed by MPO, for example 3-chlorotyrosine, in human atherosclerotic plaques (Hazen and Heinecke, 1997). Other enzymes implicated in the *in vivo* oxidation of LDL include 15-lipoxygenase, expressed in certain smooth muscle cells (Hugou *et al*, 1995) and foamy macrophages (Yla-Herttuala *et al*, 1990). Transgenic *apoe-/-* mice lacking the 12/15 lipoxygenase gene have diminished atherosclerosis and autoantibodies to OXLDL, implying that 12/15 lipoxygenase is an important source of ROS involved in the oxidation of LDL *in vivo* (Cyrus *et al*, 1999). Lipoxygenase is capable of inserting molecular oxygen into fatty acids such as hydroperoxyeicosatetraenoic acid (HPETE), this including other lipoxygenase mediated LOOH formed can be transferred across the cell membrane and 'seed' the oxidation of extracellular LDL (Benz *et al*, 1995; Ezaki *et al*, 1995).

As a result of extensive research, there are now a whole host of factors proposed to be responsible for LDL oxidation *in vivo*. These include transitional metal ions (Heinecke *et al*, 1984), caeruloplasmin (Lamb and Leake, 1994), hydrogen peroxide, superoxide (Hiramatsu *et al*, 1987), thiols (Heinecke *et al*, 1987), metmyoglobin (Dee *et al*, 1991) and methaemoglobin (Paganga *et al*, 1992).

6.2 METHODS

Apolipoprotein A-IV (apoA-IV) inhibits lipid peroxidation, a potential mechanism describing its anti-atherogenic properties (Ferretti *et al*, 2002; Qin *et al*, 1998). The aim of this study was to investigate how the inhibition of low density lipoprotein (LDL) oxidation was influenced by the common apoA-IV isoforms , S347, H360 and S127.

6.2.1 Experiments to assess the anti-oxidative properties of recombinant apoA-IV

All assays used in this chapter are fully described in Section 2.6.5. The recombinant apoA-IV proteins used in these experiments were produced as described in section 2.5.2.

6.2.2 Statistical analysis

The data are expressed as mean ± standard error (SE). Statistical analysis was performed using the Student's *t*-test and differences were considered significant at $P<0.05$. All statistical analysis in this chapter was performed using Microsoft® Excel 2000 and SPSS 12.0.1.

6.3 RESULTS

6.3.1 Testing the suitability of recombinant apoA-IV for the conjugated diene assay

Due to the propensity of apoA-IV to aggregate in the absence of denaturants (Weinberg and Spector, 1985b), the following experiment was carried out to ensure accurate readings were possible at 234nm and that no light scattering occurred. An aliquot of various recombinant apoA-IV proteins were analysed at 234nm for over 200 mins at 37°C. The addition of 200μgs of wild type and S347 protein in PBS buffer did not cause light scattering (figure 6.4) and the recombinant proteins were deemed suitable for the conjugated diene assay.

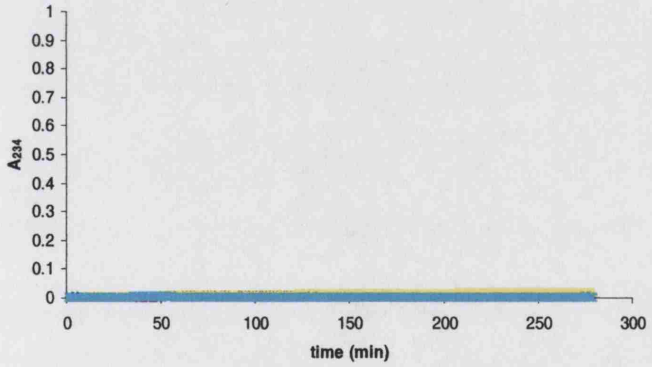


Figure 6.4: The wild type and S347 recombinant apoA-IV protein analysed at 234nm for over 250 mins at 37°C did not result in light scattering.

6.3.2 The recombinant apoA-IV concentration dependent inhibition of copper mediated LDL oxidation

The second preliminary experiment was to ensure that recombinant wild type apoA-IV, similarly to apoA-IV isolated from plasma was capable of inhibiting the copper mediated oxidation of LDL. To do this various concentrations of apoA-IV protein were mixed with LDL (50μg protein/ml) in the presence of $CuSO_4$ (5μM) and subjected to the conjugated diene assay. The results shown in figure 6.5 clearly demonstrate the apoA-IV concentration dependent inhibition of copper mediated LDL oxidation compared to the control (50μg protein/ml LDL + 5μM $CuSO_4$). Therefore, like apoA-IV isolated from plasma, recombinant apoA-IV also displays antioxidant qualities.

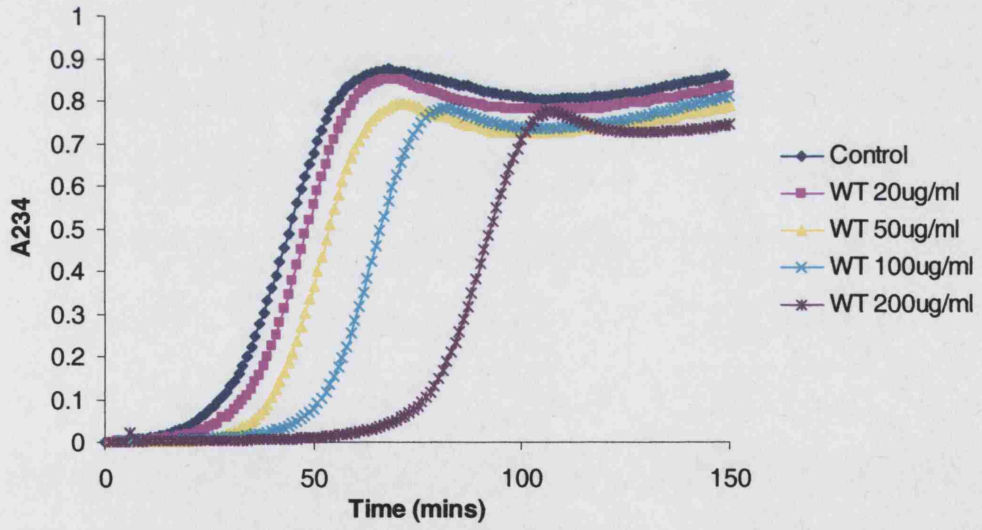


Figure 6.5: Graph demonstrating the apoA-IV concentration dependent inhibition of conjugated diene formation as a function of time. LDL (50μg LDL protein/ml) was oxidised with 5μM $CuSO_4$ at 37°C in PBS buffer, with various concentrations of apoA-IV. The experiments were done in the same spectrophotometer at 37°C using LDL of the same batch.

6.3.3 The ability of the different apoA-IV isoforms to inhibit copper mediated LDL oxidation was dependent upon the age of the LDL preparation

To examine whether there were any differential effects of the common apoA-IV isoforms on antioxidant status, the ability of the three common apoA-IV isoforms, apoA-IV-S347, apoA-IV-H360 and apoA-IV-S127 to inhibit the oxidation of LDL by Cu^{2+} ions was investigated in comparison to wild type apoA-IV using the conjugated diene assay. Initial conjugated diene experiments performed on LDL that was weeks old yielded inconsistent results (see figure 6.6), thus all further experiments were performed on LDL that was freshly isolated (less than 4 days old).

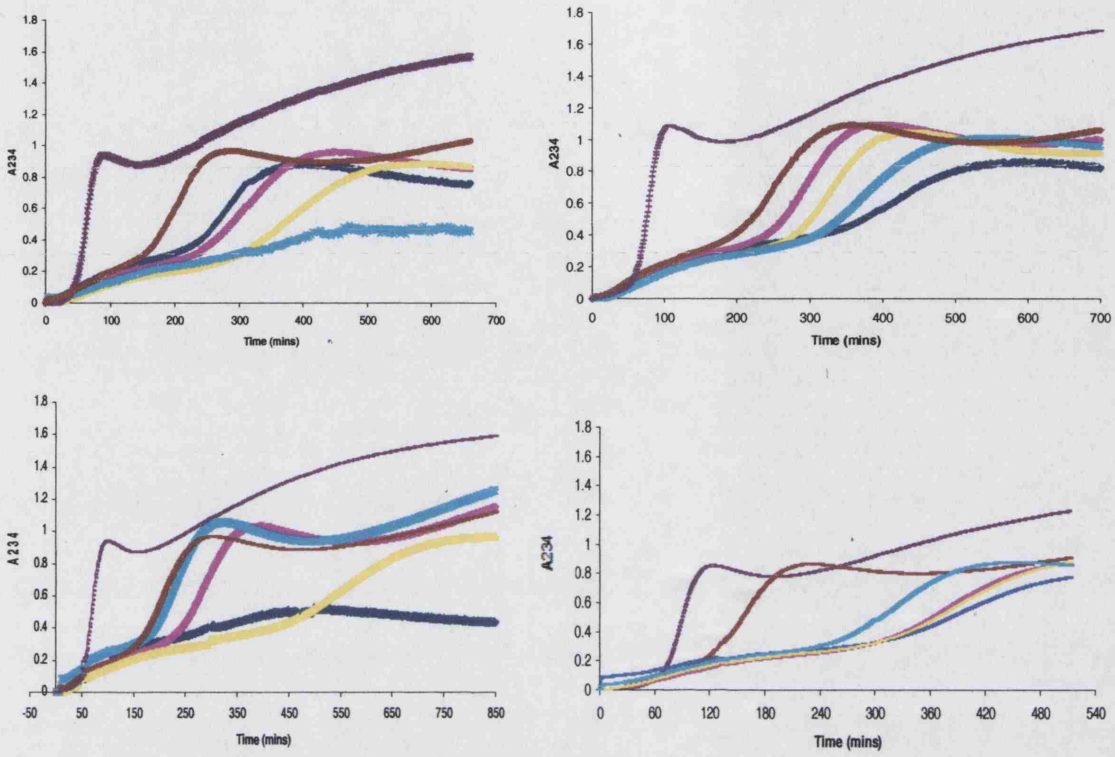


Figure 6.6: A comparison of the abilities of the different apoA-IV isoforms to inhibit the copper mediated oxidation of LDL, using weeks old LDL preparations. The oxidation of LDL (50μg protein/ml) by $CuSO_4$ (5μM) at 37 °C was monitored in the presence of 100μg/ml of wild type-apoA-IV, apoA-IV-S347, apoA-IV-S127 and apoA-IV-H360 and compared to the positive control (50μg protein/ml LDL + 5μM $CuSO_4$) and negative control (50μg protein/ml LDL+ 100μg/ml BSA) using the conjugated diene assay.

6.3.4 The ability of the various apoA-IV isoforms to inhibit LDL oxidation by copper using freshly isolated LDL

Although the absolute values varied slightly between experiments, the relative rankings of the isoforms remained constant (table 6.1) when the identical conjugated diene experiment from section 6.3.3 was carried out using freshly isolated (less than 4 days old) LDL, from three different healthy donors.

By comparison to the control (50μg protein/ml LDL + 5μM $CuSO_4$), the positive control (which included 100μg/ml BSA) and the wild type apoA-IV (100μg/ml) increased the time for 50% conjugated diene formation ($T_{1/2}$) by 2.5 fold ($P < 0.005$) and 4.7 fold ($P < 0.0005$) respectively. As shown in figure 6.7 and table 6.1, compared to the wild type apoA-IV, the $T_{1/2}$ was significantly lower when the experiment was performed in the presence of apoA-IV-S347 (374 ± 22 vs 318 ± 15 minutes $P < 0.05$). In contrast, compared to the wild type isoform the addition of apoA-IV-H360 significantly increased $T_{1/2}$ (374 ± 15 vs 442 ± 32 minutes $P < 0.05$), while the apoA-IV-S127 isoform had a anti-oxidant capacity similar to wild type apoA-IV. Furthermore it was also noted that in the presence of apoA-IV or BSA the reaction kinetics of LDL oxidation changed from conventional kinetics (Esterbauer1989) of a lag phase followed by a propagation phase to more complex kinetics. The complex kinetics consisted of a lag phase followed by a propagation phase, followed by a second lag phase and second propagation phase (Ziouzenkova *et al*, 1998). Due to these complex kinetics we measured the time taken to reach half the maximal levels of conjugated dienes, rather than the more conventional lag times (Esterbauer 1989).

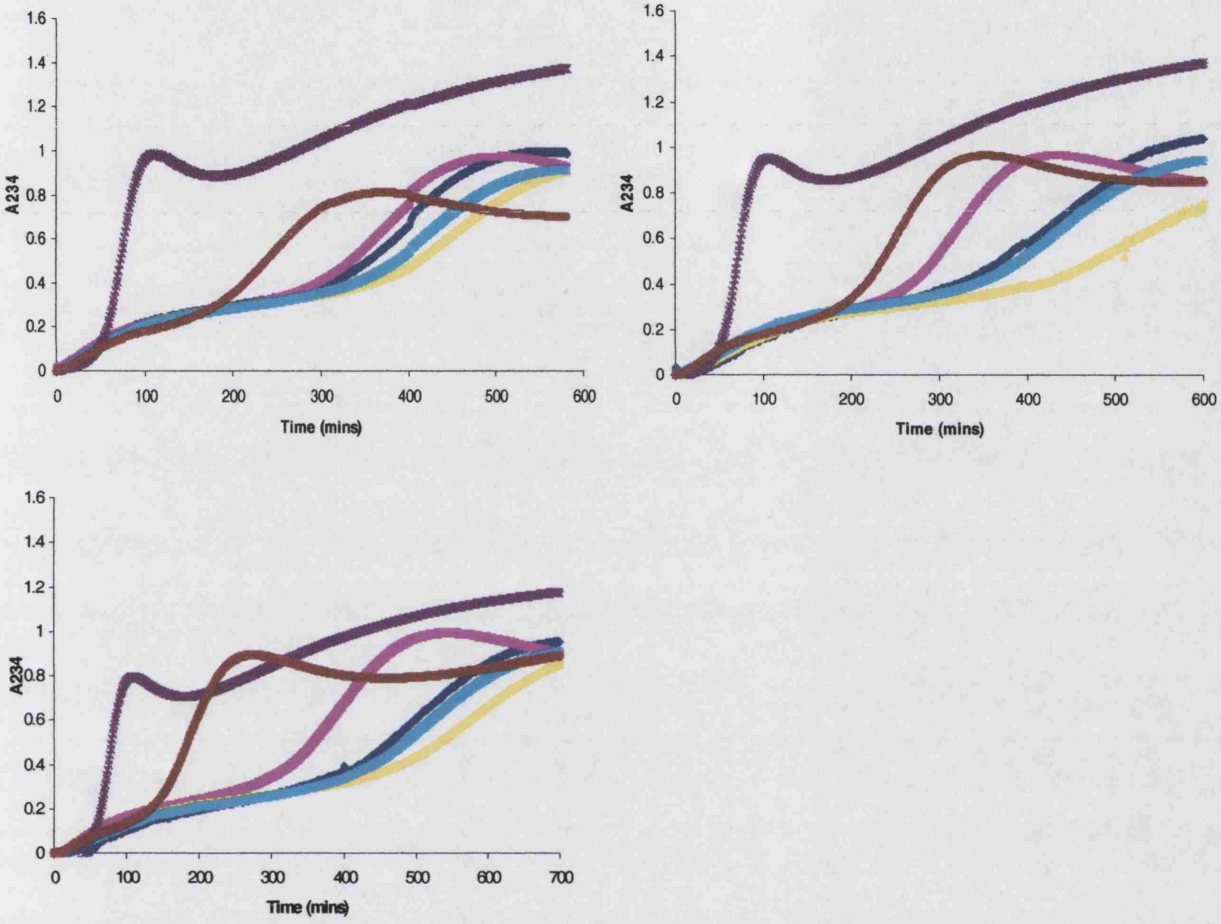


Figure 6.7: A comparison of the abilities of the different apoA-IV isoforms to inhibit the copper mediated oxidation of LDL, using freshly isolated LDL. 5 independent conjugated diene experiments were performed of which 3 are shown. The oxidation of LDL (50μg protein/ml) by $CuSO_4$ (5μM) at 37 °C was monitored in the presence of 100μg/ml of wild type-apoA-IV, apoA-IV-S347, apoA-IV-S127 and apoA-IV-H360 and compared to the positive control (50μg protein/ml LDL + 5μM $CuSO_4$) and negative control (50μg protein/ml LDL+ 100μg/ml BSA) using the conjugated diene assay. Each graph represents an independent experiment.

Sample	$T_{1/2}$ values for conjugated diene formation (mins)*	Percentage difference compared to wild type	Significance vs wild type apoA-IV (P-value)
Wild type apoA-IV	374 ± 22	-	-
ApoA-IV-S347	318 ± 15	- 15	0.036
ApoA-IV-S127	354 ± 20.6	+ 5	0.545
ApoA-IV-H360	442 ± 32	+ 18	0.046
BSA	197 ± 14	- 47	0.003

Table 6.1: The $T_{1/2}$ lag times (means ±SE) for conjugated diene formation, when 50μg/ml LDL was oxidised using 5μM $CuSO_4$ in the presence of the various apoA-IV isoforms (100μg/ml). *The $T_{1/2}$ values are expressed as mean ± SE of 5 independent experiments.

6.3.5 The tri-iodide assay used to assess the anti-oxidant abilities of the common apoA-IV isoforms

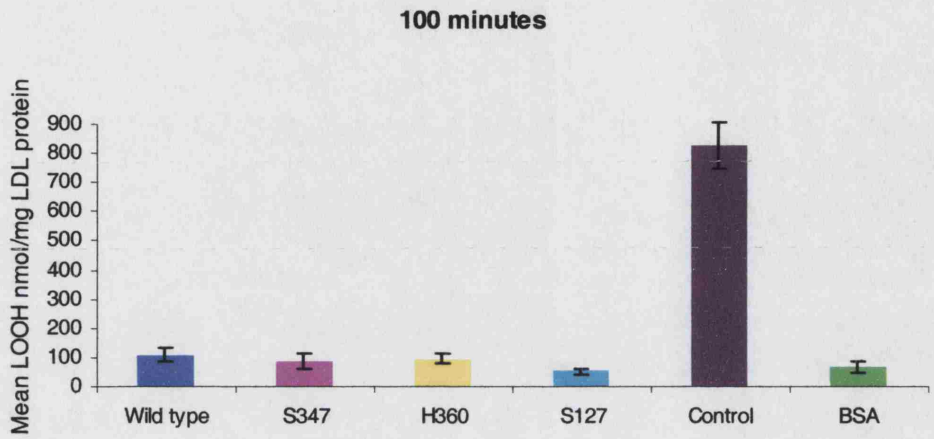
In order to further examine the ability of the apoA-IV isoforms to act as an antioxidant, the capacity of the common apoA-IV isoforms to inhibit the copper-mediated formation of hydroperoxides in LDL was assessed. LDL was oxidised at a concentration of 50μg LDL protein/ml in PBS buffer by 5μM copper at 37°C, in the presence of the various apoA-IV isoform (100μg/ml). Samples were removed from the oxidising LDL after 100 and 400 minutes of incubation and the oxidation reactions stopped by the addition of 1mM EDTA and 20mM BHT. Each sample was then evaluated in terms of LOOH concentration by the colourimetric assay (see section 2.6.5.3). LOOH concentrations were used as an indication of the extent to which LDL oxidation had taken place. The

times of 100 and 400 minutes were chosen based upon the previous conjugated diene results (section 6.3.4) showing clear distinctions in the degree of LDL oxidation between the samples at these times. All results are summarised in figure 6.8 and table 6.2.

Similarily the LOOH levels did mirror the conjugated diene pattern in terms of apoA-IV isoform anti-oxidant capabilities. As illustrated in figure 6.8A, all recombinant apoA-IV isoforms (100μg/ml) significantly inhibited copper-mediated (5μM $CuSO_4$) lipid peroxidation of LDL (50μg protein/ml), resulting in an 8 fold decrease in hydroperoxide generation ($P < 0.005$) when maximal absorbance at 234 nm (T_{max}) for the control (containing only 5μM $CuSO_4$ and 50μg/ml LDL) was reached after 100 minutes.

The relative rankings of the isoforms in terms of their ability to inhibit the copper mediated oxidation of LDL remained similar to that found in the conjugated diene experiment (figure 6.8B). Compared to the wild type apoA-IV after 400 minutes of incubation, the LOOH levels were significantly higher when the experiment was performed in the presence of apoA-IV-S347 (312.6 ± 97.8 vs 487 ± 96.2, P= 0.02). Similarly the presence of the apoA-IV-S127 isoform compared to the wild type isoform did not significantly affect LOOH formation. However in contrast to the conjugated diene data, compared to the wild type isoform the addition of apoA-IV-H360 did not protect the LDL against copper mediated oxidation (table 6.2). The reduced LOOH levels after 400 minutes in the control sample further confirms that copper induced lipid peroxidation of LDL results in the extensive decomposition of LOOH, most likely into aldehydic fragmentation products (figure 6.2) (Carpenter *et al*, 1994).

A.)



B.)

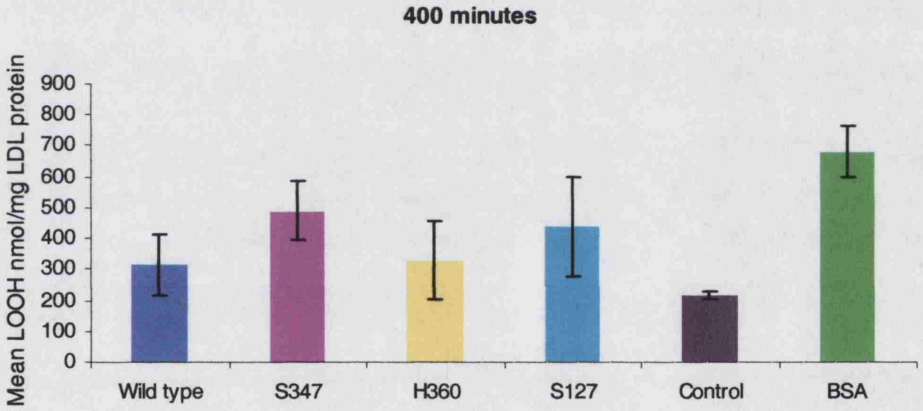


Figure 6.8: LDL (50μg protein/ml) in the presence of $CuSO_4$ (5μM) at 37 °C, was incubated separately with the various apoA-IV isoforms (100μg/ml wild type-apoA-IV, apoA-IV-S347, apoA-IV-N127 and apoA-IV-H360) and the anti-oxidant BSA (100μg/ml). Total LOOH concentrations in each tube was determined iodometrically after 100 and 400 minutes of incubation and compared to the positive control (50μg/ml LDL + 5μM $CuSO_4$). The mean LOOH values are expressed as mean ± SE of 5 independent experiments.

Sample	Mean hydroperoxide (nmol/mg LDL protein)* at 100 mins.	Significance vs wild type apoA-IV (P-value)	Mean hydroperoxide (nmol/mg LDL protein)* at 400 mins	Significance vs wild type apoA-IV (P-value)
Wild type apoA-IV	107 ± 22	-	312.6 ± 97.8	-
ApoA-IV-S347	89.3 ± 26.5	0.055	487.8 ± 96.2	0.02
ApoA-IV-S127	49.6 ± 10.7	0.545	438.6 ± 161.7	0.42
ApoA-IV-H360	95.8 ± 17.4	0.147	329 ± 125.5	0.61
BSA	67.5 ± 18.4	0.155	679.6 ± 84.4	0.03
Control (50μg protein/ml LDL + 5μM $CuSO_4$).	828.7 ± 80	0.009	215 ± 12.73	0.068

Table 6.2: The LOOH levels (means ±SE), when 50μg/ml LDL was oxidised using 5μM $CuSO_4$ in the presence of the various apoA-IV isoforms after 100 and 400 minutes of incubation at 37 °C. *The mean LOOH values are expressed as mean ± SE of 5 independent experiments.

6.3.6 The effect of apoA-IV on the peroxidation of LDL, induced by either excess copper or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)

One possible mechanism for the anti-oxidative effect of apoA-IV could be that it functions as a metal ion chelator, leaving little if any free Cu^{2+} ions to initiate lipid peroxidation. To assess this, the ability of apoA-IV to inhibit the lipid peroxidation of LDL at an excess concentration of copper was examined. The experiment was performed by monitoring conjugated diene formation in LDL (50μg/ml) subjected to oxidation by excess $CuSO_4$ (100μM), in the presence of wild type apoA-IV (100μg/ml). As indicated

in figure 6.9 recombinant apoA-IV did not significantly inhibit LDL oxidation in the presence of excess copper, as shown by the insignificant increase in $T_{1/2}$ (41.25 ± 9 vs 31.25 ± 3 minutes, P= 0.09) compared to the control. To test whether LDL oxidation by excess $CuSO_4$ (100μM) was inhibitable, this experiment was repeated replacing apoA-IV with probucol (10μM; a lipid-soluble free radical scavenger). Probucol (10μM) significantly increased $T_{1/2}$ (108 ± 17 vs 70 ± 21 minutes, $P < 0.02$) for the formation of conjugated dienes (figure 6.10), revealing that while apoA-IV can act as a copper ion chelator, at a high concentration of $CuSO_4$ all available metal ion binding sites on apoA-IV become saturated rendering apoA-IV incapable of preventing LDL oxidation.

Having identified a possible mechanism by which recombinant apoA-IV could act as an anti-oxidant, we tested whether apoA-IV could inhibit the free radical mediated peroxidation of LDL by AAPH (a model for peroxyl radical attack on LDL). The results demonstrated that apoA-IV (100μg/ml) inhibited the AAPH-mediated peroxidation of LDL, by the significant increase of the $T_{1/2}$ for conjugated diene formation compared to the control (174 ± 29 vs 87 ± 10 minutes, $P < 0.05$, figure 6.11). This implies in addition to acting as a metal ion chelator, apoA-IV could also function as a free radical scavenger.

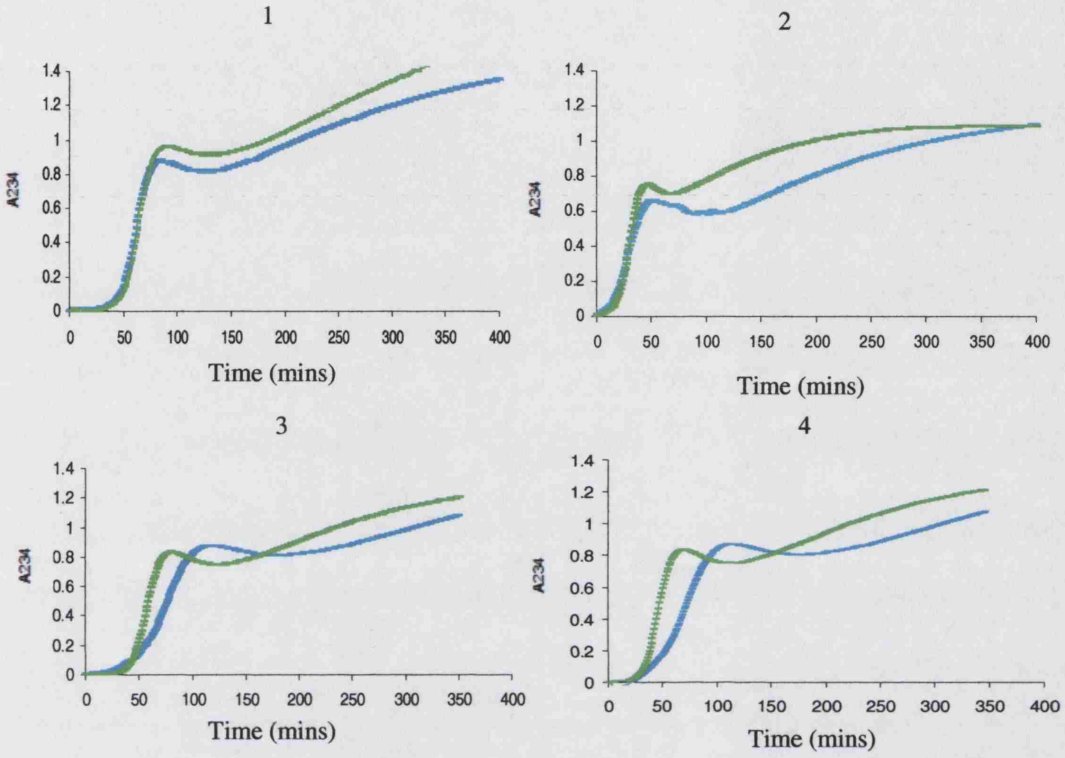


Figure 6.9: The oxidation of LDL was monitored using the conjugated diene assay as described in section 2.6.5.1. LDL (50μg protein/ml) was oxidised by excess $CuSO_4$ (100μM) in the presence (ApoA-IV) or absence (Control) of wild type apoA-IV (100μg/ml). Each graph represents an independent experiment.

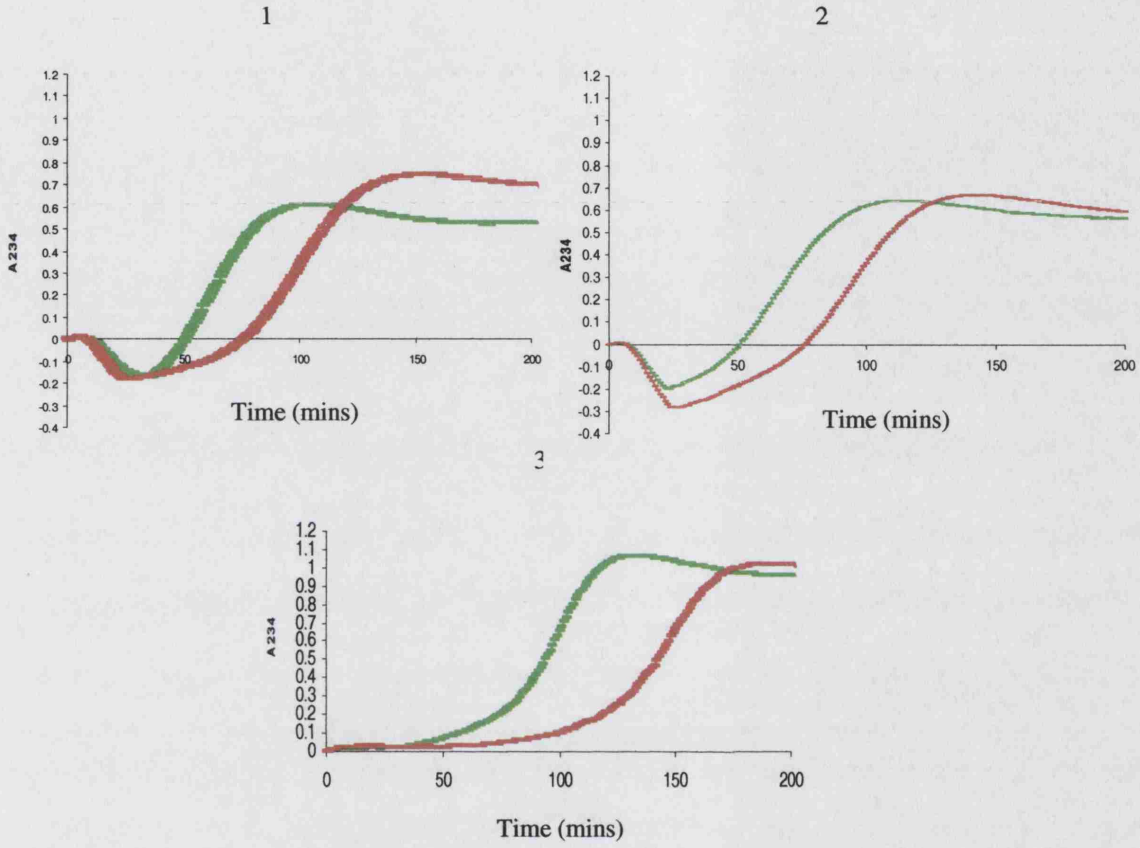


Figure 6.10: The LDL (50μg protein/ml) was oxidised by $CuSO_4$ (100μM) in the presence or absence (Control) of probucol (10μM). Each graph represents an independent experiment.

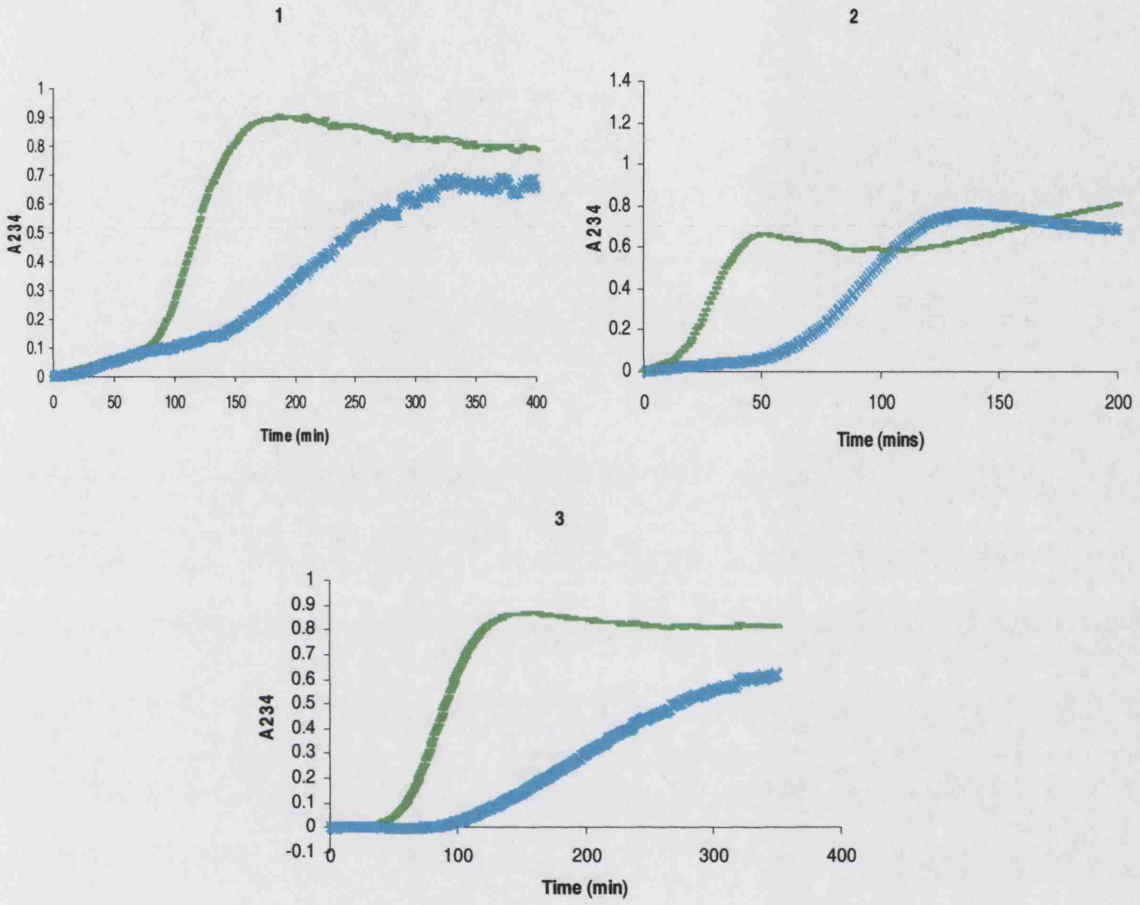


Figure 6.11: The oxidation of LDL was monitored using the conjugated diene assay as described in section 2.6.5.1. LDL (50μg protein/ml) was oxidised by AAPH (1mM) in the presence (ApoA-IV□) or absence (Control□) of wild type apoA-IV (100μg/ml). Each graph represents an independent experiment.

6.3.7 Comparison of the antioxidant properties of recombinant apoA-IV protein with or without the N terminal 6X histidine tag (his-tag)

The his-tag was removed as described in section 2.5.3 and the successful cleavage of the his-tag was examined using his-tag mouse monoclonal antibodies via the western blot protocol (section 2.5.4). First a series of optimisation experiments were performed involving cutting 10μg of apoA-IV with a series of biotinylated thrombin dilutions for 2, 4, 8 and 16 hours at 4 °C to find the most suitable conditions (figure 6.12). Once the optimal concentration of biotinylated thrombin (0.25 U/μl) was found, 500μg of recombinant apoA-IV was cut at a time (0.25 U/μl enzyme at 4 °C for 16 hours) and analysed using his-tag mouse monoclonal antibodies via the western blot protocol.

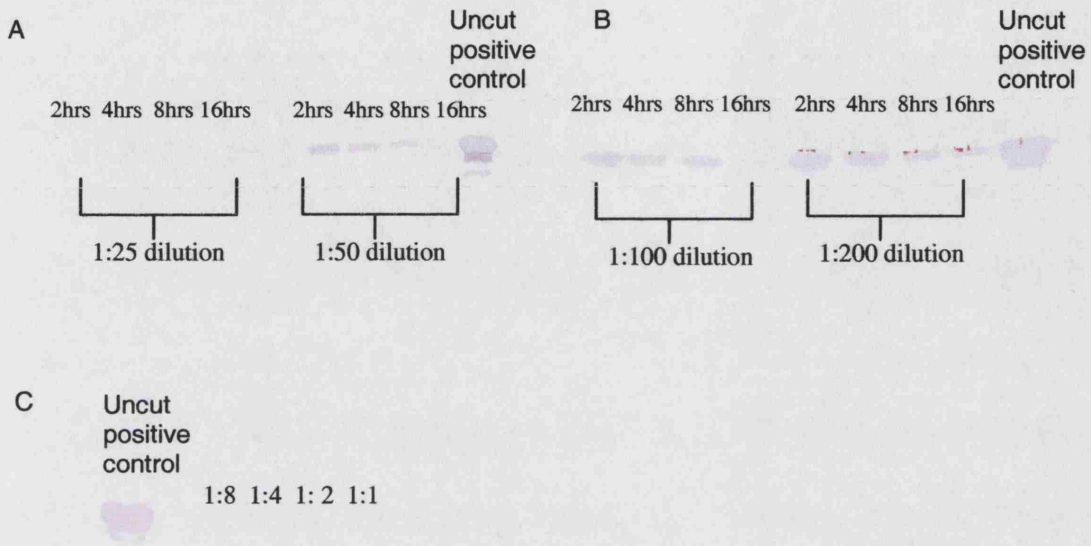
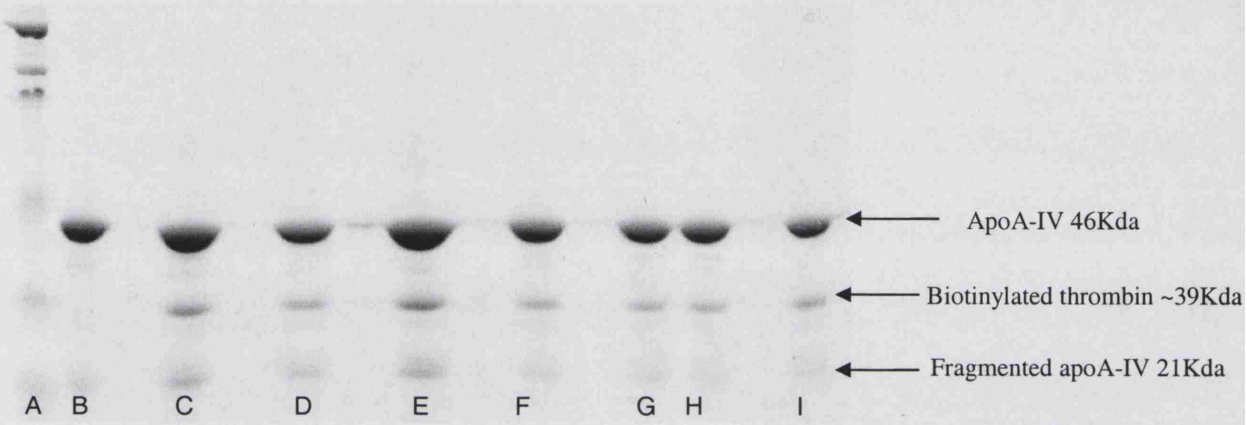


Figure 6.12: Various thrombin cleavage optimisation experiments carried out and analysed using his-tag monoclonal antibodies (section 2.5.4) A.) 0.02 U/μl (1:25) and 0.01 U/μl (1:50) of biotinylated thrombin incubated with 10μg of apoA-IV at 4 ^{o}C for 4, 8 and 16 hours. B.) 0.005 U/μl (1:100) and 0.0025 U/μl (1:200) of biotinylated thrombin incubated with 10μg of apoA-IV at 4 ^{o}C for 2, 4, 8 and 16 hours. C.) 0.063 U/μl (1/8), 0.125 U/μl (1/4), 0.25 U/μl (1/2) and 0.5 U/μl of biotinylated thrombin incubated with 10μg of apoA-IV at 4 ^{o}C for 16 hours.

Once the his-tag had been successfully cleaved the apoA-IV protein was subjected to SDS-PAGE to ensure the protein had not been degraded. Following this, the biotinlyated thrombin was removed using streptavidin agarose beads and again subjected to SDS-PAGE (for full detail please refer to section 2.5.3 and 2.5.2.2).



Calibrated molecular weights of prestained SDS-PAGE standards (BioRad)

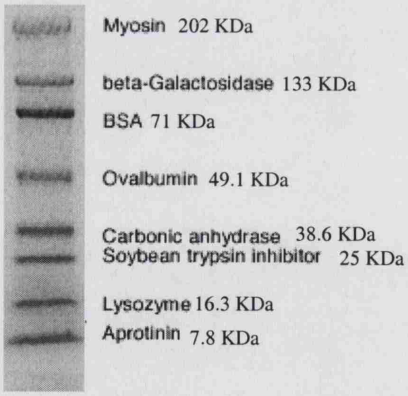
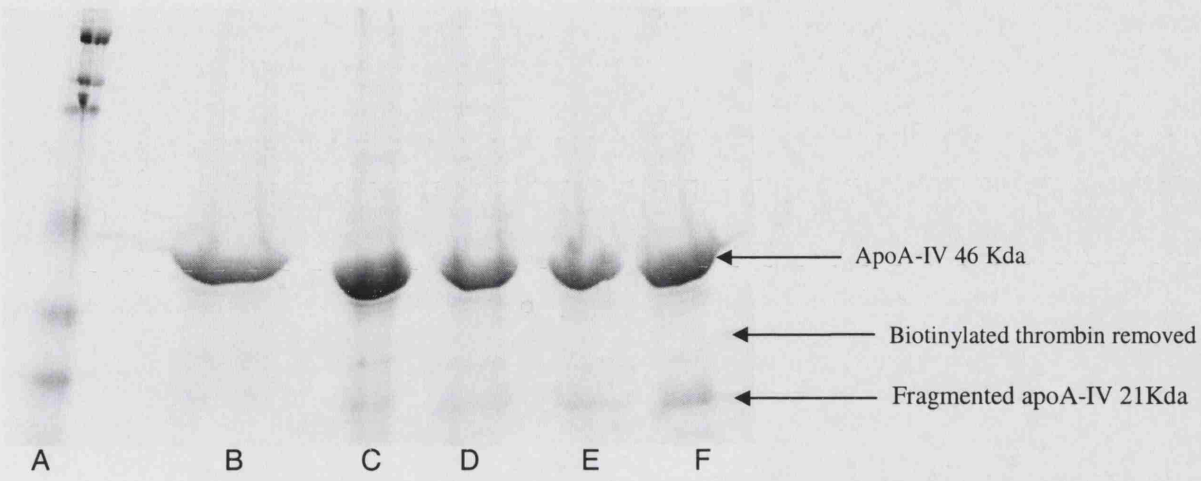


Figure 6.13: SDS PAGE analysis of the apoA-IV proteins cleaved with biotinylated thrombin. A=SDS PAGE standards B= uncut apoA-IV, C-I= cut apoA-IV.



Calibrated molecular weights of prestained SDS-PAGE standards (BioRad)

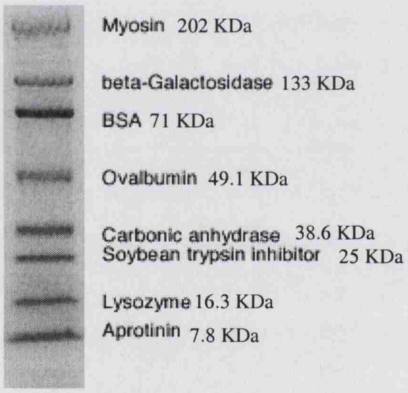


Figure 6.14: SDS PAGE of the pooled apoA-IV his-tag cut samples, incubated for half an hour at room temperature with streptavidin agarose to remove the biotinylated thrombin A= SDS PAGE standards B=uncut apoA-IV, C-F= his-tag cleaved apoA-IV incubated with biotinylated thrombin (16 μl of resin per unit of enzyme).

To assess whether or not the recombinant apoA-IV retained its antioxidant capabilities following the removal of the N terminal his-tag, the antioxidant status of recombinant apoA-IV with or without the his–tag was compared using both the conjugated diene and

tri-iodide assay (see sections 2.6.5.1 and 2.6.5.3 respectively). Recombinant apoA-IV wild type protein, with or without the his-tag significantly inhibited the copper (5μM) induced oxidation of LDL (50μg protein/ml) by 2.18 fold (P=0.001) and 3.54 fold (P=0.003), respectively, although the presence of the his-tag did result in significantly greater antioxidant potential (P=0.024, figure 6.15). Both apoA-IV proteins with or without the his-tag greatly decreased hydroperoxide generation (a greater than 90% reduction in LOOH generation (P<0.0001) was achieved, when T_{max} for the LDL only control was reached after 100 minutes, figure 6.16).

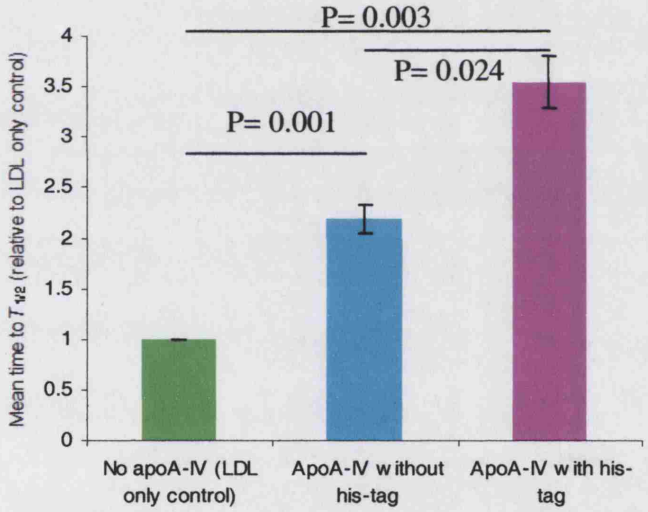


Figure 6.15: Both the recombinant apoA-IV with ☐ or without ☐ the his–tag, were incubated with LDL (50μg protein/ml) oxidised by $CuSO_4$ (5μM) at 37 °C and subjected to the conjugated diene assay. The $T_{1/2}$ for conjugated diene formation was compared to the control ☐ containing 50μg protein/ml LDL and 5μM $CuSO_4$ only. The results here represent the mean ± SE of 5 independent experiments.

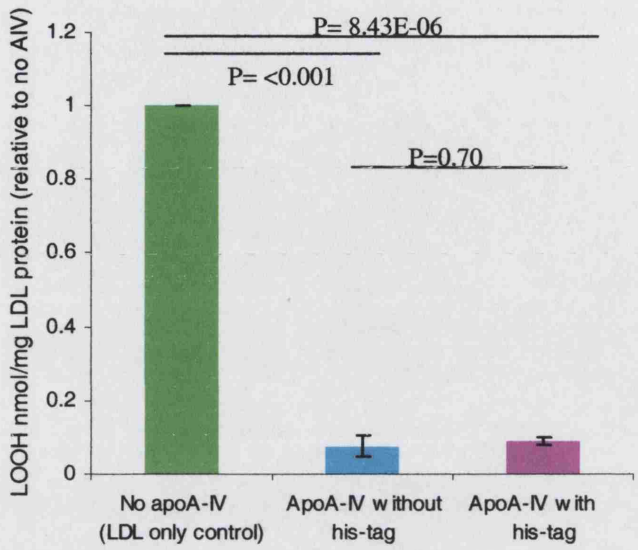


Figure 6.16: LDL (50μg LDL protein/ml) was incubated in PBS buffer with 5μM copper at 37°C in the presence of apoA-IV (100μg/ml) with ☐ or without ☐ the his-tag. After 100 minutes the reaction was halted and LOOH levels determined iodometrically as in section 6.3.5. Both apoA-IV proteins significantly inhibited the formation of LOOH at the time of maximal conjugated diene formation for the control (50μg LDL protein/ml without apoA-IV); P<0.0001. The results here represent the mean ± SE of 5 independent experiments.

6.3.8 Measuring the relative electrophoretic mobility of LDL subjected to copper induced oxidation in the presence of the various apoA-IV isoforms

The various isoforms of apoA-IV (100μg/ml) were incubated at 37°C with LDL (50μg/ml) and $CuSO_4$ (5μM). After 1 hour samples (120μl) were removed from the oxidising LDL, oxidation was blocked by the addition of 1mM EDTA and 20mM BHT and the samples subjected to electrophoresis on agarose Lipogels (according to section 2.6.5.5).

Unlike the conjugated diene and LOOH data, the agarose gel data suggests that all the apoA-IV isoforms failed to protect LDL from copper mediated oxidation, because every LDL sample regardless of which apoA-IV isoform present demonstrated an increased relative electrophoretic mobility (REM) after a 1 hour 37°C incubation. Only the BSA result reflected that of the conjugated diene and LOOH data by remaining as a potent antioxidant (figure 6.17).

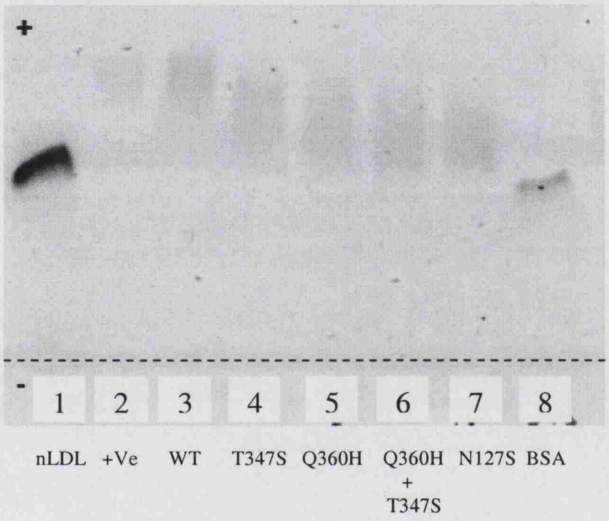


Figure 6.17: Analysis of the relative electrophoretic mobility (REM) after a 1 hour, 37°C incubation of (1) native LDL (50μg/ml) (2) native LDL (50μg/ml) + $CuSO_4$ (5μM), native LDL (50μg/ml) + $CuSO_4$ (5μM) + 50μg/ml of (3) wild type apoA-IV (WT) (4) apoA-IV-S347 (5) apoA-IV-H360, (6) apoA-IV-S347 + apoA-IV-H360 (7) apoA-IV-S127 (8) BSA (50μg/ml) using agarose gel electrophoresis as described in the methods section 2.6.5.5. The dotted line indicates the origin.

Therefore we next investigated whether the apoA-IV isoforms could directly increase the REM of native LDL by incubating the apoA-IV isoforms (100μg/ml) with LDL (50μg/ml) for 15 minutes and then subjecting the samples to electrophoresis on agarose Lipogels (according to section 2.6.5.5). Compared to native LDL alone, native LDL incubated with the apoA-IV isoforms demonstrated an increased REM implying that apoA-IV increased the net negative charge of LDL perhaps by binding to the LDL, indicating that apoA-IV could therefore have the potential to protect LDL against copper-induced oxidation in a site-specific manner (figure 6.18).

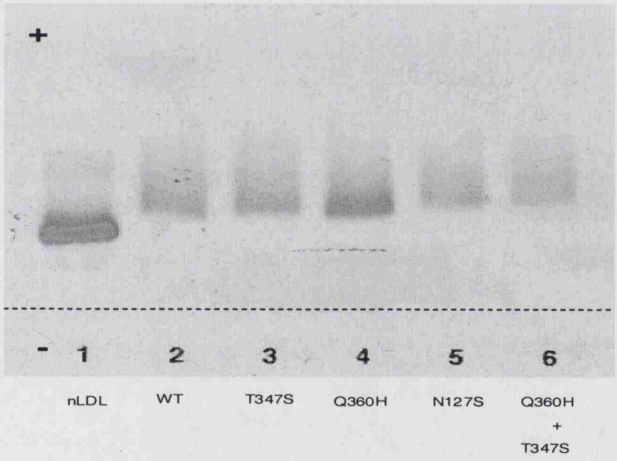


Figure 6.18: ApoA-IV (100μg/ml) alters the electrophoretic mobility of native LDL (50μg/ml) even only after a 15 minutes incubation at 37^{o}C. Analysis of the relative electrophoretic mobility (REM) of (1) native LDL, or native LDL incubated with (2) wild type apoA-IV (WT) (3) apoA-IV-S347 (4) apoA-IV-H360, (5) apoA-IV-S127 (6) apoA-IV-S347 + apoA-IV-H360 using agarose gel electrophoresis as described in the methods section 2.6.5.5. The dotted line indicates the origin.

6.4 DISCUSSION

There is now substantial evidence that apoA-IV can inhibit lipid peroxidation (Ferretti *et al,* 2002; Ostos *et al,* 2001). Our results further substantiate the anti-oxidant properties of apoA-IV, and further demonstrate that recombinant apoA-IV can significantly inhibit the generation of LDL hydroperoxides during copper catalyzed oxidation. Analogous to human apoA-IV (Ferretti *et al,* 2002), recombinant apoA-IV inhibited the oxidation of LDL by both AAPH and Cu^{2+} ions. The alteration in the REM of native LDL when mixed with apoA-IV indicates that apoA-IV can bind to LDL, implying that under certain conditions apoA-IV could perhaps bind to LDL *in vivo* protecting it against oxidation. This agrees with a previous study demonstrating that apoA-IV can sometimes bind LDL *in vitro* (Weinberg and Spector, 1986).

Copper ions are known to bind to apoB-100 on LDL particles with histidine residues, at least in part serving as ligands (Kuzuya *et al,* 1992; Retsky *et al,* 1999; Roland *et al,* 2001). Data presented here showing that apoA-IV cannot inhibit LDL oxidation by a high concentration of copper ions in great excess of that required to saturate all of the binding sites on LDL (figure 6.9) (Roland *et al,* 2001), implies that apoA-IV could also act as a chelating agent complexing with Cu^{2+} ions, therefore competing with apoB-100 for Cu^{2+}. Primary structural analysis of apoA-IV reveals that it is an ideal copper ion chelator, despite the absence of cysteine residues, apoA-IV is comprised of a significant portion of amino acids (46.4%) that can form complexes with Cu^{2+} with stability constants of 6 or greater (A,D,E,G,H,V) (Dawson *et al,* 1986). Consistent with this was the change in the kinetics of LDL oxidation from the conventional type seen with fairly high copper

concentrations, to a more complex type seen with lower copper concentrations (figure 6.7) (Ziouzenkova *et al*, 1998). Furthermore, the copper bound to apoAIV may be redox-inactive rendering it incapable of promoting the oxidation of the lipids in LDL. Our data (figure 6.11) also suggests that apoA-IV may protect against LDL oxidation by scavenging free radicals in transit between the water soluble azo initiator AAPH and the surface of the LDL particles. ApoA-IV cannot however inhibit LDL oxidation by the high concentration of copper ions, indicating that apoA-IV cannot scavenge free radicals within the LDL particles, unlike probucol (figure. 6.10).

The key novel finding from our study is that in comparison to the apoA-IV wild type isoform, apoA-IV-S347 is an inferior and apoA-IV-H360 a superior inhibitor of LDL copper catalyzed oxidation. This was demonstrated by apoA-IV-S347 and apoA-IV-H360 showing significantly lower and higher $T_{1/2}$ respectively compared to the wild type apoA-IV isoform, while the apoA-IV-S127 isoform had a similar antioxidant capacity compared to wild type apoA-IV. The relative rankings of the isoforms in terms of their ability to inhibit the copper mediated formation of lipid hydroperoxide (LOOH) remained similar to that found in the conjugated diene experiment. Compared to the wild type apoA-IV after 400 minutes of incubation, the LOOH levels were significantly higher when the experiment was performed in the presence of apoA-IV-S347, similarly the presence of the apoA-IV-S127 isoform in comparison to the wild type isoform did not significantly affect LOOH formation. However in contrast to the conjugated diene data, compared to the wild type isoform, the addition of apoA-IV-H360 did not protect the LDL against copper mediated oxidation. A detailed analysis of the kinetics of LOOH

formation in relation to conjugated diene formation during the copper mediated lipid peroxidation of LDL would elucidate this discrepancy between the two assays.

Secondary structure predictions of the apoA-IV-S347 isoform indicate that the threonine residue at position 347 is at the end of 13 amino acids within a helical conformation (Garnier *et al*, 1978). Serine at position 347 is predicted to extend an adjacent region containing a stretch of four residues in coil conformation to five (Lohse *et al*, 1991). These structural predictions imply that the apoA-IV-S347 isoform could have reduced lipid affinity (Lohse *et al*, 1991), which has since been demonstrated *in vitro* (Weinberg, R.B, unpublished observations). Therefore compared to wild type apoA-IV, apoA-IV-S347 may be less efficient at inhibiting the binding of copper ions to LDL, or alternatively, at scavenging apo B and lipid free radicals at the LDL surface. The reduced antioxidative abilities of the apoA-IV-S347 isoform, provides a possible explanation for the association of the *APOA4* T347S variant with reduced total antioxidant status and increased CHD risk (Wong *et al*, 2003; Wong *et al*, 2004).

Given our observation that the apoA-IV-H360 variant was a better inhibitor of LDL oxidation compared to the wild type isoform, it would follow that individuals carrying the variant allele would be at reduced risk for CHD. However, due to the low population frequencies of the H360 allele ranging from 0.03 to 0.10 no study to date has been powerful enough to assess this association (Hixson and Powers, 1991; von Eckardstein *et al*, 1992; Zaiou *et al*, 1994). From our previous haplotype analysis of the *APOA5/A4/C3* data (Wong *et al*, 2003) the haplotype defined by *APOA4* H360 was associated with a

lower percentage than the sample mean for CHD events, although the protective effect did not reach statistical significance. Nonetheless, our data does provide support for the notion that the H360 isoform could be cardioprotective. By comparison to wild type apoA-IV, apoA-IV-H360 has a higher percentage of α-helices in the native state and displays greater affinity for lipid surfaces (Weinberg *et al*, 1990b). This could result in a greater ability for the apoA-IV-H360 isoform to bind to and protect LDL against oxidation compared to wild type apoA-IV.

The apoA-IV-S127 isoform demonstrated no significant difference in the ability to inhibit the copper-catalyzed peroxidation of LDL compared to wild type apoA-IV. This is perhaps not surprising, for an Edmunson wheel plot of residues 117-139 reveals that asparagine 127 lies within the polar face of an atypical class A amphipathic helice (figure 6.19), an asparagine to serine substitution at position 127 would not be expected to significantly alter the charge distribution or hydrophobicity of helix 117-139 or alter the global confirmation of apoA-IV. Thus the interfacial binding properties of apoA-IV-S127 are predicted to be similar to that of wild type apoA-IV.

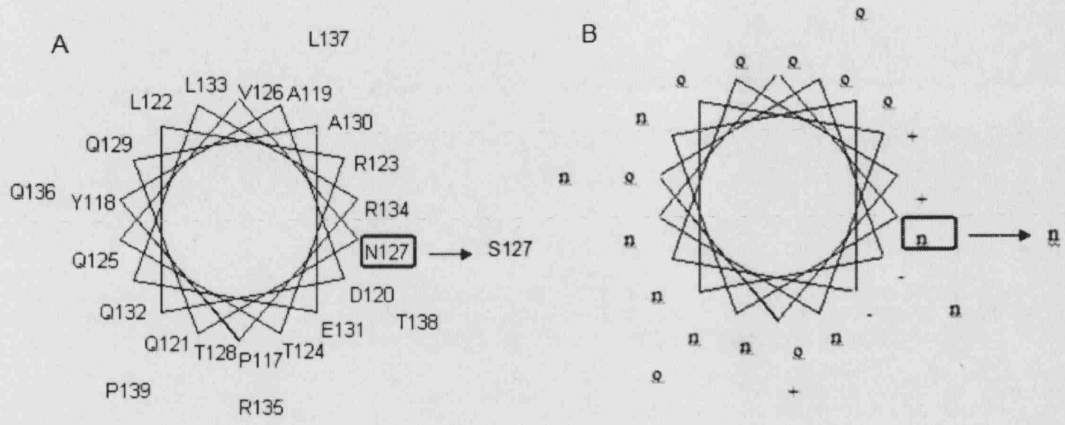


Figure 6.19: A.) An Edmundson wheel representation of an atypical class A amphipathic helice of apoA-IV (residues 117-139), the N127S substitution is highlighted. B.) The charge distribution representation of the helix (residues 117-139). o = hydrophobic, + = positive charge, - = negative charge and n = neutral charge, with the N127S substitution highlighted.

Histidine can protect LDL against oxidation by copper (Kalant *et al*, 1991; Patterson and Leake, 1998) and therefore the presence of the N terminal his-tag in the recombinant apoA-IV could account for some of its antioxidant activity. To examine whether expressed apoA-IV still retained its antioxidant activity after removal of the his-tag, the ability of expressed wild type protein with or without the his-tag to inhibit LDL oxidation by copper using the conjugated diene assay was compared. Wild type apoA-IV still retained its antioxidant effect after removal of the his-tag and significantly inhibited copper induced oxidation, although the presence of the N-terminal his-tag did confer a 62% greater antioxidant activity to recombinant apoA-IV. Since all recombinant isoforms

compared in these experiments had exactly the same concentration of N-terminal his-tag present, all differences observed are due to the amino acid substitutions arising from the common genetic polymorphisms of *APOA4*.

The ability of the different isoforms to inhibit the copper induced oxidation of LDL was sensitive to the age of the LDL. When the conjugated diene experiment was performed using LDL older than 4 days, inconsistent data was produced. This may be because LDL of that age consists to a greater extent of a complex mixture of lipid peroxidation products each reacting with the various apoA-IV isoforms to a different extent.

Under our experimental conditions, the protection against hydroperoxide generation exerted by apoA-IV was at a concentration (100μg/ml) comparable to those reported in human plasma (140 to 370μg/ml). Despite this the apoA-IV:apoB mass ratio in these experiments was 10-14 fold higher than the physiological range. The accumulation of apoA-IV within the atherosclerotic lesions of *APOA4*/apoe-/- mice (Ostos *et al*, 2001) indicates that apoA-IV, like paroxonase, clusterin and apoA-I, can accumulate in diseased artery walls to help protect against lipid peroxidation (Mackness *et al*, 1997). It is within the plaque, due to the retention of LDL by proteoglycans (Anber *et al*, 1996) that LDL concentration may well be higher, and hence where the greater apoA-IV:apoB mass ratio (2:1) in our experiments could thus be applicable. Given the presence of potent aqueous phase antioxidants (such as uric acid, ascorbic acid and bilirubin) (Esterbauer *et al*, 1992), it is unlikely that apoA-IV functions as an aqueous phase free radical scavenger within the plasma. The antioxidant effect of apoA-IV could be mediated by its ability to

bind to the surface of the abundant LDL within atherosclerotic plaques, where it is exposed to an environment (which includes the presence of Cu^{2+} ions) that favours extensive LDL oxidation (Lamb and Leake, 1994; Swain and Gutteridge, 1995).

However despite transgenic mouse studies and *in vivo* experiments demonstrating the anti-oxidative potential of apoA-IV, this per se does not prove that the anti-atherogenic effect of apoA-IV *in vitro* is a result of antioxidant potential (Ostos et al, 2001; Ferretti *et al*, 2002). The physiological role of apoA-IV as an anti-oxidant can be questioned, given the far greater presence of the potent anti-oxidant albumin (32-50g/L) in the human plasma and in the normal human aortic intima (17-27g/L) (Smith *et al*, 1982). Albumin has a vast capacity for ligand binding due to the large number of charges and the large quantity of the protein in serum. For example albumin binds and solubilises non-polar compounds such as bilirubin (Stocker *et al*, 1987), a physiological anti-oxidant in the plasma. In addition as shown in figure 6.15, at least 60% of the anti-oxidant capacity of apoA-IV was due to the presence of the 6x his-tag, thus weight for weight *in vitro* one would expect BSA to be the more potent anti-oxidant.

In order for copper mediated lipid peroxidation of LDL to proceed at a reasonable rate, Cu^{2+} ions must be converted to the active reduced form Cu^{+} (Burkitt, 2001). Copper (Cu^{2+}) ions tend to preferentially bind to the apoB component of the LDL particle, with histidine residues serving as ligands (Kuzuya *et al*, 1992; Retsky *et al*, 1999). The metal ion bound to apoB can be reduced by endogenous LDL α-tocopherol to form Cu^{+} ions (Iwatsuki *et al*, 1995), these Cu^{+} ions can then reduce preformed LOOH present yielding

alkoxyl radicals ($LO^{\bullet}$) initiating lipid peroxidation (Thomas and Jackson, 1991). Thus apoA-IV could inhibit copper mediated lipid peroxidation of LDL by competing with Cu^{2+} ions for apoB binding sites preventing the α-tocopherol induced reduction of Cu^{2+} ions and the formation of $LO^{\bullet}$ radicals. Data presented here and in a previous report (Ferretti *et al*, 2002) demonstrates apoA-IV could possibly act as chelating agent complexing with Cu^{2+} ions, interfering with Cu^{2+} ions binding to LDL and therefore preventing the formation of Cu^{+} ions. Both mechanisms could act in synergy to protect LDL against lipid peroxidation.

Evidence for the involvement of metal ions like Cu^{2+} as mediators of lipid peroxidation is conflicting. Early work demonstrated that high concentrations of copper can oxidise LDL *in vitro*, and that copper ions are a potent inducer of lipid peroxidation with micromolar concentrations enabling cultured cells to oxidise LDL (Heinecke *et al*, 1984). Free catalytically active copper capable of oxidising human LDL has been detected in human atherosclerotic plaques (Swain and Gutteridge, 1995). Ceruloplasmin (the major copper containing protein in the plasma) has been reported to catalyse the macrophage-mediated oxidation of LDL, a process that is enhanced under acidic conditions found within atheroscerotic plaques (Ehrenwald *et al*, 1994; Lamb and Leake, 1994).

Epidemiological evidence suggests that high levels of Ceruloplasmin and serum copper levels (>1.02 mg/l) has been associated with higher risk of myocardial infarction (MI) (Reunanen *et al*, 1992; Salonen *et al*, 1991). Although not a consistent finding, elevated serum copper concentrations have been reported in diabetics with glucose at pathophysiological concentrations enhancing both copper and Ceruloplasmin mediated oxidation of LDL (Chen *et al*, 1995; Leoni *et al*, 2002; Williams *et al*, 1995). However pathology studies in hemochromatosis and Wilson's disease patients with elevated iron and copper levels have shown that these individuals have less extensive coronary artery disease compared to controls (Miller and Hutchins, 1994). Additionally levels of distinctive oxidised amino acids resulting from LDL exposed to Cu^{2+}, for example orthotyrosine, have been found to be no higher in early or intermediate lesions compared to samples from healthy artery walls (Leeuwenburgh *et al*, 1997), indicating that metal ions may play no role in the initiation of LDL oxidation *in vivo*. Despite the physiological controversies, the analysis of LDL oxidation mediated by Cu^{2+} ions is convenient and produces consistent, reproducible results. In addition, the by-products generated from the copper-mediated oxidation of LDL are comparable to those induced by cell mediated oxidation of LDL in culture (Rice-Evans *et al*, 1996).

CHAPTER 7

CHAPTER 7: DISCUSSION AND CONCLUSION

7.1 DISCUSSION AND CONCLUSION

Lipid disorders and alterations in lipid metabolism have been implicated in the development of atherosclerosis, this can be observed by the presence of lipid deposits (fatty streaks) in early atherosclerotic lesions (Nordestgaard and Nielsen, 1994). Apolipoproteins are lipid binding proteins involved in the transport of lipoprotein particles in the plasma. Defects in apolipoprotein structure or synthesis may result in disorders of lipid metabolism and the development of coronary artery disease (CAD) (Herbert *et al* , 1982). Although the precise physiological role of apoA-IV has yet to be deduced, based upon *in vivo* studies carried out in transgenic mice (Cohen *et al*, 1997; Duverger *et al*, 1996) and the observation by Kronenberg *et al* that low plasma apoA-IV levels is associated with CHD in men (Kronenberg *et al*, 2000), apoA-IV is considered to be an anti-atherogenic apolipoprotein and thus perhaps a determinant of CHD risk. The main aim of my thesis was to investigate whether genetic variation in apoA-IV could influence the risk of CHD and to use molecular biology techniques to further investigate the structure/function relationship of apoA-IV.

7.1.1 *APOA4*: association with CHD risk and functional studies to examine this

Although no novel *APOA4* variants were discovered when the *APOA4* promoter (-1550 to +244, from the start of translation) was scanned by SSCP, the analysis of several common SNPs in both case-control Caucasian cohorts and a prospective Caucasian study yielded very interesting results. Examination of both the Q360H and T347S *APOA4* variants in the Kronenberg study revealed a possible association of the S347 allele with

reduced apoA-IV plasma levels, a factor known to be associated with CHD (Kronenberg *et al*, 2000). This was confirmed in the larger EARS cohort where individuals homozygous for the S347 had significantly lower apoAIV plasma levels (13.68 ± 0.59 mg/dL) compared to carriers of the T347 allele (14.90 ± 0.12 mg/dL) (P=0.035). In addition haplotype analysis of 9 SNPs within the *APOA1/C3/A4/A5* cluster revealed that the *APOA4* T347S variant was associated with increased CHD risk in the Northwick Park Heart II study (n= 3020) [hazard ratio (HR) of 2.07 (95%CI 1.04 to 4.12)] independent of any effects on lipid levels. The S347 allele was also associated with reduced total anti-oxidant status (TAOS) in the University College Diabetes and Cardiovascular Study (n=1014). Amongst individuals with CVD, those homozygous for the S347 had significantly lower TAOS compared to T347 carriers (31.2% ± 9.86 vs 42.5% ± 13.04, P=0.0024), implying the presence of greater oxidative stress in S347 homozygous individuals with CVD.

One possible pathological mechanism to explain the reported inverse association between apoA-IV levels and CHD is the ability of apoA-IV to act as an effective inhibitor of lipid peroxidation (Ferretti *et al*, 2002), as suggested by the association of the S347 allele with increased CHD risk, reduced TAOS and lower apoA-IV plasma levels. To consider this possibility the common apoA-IV isoforms (Q360H, T347S and N127S) were generated by *in-vitro* expression in *E.coli* and their ability to inhibit the copper mediated oxidation of LDL was measured using the conjugated diene assay. Compared to wild type apoA-IV, apoA-IV-S347 decreased the time of 50% conjugated diene formation ($T_{1/2}$) by 15% (P=0.036) and apoA-IV-H360 increased $T_{1/2}$ by 18% (P=0.046), while the S127 isoform

had an anti-oxidant capacity similar to wild-type apoA-IV. Although not statistically significant the tri-iodide assay gave comparable results. All apoA-IV isoforms produced increased the relative electrophoretic mobility (REM) of LDL, indicating that apoA-IV could bind to LDL and protect it against lipid peroxidation in a site specific manner. Further experiments revealed that apoA-IV could function as a chelating agent complexing with Cu^{2+} ions and free radicals protecting LDL from oxidation. But unlike probucol, apoA-IV is unable to scavenge free radicals within the LDL particles. The anti-oxidant properties of apoA-IV were still significant once the N terminal histidine tag was removed (P=0.001). Despite the H360 apoA-IV isoform having been found to be a significantly more potent inhibitor of LDL oxidation, this finding has not been reflected in any association studies. A possible explanation for this is that no study to date has been powerful enough to assess this association. Nonetheless from our previous haplotype analysis of the *APOA5/A4/C3* data (Wong *et al*, 2003), the haplotype defined by *APOA4* H360 was associated with a lower than the sample mean percentage of CHD events (figure 3.7A). This supports the notion of H360 being protective, although this protective effect was not statistically significantly different from the common haplotype. Thus the genetic data in combination with functional studies within this thesis imply that apoA-IV is a mediator of CHD with the S347 allele being associated with increased CHD risk, perhaps due to its reduced antioxidant potential compared to the wild type apoA-IV isoform.

7.1.2 Studies to examine the structure/function relationship of apoA-IV

Protein modelling was used to identify important domains within apoA-IV which were then probed using site directed mutagenesis and biophysical studies to obtain further insight into the structure/function relationship of apoA-IV. In total 20 apoA-IV isoforms were successfully synthesised using the pET14b system. Despite the lack of the three arginine apoA-I LCAT activation motif (R149, R153 and R160) in apoA-IV, when the apoA-IV LCAT activation region was subjected to helical wheel analysis, two atypical class A amphipathic helices between residues 117-138 and 146-161 respectively were isolated. Based upon the apoA-I LCAT activation motif containing three critical arginine residues (R149, R153 and R160), three interesting amino acid residues within the polar faces of each of the apoA-IV helices, helix 117-138 and helix 146-161 were isolated (Roosbeek *et al*, 2001). However due to time constraints only the three amino acid residues (R123, R134Q, N127) within the polar face of helix 117-138 were investigated, with only helix 117-138 mutant proteins complexed with 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) and subjected to further structural/functional studies.

All helix 117-138 mutant proteins generated (R123Q, R134Q, N127R, R123Q+R134Q, R123Q+N127R, N127R+R134Q) similarly to wild type apoA-IV gave a characteristic α-helix CD spectra with two minima at 208 and 222nm that increased upon the formation POPC complexes. On average wild type apoA-IV was more α helical compared to the apoA-IV variants in the lipid free state, but once complexed with POPC all recombinant apoA-IV proteins had very similar secondary structures. The LCAT activation properties of wild type and helix 117-138 apoA-IV mutants were next assessed. Calculation of the

catalytic efficiency V_{max}/K_m clearly demonstrated that mutations within the predicted LCAT activation motif of helix 117-138 did not significantly affect apoA-IV LCAT activation. The minor differences in V_{max} and K_m of the mutant proteins compared to wild type apoA-IV were probably attributable to slight differences in composition, size and structure of the apoA-IV mutant complexes. This is exemplified in the R127 variant designed to mimick the three critical LCAT activating arginine residues (R149, R153 and R160) of apoA-I, which yielded comparable LCAT activation properties to the wild type protein. These results indicate that the two arginine residues, R123 and R134, within helix 117-138 of apoA-IV are not critical for LCAT activation and that the apoA-I three critical arginine LCAT activation motif does not reside between residues 117-138 in apoA-IV. Another possibility is that apoA-IV could perhaps activate LCAT via a different mechanism compared to apoA-I.

7.2 FUTURE WORK

7.2.1 *APOA4* and the risk of CHD

Due to the strong linkage disequilibrium (LD) existent across the *APOC3-A4-A5* gene cluster (figure 3.6) in order to fully deduce any associations of apoA-IV with the risk of CHD within NPHSII, haplotype analysis should be carried out on all common apoA-IV Caucasian polymorphisms (Q360H, $CTGT_{3/4}$, T347S and N127S). Functional studies to assess whether the 3' UTR $CTGT_{3/4}$ polymorphism could affect mRNA stability would be interesting. This could be performed using one of the various transcriptional pulsing techniques, analysing the mRNA turnover in mammalian cells (Loflin *et al*, 1999). Transcriptional pulsing relies on the use of tightly regulatable promoters (such as c-*fos*) that are subjected to transient induction. A construct is created containing the gene of interest under the control of a highly regulatable promoter, which is then transfected into an ideal mammalian cell. Following this a stimulus is provided that leads to a burst of mRNA synthesis which when removed allows for the monitor of mRNA decay, involving the isolation of cytoplamic RNA subjected to Northern blott analysis using an appropriate probe at various time intervals.

An experiment to confirm that the results obtained from the lipogels were in fact due to apoA-IV binding to LDL and altering nLDL REM, would be to radiolabel the apoA-IV before subjecting it to REM analysis of LDL and visualising the apoA-IV bound LDL with increased REM on a phosphor-imager. It would also be interesting to investigate the conformational changes of apoA-IV once bound to copper in the lipid bound state, by

performing CD on H360, S347 and S127 rHDLs dialysed for 400 minutes with 5μM Cu^{2+}.

7.2.2 Additional structural/functional studies on apoA-IV

7.2.2.1 Glycosylation and apoA-IV

Glycosylation is the process or result of the addition of saccharides to proteins and lipids. Although it is the most prevalent post-translational modification in eukaryotic organisms, a well defined universal purpose has yet to be defined. The biological activities of many glycoproteins are not detectably different if the carbohydrates are removed, with functions assigned to the carbohydrates so far seeming to be specific to each protein (Creighton, 1996). There are two types of glycosylation, N-type and O-type; N-type glycosylation occurs on the nitrogen atoms of asparagines side chains, while O-type glycosylation occurs on the oxygen atoms of hydroxyl present on serine and threonine residues.

The oligosaccharides present and their properties in native apoA-IV have yet to be characterised, hence the effect of glycosylation in apoA-IV is unknown. Once found the N and O glycosylation sites of apoA-IV could be mutated and the mutant variants expressed in appropriate human colonic carcinoma cell lines (CACO). A whole range of experiments could then be performed to deduce the role of glycosylation in apoA-IV, including monitoring the synthesis rate and intracellular distribution of apoA-IV. The effect of glycosylation on apolipoprotein function seems to be specific for each apolipoprotein, for example the N-type glycosylation of apoB seems to play an important

role in the assembly and secretion of VLDL (Vukmirica *et al*, 2002), whereas O-type glycosylation has no effect at all on apoCIII secretion or lipid binding (Roghani and Zannis, 1988).

7.2.2.2 Self association determination of apoA-IV

Previous work on apoC-I indicates that protein concentration significantly effects melting curves produced, with protein concentrations at greater than 10μg/ml leading to protein aggregation and a reduction of transition temperature not indicative of decreased thermal stability, but reflecting oligomer dissociation into stable monomers (Gursky and Atkinson, 1998). No apoA-IV thermal stability studies to date have been carried out on monomeric apoA-IV, and this would be very interesting to see if the extent of apoA-IV aggregation has any effect on T_m.

7.2.2.3 Subjecting the apoA-IV samples differential scanning calorimetry

Differential Scanning Calorimetry (DSC) would be an ideal tool to visualise the change in heat capacity (ΔCp) of apoA-IV upon heating. Sequential scans using different temperature increments have been used to analyse LDL and apoE (Acharya *et al*, 2002; Jayaraman *et al*, 2005). The resultant DSC transition scans yield valuable information about unfolding behaviour, for example they can be used to analyse the effect of aggregation on thermal stability and the kinetic barriers that modulate the thermal stability of apoA-IV.

7.2.2.4 Further examination of the LCAT activation properties of apoA-IV

It would be very interesting to analyse the effects of the apoA-IV helix 146-161 variants on apoA-IV secondary structure and apoA-IV LCAT activation, to investigate if the apoA-I LCAT activation motif could reside between residues 146-161 of apoA-IV. Alternatively apoA-IV LCAT activation could occur through cooperative segments, an ideal experiment to assess this possibility would be to analyse the ability of apoA-IV mutants with or without helix 117-138 and helix 146-161 to activate LCAT.

LCAT possess enzymatic antioxidant activity through its ability to convert estrogens to lipophilic estrogen esters which can be incorporated into HDL, increasing the antioxidant potential of this lipoprotein fraction (Hockerstedt *et al*, 2004). LCAT activity alone can also give rise to increased antioxidant capacity by preventing the accumulation of oxidised lipid in LDL. The anti-oxidant activity of LCAT has been postulated to be due to the catalytic serine residue 181, which is capable of acting as a reusable proton donor neutralising free radicals, as well as mediating LCAT hydolysis of oxidised phosphatidylcholine (OXPC) generated during LDL oxidation (Goyal *et al*, 1997; Vohl *et al*, 1999). OXPC has well known proatherogenic properties, for example it can act as a potent chemotactic factor for monocytes (Quinn *et al*, 1991) and induce the upregulation of adhesion molecules (Sugiyama *et al*, 1994), therefore its removal is essential in preventing the proatherogenic effects of OXLDL. It would be fascinating to investigate if the activation of LCAT via the various apolipoproteins such as apoA-I, apoE and apoA-IV affects the antioxidant properties of LCAT.

7.3 CONCLUSION

In conclusion the association data and subsequent functional work completed in this thesis suggest genetic variation in the *APOA4* gene may influence the risk of CHD, by directly modulating the anti-oxidant properties of apoA-IV. In addition further research and biophysical studies using recombinant apoA-IV revealed that the critical three arginine LCAT activation motif does not reside in the helix spanning residues 117-138 of apoA-IV.

APPENDIX

CONTRIBUTIONS TO THIS WORK

Contributions to this work by other researchers are as follows:

1. Genomic DNA from the participants of the EARS, NPHSII and UDACS study had been isolated previously by co-workers in the CVG laboratory. Genotyping for the T347S and Q360H *APOA4* variants in NPHSII was completed by co-workers in the CVG laboratory.
2. Statistical analysis of the EARS genotyping data was conducted by our collaborators in France (Viviane Nicaud).
3. Statistical analysis of the UDACS study was performed with the help of Dr Jeffrey Stephens.
4. Statistical analysis and haplotype construction using the *APOC3-A4-A5* gene cluster data was conducted by the statistician of the CVG department (Emma Hawe).
5. Non-linear regression of the LCAT assay data using GraphPad Prism 4.0 computer software was performed by our collaborators in Australia (Professor Kerry Ann Rye)
6. Molecular modelling of apoA-IV was performed by our collaborators in Belgium (Dr Frank Peelman)
7. Large scale production of recombinant apoA-IV was performed with the assistance of Wendy Putt and Jane Roberts.

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