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Μεταπτυχιακή Διατριβή

"ΜΕΛΕΤΗ ΤΟΥ ΡΟΛΟΥ ΤΗΣ ΑΠΟΛΙΠΟΠΡΩΤΕΙΝΗΣ Ε ΣΤΗΝ ΑΘΗΡΟΣΚΛΗΡΩΣΗ"

Μεταπτυχιακή Φοιτήτρια: **Σερέτη Κωνσταντίνα-Ιωάννα**

Υπεύθυνος Καθηγητής: **Ζαννής Βασίλης**

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Master Thesis

"ROLE OF APOLIPOPROTEIN E IN ATHEROSCLEROSIS"

Graduate Student: **Sereti Konstantina-Ioanna**

Supervising Professor: **Dr Zannis Vassilis**

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Περίληψη:

Η απολιποπρωτεΐνη Ε (αποΕ) αποτελεί μια πρωτεΐνη κλειδί στο μεταβολισμό των λιποπρωτεϊνών και παίζει σημαντικό ρόλο στη παθογένεση της αθηροσκλήρωσης και της νόσου του Alzheimer. Η ανάλυση του γονιδίου της αποΕ αποκάλυψε την ύπαρξη τριών κοινών ισομορφών (Ε2, Ε3 και Ε4) καθώς και ορισμένων σπάνιων μορφών.

Η αποΕ παράγεται από ποικίλους ιστούς όπως το ήπαρ και ο εγκέφαλος καθώς και από κύτταρα όπως τα μακροφάγα και συμβάλλει στη μεταφορά της χοληστερόλης και άλλων λιπιδίων. Πιο συγκεκριμένα, η αποΕ συνδέεται με τα υπολείμματα των χυλομικρών και της VLDL και συμμετέχει στην απομάκρυνσή τους από τη κυκλοφορία του αίματος μέσω του υποδοχέα της LDL. Επίσης, η αποΕ συνδέεται με HDL σωματίδια και μέσω αλληλεπίδρασης με τον υποδοχέα SR-BI επιτρέπει τη μεταφορά της χοληστερόλης στο ήπαρ.

Διάφορες μελέτες έχουν δείξει ότι υψηλά επίπεδα ή απουσία της αποΕ αυξάνει τις πιθανότητες ανάπτυξης της αθηροσκλήρωσης.

Πρόσφατα δημιουργήθηκε μια μεταλλαμένη μορφή της αποΕ η οποία ονομάζεται αποΕ4-mut1 και παρουσιάζει σημαντικά πλεονεκτήματα. Με τη βοήθεια της τεχνολογίας των αδενοιών η αποΕ4-mut1 χορηγήθηκε σε ποντίκια που έχουν έλλειψη της αποΕ ($αποΕ^{-/-}$). Η χοληστερόλη του πλάσματος των ποντικών μειώθηκε σημαντικά ενώ δεν παρουσίαστηκε υπερτριγλυκεριδαιμία.

Σκοπός της εργασίας αυτής είναι η παραγωγή μεγάλης ποσότητας της πρωτεΐνης αποΕ4-mut1 και η χρησιμοποίησή της για την παραγωγή προτεολιποσωμάτων τα οποία στη συνέχεια θα χορηγηθούν σε ποντίκια $αποΕ^{-/-}$. Η μελέτη αυτή στοχεύει στον καθορισμό της επίδρασης της αποΕ4-mut1 στην ανάπτυξη της αθηροσκλήρωσης.

Παρόμοια πειράματα στα οποία χρησιμοποιήθηκε μια μορφή της πρωτεΐνης αποΑ-Ι που περιέχει μια φυσική μετάλλαξη και ονομάζεται αποΑ-Ι milano έδειξαν ότι η ανάπτυξη της αθηροσκλήρωσης σταμάτησε μετά από τη χορήγησή της πρωτεΐνης σε ποντίκια $αποΕ^{-/-}$ με τη μορφή πρωτεολιποσωμάτων.

Στην εργασία αυτή έγιναν παράλληλα πειράματα χρησιμοποιώντας τις πρωτεΐνες αποΕ4-mut1 και αποΑ-Ι milano έτσι ώστε να συγκριθούν οι δράσεις τους.

Συνολικά παρήχθησαν 1000mg απολιποπρωτεΐνης E4-mut1 μέρος των οποίων χρησιμοποιήθηκε για το σχηματισμό 240mg πρωτεολιποσωμάτων. Τα πρωτεολιποσώματα αυτά ενέθηκαν σε ποντίκια απο$E^{-/-}$ τα οποία βρίσκονταν υπό δίαιτα πλούσια σε λίπη.

Σε αντίθεση με τη αποE4-mut1, η παραγωγή και ο καθαρισμός της αποA-I milano καθώς και ο σχηματισμός πρωτεολιποσωμάτων παρουσίασαν ιδιαίτερες δυσκολίες.

I. INTRODUCTION

A. Apoprotein E

Apolipoprotein E (ApoE) is a key protein in lipoprotein metabolism and it plays an important role in the protection from or the pathogenesis of atherosclerosis and Alzheimer's disease.

ApoE gene, protein synthesis and sites of expression

The human ApoE gene resides in a 50kb gene cluster (figure 1) [2] with apoCI, an apoCI pseudogene, apoCIII and apoCIV which is found on the long arm of chromosome 19 [5 6]. The apoE gene is 3.7kb long and contains four exons and three introns [6]. The primary translation product consists of 317 amino acids including an 18 amino acid signal peptide [7]. ApoE is modified in Golgi by O-linked glycosylation [8]. The secreted (mature) apoE is composed of 299 amino acids.

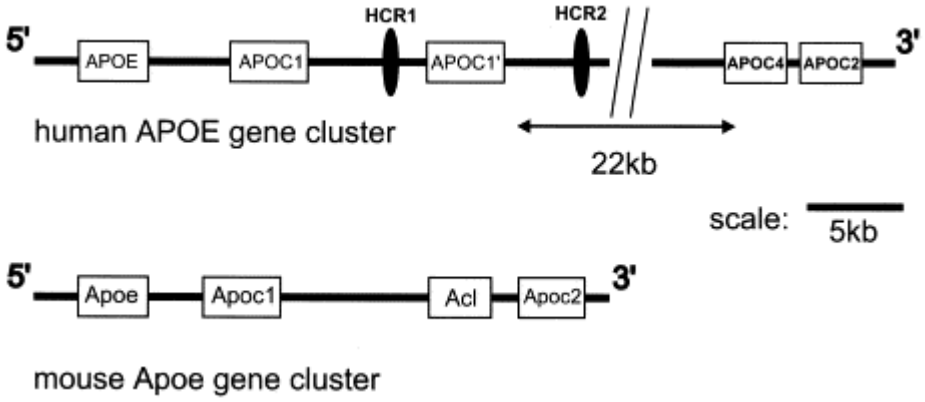


Figure 1: Human and mouse APOE gene cluster. APOC1′ is a pseudogene. Hepatic Control Regions 1 and 2 (HCR1 and HCR2) control gene expression in the liver[2].

ApoE is produced by various tissues and cells such as liver, brain, kidney, adrenal gland, ovary, spleen, muscles, lung and macrophages. Most of the plasma apoE derives from the

liver whereas about 20-40% of the plasma apoE is produced from extra-hepatic tissues and cells such as the macrophages.

ApoE gene expression is regulated in a tissue specific fashion and is affected by several cellular and extracellular factors [9]. ApoE expression in macrophages can be modulated by cholesterol loading [10].

structure

Physical and biochemical analysis of the protein's secondary structure suggested that apoE consists of two independently folded domains linked by a loop [11, 12]. The amino-terminal moiety (residues 1-191) contains the receptor- and heparin-binding domains. It consists of four anti-parallel amphipathic α helices arranged with their hydrophobic domains facing the interior of the bundle. A fifth short helix connects helices 1 and 2 [13]. The receptor and heparin-binding domains are located in helix 4. Mutational analysis as well as chemical modification has demonstrated that an arginine- and lysine-rich region (residues 136-150) of helix 4 is essential for the interaction with the receptors that recognize apoE, such as the low density lipoprotein (LDL) receptor (LDLR (figure 2). [14] Recently, it has been shown that the amino-terminal domain of apoE contains a binding site for the scavenger receptor class B [15, 16].

The lipid-binding domain of apoE is found in the carboxy-terminal region (residues 216-299) which is also believed to contain amphipathic α-helices. Recent data demonstrate that a segment of the carboxy-terminal domain adopts a coiled-coil conformation which most likely is essential for the clustering of heparin-binding sites and/or the isolation of the lipid-binding domain in the lipid-free state [17].

It has been shown that lipidation of apoE is required for its interaction with the receptors [14, 16]. When lipids bind to the amino-terminal domain the bundle opens exposing hydrophobic lysines 143 and 146 to the aqueous phase and thus triggering a helical configuration of residues 165-169.[9] In this conformation apoE is able to bind with high affinity to the LDLR [9, 18].

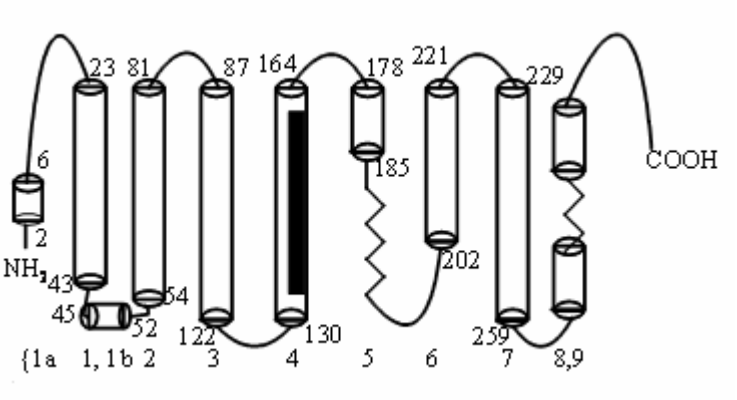


Figure 2: Secondary structure of human apoE

Polymorphisms

Analysis of the apoE gene revealed three common isoforms (E2, E3 and E4) and few number of rare variants[19]. The three frequently occurring phenotypes are products of three alleles (ε2, ε3 and ε4) at a single genetic locus (figure 3). The six possible phenotypes, three homozygous (E2/2, E3/3 and E4/4) and three heterozygous (E2/3, E2/4 and E3/4) are all detectable in humans with different gene frequencies [20]. These three isoforms differ only in residues 112 and 158 with apoE2 having cysteine in both residues, apoE3 having cysteine and arginine in residues 112 and 158 respectively and apoE4 having arginine in both residues [19].

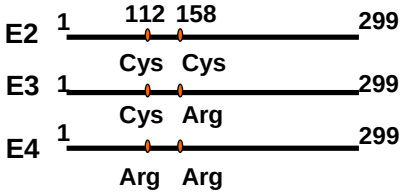


Figure 3: Amino acid differences between the ApoE isoforms.

Other, non-genetically determined apoE forms result from differential post-translational glycosylation. The sialylated isoforms account for 10-20% of plasma apoE but their functional relevance is still unknown [19].

Even though, the three genetically determined apoE isoforms differ in only one amino acid, they have important structural and functional differences. For instance, they demonstrate different chemical and thermal stabilities (E4<E3<E2) [21]. Furthermore, the amino acid substitution in apoE2, compared with apoE3, alters salt bridges in helices 3 and 4 and reduces the positive ion potential of the region between residues 140-150, where the receptor-binding site resides. Similar changes occur also in apoE4 compared with apoE3. The presence of arginine at position 112 in apoE4 results in the formation of a salt bridge between residues 109 (glutamic acid) and 112 in helix 3 and drives the side chain of arginine 61 to a new exposed position in helix 2 that allows interaction with residue 255 (glutamic acid) (figure 4) [22-24].

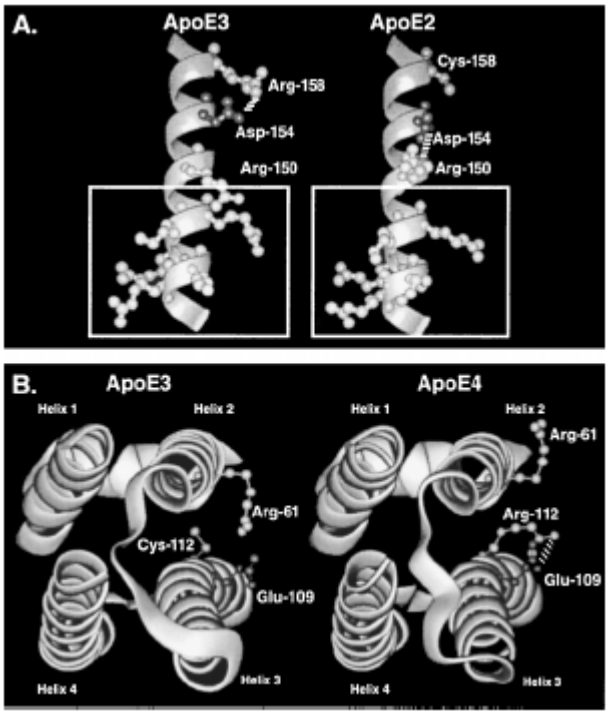


Figure 4: Three-dimensional structure of apoE regions. A) A modified salt bridge in helix 4 of apoE2 reduces the positive ion potential of the LDLR binding region between residues 140-150 [4],
B) The substitution of arginine for cysteine at position 112 of helix 3 of apoE4 creates a salt bridge between residues 109 and 112 and exposes the side chain of arg61 so that it can interact with glu255 [4].

The receptor-binding affinity also varies between the three isoforms. In cultured cells, the low density lipoprotein receptor binds with the same affinity to apoE3 and apoE4, whereas apoE2 has 2% less affinity [21, 25]. This lower binding affinity is thought to be due to the lower positive ion potential at position 158 that alters the salt bridges within helix 4

and between helices 3 and 4 which are important for the maintenance of the conformation of the receptor binding domain [26].

Furthermore, the amino acid differences between the three apoE isoforms affect their lipid-binding affinities as well. ApoE3 and apoE2 preferably bind to smaller, high density lipoproteins (HDL), whereas apoE4 has a greater affinity for larger, very low density lipoproteins. It is believed that the interaction of Arg61 with Glu255 is responsible for this difference in lipoprotein affinity [27].

Functions

ApoE and metabolic pathways

"Endocrine-like function": apoE is synthesized in several sites and contributes in the transport of cholesterol and other lipids to the cells of other organs.

- Upon dietary fat and cholesterol uptake the dietary lipids in the intestine associate with $apoB_{48}$ and form the chylomicrons which are then secreted to the mesenteric and thoracic duct lymph. The formation and the secretion of chylomicrons require the action of the microsomal triglyceride transfer protein (MTP). In the lymph, lipoprotein lipase (LPL) is activated by apoCII and hydrolyzes the triglycerides of chylomicrons which become rich in cholesteryl esters and are referred to as chylomicron remnants. Subsequently, they associate with apoE and they are rapidly cleared by the liver through receptors that recognize apoE. Recent findings from Kypreos & Zannis have shown that this task is accomplished through the LDL receptor (LDLR) alone in mice and that other apoE-recognizing receptors have negligible role [28]. However, in humans, patients with familial hypercholesterolemia that lack or have defective LDL receptor have the capacity to clear the remnants from their plasma (figure 5) [3, 29].

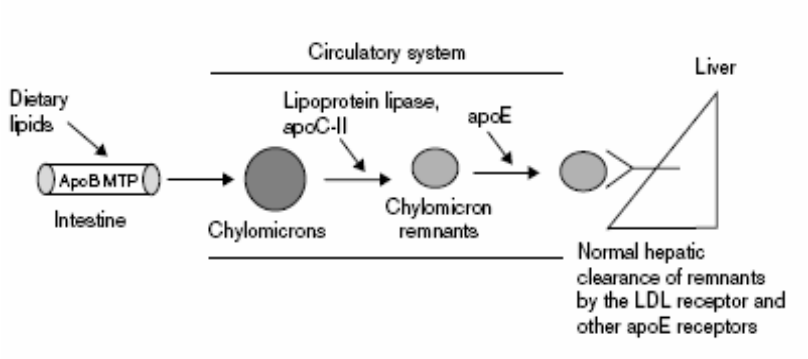


Figure 5: The chylomicron biosynthesis and catabolism pathway by the liver[3].

- Similarly, apoE contributes to the transport of endogenous lipids from the liver to the peripheral tissues. Hepatocytes synthesize and secrete very low density, triglyceride-rich lipoproteins (VLDL) that contain apoE and apoB100. When they are secreted to the plasma, lipoprotein lipase (LPL) catalyzes their hydrolysis resulting in smaller and more cholesterol enriched particles referred to as VLDL remnants. Further hydrolysis leads to the formation of intermediate density lipoproteins (IDL). Both VLDL remnants and IDL are cleared from the plasma trough the LDL receptor. The clearance is primarily mediated by apoE. As the particles undergo further hydrolysis and become more LDL-like they lose apoE and apoB100 becomes the ligand. (figure 6) [3].

- Moreover, apoE plays a significant role in the transfer of excess cholesterol to the liver for excretion, a process named "reverse cholesterol transfer" [30, 31]. ApoE is a component of a subclass of small HDL particles that accept cellular cholesterol very efficiently and through association of apoE with SR-BI they deliver it to the liver. This uptake is mediated by the interaction of the amino-terminal 1-165 residues of apoE with SR-BI [32, 33]. POPC particles containing WT or truncated forms of ApoE are able to promote cholesterol efflux through SR-BI, whereas non-lipidated apoE does not bind to SR-BI and cannot promote the efflux [32]. This capacity of apoE to participate to the delivery as well as the efflux of cholesterol may be responsible for its atheroprotective role that will be discussed later.

ApoE is also capable to contribute to the transfer of lipids within a tissue and thus also has a "paracrine-like" function [10, 29].

It has been suggested that in the space of disse, apoE-containing lipoproteins bind to heparin-sulfate proteoglycans (HSPG) rich cell surfaces. Following binding, apoE-containing lipoproteins are subject to lipolysis and are taken up by the HSPG/LRP complex and transferred to the LDLR for internalization and uptake. [34, 35]

ApoE3, the most common of the three isoforms, contributes little to the variation of plasma lipids. ApoE2 is usually associated with high apoE and triglyceride levels and low apoB and cholesterol levels, whereas, apoE4 is associated with low apoE and high apoB and cholesterol levels [4].

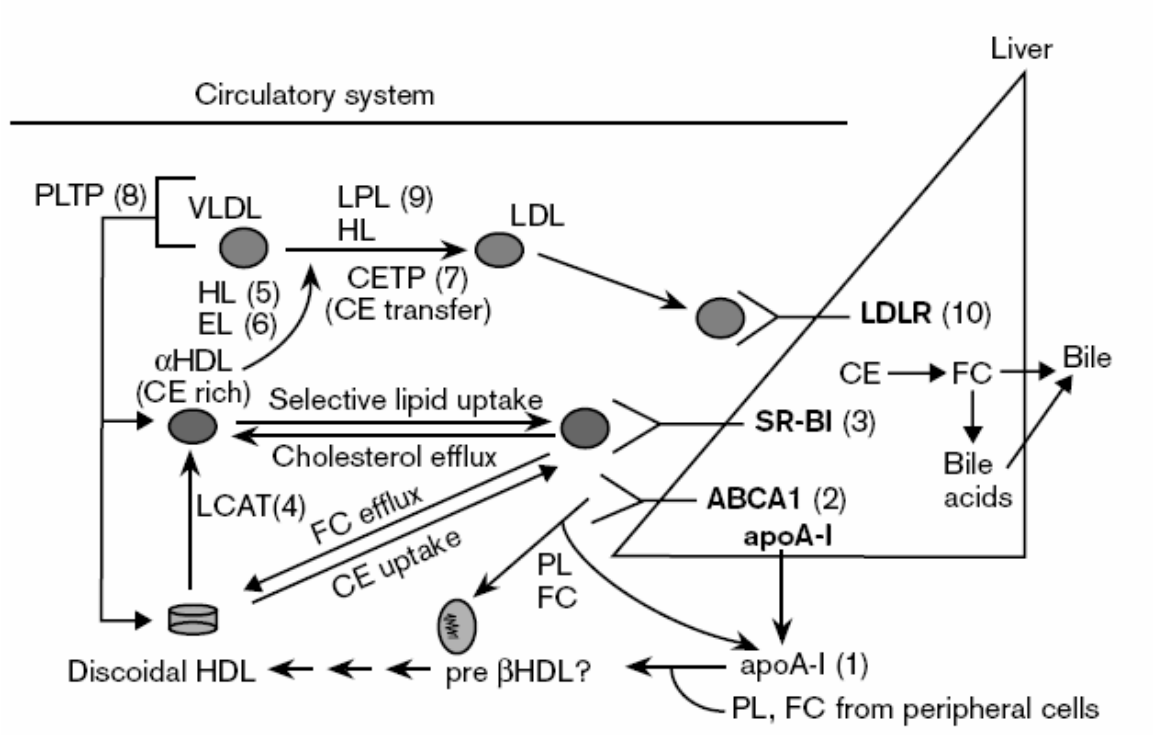


Figure 6: The HDL biosynthesis and catabolism pathway by the liver[3].

ApoE and atherosclerosis

Type III hyperlipoproteinemia: There is a plethora of findings associating the homozygous E2/2 phenotype with type III hyperlipoproteinemia. The E2/2 phenotype is defective in lipoprotein receptor-binding which causes delayed remnant-clearance. Type III HLP is an adult-onset disorder characterized by elevated plasma cholesterol and triglyceride levels (approximately >300mg/dl) and the presence of abnormal lipoproteins called β-VLDL. These particles are cholesterol- and apoE-enriched remnants originating from the intestine and liver. The increased lipids in type III HLP are mostly due to high β-VLDL since HDL and LDL levels are low. Type III HLP predisposes to premature atherosclerosis and its main clinical feature is xanthomas [4].

Type III HLP is essentially a recessive disorder but some other rare apoE variants are associated with dominant forms. Interestingly, most of the humans with the E2/2 phenotype have normal or low lipid levels indicating that other factors (hormonal, environmental or genetic) are necessary for the development of overt type III HLP [4].

Cardiovascular disease: a number of studies have examined the role of apoE isoforms in heart disease. It would be reasonable to think that since apoE2 is associated with type III HLP, it is also the one related with the higher risk for cardiovascular disease. Surprisingly, this is not the case. The various findings are often conflicting indicating that factors such as geographic and ethnic background, sex, allele frequency, potential interaction between genes, environment and the study design probably affect the outcome of the studies [36, 37].

A number of studies support that apoE4 is the isoform that presents the most risk for cardiovascular disease. As mentioned earlier apoE4 is associated with high LDL cholesterol and apoE levels which constitute significant risk factors for heart disease. Additionally, clinical studies revealed that in patient populations with hyperlipidemia and heart disease, apoE4 is very frequent [4, 37-39]. The study of middle-aged men from different populations showed that the individuals carrying the ε4 allele had a 40% greater risk for dying from coronary heart disease than those carrying the ε2 or ε3 allele [38].

Protection from atherosclerosis: it is generally believed that apoE protects against atherosclerosis and this effect depends on the apoE isoform, the site of synthesis and secretion and the total plasma apoE level.

The role of apoE in lipoprotein remnant clearance and reverse cholesterol transport is in agreement with its atheroprotective effects. ApoE deficiency in humans results in a pro-atherogenic lipoprotein profile and severe atherosclerosis [40, 41]. In apoE null mice ($apoE^{-/-}$) cholesterol levels are elevated due to the accumulation of lipoproteins in the VLDL / IDL region and atherosclerotic lesions develop within 10 weeks even when mice are fed with a normal chow diet [42-46]. Atherosclerosis can be reduced in these mice by apoE gene transfer through recombinant adenovirus [47, 48], expression of apoE transgenes [49] or delivery of an apoE mimic peptide [50].

Furthermore, a number of studies have shown that apoE has an additional atheroprotective role that is separate from its role in cholesterol homeostasis. ApoE is present in high quantities in atherosclerotic plaques and it is considered to be mainly derived from resident macrophages [51]. The beneficial effect of its expression from macrophages has been well documented with several animal studies. Bone marrow transplantation has revealed that apoE synthesized by macrophages is anti-atherogenic and that this property is independent of its effect on plasma lipoprotein levels [52-55]. Moreover, mice with macrophages as their only apoE source were protected against atherosclerosis although they had low plasma apoE levels and high cholesterol [56]. In addition when apoE was normally expressed in all tissues except in macrophages, the mice were more susceptible to atherosclerosis [53].

Macrophage-derived apoE has local cytokine- and hormonal-like effects which contribute to its anti-atherosclerotic role. ApoE inhibits platelet aggregation and the expression of vascular cell adhesion molecule1 (VCAM-1) on epithelial cells by interacting with apoER2 and activating the nitric oxide synthesis pathway [57-59].

ApoE can also inhibit lipid oxidation which might hinder the accumulation of oxidized LDL [60]. ApoE null mice exhibit oxidative stress and accumulate oxidized LDL in the

aortic lesions [61], their plasma lipoproteins are more oxidized and more oxidation-prone in vitro than in wild type animals [62].

ApoE is also able to inhibit the activation and proliferation of T-lymphocytes probably by preventing intracellular calcium accumulation and phosphatidylinositol turnover [63-65] as well as the migration and proliferation of SMC [66-68].

Finally, a part of macrophage-produced apoE is retained in the pericellular proteoglycan matrix. Proteoglycan-bound apoE interferes with the traffic of lipoproteins into the vessel wall and also controls the availability of cytokines and growth factors [69].

However, there is evidence showing that apoE can also be pro-atherogenic under specific conditions. For example, as mentioned earlier, some homozygotes for the ε2 allele or individuals carrying other rare apoE variants develop type III HLP which is characterized by premature atherosclerosis [4]. Additionally, the apoE4 isoform is associated with high cholesterol levels which consists a significant risk factor for the development of atherosclerosis.

In conclusion, it is undeniable that absence of apoE increases the risk for atherosclerosis but on the other hand high levels of the protein is also associated with increased risk. Most likely, an optimal range of apoE plasma concentration exists and that levels below or above that range consist a risk for the development of atherosclerosis [70].

ApoE and Alzheimer's disease

Alzheimer disease consists of the most common type of progressive dementia in the elderly. It is a neurodegenerative disorder characterized by intracellular neurofibrillary tangles (NFT) and extracellular amyloid plaques that accumulate in the brain. Their major component is the amyloid Aβ peptide [71]. In AD, hippocampal and neocortical neurons are destroyed resulting in gradual dementia and finally death [72].

ApoE is the only apoprotein expressed locally at high levels in the brain [29, 73]. ApoE plays a significant role in the maintenance and repair of neurons. However, each of the three apoE isoforms has different effects in neurobiology. In 1993, Corder et al discovered that apoE4 is associated with 40-65% of cases of late onset and sporadic Alzheimer's disease[74]. Increasing doses of apoE4 augmented the risk for AD and lowered the mean

age of onset in families with familial late onset AD [74]. Since then, many studies in different populations have established apoE4 as the main known risk factor for AD [75].

Neuronal repair and protection: in order to maintain the synapto-dendritic connections, neurons need to be protected and repaired and this requirement increases with age. ApoE3 and apoE2 are considered to be able to contribute to this process, whereas apoE4 is unsuccessful to promote efficient neuronal repair [75]. In vitro studies have demonstrated that a lipoprotein vehicle transfers apoE3 and apoE2 to neurons where they stimulate the production of long neurites through a cell-surface receptor pathway. In contrast apoE4 inhibits neurite outgrowth by enhancing microtubule depolymerization [76]. It was proposed that this effect may be accomplished though interaction with a microtubule-stabilizing protein termed tau. In vitro experiments have shown that apoE3, but not apoE4 can associate with tau and prevent its hyperphosphorylation which would inhibit its property to stabilize microtubules [77, 78].

ApoE4 and AD pathology: as mentioned earlier, a number of studies have demonstrated that apoE4 is strongly related to AD. It has been proposed that apoE4 interacts with the Aβ peptide, enhances its deposition in plaques and reduces its clearance rate [75, 79]. Lipid-free apoE interacts through its lipid-binding domain with the Aβ peptide in vitro. It was found that binding follows the order apoE2 > apoE3 >> apoE4[80]. Lipidated apoE interacts stronger than lipid-free apoE[80].

Moreover, apoE4 increases Aβ-mediated lysosomal cell death and apoptosis to a greater degree than apoE3 [81, 82].

It has been shown that apoE4 enhances Aβ secretion by cultured neuronal cells. Aβ secretion was reduced by inhibition of LRP by siRNA or by the receptor associated protein as well as by replacing Arg61 with threonine in apoE4 [79].

Recent studies have shown that apoE fragments, which result from proteolytic cleavage of apoE synthesized by neurons, hinder the protection/repair process [83-85]. In patients with AD and apoE transgenic mice fragments of the protein lacking the c-terminal 27 amino acids were observed [83]. Because of its conformation apoE4 is more prone to proteolysis than apoE3. The enzyme responsible for this cleavage is a chymotrypsin-like serine protease that is neuron-specific and cuts the protein at Met272 and/or Leu268 [83, 84]. Mutation of Arg61 (or Glu255) and thus inhibition of the domain interaction lowers the

susceptibility of apoE4 to proteolytic cleavage. Further studies have shown that apoE4 lacking the c-terminal 27 amino acids is toxic to cultured neurons, translocates into the cytosol and accumulates in neurofibrillary tangle-like structures into the cytosol and in mitochondria [85, 86].

ApoE and Immunoregulation

As indicated above, apoE can also affect the activation and proliferation of T-lymphocytes. It has been shown that low concentrations of apoE-containing lipoproteins can increase the sensitivity of T-lymphocytes to mitogens and thus their proliferation, by binding to the LDL receptor [87]. However, when these lipoproteins bind to other receptors (immunosuppressive receptor) they suppress T-cell activity [4]. ApoB-containing lipoproteins can also inhibit this process but apoE is four times more efficient [88]. ApoE is able to inhibit both CD4 and CD8 lymphocytes by decreasing the production of interleukin-2 [65].

Several data support that this immunosuppression does not occur through the LDL receptor, but through another receptor that hasn't been identified yet [4]. Two possible candidates for this function are the HSPG and the transferrin receptor and the region that is most likely responsible for this interaction is the LDL receptor- and heparin-binding domains [4, 89].

ApoE and Infectious diseases

Accumulating evidence shows that apoE could be implicated in protecting from infectious agents such as bacteria, viruses and protozoan parasites.

Firstly, apoE knockout mice are more susceptible to bacterial infection than wt mice [90, 91] and this is not due to the hyperlipidemia observed since LDLR knockout mice which are also hyperlipidemic are protected from bacterial infection [92].

Secondly, it is possible that apoE inhibits the binding of several viruses to the HSPG through competition for the binding. Three viruses are known to be able to bind to the HSPG: the herpes simplex virus (HSV), the human immunodeficiency virus (HIV and the Dengue virus). The fact that apoE can inhibit the proliferation of HIV-related Kaposi's sarcoma cells supports the existence of a protective role for apoE [93]. Moreover, HSV-1 is

related with increased risk for Alzheimer's disease and in infected AD patients the ε4 allele is overpresented [94].

Thirdly, apoE has also been implicated in protection against malaria. The malaria circumsporozoite protein enters the hepatocytes through the HSPG/LRP pathway and inhibition of this pathway can prevent the cell invasion [95].

Recently, van den Elzen et al have shown that apoE secreted by antigen presenting cells (APC) is able to bind serum born lipid antigens and deliver them to the APCs by receptor mediated uptake. This represents a novel mechanism by which apoE secreted by the APCs surveys the environment for the presence of antigens or microbial lipids from infected cells [96].

Finally, several data suggest that environmental and other genetic factors most likely interact with the apoE isoforms. Thus, factors such as smoking, obesity, diet, physical activity, gender, alcohol consumption and cholesterol lowering drugs probably contribute to the different effects attributed to the apoE phenotype [9].

B. Apoprotein A-I

ApoA-I and Reverse cholesterol transport

ApoA-I is the principal protein of high density lipoprotein (HDL). It consists of 243 residues which are organized in 22- and 11-amino acid repeats that form mainly amphipathic α-helices (figure 7) [1].

A number of belt-like and hairpin-shape models have been proposed to describe the association of apoA-I to lipids and the formation of discoidal and spherical HDL [97, 98].

The pathway of HDL formation and catabolism is very complex and involves several proteins which interact with apoA-I along different steps of this pathway. Firstly, on the surface of hepatocytes and other cell types the ABCA1 (ATP-binding cassette transporter AI) transporter transfers phospholipids from intracellular membrane pools to extracellular apoA-I where with the addition of more lipids, discoidal HDL is formed. Following esterification by LCAT (lecithin: cholesterol acyltransferase) of free cholesterol, the discoidal HDL is converted to spherical. These particles interact with SR-BI which

mediates selective lipid uptake and efflux of excess cholesterol [3]. Exchange of lipids between HDL and other lipoproteins (VLDL) is mediated by PLTP (phospholipid transfer protein) and CETP (cholesteryl ester transfer protein), whereas lipolysis of residual triglycerides of HDL occurs through the action of hepatic lipase (HL) and endothelial lipase (EL) [1].

More than half of the 46 naturally occurring mutations of apoA-I are associated with low levels of HDL [99].

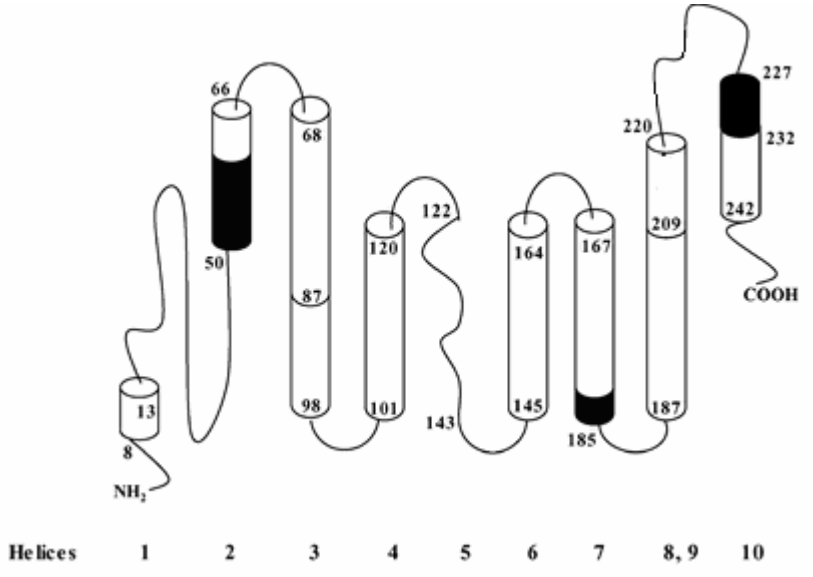


Figure 7: Secondary structure of ApoA-I [1].

Several studies have established that apoA-I has atheroprotective functions. It has been shown that apoA-I and HDL have anti-oxidant and anti-inflammatory function, affect prostacyclin levels, platelet function and nitric oxide production [100]. Moreover, human apoA-I over expression through a helper-dependent adenovirus in LDLR knockout mice placed under atherogenic diet, resulted in more than 50% decrease in the development of atherosclerosis [101]. However, apoA-I knockout mice do not develop atherosclerosis [102].

ApoA-I milano

ApoA-I milano is a natural variant found in the population of a village in northern Italy, close to the city of Milano. Individuals carrying apoA-I milano have low HDL

cholesterol and high triglyceride levels, develop less atherosclerosis than expected and they live longer [103, 104].

ApoA-I milano has a cysteine for arginine substitution at residue 173 in the amino acid sequence [105]. This substitution results in the modification of the amphipathical structure of the helix 7 of apoA-I (figure 7). Moreover, this variant binds phospholipids more efficiently than WT apoA-I which probably accounts for the more effective uptake of tissue lipids [106].

Studies showed that administration of recombinant ApoA-I milano/phospholipid complexes to mice and rabbits with atherosclerosis resulted in reduction of the atherosclerotic plaques [107-110].

The beneficial properties of apoA-I led to the design and execution of a small randomized controlled clinical trial. In this study administration of ETC-216, a complex of apoA-I milano associated with naturally occurring phospholipids that mimics HDL to patients with acute coronary syndrome. This treatment reduced the atheroma volume by 4.2% from baseline. [111].

C. Goal of this study

Previous findings

ApoE knock-out mice exhibit high cholesterol levels and the phenotype could not be corrected by over expression of full length apoE delivered through adenovirus-mediated gene transfer. On the contrary, VLDL triglyceride secretion was increased and the mice developed hypertriglyceridemia. Moreover, hypercholesterolemia was induced in apoE2 knock-in mice infected with apoE3- or apoE4-expressing adenoviruses [3, 112-116]. In addition, normal C57BL6 mice infected with adenovirus expressing full length apoE exhibited high cholesterol and triglyceride levels [114].

Adenoviruses expressing apoE forms with different deletions of the carboxy-terminal domain corrected the high cholesterol levels observed in the apoE knock-out and apoE2 knock-in mice and did not alter the cholesterol and triglyceride levels of normal C57BL2 mice [3, 112-116]. Further experiments in apoE and LDLR double knock-out mice showed that the truncated forms of apoE although they did not correct the elevated cholesterol

levels, they did not cause high triglycerides. These results point out that the carboxy-terminal domain of apoE is responsible for the hypertriglyceridemia [3, 113]. This region extends from residue 260 to 299 and contains two sections (between residues 261-269 and 276-283) with hydrophobic amino acids [3, 112-117].

Recently, Kypreos et al, created two adenoviruses expressing mutant apoE forms, termed apoE4-mutant1 and apoE4-mutant2, containing substitutions of residues of both sections to alanines and studied their functions in vivo in apoE$^{-/-}$ mice. The adenovirus expressing ApoE4-mut1 (L261A, W264A, F265A, L268A and V269A) corrected the plasma cholesterol profile and did not cause hypertriglyceridemia, whereas AdGFPApoE4-mut2 (W276A, L279A, V280A and V283A) had the opposite effects. Moreover, WT apoE4 and apE4-mut2 displaced apoA-I from HDL and resulted in the formation of discoidal HDL particles, while apoE4-mut1 did not affect the presence of apoA-I in the HDL region and produced spherical particles [117].

Goal

As indicated earlier, apoE has atheroprotective functions and constitutes a very prominent target for the development of therapeutic strategies. Moreover, it is considered more effective protector than apoA-I since apoE null mice develop atherosclerosis. Additionally, the recent production from Kypreos et al of an apoE variant with improved biological properties is an important discovery of a prominent therapeutic tool. This apoE form named apoE4-mut1clears apoE from apoE$^{-/-}$ mice without induction of hypertriglyceridemia and promotes the formation of apoE-containing HDL[113, 117]. Based on these findings the present work aims to produce large amounts of the apoE4-mut1 protein and use it to generate proteoliposomes which will be injected in apoE deficient mice. The ultimate goal is to determine the effects of this mutant protein to the development of atherosclerosis. Whether it prevents the development of atherosclerotic lesions or is able to promote their regression.

Furthermore, a similar work was performed by Shah et al using proteoliposomes containing the apoA-I milano protein and showed that the progression of aortic atherosclerosis was prevented and the presence of lipids and macrophages in plaques was significantly reduced in apoE deficient mice [108].

Parallel experiments using apoE4-mut1 and apoA-I milano will be performed in order to compare the effects of both proteins on the protection from or regression of atherosclerosis.

II. MATERIALS AND METHODS

Expansion, purification and titration of adenoviruses:

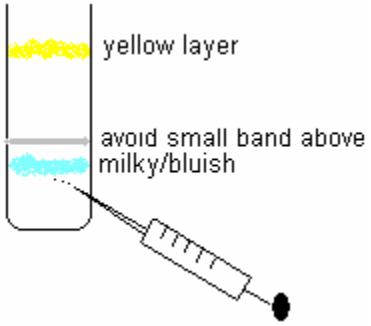
Plating triples: HEK293 (Human Embryonic Kidney) cells in T175 flasks are grown to confluence. The medium is removed and cells are washed with 4ml of D-PBS (Dulbecco's Phosphate-Buffered Saline) per flask. Then, 4ml of trypsin are added and cells are incubated for 5min at room temperature. Following, 6ml of complete medium (Leibovitz's L-15 medium 10% FBS-Fetal Bovine Serum, 1% Penicillin / Streptomycin) are added and cells are mixed thoroughly to break the cell clumps. The content of 2.5 flasks is transferred into one bottle of complete medium which is then poured into five triple flasks. Cells must be incubated at 37^{o}C for 4-5 days to reach approximately 70% confluence (no CO_2).

Infection of triple flasks: For five triple flasks 1lt of medium (Leibovitz's (L-15) with 2% Heat Inactivated Horse Serum-HIHS and 1% P/S) is prepared by adding as much adenovirus stock as necessary to have a multiplicity of infection of 5 (MOI: number of plaque forming units per cell). Then, the complete medium in the triple flasks is replaced by new medium containing the virus. The infected cells are then incubated at 37^{o}C for 2-3 days.

Harvesting triple flasks: When all cells have been infected they are collected in 500ml (for five flasks) centrifuge tubes and spun for 10min at 1000rpm. The supernatant is removed and the precipitate is resuspended in 2ml of medium, then transferred to 50ml falcon tubes and stored at -80^{o}C.

Virus purification by CsCl gradient: The harvested pellet must be frozen (dry ice for 30min) and thawed three times (37^{o}C water bath for 10min) and then spun for 10min at 3000rpm in a Beckman CS6 centrifuge. During the spin two ultra clear Beckmann centrifuge tubes are prepared by carefully overlaying 2ml of CsCl 1.379 density (0.619g/ml in TE pH 8.0) followed by 5ml of CsCl 1.353 density (0.277g/ml in TE Ph 8.0). When the spin is over the adenovirus supernatant is removed and each tube is overlaid with an equal volume. Then, the samples are spun in a Beckmann ultracentrifuge for 90min at 30.000rpm at 4^{o}C using the SW41 swinging bucket rotor.

When the spin is over different bands are formed. The one containing the virus is a milky bluish layer about 1" from the bottom of the tube.



Before the spin has ended one quick seal Beckmann tube is prepared by adding 2/3 of CsCl 1.363 density (0.450g/ml in TE pH 8.0). Using a 5ml latex free syringe with an 18g needle the band is removed and transferred into the quick seal tube. Following, more CsCl 1.363 density is added in the tube until half of the tube's neck is filled. The tube must then be sealed using a Beckman tube sealer and placed in the Ti70 Beckmann rotor and spun for 18h or more at 55.000rpm at 4^{o}C. The lower band formed contains the virus and is removed again with a syringe and inserted into a slide-a-lyzer dialysis cassette (PIERCE 10000MWCO, volume 0.5-3ml). The virus preparation is dialyzed in dialysis buffer (0.137M NaCl, 5mM KCl, 0.73mM Na_2HPO_4, 25mM Tris-Base, 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$, pH 7.8) with one change after two hours and another one after 18h. Two hours after the last buffer change the dialysis cassettes are transferred to a 5% glucose buffer for 3h. Finally, the virus preparation is aliquoted and stored at -80^{o}C.

Virus titration: In a six-well plate, 1*10^{6} 911 (Human Embryonic Retinoblastoma) cells in DMEM-10% FBS medium are grown to confluence. Serial dilutions are prepared in the following way: 20µl of the purified adenovirus stock are transferred in 1ml of medium (L-15, 2%HIHS and 1% P/S). From this dilution (1/50) 10µl are transferred to 1ml of medium in a second tube (1/5000 dilution), 10µl are taken from the latter and mixed with 1ml of medium in a third tube (5*10^{-5} dilution). In the same way dilutions 5*10^{-6}, 5*10^{-7}, 5*10^{-8} and 5*10^{-9} are prepared. The highest four dilutions are used to infect the 911 cells which are then incubated at 37^{o}C for 20min. Meanwhile, a mixture of one volume of a 1.5% agarose solution (1.5% agarose and 0.04M Hepes pH 7.4) and one volume of medium (2xMEM, 0.025M $MgCl_2$ and 4% HIHS) is prepared and placed at

55°C. Next, the infection media is removed from the six-well plate and is replaced by 2-3ml of the prepared agarose solution. The plates are incubated at 37°C for several days until plaques are visible either under the microscope that detects the fluorescence of the GFP (AdGFP) or by eye. Then the plaques from the highest dilution are counted and the titer in plaque forming units (pfu) per ml is calculated by the following equation:

$$\textbf{Titer}\ (\text{pfu}/\mu\text{l}) = \frac{\text{Number of plaques at the highest dilution}}{20} * 1 / \text{dilution}$$

Protein Production by Large-Scale Growth of HTB-13 cells in Roller Bottles:

Plating and growing HTB-13 cells in 1750cm^2 roller bottles: HTB-13 (Human Astrocytoma) cells in four T175 flasks are grown to confluence in complete medium (Leibovitz's L-15 medium 10% FBS-Fetal Bovine Serum, 1% Penicillin / Streptomycin). Then, they are transferred to four 1750cm^2 roller bottles with expanded surface and grown to confluence in 100ml of complete medium with medium changes every five days. When confluence is reached, cells are transferred to twenty 1750cm^2 roller bottles in 100ml of complete medium. The cells are incubated at 37°C and the medium is renewed after seven days and incubated for additional four to five days. The cells are then ready to be infected.

Infection of HTB13 cells for protein production: The complete medium is replaced by 50ml of Leibovitz's L-15 medium with 2% HIHS and 1% P/S. Adenovirus is added in each roller bottle in order to have a multiplicity of infection (MOI) of 20 for AdGFPapoE4-mut1 and 30 for AdGFPapoA-I milano or AdapoA-I milano. The volume of virus used to infect each roller bottle is calculated by the following equation:

$$\mathbf{V} = \frac{\text{MOI} * \text{Number of cells}}{\text{Titer}}$$

(Number of cells per confluent roller bottle: $1.2 * 10^8$)

After 24h the infection medium is removed, cells are washed with 100ml of D-PBS / roller bottle and are incubated in 50ml of serum-free medium (Leibovitz's L-15 medium with 1% Penicillin / Streptomycin).

Harvesting: After 24h the medium is harvested using sterile vacuum flasks and cells are incubated for another 24h in 50ml of fresh medium. The harvested medium is then transferred to two 500ml centrifuge tubes and spun for 10min at 1000rpm. Next, the supernatant is filtered with 0.2μM pore size filters and stored at -80^{o}C. This process is repeated for six days for apoE4-mut1, while for apoA-I milano two harvests are performed the first day with a 12h interval and one for each of the two following days. To evaluate the protein production 20μl of medium from each roller bottle are analyzed by SDS-PAGE (13%) and Coomassie staining. For the first harvest, samples from each roller bottle are analyzed, whereas for the next harvests every two roller bottles are tested.

ApoE4-mut1 purification through Heparan-Sepharose ion-exchange chromatography: The protein purification is performed with an Acta Purifier from Amersham Biosciences. The harvested media from each day is loaded on a Heparan-Sepharose column (98ml) and the protein is eluted in several fractions. The column is first equilibrated with buffer A (20mM Tris-HCl, 0.2M NaCl, 0.01% Sodium Azide, pH 7.4) (10ml/min) for one column volume (CV). Then, the medium is loaded on the column at a speed of 5ml/min followed by a one CV wash of buffer A. The continuous gradient elution begins with 100% buffer A and ends with 100% buffer B (20mM Tris-HCl, 1M NaCl, 0.01% Sodium Azide, pH 7.4) until three CVs have passed through the column. Another 100ml of buffer B passes in order to clean any amount of protein that remained attached to the column. Finally, the column is washed with two CVs of buffer B followed by three CVs of buffer A (10ml/min). Totally, 98 fractions of 5ml are collected

ApoE4-mut1 elution analysis by SDS-PAGE and Coomassie staining: Every three consecutive fractions are pooled and 20μl are loaded on a gel for SDS-PAGE analysis. The first gel run has shown that apoE4-mut1 is eluted between fractions 43 and 81 so for the following purifications only those fractions were tested. The fractions containing the pure protein are then pooled together and dialyzed.

Dialysis: 25000MWCO membrane tubes are used for the dialysis against ammonium bicarbonate (NH_4HCO_3). The pooled fractions from each elution were dialyzed in 3.6lt of 0.05M NH_4HCO_3 for two days during which the buffer was renewed three times.

Lyophilization: The dialyzed medium was then frozen in aliquots of 30ml in 50ml falcon tubes and lyophilized. The tubes were then stored at -80^{o}C.

ApoA-I milano purification by ion-exchange chromatography: The protein purification is performed using a classic FPLC system from Pharmacia. The harvested media is dialyzed against buffer A (0.01M Tris-HCl pH 8.0) for two days with three buffer changes using a 12000-14000 MWCO membrane. It is then loaded on the equilibrated column with 2.5ml/min. The column used is a HiTrap Q FF-5ml column and is equilibrated by passing through 5ml/min buffer A for 5min. After the medium is loaded, five more volumes (25ml) of buffer A pass through the column (1ml/min) and the continuous gradient elution begins. It starts with 100% buffer A and ends with 100% buffer B (1M NH_4HCO_3) until 15 column volumes (75ml) are passed through (1ml/min). Finally, the column is washed with 25ml of buffer B (1ml/min). Totally forty fractions containing 3ml were collected.

ApoA-I milano elution analysis by SDS-PAGE and Coomassie staining: The first gel run showed that the protein is eluted between fractions 11-34, so 20μl from each fraction are loaded on a gel for SDS-PAGE analysis.

ApoA-I milano purification through ethanol precipitation: One volume of eluted sample is mixed with one volume of 100% ethanol and frozen in dry ice with 95% ethanol for 2h. Then the mix is thawed and spun at 14.000rpm for 10sec. Following, the precipitate is dried and resuspended in 50μl 0.15M NH_4HCO_3. To check the success of the method, 20μl from a 1 to 20 dilution of the resuspension mix is analyzed by SDS-PAGE.

Medium concentration:

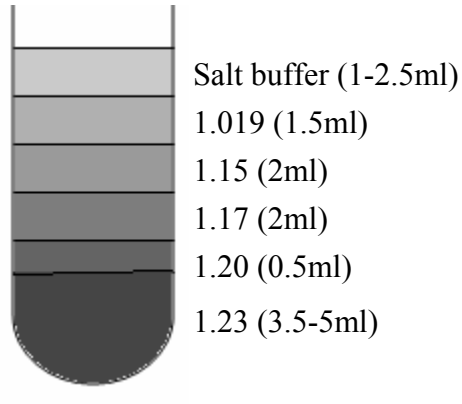
By Filtering: The harvested media containing apoA-I milano was extensively dialyzed against salt buffer (10mM Tris-HCl pH 8.0, 0.15M NaCl, 0.01% EDTA) for two days with three buffer changes. The medium was then split to four Millipore/Amicon Centricon plus 70 centrifugal filters that retain molecules with MW > 10000KDa and was concentrated to 4ml by centrifugation at 3000rpm.

By Lyophilization: The harvested medium containing apoA-I milano was extensively dialyzed against 4lt of 0.05M NH_4HCO_3 for 2 days with three buffer changes using 12000-14000MWCO dialysis tubing. Then it was filtered and frozen in 900ml lyophilization flasks. After lyophilization the protein was stored at -80^oC.

Proteoliposome formation: In a 30ml COREX tube, DPPC (1,2 Dipalmitoyl-sn-Glycero-3-Phosphocholine) (150moles DPPC for 1mole of protein or 4.4043mg of DPPC for every mg of protein) are dissolved by adding a chloroform:methanol (2:1 ratio) solution so that their final concentration is 20mg/ml. The solution is then dried under N_2 and 250μl of salt buffer (10mM Tris-HCl pH 8.0, 0.15M NaCl, 0.01% EDTA) per mg of protein are added. Next, the tube is placed at 44^oC and mixed by vortexing at small intervals. When the solution becomes uniformly cloudy (10-20min) sodium cholate is added (51μl from 30mg/ml stock solution per one mg of protein) and the mixture after it has been covered with N_2, is placed at 44^oC for 16-20h. Lyophilized protein is then diluted in salt buffer (final concentration 5mg/ml) and mixed with the proteoliposomes solution. The final protein concentration must be 2mg/ml. The tube is covered again with N_2 to prevent oxidation and is placed back at 44^oC for 1h. Finally, the mixture is dialyzed against salt buffer using a 50000MWCO dialysis membrane, for two days with three buffer changes. The proteoliposomes are then stored at 4^oC under N_2.

Density gradient ultracentrifugation (for ApoA-I milano): The proteoliposomes solution (2mg/ml) is mixed with KBr so that the KBr density is 1.23 and placed at the bottom of a 12ml, 14*89mm Beckmann tube (max 8mg of proteoliposomes per tube). Subsequently, layers of decreasing KBr density are overlaid as shown in the following

figure and the gradient is spun in a Beckmann ultracentrifuge for 26-30h at 30000rpm at 4°C using the SW41 swing bucket rotor.



The amount of KBr used to obtain specific density is calculated through the following equation:

$$\text{KBr (g)} = \frac{\text{Volume (density-1)}}{\text{1-(0.298 * density)}}$$

At the end of the spin fractions of 1ml each are removed from the top of the gradient and transferred to a glass tube. Approximately 12 fractions are collected. An aliquot of 100µl from each fraction is dialyzed separately against EM buffer (0.125M NH_4COOH, 2.6mM $(NH_4)_2CO_3$, 0.26mM tetra sodium EDTA pH 7.4) and stored for Electron Microscopy analysis. The refractive index of each sample is then measured using an ABBE refractometer from American Optical Corp. and their density is determined based on a calibration curve. The fractions are then dialyzed against salt buffer with three buffer changes every 12h. An aliquot of 10µl of each fraction is analyzed by SDS-PAGE and the fractions containing the proteoliposomes are pooled and stored at 4°C. The concentration must be 2mg/ml. The proteoliposome fractions are then concentrated by centrifugation in centrifugal filters that retain molecules with MW>10000KDa, to achieve 2mg/ml concentration.

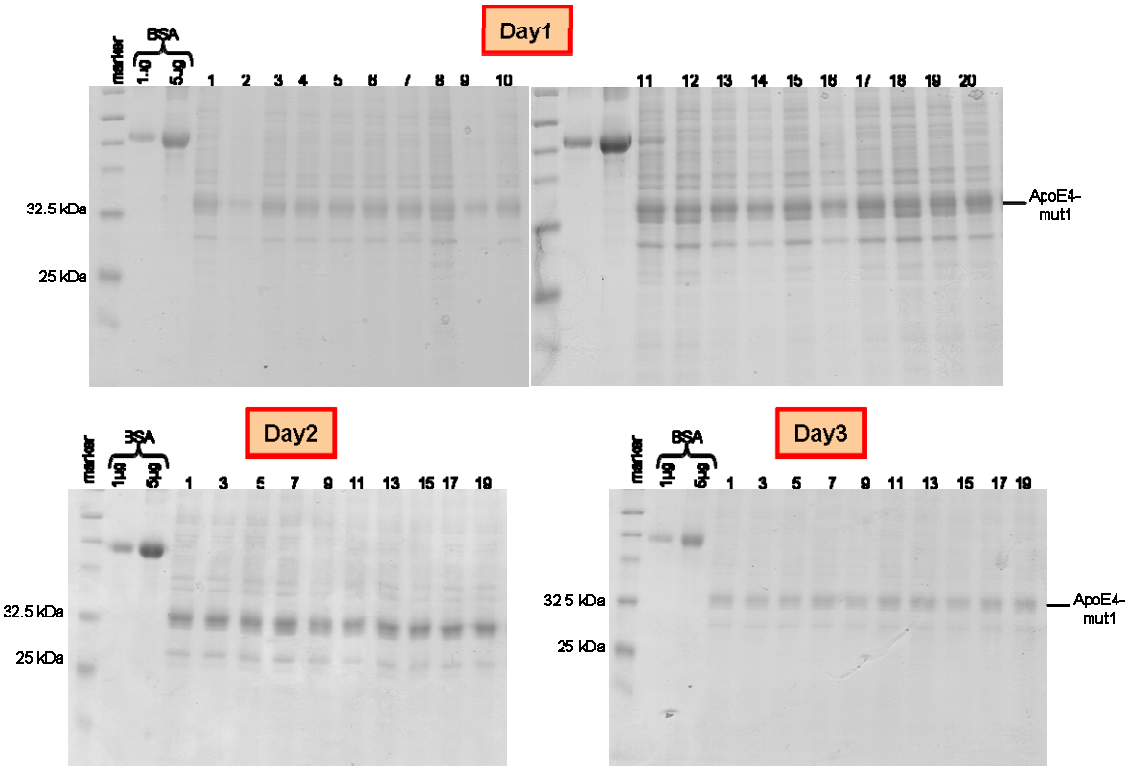
Animal studies: 1) Forty female 10-week old apoE deficient mice were placed on atherogenic diet (standard western diet). After six weeks ten mice were sacrificed to determine the atherogenic lesions (baseline), ten mice were injected intravenously through the tail vein with 1mg of proteoliposomes containing apoE4-mut1, ten other mice were injected only with liposomes and ten more mice didn't receive any treatment. The injections were repeated every two days for five weeks while the mice remained on atherogenic diet. At the end of the treatment the mice were sacrificed in order to measure the development of atherogenic lesions. 2) A duplicate experiment was performed with the only difference that after the six weeks of atherogenic diet the mice were swiched back to normal (chow) diet.

III. RESULTS

A. ApoE4-mut1

- *Protein production*

As described in the materials and methods section, HTB13 cells grown to confluence in 20 roller bottles were infected with a virus expressing apoE4-mut1 (AdGFP-ApoE4-mut1) at a multiplicity of infection 20. The medium was collected for six consecutive days with the amount of produced protein decreasing as days passed (figure 8). This procedure was repeated several times. Each batch (set of 20 roller bottles) produced in average 100mg of apoE4-mut1. Totally, ten batches were set up and approximately 1000mg of protein was collected.



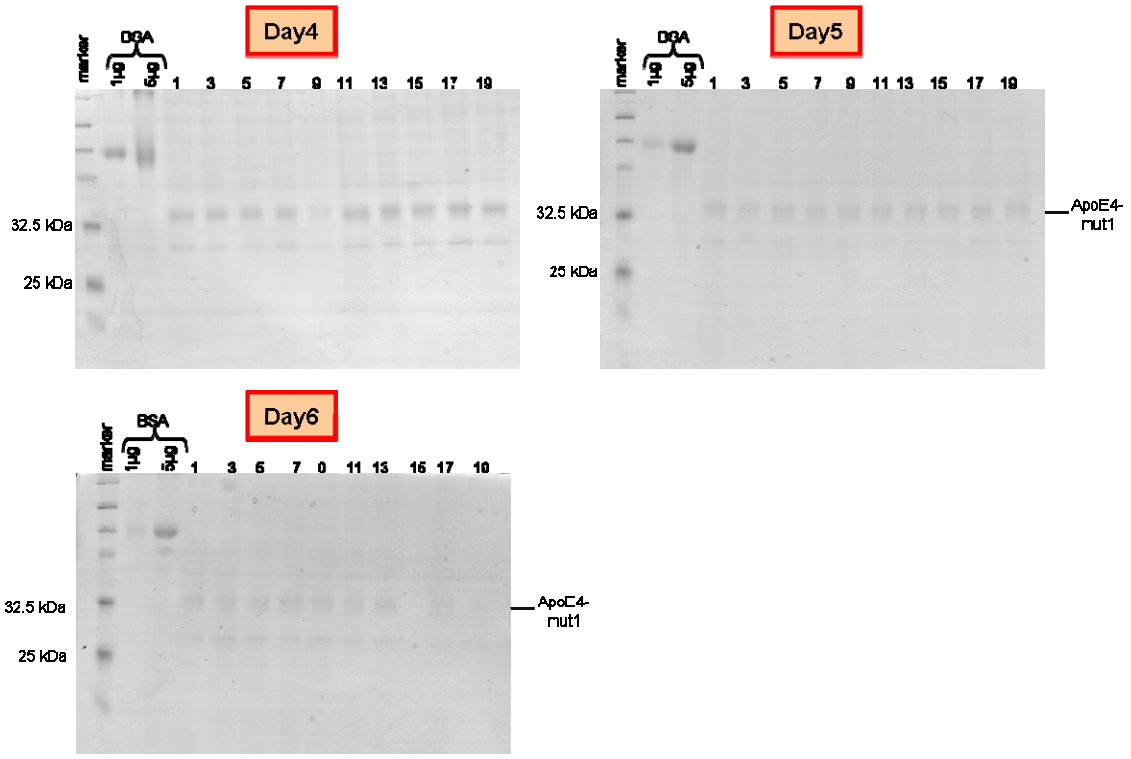


Figure 8: ApoE4-mut1 production. Numbers 1 to 20 indicate each roller bottle. BSA standards of 1µg and 5µg are used to calculate the amount of protein in each lane. For example in day1 by comparing the bands corresponding to apoE4-mut1 from each roller bottle with the BSA we can say that the 20 fractions contain approximately 1µg of protein in average and since 20µl of sample have been loaded in each well, the concentration of the protein harvested is 0.05mg/ml. In 1lt of harvested medium a total of 50mg of protein is collected.

- *Protein purification*

The protein collected was then purified by ion exchange chromatography. The yield was estimated by SDS-PAGE. The total protein eluted in each batch was approximately 100mg. For example in Day1 by comparing again the bands corresponding to the eluted protein with the BSA standards the amount of protein in average is estimated to be approximately 5µg per lane. Since 20µl of sample have been loaded in each well the concentration of the protein is 0.25mg/ml. The nine wells that were selected correspond to 27 fractions of 5ml each. Thus the total protein eluted is 33.75mg. These estimations were also verified by Lowry.

Almost all the protein was eluted relatively pure. Figure 9 demonstrates the elution profile of an entire batch.

Furthermore, the fractions containing the purified protein were collected, pooled and lyophilized.

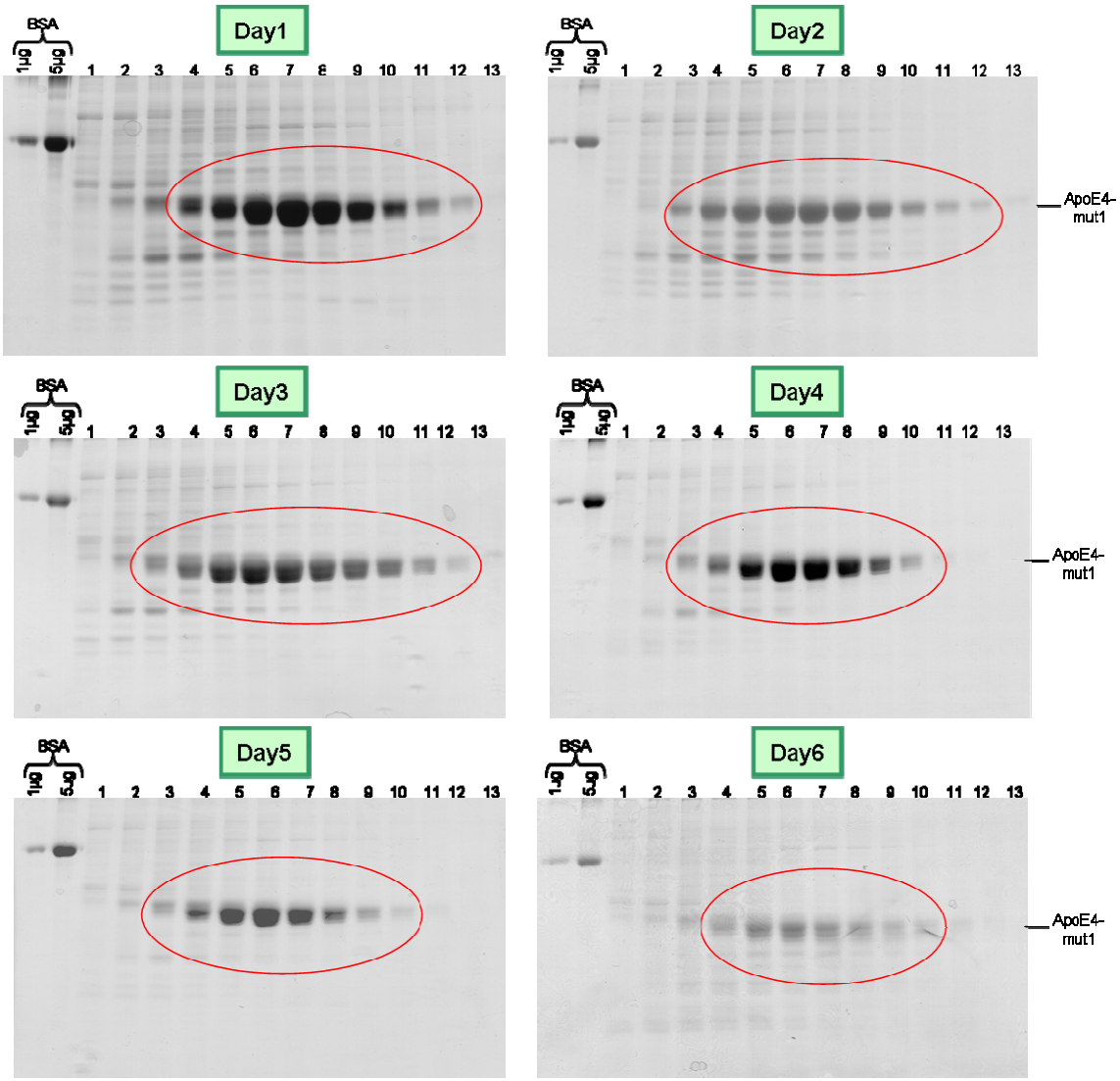


Figure 9: ApoE4-mutt1 elution profile. Purification by ion-exchange chromatography. Each number from 1 to 13 corresponds to three consecutive eluted fractions pooled together. The first fraction analyzed is fraction 43 and the last one is 81. Circled in red are the fractions containing the purified apoE4-mut1.

- *Proteoliposome formation*

In order to inject apoe4-mut1 into the mice, the protein had to be associated with DPPC liposomes). Proteoliposomes were prepared as described in the experimental procedures. Figure 10 shows that the particles formed contain apoE4-mut1 and that the solution that will be injected does not contain any other proteins. In addition, EM analysis revealed that the particles were discoidal.

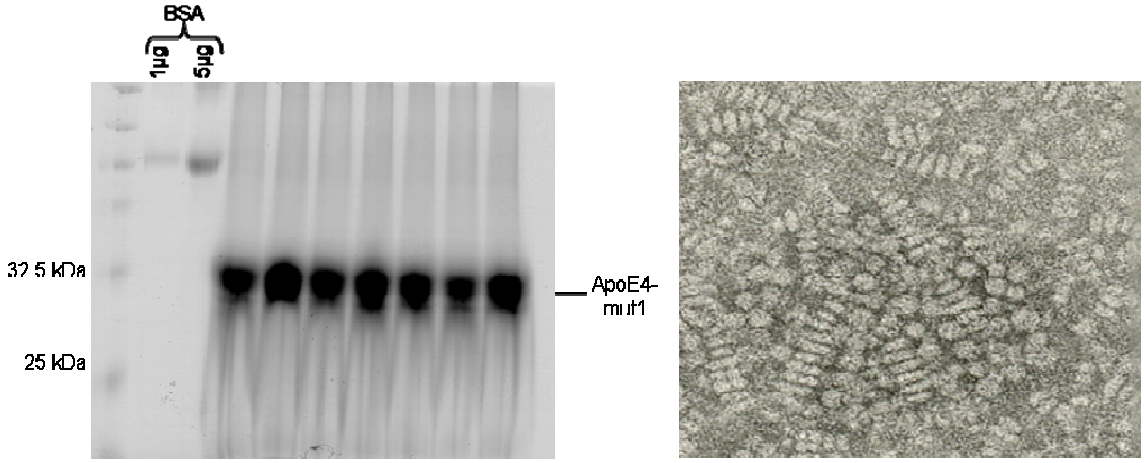


Figure 10: A) Analysis of the proteoliposomes by SDS-PAGE. An aliquot of 10µl from different proteoliposome preparations were loaded in each well. Compared to the BSA standards each lane contains approximately 20µg of particles and the proteoliposome concentration is 2mg/ml.B) Analysis of proteoliposomes by EM analysis.

Totally 240mg of proteoliposomes were generated. ApoE-/- mice fed with atherogenic diet were injected with 1mg of the preparation every two days (see material and methods). After five weeks of injections the mice were sacrificed and their aortic arch and the subclavian and carotid arteries were prepared for sectioning. The evaluation of the development of atherosclerosis through oil red-O-staining is still in process.

B. ApoA-I Milano

In contrast to the manipulations of apoE4-mut1, with apoA-I milano we run into major obstacles starting with the quantity of protein produced, its purification and finally with the proteoliposomes formation.

- *Adenovirus containing GFP*

As described in the materials and methods section, 20 roller bottles containing HTB13 cells were infected with the adenovirus AdGFP-apoA-I milano at a multiplicity of infection 25. Medium was collected for five days. The amount of protein produced wasn't satisfying and the cells stopped producing apoA-I milano after the third day. Moreover, it seems like the cells were undergoing lysis since GFP appeared on the polyacrylamide gel (Coomassie staining) with increasing quantity as days were passing (figure 11).

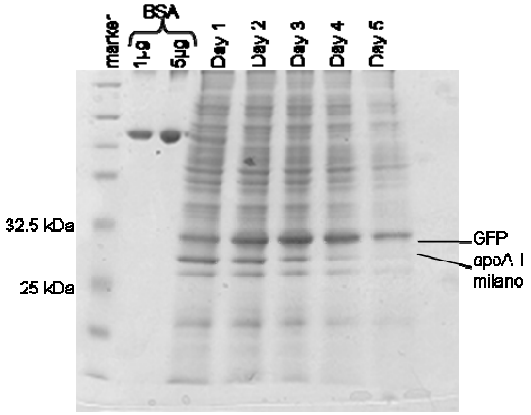


Figure 11: ApoA-I milano production for five consecutive days. Cells were infected with AdGFP-ApoA-I milano. Each lane corresponds to one day of harvest. One harvest per day.

Therefore, it was decided that media would be collected twice the first day of harvest with an interval of 8 hours and once the next two days. The quantity of protein produced was approximately 25mg/harvest as evaluated by Lowry assay and SDS-Page (figure 12). To overcome the cell lysis and degradation problem we provided the cells with anti-oxidants, vitamin C and DTT but the outcome was the same. We next tried to infect two

different cell lines the CHO (Chinese Hamster Ovary) and HeLa (Henrietta Lacks) but cell lysis was still observed.

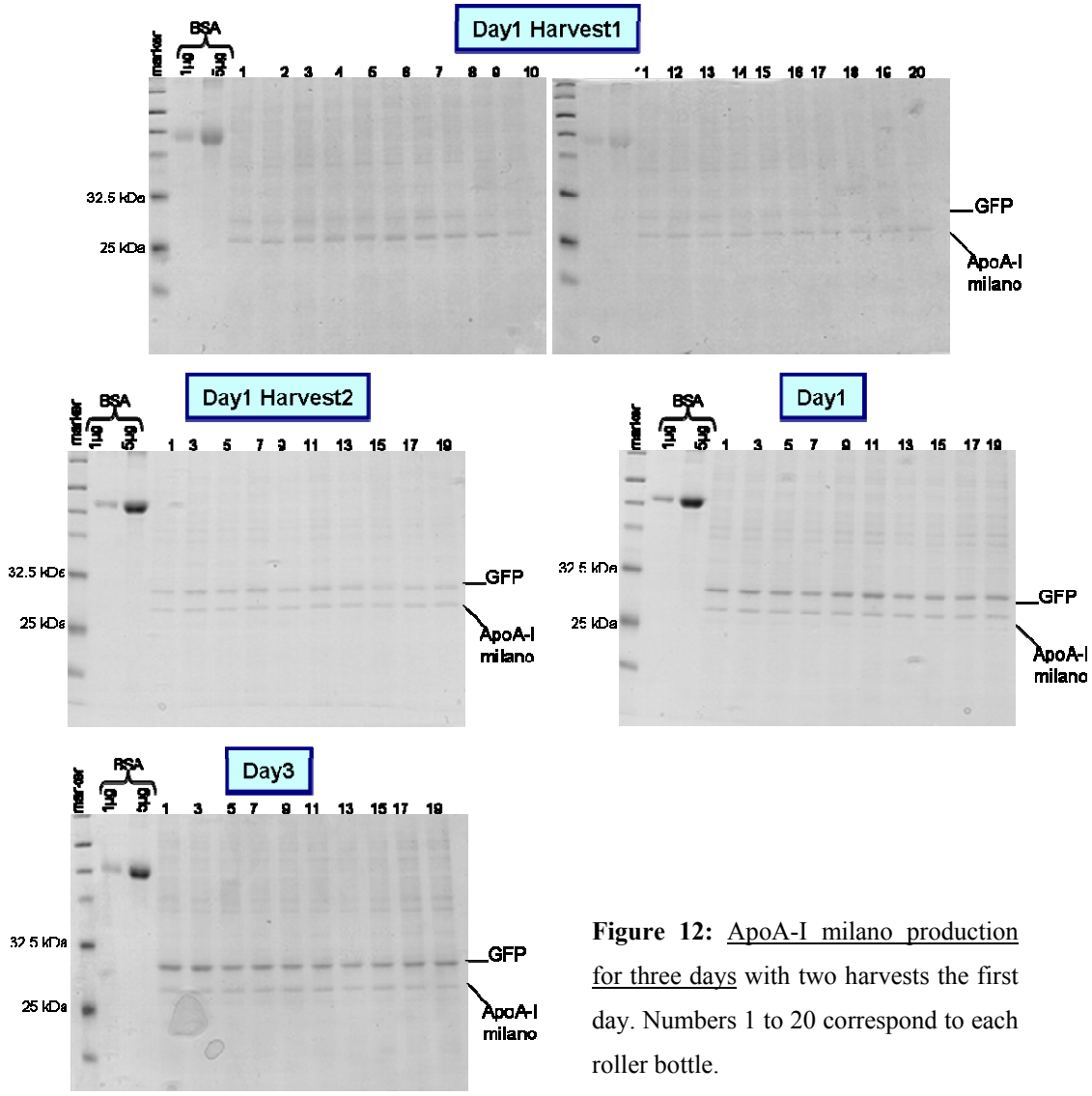


Figure 12: ApoA-I milano production for three days with two harvests the first day. Numbers 1 to 20 correspond to each roller bottle.

The following step was the purification of the medium from each harvest (1Lt/harvest) through ion-exchange chromatography. During the elution almost all the protein was lost, probably retained in the column or degraded. Furthermore, GFP was eluted in the same fractions as apoA-I milano so further purification was necessary (figure 13). Figure 13 shows that apoA-I milano and GFP are eluted between fractions 9 and 14. Using the BSA as standard, the amount of protein present in each lane of the gel can be calculated. Thus,

these six fractions contain approximately 1.3µg of protein in average (1µg in fraction 9, 2 µg in each of the fractions 10 to 12 and 1µg in both the remaining fractions) and since

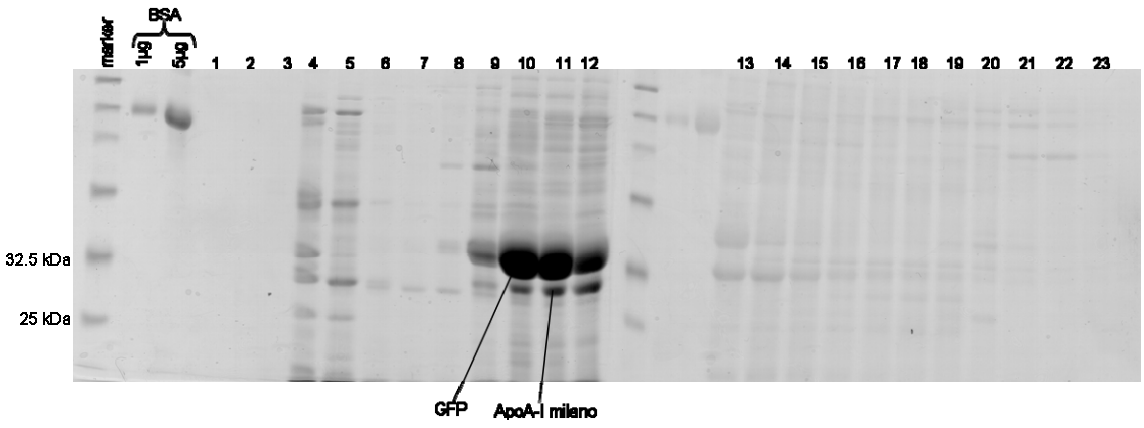


Figure 13: ApoA-I milano elution profile. Purification by ion-exchange chromatography.

20µl of sample have been loaded in each well, the concentration of the protein in all the fractions is 1.3 / 20 = 0.06mg/ml. Taking into account that every fraction has a volume of 2ml, the total purified protein is 0.8mg.

Next, in order to separate the protein of interest from GFP, ethanol precipitation was performed. The fractions containing the most protein (10, 11 and 12) were precipitated with ethanol in a 1:1 ratio. This method was not successful either since GFP precipitated with apoA-I milano (figure 14)

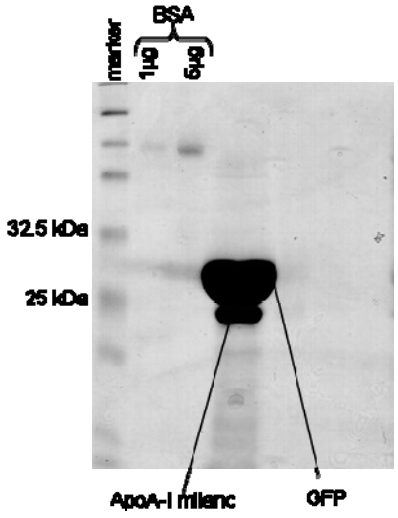


Figure 14: ApoA-I milano purification through ethanol precipitation.

- *Adenovirus without GFP*

In order to overcome the obstacle of GFP, an adenovirus expressing only apoA-I milano without GFP was generated. HTB13 cells were infected with the new virus at a multiplicity of infection 25 and the protein was harvested as described previously.

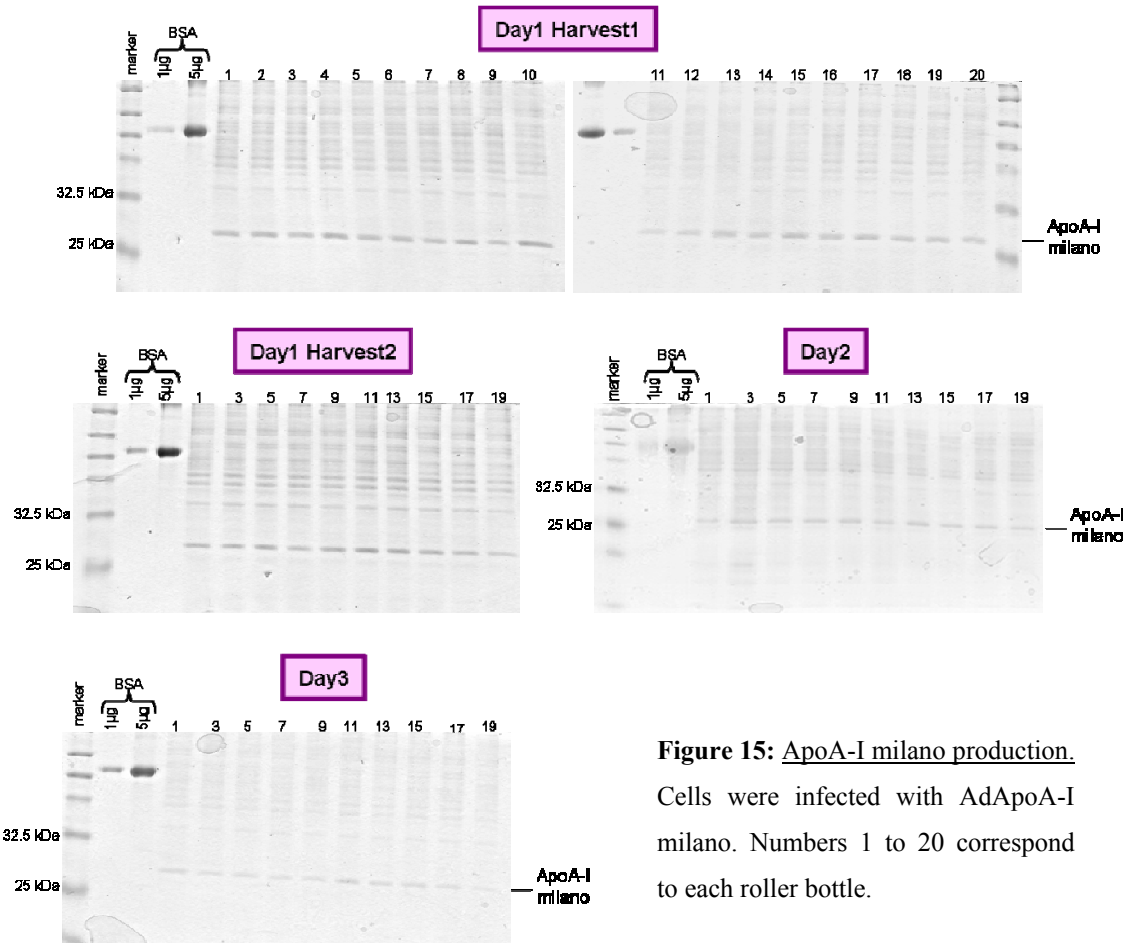


Figure 15: ApoA-I milano production. Cells were infected with AdApoA-I milano. Numbers 1 to 20 correspond to each roller bottle.

Figure 15 shows clearly that apoA-I milano production diminishes progressively 2 and 3 days post infection. Thus, the first two harvests give about 20mg each and the harvests of day2 and day3 give less than 10mg each.

Next, the harvested media was dialyzed as described in the experimental procedures and loaded on the ion-exchange chromatography column in order to purify apoA-I milano. During the elution the majority of the protein was lost and impurities were still present in

the fractions. The following figure shows the elution profile of the first and last harvests (figure 16).

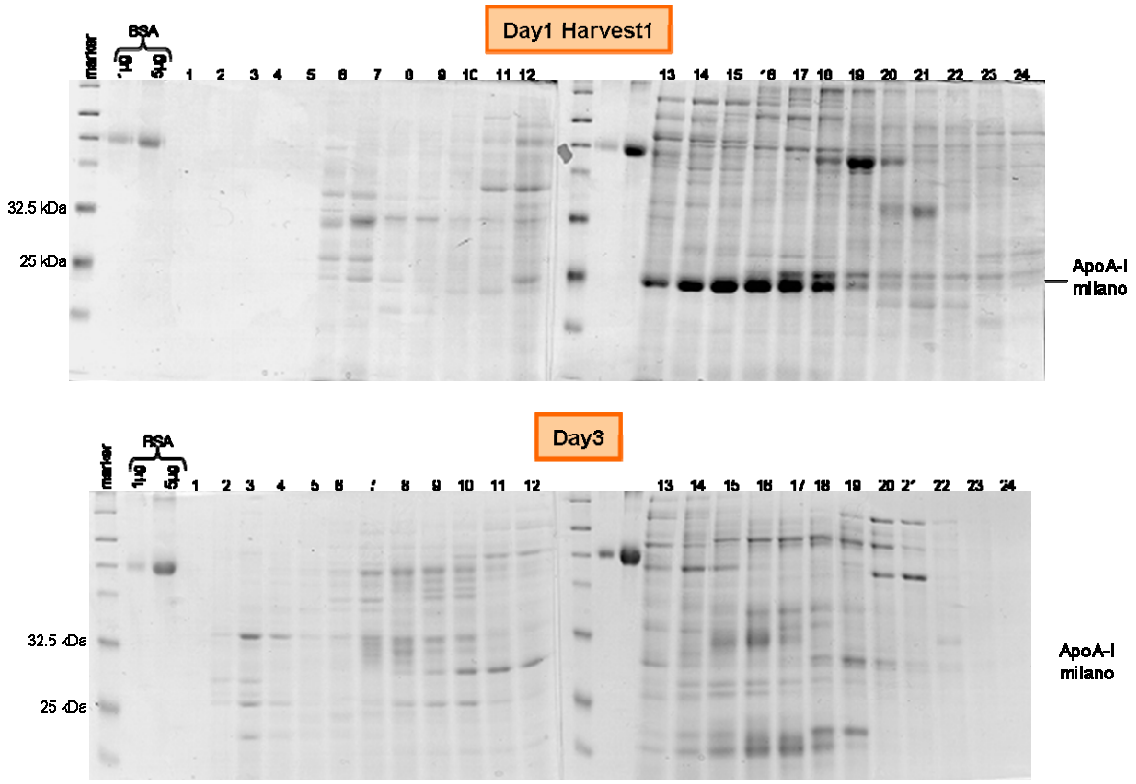


Figure 16: ApoA-I milano elution profile. Purification by ion-exchange chromatography. Numbers 1 to 24 correspond to fractions 11 to 34. A) Purification of the first harvest, B) purification of the last harvest

Following these results the purification through ion-exchange chromatography was abandoned.

The next method that was tested was the concentration by centrifugation of the harvested media through Millipore/Amicon Centricon plus 70 centrifugal filters. Thus, each liter of medium containing the protein from the daily harvest was concentrated to 4ml. Through this process approximately 1/4 of the produced protein was recovered which still is very little. The amount of protein after concentration is shown in figure 17. A small fraction of the concentrated media was diluted 10 times and 1, 5 and 10µl were loaded on the SDS PAGE (figure 17).

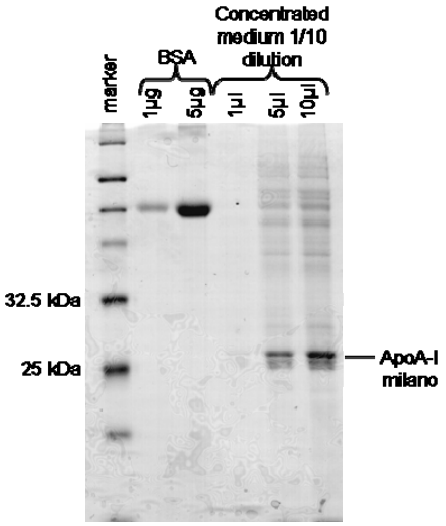


Figure 17: Quantification of ApoA-I milano after concentration of the media through centrifugation.

- *Proteoliposomes formation*

The concentrated medium contains not only apoA-I milano but several other proteins ("impurities") too. An alternative purification method that was chosen was the generation of apoA-I milano-containing proteoliposomes and subsequent flotation through density gradient ultracentrifugation. It is expected that during this process only apoA-I milano will form proteoliposomes and that the other proteins contained in the concentrated

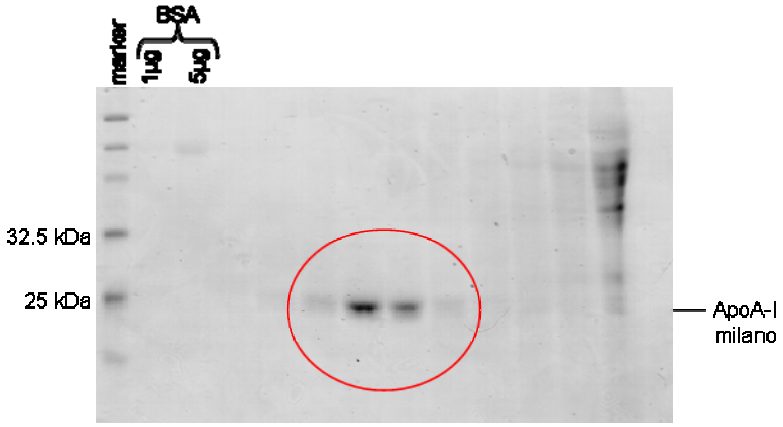


Figure 18: SDS-PAGE showing the fractions from the density gradient ultracentrifugation. The bottom fractions containing the "impurities" are on the right side of the figure and the upper fractions containing apoA-I milano are circled in red. An aliquot 20µl from a 1/20 dilution of each fraction were loaded in each well. Each of the bands circled in red corresponds to approximately 1.625µg of protein in average. Thus the total amount of proteoliposomes contained in these four fractions of 1ml each is 6.5mg.

medium will not. The apoA-I milano-proteoliposomes due to their large size are expected to float in the upper fractions.

Indeed, apoA-I milano appeared in the upper fractions of the gradient, whereas the free proteins remained at the lower fractions (figure 18). Moreover the particles formed were discoidal as revealed by the EM analysis (figure 19).

The problem that still remained to be solved was the important loss of protein during the concentration of the media. Therefore, a new way of concentration was adopted. The harvested medium was extensively dialyzed against ammonium bicarbonate and then lyophilized. To evaluate the efficiency of this method, the protein obtained from the lyophilization of 500ml medium was resuspended in 2ml 1x salt buffer (see materials and methods) and quantified by SDS-PAGE.

Figure 19: EM analysis of the discoidal particles containing apoA-I milano

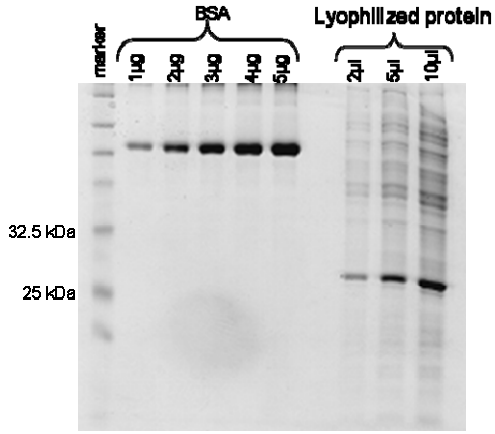


Figure 20: Quantitation of apoA-I milano after concentration of the media by lyophilization. Lyophilized protein from 500ml of medium was resuspended in 2ml of 1x salt buffer. 2µl, 5µl and 10µl from a 1/20 dilution were loaded on the gel and compared to different amounts of BSA (1-5µg). A total of 8mg of protein were recovered.

The recovery of protein was better than with any other method tested. From 12.5mg of apoA-I milano contained in 500ml of medium from the first harvest we recover 8mg of protein (figure 20).

The next step was to separate apoA-I milano form the rest of the proteins contained in the medium by formation of proteoliposomes and flotation. ApoA-I milano appeared in the upper fractions of the gradient as shown in figure 21 and the particles formed were discoidal.

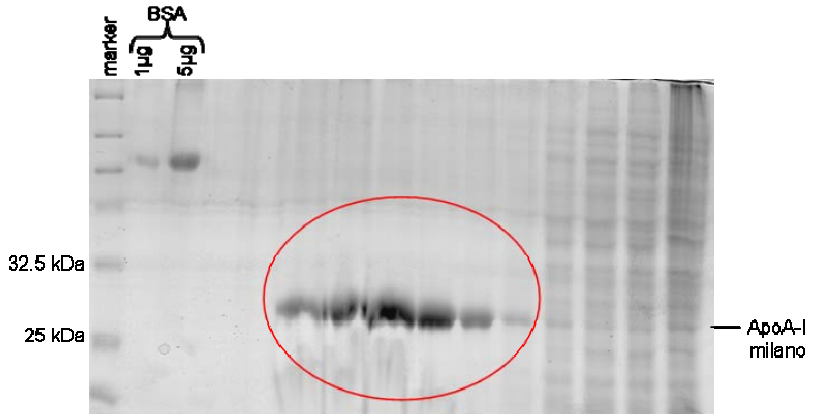


Figure 21: Fractions of the density gradient ultracentrifugation. The red circle indicates the upper fractions containing apoA-I milano. An aliquot of 20μl from a 1/20 dilution of each fraction were loaded in each well. Each of the bands circled in red corresponds to approximately 2μg of protein in average. Thus the total amount of proteoliposomes contained in these six fractions of 1ml each is 12mg (2mg/ml).

Since this method seemed to have positive results we proceeded in the resuspension of more protein and the formation of a larger quantity of proteoliposomes. Thus, 54mg of protein were processed to form proteoliposomes. Surprisingly, the experiment worked only partially since apoA-I milano was not concentrated in the upper fractions. On the contrary, it was dispersed in upper and lower fractions. Probably, the gradient that was used needs to be changed since apparently it is only appropriate for small amounts of protein (figure 22).

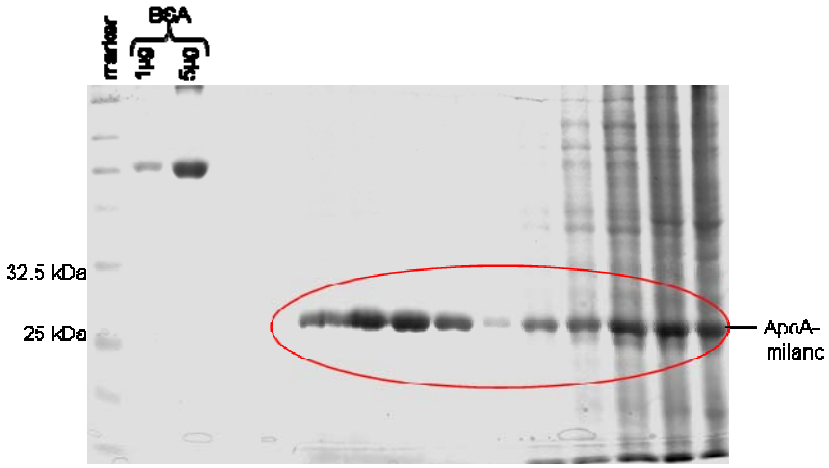


Figure 22: Fractions from the density gradient ultracentrifugation. Flotation of proteoliposomes containing apoA-I milano. Both upper and lower fractions containing apoA-I milano are circled in red. An aliquot of 20µl from a 1/10 dilution of each fraction was loaded in each well. Each of the bands circled in red corresponds to approximately 3.6µg of protein in average. Thus the total amount of proteoliposomes contained in these ten fractions of 1ml each is 18mg (1.8mg/ml). Totally, 54 mg of protein was processed in three tubes which all produced the same flotation profile.

IV. DISCUSSION

As already mentioned, ApoE4 has undoubtedly atheroprotective properties [40, 41, 43, 45-50]. The structural and functional analysis of this molecule has given many encouraging results for its use in therapy. In particular the apoE variant, apoE4-mut1 which has beneficial effects on the cholesterol and triglyceride levels of apoE deficient mice is a very promising apoE derivative with potential therapeutic properties [117].

In this study, large amounts of apoE4-mut1 protein have been produced, purified and associated to liposomes. The protocol used was successful since the quantity of protein produced was more than satisfying, the purification method was very efficient and the proteoliposomes formed were of good quality.

Moreover, the results of a pilot experiment performed by Kypreos et al (unpublished results) supported the expected atheroprotective effects of apoE4-mut1 on the development of atherosclerosis. In this experiment 1mg of proteoliposomes containing apoE4-mut1 or WT apoA-I was administered to atherosclerosis prone apoE / apoA-I double deficient mice every second day, in order to compare their atheroprotective properties. One and three weeks later, plasma was isolated in order to analyze the lipid levels and their lipoprotein profile. At the end of the forth week the mice were sacrificed and the development of atherosclerosis was determined by Sudan IV staining. The plasma analysis revealed that apoE4-mut1 corrects the high cholesterol levels of $apoE^{-/-}$ mice and causes a transient increase in plasma triglycerides[117]. Most important are the results from the Sudan IV staining of the aortic arch. As shown in figure 23 , mice treated with WT apoA-I-containing proteoliposomes develop atherosclerotic lesions, whereas apoE4-mut1 treatment resulted in inhibition of atherosclerosis.

These interesting results need to be verified in the larger scale study that was described in the materials and methods. It will be interesting to determine whether ApoE4-mut1 will prevent the progression of atherosclerosis or even better, it will reverse the disease. In the case that apoE4-mut1 shows the desired properties it could be used instead of apoA-I milano for treating patients with acute coronary syndrome.

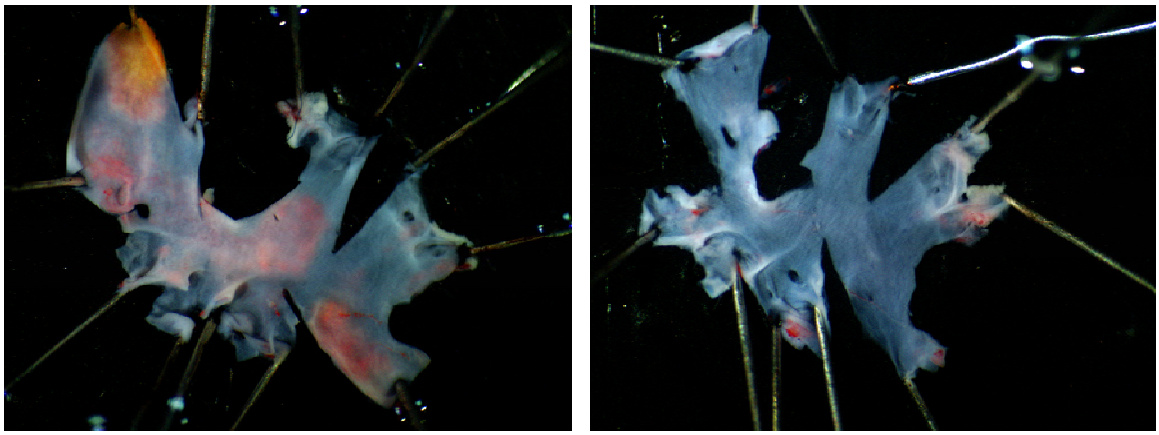


Figure 23: Sudan IV staining of the aortic arch of APOE$^{-/-}$; APOA-I$^{-/-}$ mice treated with proteoliposomes containing wt apoA-I or apoE4-mut1. Areas with atherosclerosis are stained in red.

As described previously, this apoE variant contains five amino acid substitutions (L261A, W264A, F265A, L268A and V269A), which might affect significantly the protein's conformation and could elicit an immunological response. For this reason, Drosatos et al have generated another apoE variant, apoE4-mut13, which contains only three amino acid substitutions (L261A, W264A, F265A) and has similar effects with apoE4-mut1 (unpublished results). An analogous study can be performed with this mutant, which is closer to the wild type protein.

Moreover, as already mentioned administration of apoA-I milano / phospholipid complexes results in reduction of atherosclerosis in mice, rabbits and human patients [107-111]. It is thus imperative to compare the apoE4-mut1 effects on the development of atherosclerosis with those of apoA-I milano. Further experiments need to be performed so as to purify the protein and form proteoliposomes without decreasing the yield. Then analogous experiments in mice will indicate whether apoE4-mut1 is indeed more atheroprotective than apoA-I milano.

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