Fachbereich Ernährungs- und Haushaltswissenschaften der Justus-Liebig Universität Giessen

EFFECT OF APOLIPOPROTEIN E, A-I, A-IV, AND LIPOPROTEIN LIPASE GENOTYPES ON FASTING GLUCOSE, LIPID, LIPOPROTEIN, AND APOLIPOPROTEIN LEVELS, AND THEIR RESPONSE TO LIFESTYLE INTERVENTION

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von Ilona Larson
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Abbreviations

°C	Degree Celsius
μL	Microliter
μM	Micromole
A	Adenine
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
Apo	Apolipoprotein
BMI	Body Mass Index
bp	Base Pair
C	Cytosine
CAD	Coronary Artery Disease
CETP	Cholesterol Ester Transfer Protein
CHD	Coronary Heart Disease
cm	Centimeter
CV	Coefficient Variation
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FCR	Fractional Catabolic Rate
G	Guanine
GLM	General Linear Model
Gln	Glutamine
HDL-C	High Density Lipoprotein Cholesterol
His	Histidine
HRP	Horseradish Peroxidase
IDL-C	Intermediate Density Lipoprotein Cholesterol
IEF	Isoelectric Focusing

kDa	Kilo Dalton
kg	Kilogram
kJ	Kilo Joule
LCAT	Lecithin:Cholesterol:Acyltransferase
LDL-C	Low Density Lipoprotein Cholesterol
LPL	Lipoprotein Lipase
LRP	LDL-Receptor Related Protein
mA	Milliampere
mg	Milligram
mg/dl	Milligram/Deciliter
min	Minute
mM	Millimole
mmol/L	Millimole/ Liter
MUFA	Monounsaturated Fatty Acid
NCEP	The National Cholesterol Education Program
ng	Nanogram
nm	Nanometer
NTP	Nucleotide Triphosphate
OD	Oligonucleotide Density
P/S	Ratio of Polyunsaturated to Saturated Fat
PCR	Polymerase Chain Reaction
POLY	Polyunsaturated Fatty Acid
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
sec	Second
Ser	Serine
SFA	Saturated Fatty Acid
SNP	Single Nucleotide Polymorphism
T	Thymine
TC/HDL	Ratio of Total Cholesterol to High Density Lipoprotein
Thr	Threonine

TMB	Tetramethyl-Benzidine
U	Unit
V	Volt
VLDL	Very Low Density Lipoprotein

Introduction

Coronary heart disease (CHD) is a major cause of death in many of the developed countries of the world including Germany and the USA (1). As many women die of this disease as men, but on average, they do it about seven years later. The disease is caused by atherosclerosis of the coronary arteries, which progressively narrows the arteries until they become totally occluded. At that point in time, there is myocardial infarction (death of heart muscle), also known as a heart attack.

Therapy for this disease includes coronary artery bypass surgery or balloon angioplasty to treat the disease when it occurs. An alternative approach is, of course, CHD prevention. In this regard, epidemiological as well as experimental studies have clearly shown that the major risk factors for CHD, in addition to a sedentary lifestyle and diet high in saturated fat and cholesterol, include: family history of premature heart disease ($\leq$ 55 years old in a male first degree relative and $\leq$ 65 in a female first degree relative), advancing age (being a male $\geq$ 45 or a female $\geq$ 55 years old), high blood pressure, diabetes mellitus, cigarette smoking, elevated low density lipoprotein (LDL) cholesterol (over 160 mg/dl, 4.1 mmol/L) and decreased high density lipoprotein (HDL) cholesterol (less than 35 mg/dl or 0.9 mmol/L) (2-4). The current view is that with aging and hypertension, as well as diabetes and smoking, there is progressive damage to the endothelial cells lining the arteries, causing excess deposition of LDL into the artery wall, resulting in higher levels of "foam cells." The role of HDL appears to promote cholesterol efflux from tissue, including the artery wall, and high levels of HDL cholesterol (HDL-C) have been shown to be protective, while low levels of HDL-C have been shown to be a significant risk factor for heart disease (5).

The cornerstone of prevention of CHD continues to be altering one's lifestyle, specifically diet and exercise (2-4). Prospective studies with diet, or diet and drug therapy associated with LDL cholesterol (LDL-C) lowering have been shown to markedly reduce the risk of heart disease (6-8). Diet therapy is indicated in patients if their LDL-C levels are $\geq$160 mg/dl in the absence of heart disease risk factors, $\geq$130 mg/dl in the presence of two or more risk factors as previously mentioned, and $\geq$100 mg/dl if the patient has established heart disease. For patients with LDL-C values even 30 mg/dl higher than those cut points as mentioned for diet, additional drug therapy is indicated. The US National Cholesterol Education Program (NCEP), however, has emphasized that lifestyle modification should be the primary treatment in lowering cholesterol values, with

drug therapy reserved for those patients where lifestyle modification is ineffective. Modifications contain dietary changes, regular aerobic exercise, and normalization of body weight.

With regard to dietary changes, the NCEP recommendations to reduce CHD risk for the general population include restriction of total fat intake to ≤ 30% of daily calories, saturated fat to less than 10% of daily calories, and dietary cholesterol to ≤ 300 mg/day (Step 1 diet). For individuals with elevated LDL-C levels, further restrictions for saturated fat to < 7% of daily calories and dietary cholesterol to < 200 mg/day are emphasized (Step 2 diet). However, the results of such cholesterol lowering diets are variable. When such diets are imposed on people and all the food is given to them under controlled settings as compared to an average Western diet, reduction of LDL cholesterol is generally in the order of 15-20%, but there is a wide variation in response to such a diet, ranging from virtually no reduction to 50% reduction (9-13). One factor clearly affecting this variation in response is the genetic differences within various proteins involved in lipoprotein metabolism. In the free-living state, diet counseling by a dietitian generally results in about a 5% reduction in LDL-C, largely because of decreased compliance (8). An alternative approach is for patients to go into a very intensive program where they actually live at a center and have the food provided to them, such as at the Pritikin Longevity Center. In this residential program, patients are provided with a diet rich in food of plant origin and very low in fat, saturated fat, and cholesterol. In such a setting, significant reductions in LDL cholesterol, triglyceride, glucose and body weight have been reported (13). The question of course is what the long-term effects of these diets are, and do the patients or clients stay on such programs when they are back at home in the free-living situation.

A quantitative relationship has been established between changes in dietary intake of fatty acids and cholesterol and the resulting serum cholesterol change (14-16). Other mechanisms of predicting HDL and LDL cholesterol response have been established. These relationships are well founded and predictable for groups (17-19); however, on an inter-individual basis, a striking variability in serum cholesterol response to diet has been known for years (20). Therefore, this variability in response has been discussed in several previous reports (19, 21-23). While in some individuals, plasma cholesterol levels dramatically decreased following consumption of a low fat diet, it remained unchanged in others. Jacob et al. (22) demonstrated under a variety of controlled dietary conditions that 3% were nonresponders, 9% were hyporesponders, and 64% responded within 30% of prediction using the Keys-Minnesota equation. The remaining 9% were considered hyperresponders. Furthermore, Katan et al. (20) described a consistency of response on intra-individual basis. In their study, they observed that hypo- and hyperresponders, based on their

response to dietary cholesterol intake, remained in the same group, even though their diets were modified several times. This would indicate that lipid alterations induced by dietary intervention might partly be controlled by genetic factors. Animal experiments (24-27) already revealed that response of serum lipoprotein to dietary modifications may have a strong genetic component. In humans, the evidence is still unclear, even though several gene loci have been suggested to be involved in the individual lipid response to dietary modifications. If such a genetic variability is proven to be true, it could have a significant impact on the development and success of public health policies and individual therapeutic interventions.

Several genes coding for apolipoproteins have been identified to be good candidates for investigation regarding lipid variation and dietary response. Apolipoproteins are structural components of lipoproteins, which are primarily synthesized in the liver and intestine. They play an important role in the transport and redistribution of dietary and endogenously derived lipids in the body (28-30). Apolipoproteins, are also cofactors of enzymes involved in lipid metabolism and ligands for cell surface receptors involved in lipoprotein uptake (28). So far, several different apolipoproteins, designated apo A-I, apo A-II, apo A-IV, apo B-48, apo B-100, apo C-I, apo C-II, apo C-III, apo D, and apo E, have been found. For most of those proteins, genetic variability has been described in humans (29). Some of these gene variations have been shown to be responsible for abnormal lipid profiles, which may contribute to the pathogenesis of atherosclerosis. Therefore, studies of the impact of genetic variability at these gene loci on plasma lipid levels and their interaction with other genes and environmental factors are of great interest.

In the present study, we investigated specific common mutations at the apolipoprotein E, A-I, A-IV, and lipoprotein lipase candidate gene loci, and their effects on plasma lipid levels at baseline and after an intervention program including diet therapy and exercise. Genetic variations at these gene loci are frequent enough to have a potential impact not only on an individual's levels, but also in the general population.

The following section includes an overview of each of those apolipoproteins and lipoprotein lipase, as well as their gene polymorphisms investigated in this study.

Apolipoprotein E

Apolipoprotein (Apo) E polymorphism is supposed to rank among the major factors involved in determining interindividual differences in the initiation and progression of atherosclerosis due to

its influence on plasma lipoprotein concentrations. Apo E circulates in the plasma as a protein constituent of intestinally derived chylomicron remnants, hepatic derived very low density lipoproteins (VLDL) and their remnants as well as a subclass of HDL (31). When associated with these lipoproteins, apo E serves as a ligand for the apo B,E-receptor and the LDL-receptor related protein (LRP) (32-34).

The gene for apo E is located on chromosome 19 (35). It is in close linkage to the genes for apo C-I and C-II and more distantly linked to the gene for the LDL-receptor (36). In humans, the structural gene locus for plasma apo E is polymorphic (37-40): three common alleles, designated as ε2, ε3, and ε4 which code for three major apo E isoforms E2, E3 and E4, respectively. Therefore, this polymorphism leads to six different phenotypes which have been distinguished by isoelectric focusing (IEF) and immunoblotting (41): three homozygous (E2/2, E3/3, and E4/4) and three heterozygous (E2/3, E2/4, and E3/4). The isoforms differ from each other by a single amino acid substitution. While apo E3 contains a cysteine at residue 112 and an arginine at residue 158, apo E2 contains two cysteines and apo E4 two arginines at each residue, respectively (42,43).

Figure 1: Apo E Alleles Caused by an Inter-Exchange of the Amino Acids Arginine and Cysteine

ARG: Arginine, CYS: Cysteine at Amino Acid Positions 112 and 158.
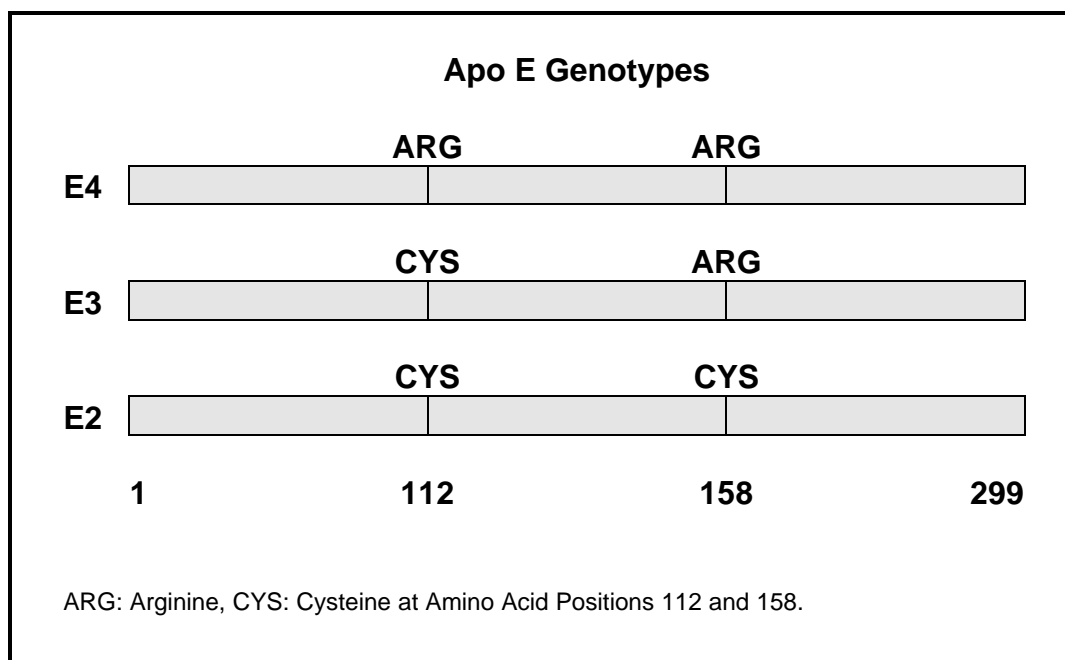
As a result, apo E4 has one more positive charge compared to E3, and E2 has one less (44). Considering that the receptor-binding site of apo E is located between residue 136 and 158, this difference in charge is responsible for their different binding affinities for the LDL-receptor. While E3 and E4 both carry an arginine at position 158, their ability to bind to the apo B,E receptor is the same compared to the markedly reduced affinity (<1%) of E2 (45). This reduced binding capacity results in a slower clearance of apo E2 lipoproteins, which subsequently leads to an up-regulation of the LDL-receptors. Therefore, a majority of individuals with the E2 allele have reduced serum cholesterol concentration (46). However, 1-10% of individuals with the E2/2 genotype develop type III hyperlipoproteinemia (47), a condition characterized by the accumulation in the plasma of triacylglycerol-rich lipoprotein remnants. Such individuals have a strong predisposition for the development of premature and accelerated atherosclerosis (48).

Apo E4, on the other hand, has an amino acid substitution at codon 112 which appears to reduce disulfide bonding of apo E with other sulfhydryl-containing proteins (49). As a consequence, the transfer of apo E4 from HDL to chylomicron remnants may be accelerated compared to apo E3, resulting in an enhanced receptor-mediated clearance of LDL, accumulation of hepatic cholesterol, down-regulation of LDL-receptors, which leads to elevated concentrations of serum cholesterol (46).

Apo E polymorphism has usually been ascertained by isoelectric focusing, which may be subjected in some cases to a significant amount of uncertainty as to the precise phenotype. One of the reasons is that the apo E protein contains a considerable amount of carbohydrate, and the presence of this carbohydrate can alter the isoelectric point (50). This is especially true in subjects with diabetes, where the amount of sialic acid on apo E is increased (51). Moreover, there are other mutations within the apo E gene that can affect the isoelectric point other than that at the 112 and 158 positions. More recently, apo E genotyping by PCR has allowed a faster and more definitive screening for the presence or absence of mutations at residue 112 or 158 (52-54).

Many population studies have investigated the role of apo E phenotype as assessed by IEF in affecting lipoprotein alterations (41,49,55-69). These studies have generally shown that the presence of the apo E4 allele in the heterozygous state is associated with elevations in LDL cholesterol, while the presence of the apo E2 allele in the heterozygous state is associated with decreased levels of LDL cholesterol. It has been estimated in the general population that the apo E alleles may account for as much as 14% of the normal interindividual variation in plasma total and LDL cholesterol (49). However, data from the Framingham Offspring study (60) revealed that only 1% of the LDL variation was accounted for by the apo E isoforms in male and females

being pre-menopausal, and 5% in females being post-menopausal. A meta-analysis has shown that subjects carrying the apo E2 and E4 alleles are associated with higher triglyceride levels compared to apo E3/3 homozygotes (58). Moreover, there have been reports that the presence of the apo E4 allele is associated with CHD, Alzheimer's disease, and all-cause dementia (70-75).

Several investigators have recently focused on the interrelationship of lipoprotein responsiveness to dietary changes and apo E phenotypes (46,76-96), which recently has been reviewed by Ordovas (97). While some studies document in individuals having the apo E4 allele a greater response in plasma lipids (12,79,80,86-90,92,94,98) others failed to note such an effect (76,81-85,93,96,99-102). However, a meta-analysis carried out by Ordovas et al. (103) supports the concept that the apo E4 allele is associated with greater LDL response to changes in dietary saturated fat and cholesterol. They also suggest that the responsiveness to diet may be dependent on age, with genetic variation having a greater impact with increasing age.

Apolipoprotein A-I

Apolipoprotein A-I (apo A-I) is the major protein constituent of plasma HDL and plays a crucial role in lipid transport and metabolism. Several epidemiological studies have demonstrated that HDL-C and apo A-I levels are inversely correlated with the risk of developing coronary heart disease (104-106). Some investigators (107,108) believe that the plasma level of apo A-I is a better discriminator for angiographically assessed coronary heart disease than the level of HDL-C. In the absence of apo A-I, as in familial apo AI-CIII-AIV deficiency or apo AI-CIII deficiency, a virtual absence of HDL from plasma is observed, and these patients are at substantially increased risk for premature atherosclerosis (109-111). It has been suggested that apo A-I mediates the reverse cholesterol transport by acting as a ligand for the HDL-receptor (112) and promoting cholesterol efflux from peripheral tissue back to the liver (113). Furthermore, it is the major in vivo activator of the lecithin:cholesterol:acyltransferase (LCAT), an enzyme that catalyzes the esterification of cholesterol in plasma (114).

Although various environmental factors such as diet, exercise, alcohol, smoking, sex hormones, and certain drugs can significantly influence the levels of HDL-C and apo A-I (115-118), family and twin studies demonstrated a strong genetic heritability, accounting for up to 66% of the variability of HDL-C and apo A-I levels (119,120).

The gene coding for apo A-I is clustered with the genes for apo C-III and apo A-IV on the long arm of chromosome 11 (121-124). Several restriction fragment length polymorphisms (RFLPs) in

the apo AI-CIII-AIV gene cluster have been identified (125-128) and associations with lipid abnormalities and the risk of CAD have been shown (129,130).

In this study, we investigated the promoter polymorphism located 75 base pairs (bp) upstream from the apo A-I transcription start site as well as the +83/84 bp mutation located in the first intron of the apo A-I gene. While the former mutation at -75 bp is due to a guanine (G) to adenine (A) substitution (131,132), the latter is created by a cytosine (C) to thymine (T) (+83 bp) and/or a G to A (+84 bp) interchange which occur either independently or together (133).

With regard to the -75 bp promoter polymorphism, which occurs at a frequency of 0.15 to 0.20 in Caucasian populations, several controversial results have been published in the literature (see Table 1). While some studies (131,134-136) reported in individuals carrying the rare A-allele higher levels of HDL-C and A-I compared with the G/G wildtype, others (132,137-142) documented associations only with one of those parameters. More recent studies (143-148), however, could not detect an association of the G/A mutation on HDL-C or apo A-I levels at all. Furthermore, the magnitude and gender distribution of the effects found, differed among studies.

Table 1: Literature Overview for Apolipoprotein A-I -75 bp Genotype

Author	Year	Study Subjects	Results
Pagani et al.	1990	136 females, 108 males from Italy	Division of HDL-C into 3 deciles. In women the frequency of the A-allele increased significantly from the lowest to the highest decile (0.10, 0.14, 0.27). No effect in men.
Jeenah et al.	1990	96 males from England	Males having the A-allele had significantly higher HDL, HDL$_2$, and apo A-I levels. Frequency of the A-allele increased from 0.11 to 0.25 in men with serum apo A-I concentrations greater than 180mg/dl.
Hayase et al.	1992	162 boys and young men from Belgium	Boys and young men having the A-allele had 4.5% higher apo A-I levels compared to G/G genotype. Gene-dosage-effect: Increase in A-I levels from G/G< G/A< A/A.
Sigurdsson et al.	1992	149 males, 166 females from Iceland	Significant association between genotype and HDL-C and apo A-I only in non-smoking men with G/A having higher levels than G/G. No association was noted in smokers and females.
Xu et al.	1993	111 boys, 93 girls from Italy	Boys with the A-allele had significantly higher levels of total cholesterol (9%), LDL-C (11%), apo B (10%), and apo A-I (7%) than G/G homozygotes. No significant effect on any lipid traits was observed in girls.

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Author	Year	Study Subjects	Results
Civeira et al.	1993	60 males, 65 females from Spain	No significant association between the G/A mutation and levels of HDL-C and apo A-I.
Talmud et al.	1994	825 males, 818 females from Europe	Females, having the A-allele had significantly higher apo A-I levels than G/G homozygotes, but no effect was seen on HDL-C. In males, no significant effect on apo A-I or HDL-C was observed.
Barre et al.	1994	204 males, 205 females White	No significant effect of the genotype on HDL-C levels in either gender.
Saha et al.	1994	148 males, 139 females from China	Non-smoking men having the A-allele had significantly higher apo A-I levels (20%) than G/G homozygotes. No effect in smokers and females. Furthermore, no effect of the A-allele on HDL-C in either gender.
Lopez-Miranda et al.	1994	50 males from Spain	At baseline, no significant association between the genotype and lipid traits was observed.
Minnich et al.	1995	345 males, 308 females from Canada	Females having the A-allele had significantly higher levels of HDL-C (12%) and apo A-I (10%) than G/G homozygotes. They suggest no direct effect of A-allele on HDL-C: 1) no gene dosage effect. 2) bimodal distribution of HDL-C. 3) Hyperalphalipoproteinemia only in a subset of individuals with the A-allele. In men, no effect of the A-allele was noted.
Matsunaga et al.	1995	120 CAD patients, 125 controls, 199 students from Japan	Identical frequency of the A-allele between CAD patients and controls. No effect of the A-allele on HDL-C, apo A-I, or any other lipid trait in either group. Students and controls having the G/A genotype had significantly lower plasma levels of apo A-I.
Akita et al.	1995	168 from Japan	No significant effect of the genotype on HDL-C levels regardless of gender or CETP status.
Wang et al.	1996	118 males, 125 females from Australia	Adult males and females with the A-allele had higher HDL-C levels compared to G/G homozygotes. No effect was observed in children.
Wang et al.	1996	467 males, 177 females CAD patients	CAD patients with the A-allele did not have higher levels of HDL-C and apo A-I, but males tended to have more severe CAD.
Kamboh et al.	1996	252 males, 282 females US-Whites	Significant raising effect of the A-allele on apo A-I levels in male non-smokers; no effect in male smokers or in females. No effect on HDL-C levels in either gender.
Meng et al.	1997	42 males, 44 females from Finland	Men having the A-allele had significantly higher levels of HDL-C and apo A-I than G/G homozygotes. No effect was observed in females.

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Author	Year	Study Subjects	Results
Carmena-Ramon et al.	1998	25 males, 44 females from Spain	G/A heterozygote males and females had significantly lower levels of total cholesterol, LDL-C, and apo B compared to G/G homozygotes. No effect on HDL-C levels.
Mata et al.	1998	26 males, 24 females from Spain	G/A heterozygotes had significantly higher levels of plasma cholesterol, LDL-C, and triglyceride than those with the G/G genotype did. No effect on HDL-C.
Larson et al.	1999	373 males, 361 females from America	Significant raising effect of the A-allele on levels of apo B, total cholesterol, LDL-C, and TC/HDL-C ratio in women, but not in men. No effect on HDL-C levels in either gender.

With regard to the +83/84 bp polymorphism, only little is known. The few studies published are not congruent (see Table 2). Wang et al. (141) reported in healthy Caucasians carrying the rare M2- allele significantly higher HDL-C levels compared to homozygotes carrying the common M2+ allele. However, investigating CAD patients, Wang et al. (146) failed to observe the positive effect of the rare +83 bp allele on HDL-C and A-I levels; instead they noted even more severe coronary artery disease. The most recent report by Carmena-Ramon et al. (148) on 69 heterozygotes for familial hypercholesterolemia (FH) failed to show an association between the +83 bp polymorphism and lipids.

Table 2: Literature Overview for Apolipoprotein A-I +83bp Genotype			
Author	**Year**	**Study Subjects**	**Results**
Wang et al.	1995	118 males, 125 females from Australia	Subjects heterozygous for the rare M2- allele had significantly higher HDL-C levels compared to the M2+/+ homozygotes.
Wang et al.	1996	467 males, 177 females CAD patients	The rare M2- allele was not associated with higher levels of HDL-C and apo A-I in either gender. In males, however, the frequency of the rare M2- allele was significantly higher in patients with more severe CAD.
Kamboh et al.	1996	252 males, 282 females US-Whites	Significant raising effect of the rare M2- allele on apo A-I levels in male non-smokers; no effect in smokers or in females. No effect on HDL-C levels in either gender.

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Author	Year	Study Subjects	Results
Carmena-Ramon et al.	1998	25 males, 44 females from Spain	No association of the rare M2- allele with altered baseline lipids nor with dietary response was observed in either gender in heterozygous FH.
Larson et al.	1999	373 males, 361 females from America	Significant raising effect of the rare M2- allele on levels of apo A-I and total cholesterol in males, and lowering effect on levels of total cholesterol in females. No effect on HDL-C levels in either gender.

Studies on response to a high fat, high cholesterol diet have shown in non-human primates that the apo A-I gene locus is responsible for up to 33% of the variation (149). In humans, Xu et al. (138) found less significant associations (6%) between the apo A-I locus and dietary induced changes in apo A-I levels. Lopez-Miranda et al. (144) studied 50 young males carrying the A-allele and reported greater responsiveness to dietary changes than in subjects being homozygous for the common G-allele. After having switched from a low fat to a high fat, high monounsaturated fat diet (40% fat, 22% MUFA), significant increases in LDL-C were observed in the G/A group versus the G/G group. On the other hand, diet modification from a saturated fatty acid (SFA) to a polyunsaturated fatty acid (POLY) diet resulted in greater total and LDL cholesterol reduction in G/A subjects as compared to G/G subjects (150). In contrast, others (136) documented no significant difference between apo A-I genotypes and lipid traits in response to a low cholesterol, low fat diet. With regard to the +83 bp polymorphism, only one study (148) has been published, documenting no effect of the genetic variation on lipid phenotypes.

Therefore, the purpose of this study was to further elucidate the physiological influence of these two apo A-I polymorphisms (-75 bp and +83 bp) separately and jointly on various quantitative traits, in particular on apo A-I levels. In addition, we wanted to investigate whether or not plasma LDL-C responsiveness to changes in dietary fat saturation and cholesterol content is partly explained by the variation at the apo A-I gene locus.

Apolipoprotein A-IV

Human apolipoprotein A-IV (apo A-IV) is a plasma glycoprotein with a molecular mass of 46 kDa, consisting of 376 amino acid residues (151-153). Apo A-IV is synthesized predominantly in the enterocytes of the small intestine during fat absorption as prepro apo A-IV. The mature protein is secreted into the lymph incorporated onto the surface of nascent chylomicrons (154,155). After entry into the bloodstream apo A-IV is found in plasma associated with very

low density lipoprotein (VLDL) (153,156), HDL (157,158), and in the lipoprotein free fraction (156,159). Early reports using ultracentrifugation, indicated that the majority of apo A-IV is located in the latter fraction, while Lagrost et al. (158) using high performance gel filtration documented that apo A-IV is primarily associated with the HDL fraction. The average level of plasma apo A-IV ranges from 14 to 37 mg/dl according to the immunological method of determination (156,157,159).

Although its precise function remains unclear, apo A-IV has been proposed to play a role in the metabolism of triglyceride-rich lipoproteins and HDLs. In vitro studies have shown that the activation of lipoprotein lipase by apo C-II can be mediated by apo A-IV (160). Furthermore, apo A-IV seems to play an important role in the reverse cholesterol transport as it can serve as an activator for lecithin:cholesterol:acyltransferase (LCAT), the enzyme responsible for cholesterol esterification (161,162). It also has been reported that apo A-IV containing lipoproteins can promote cholesterol efflux from cultured cells in vitro (163-166), and there is evidence that apo A-IV may be one of the ligands for the putative HDL-receptor (167) as well as participate in the HDL particle conversion by cholesterol ester transfer protein (CETP) (168-170). In transgenic mice overexpressing mouse apo A-IV, a protective effect was observed against the formation of diet-induced aortic lesions, which seemed to be due to the influence of apo A-IV on the metabolism and antiatherogenic properties of HDL (171). In addition, research on apo A-IV knockout mice demonstrated that apo A-IV played a role in increasing HDL-C levels by inhibiting the fractional catabolic rate (FCR) of HDL-cholesteryl ester (172).

The structural gene for apo A-IV is located on human chromosome 11q and is in close linkage with apo A-I and apo C-III genes (173,174). Genetically determined polymorphisms of apo A-IV have been detected in humans (175-177) and in other mammalian species (178,179). Using isoelectric focusing and immunoblotting (176), several isoforms of apo A-IV have been detected. The most common isoform in all population studies is apo A-IV-1 with an allele frequency in Caucasians ranging from 0.89 to 0.97. Apo A-IV-2, created by a G to T nucleotide exchange resulting in an amino acid substitution, where glutamine (Gln) replaces histidine (His) at residue 360, is the second most common isoform. The allele frequency is in the range of 0.03 to 0.11 in Caucasians (Table 3). Other rare isoforms (apo A-IV-0, apo A-IV-3 through apo A-IV-7, apo A-IV-Tokyo) have been found (176,180-183), which some of them are restricted to a particular ethnic group. Additional variation within the apo A-IV gene locus has been detected (184). Using nucleotide sequencing after polymerase chain reaction (PCR) amplification, Boerwinkle et al. (184) detected within subjects with the apo A-IV-1 isoform an A to T polymorphism that results in threonine (Thr) to serine (Ser) substitution at amino acid position 347. This mutation

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does not alter apo A-IV charge properties and has therefore not been identified by isoelectric focusing methods. The frequencies for the rare S-allele were 0.16 - 0.20 and for the more common T-allele were 0.80 - 0.84.

The impact of apo A-IV genetic variation in determining plasma lipoprotein levels has been investigated in several population studies (Table 3). The results, however, are very contradictory. Some studies observed a significant effect of the apo A-IV 360 polymorphism on HDL-C levels (185-187), others on triglyceride levels (186,188), or on glucose or insulin levels (189,190). Concerning apolipoproteins levels, associations between the 360 mutation and levels of apo A-I (191) and apo A-IV (192,193) have also been described. Conversely, several investigators failed to show any effect of the apo A-IV 360 polymorphism on glucose, lipid and lipoprotein phenotypes (193-196).

Table 3: Literature Overview of the Effects of Apolipoprotein A-IV 360 Genotype on Biochemical Parameters

Author	Year	Females/ Males	Population	Frequency of AIV-2	Findings
Menzel	1988	260/ 213	Austrian	0.08	Significant association of A-IV-1/2 phenotype with higher HDL-C levels.
De Knijff	1988	/ 1393	Dutch	0.08	No effect of A-IV phenotype on lipids and on apo A-I levels.
Menzel	1990	93/ 95	Icelandic	0.11	Apo A-IV-1/2 phenotype significantly raised HDL-C levels and lowered TG levels. No effect on A-IV levels.
Visvikis	1990	158	French	0.06	Significant association of apo A-IV-2 allele with elevated glucose levels, but no effect of apo A-IV phenotype on any lipid traits.
Hanis	1991	253/ 78	Mexican American	0.07	No effect of A-IV phenotype on glucose or lipid levels. Weak association of the apo A-IV-2 isoform with apo A-I resulting in higher levels.
Kamboh	1991	82/ 204	Diabetic Controls, U.S.	0.08	No effect of apo A-IV phenotype in diabetics, except in males, A-IV-1/2 heterozygotes had higher fasting insulin levels. In controls, females carrying the 2-allele had higher levels of LDL-C. No difference in A-IV phenotype distribution between diabetics and controls.

Continued

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Author	Year	Females/ Males	Population	Frequency of AIV-2	Findings
Kamboh	1992	188/ 238	Diabetic Controls Hispanic	0.10 0.07	No effect of A-IV phenotype on any trait in diabetics. In control group, females with the 2-allele had lower levels of HDL, HDL_2, and HDL_3 cholesterol than A-IV-1 homozygotes.
v. Eckardstein	1992	291	German	0.07	A-IV-1/2 heterozygotes had significantly higher concentrations of LDL-C and lower Lp (a) levels compared to A-IV-1/1 homozygotes. No other effect on lipid traits was found.
v. Eckardstein	1993	/ 275	CAD patients	0.08	A-IV-1/2 heterozygotes showed 30% lower Lp (a) concentration than 1/1 homozygotes. Relative frequency of Lp (a) concentration >20mg/dl was significantly increased in 1/1 phenotypes. Other parameters were not associated with apo A-IV phenotype.
v. Eckardstein	1994	188/ 426	CAD patients Germany	0.07	Males with the A-IV-1/2 phenotype had significantly higher levels of Lp A-I and A-IV, higher plasma activity of LCAT than 1/1 homozygotes. In both genders, a lower activity of CETP in apo A-IV-1/2 heterozygotes was observed. No effect on any other lipid traits.
Zaiou	1994	105	French		No effect of apo A-IV phenotypes on any lipid traits.
Ehnholm	1994	1890	EARS-I	0.05	No effect of apo A-IV genotype on HDL-C, apo A-IV, or any other lipid traits.
Verges	1994	83/ 100	NIDDM Controls		No effect of apo A-IV phenotype on any lipid traits in NIDDM subjects. In controls, apo A-IV-1/2 heterozygotes were associated with higher levels of HDL and HDL_2 cholesterol.
Carrejo	1995	119	American	0.05	No effect of the apo A-IV genotypes on lipids, apo A-I, apo C-III, or apo A-IV levels.
Malle	1996	141/ 99	Turkish	0.03	No significant influence of the A-IV phenotypes on plasma lipids, apo A-I, apo B, and apo E was observed in either gender.

Continued

Author	Year	Females/ Males	Population	Frequency of AIV-2	Findings
Wang	1997	145	Chinese	0.06	Homozygotes and heterozygotes for the apo A-IV-2 isoform exhibited a significantly higher TC concentrations compared to the apo A-IV-1/1 homozygotes. No effect on A-IV levels.
Saha	1997	/ 176	Indian	0.03	Marginal effect of the 2-allele on reduced LDL-C and increased triglyceride levels compared to 1/1 homozygotes.
Lehtinen	1998	/ 71	Saami	0.06	Significantly higher HDL cholesterol levels in Saami with apo A-IV-1/2 genotype compared to A-IV-1/1 genotype.
		/ 177	Finnish	0.11	No significant effect on any lipid trait.
Fischer	1999	772	EARS II	0.07	Significantly lower BMI values in control subjects carrying the 2-allele; a borderline significance was observed for lower total cholesterol and triglyceride levels.
Larson	1999	361/ 373	American	0.08	No significant effect of the apo A-IV genotype on lipids, lipoproteins, and apo A-IV levels. In females, significant association of apo A-IV-1/2 heterozygotes with elevated glucose levels compared to apo A-IV-1/1 homozygotes.

With regard to the apo A-IV 347 mutation, only very little is known (Table 4). While one study documented (197) a significant association of the rare S-allele with lower plasma apo B in both sexes and with lower LDL-C levels, others (195) did not note an effect on any lipoprotein and apolipoprotein levels.

With regard to the effect of genetic variation on dietary response, only little is known. For apo A-IV 360 mutation, some studies (12,144,150,198) indicated that the presence of the A-IV 2-allele resulted in reduced responsiveness of LDL-C levels to dietary cholesterol and fat restriction. In contrast, another study (199) detected a greater responsiveness of HDL-C and apo A-I levels in subjects carrying the 2-allele. Concerning the 347 mutation, differences in LDL-C and apo B response (200), as well as in triglyceride-rich lipoproteins (201) response to diet modifications have been documented.

Table 4: Literature Overview of the Effects of Apolipoprotein A-IV 347 Genotype on Biochemical Parameters

Author	Year	Females/ Males	Population	Frequency of AIV-2	Findings
v. Eckardstein	1992	291	German	0.16	The rare S-allele was significantly associated with lower plasma apo B concentrations in both sexes, and with lower LDL-C levels in men.
Tenkanen	1992	NA	Finnish	0.18	No effect of the apo A-IV-S allele on plasma lipid and lipoprotein levels in hyperlipidemic men and normal controls.
Carrejo	1995	119	Hyperlipidemics	0.17	No effects of apo A-IV genotypes on lipid traits or apo A-I, C-III, or apo A-IV levels.
Saha	1997	/ 176	Indian	0.12	Significant effect of S-allele on lower non-fasting total and LDL cholesterol compared to T/T homozygotes.
Fischer	1999	745	EARS II	0.19	In controls, significant effect of S-allele on higher values of BMI and waist/hip ratio as well as higher levels of total cholesterol, triglyceride, and apo B.
Larson	1999	361/ 373	American	0.20	No effect of apo A-IV genotypes on lipid, lipoprotein, or apo A-IV levels in both sexes.

NA = Not Available

Lipoprotein Lipase

Lipoprotein lipase (LPL) is a key enzyme of lipid metabolism and cellular energy balance. The primary function of LPL is the hydrolysis of core triglycerides of circulating chylomicrons and VLDL (202). In the presence of its cofactor apo C-II, LPL releases monoglycerides and free fatty acids which are taken up by skeletal muscle or adipose tissue for oxidation or storage (203,204). Besides its lipolytic function, LPL is believed to enhance the binding of apo E-containing lipoproteins to the low density lipoprotein (LDL) receptor-related protein (LRP), and therefore, plays an important role in the catabolism of chylomicron remnants (205). In addition, during lipolysis, surface components such as apolipoproteins and phospholipids, are transferred from triglyceride-rich lipoproteins to high-density lipoproteins 3 (HDL_3) particles to form HDL_2

particles. This transfer accounts for the positive correlation between post heparin plasma LPL activity and plasma HDL_2 concentration (206). These HDL_2 particles play a crucial part in the "reverse cholesterol transport" by taking up tissue cholesterol and transporting it to the liver for excretion. Several studies have observed an inverse relationship between HDL_2 concentrations and the extent of CHD (5,126,207). Due to its role in the maturation of HDL and the metabolism of other lipoproteins, variability of LPL activity may represent a risk factor for CHD (208). LPL is synthesized in parenchymal cells of a variety of tissues, including skeletal muscle, adipose, heart, lung, brain, and macrophages (209). Its physiological location, however, is on the luminal surface of the capillary endothelium, where it is anchored to the cell surface through ionic interaction with membrane-bound glycosaminoglycan chains (210).

The human LPL gene is located on chromosome 8p22 (211) and its gene structure and cDNA has been described (212-216). The gene spans approximately 30 kb and the first 9 of 10 exons code for a protein containing 475 amino acids, including a 27 amino acid signal peptide that is cleaved posttranslationally to yield the mature LPL with a molecular weight of approximately 60 kDa.

Several restriction fragment length polymorphisms (RFLP) in the LPL gene have been documented and associated with various lipid traits (217-220). This study focused on the LPL Hind III polymorphism in which a replacement of a thymine (T) with a guanine (G) base occurs at position +495 in intron 8 and abolishes an Hind III restriction enzyme recognition site (221). Although this polymorphism is located in an intron of the LPL gene, it has been hypothesized that the more common H+ allele (presence of cutting site) is associated with a lower LPL activity compared to the rare H- allele (absence of the restriction site). As such, it has been proposed that carriers with the H+/+ genotype have higher levels of triglyceride and lower levels of HDL versus carriers of the H-/- genotype.

Previous studies of these relationships have yielded variable results (Table 5). While in some studies the common H+ allele of the LPL Hind III polymorphism has been shown to be significantly associated with hypertriglyceridemia (222-227), hypercholesterolemia (209), lower HDL levels (209,228,229), elevated apo C-III (229) and apo B levels (226), and premature coronary heart disease (CHD) (223,226,228,229), other reports failed to note such effects (222,230-232). The inconsistency of those reports may be due to small sample sizes or heterogeneity with regard to ethnic backgrounds, age, and sex of study subjects.

Regarding response to diet changes, only two studies have been published, indicating different results. While one study (233) found a reduced responsiveness of the common H+/+ genotype on

total cholesterol, the other study (234) reported an increased reduction of VLDL triglyceride and apo B compared to subjects carrying the rare H- allele. Therefore, further research is necessary to elucidate the Hind III genotype effect on lipid response to diet in a large population.

Table 5: Literature Overview of the Effects of Lipoprotein Lipase Hind III Genotype on Biochemical Parameters

Author	Year	Females/ Males	Population	No Association	Significant Association	H+ Higher	H+ Lower	M/F	P values
Chamberlain, J.C	1989	98 /	Caucasian	TC, HDL, TG					
Heinzmann, C.	1991	190	US Whites	LDL, TG	TC		*		0.025
Peacock, R.E	1992	93 /	Swedish men	VLDL-C, VLDL-TG HDL	TG	*		M	0.030
Ahn, Y.I	1993	250 / 281	Normoglycemic	Insulin, Glucose	TG	*		M / F	<0.001
			Non-Hispanic Whites (NHW)	TC, LDL	HDL	*		M / F	0.005
		185 / 196	Hispanics (H)						
		43 / 32	NIDDM (NHW)	Glucose, TC, LDL,					
		75 / 116	NIDDM (H)	HDL, TG	Insulin		*	M	0.009
Mattu, R.J.	1994	123 /	English men	TG, HDL, Apo AI	TC	*		M	0.021
					Apo B	*		M	0.005
					LDL	*		M	0.010
Mitchell, RJ.	1994	144/	Mediterranean	LDL	TG	*		M	0.030
					HDL		*	M	0.008
Gerdes, C.	1995	446 /	Danish men	TC, Non-HDL-C, TG	HDL		*	M	0.030
Jemaa, R.	1995	74 / 162	French (obese)	TC, LDL, Lp AI, Apo B, Apo AI	VLDL-C	*		F	<0.010
					VLDL-TG	*		F	<0.050
					HDL		*	F	<0.050
					HDL$_2$		*	F	<0.050
					TG	*		F	<0.050

Continued

Author	Year	Females/ Males	Population	No Association	Significant Association	H+ Higher	H+ Lower	M/F	P values
Jemaa, R.	1995	725	European (Multicenter)	TG	Apo CIII	*			<0.010
					HDL		*		<0.050
Vohl, M-C.	1995	52 /	Caucasians	TC, LDL, HDL, HDL$_2$, HDL$_3$, TC/HDL, TG				M	
Georges, J.L.	1996	193 / 193	French Canadians	BMI, TG				M / F	
Chen, L.	1996	131 / 107	Caucasians	TC, HDL, TG				M / F	
Peacock, R.	1997	101 / 102	Icelandic non-smokers	HDL, TG, Apo CIII, Apo AI				M / F	
		47 / 58	Icelandic smokers	HDL, TG, Apo CIII, Apo AI	TG	*		F	0.001
Larson, I.	1999	341 / 342	Americans	Fast. Gluc, HDL,	TC	*		F	0.039
				TG	LDL	*		F	0.004
					HDL		*	M	0.003

M: Males, F: Females, TC: Total Cholesterol, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, HDL: High-density lipoprotein, TG: Triglyceride, Fast. Insulin: Fasting Insulin, Fast. Gluc: Fasting Glucose, Apo: Apolipoprotein

Purpose and Hypothesis

The purpose of this study was to assess the impact of genetic variants at the APOE, APOAI, APOAIV, and LPL gene loci in determining serum glucose, lipids, lipoproteins, and plasma apolipoproteins at baseline as well as in response to diet and behavioral modifications.

The aim of the study was to provide more definitive information about the interaction of genetic variants with plasma lipids (specifically low-density lipoprotein cholesterol) in order to more accurately assess potential responsiveness of individuals to dietary modification.

The hypotheses tested included:

1. Common genetic variants at the APOE gene locus are significant determinants of LDL cholesterol and apoE levels, and LDL-C lowering in response to diet.

2. The -75 bp and +83 bp polymorphisms at the APOAI gene loci are significantly associated with plasma levels of apo A-I and HDL-C, as well as LDL-C lowering in response to diet.

3. The 360 and 347 polymorphisms at the APOAIV gene loci significantly influence levels of apo A-IV, HDL-C, and triglyceride, and LDL-C lowering in response to diet.

4. The common Hind III polymorphism at the LPL gene locus has a significant impact on triglyceride and HDL-C levels, as well as LDL-C reduction in response to diet.

5. Plasma lipid levels in women are less responsive to lifestyle intervention than in men.

6. Intensive lifestyle intervention with diet and exercise has a significant effect on serum lipid response short-term, but only little effect long-term.

Materials and Methods

Subjects and Lifestyle Intervention

A total of 734 middle-aged and elderly subjects participated in this study. All 361 females and 373 males took part in a lifestyle modification program at the Pritikin Longevity Center in California. The majority of the patients entered the program to reduce their risk for heart disease as well as to reduce their body weight. All participants signed a consent form, permitting use of their data for research purposes. Data were obtained from the medical records kept on each patient.

Subjects checked into the center on a Sunday afternoon, and following the evening meal attended an orientation where they received information about the program and instructions for a 12-hour fast. On Monday morning, fasting blood was drawn between 6:30 and 7:30 AM while subjects were in a seated position. A second set of blood was drawn from all subjects on the following Monday, while on a subset of 31 participants, a third blood sample was taken after 15 days. Subjects were seen by a physician who conducted an initial medical history and physical examination, and who then sent them for a multi-stage symptom-limited treadmill test. Immediately following this test, the results were analyzed by a physician, who assigned a training heart rate for their exercise program. Furthermore, participants met with an exercise physiologist who wrote an exercise prescription for daily walking. The prescription initially called for 30-40 minutes (min) of walking, six days per week, and every other day subjects were to walk fast enough to get their heart rate just below the training heart rate. At the end of each week a new prescription was given to each subject to increase the duration and intensity of their daily walking. In addition to daily walking, the subjects also attended an organized exercise class that met for 60 min, five days per week, and consisted of stretching and flexibility activities, some muscle conditioning, and aerobic exercise on a treadmill or bicycle ergometer.

During their stay at the center, the subjects were provided with meals, which were high in complex carbohydrate and fiber, and low in total fat, cholesterol, and salt. Less than 10% of total calories were derived from fat and the ratio of polyunsaturated to saturated fat (P/S) was estimated to be 2.4. Fifteen percent of the calories were derived from protein, and the remainder from carbohydrate, of which 90% was unrefined. The diet provided 35-40 grams of dietary fiber per 420 kJ, and a total daily intake of 1.6 grams or less of sodium chloride. Cholesterol was

limited to 25 mg per day (or 0.06 mmol/day), while the subjects were at the center, and they were encouraged to limit cholesterol intake to <100 mg per day (or 0.26 mmol/day), once they left the program. The diet consisted primarily of whole grains, cereals, beans, peas, other vegetables, and fresh fruit. Protein was derived primarily from vegetable sources and, except for nonfat milk, limited amounts of nonfat cheese, fish, or poultry. The food served at the center was all natural food, most of which are readily available in the standard market.

Details concerning the diet have been previously published (13). Those individuals wishing to lose weight were instructed on how to restrict calories, while ensuring an adequate intake of required nutrients. Female subjects were instructed not to reduce caloric intake to <420 kJ per day, and male subjects to no less than 504 kJ per day. Rapid weight loss was discouraged. All subjects attended educational classes and lectures that can be divided into three different groups. The first group of classes dealt with the major diseases (hypertension, diabetes, cancer, and coronary heart disease) and the roles that diet and exercise play in their management and prevention. A second group of lectures dealt with various aspects of nutrition, and were conducted with cooking classes, while a third group of lectures dealt with lifestyle management, including understanding and coping with stress. Special smoking cessation classes were offered.

As mentioned before, a subset of 31 individuals (9 females and 22 males) who participated in the three-week program was independently analyzed. The reason was to ascertain what the effects on lipid response would have been, had the subjects been sampled at the two-week point. Therefore, blood samples were drawn at baseline, eight days, and at 15 days of the intervention.

In addition, to evaluate the long-term effect of this lifestyle modification program on plasma glucose and lipid traits, another subset of 202 participants was analyzed. These 95 women and 107 men were seen on two occasions at the Pritikin Longevity Center. They participated twice in the same program for two weeks with data analyzed after 8 days of intervention, respectively. The mean time span between visits was 1.7 years.

Blood Sampling

Fasting blood samples were drawn at admission to the Pritikin Longevity Center. One set of samples were placed into two 10-cc EDTA tubes (0.1% EDTA) for the isolation of DNA. The second set of samples was placed into two 10-ml tubes containing SST clot activating gel (Becton-Dickinson vaccutainer system). These serum sample tubes were allowed to clot and the serum was separated by high-speed centrifugation for 15 min. Serum was used for the

determination of serum lipids and glucose. Non-HDL lipoproteins were precipitated using the sodium phosphotungstic acid reagent. Total cholesterol, HDL-C, triglyceride, and glucose levels were measured using standard automated enzymatic procedures on an Olympus Autoanalyzer (Smith-Kline Beecham Laboratories). LDL cholesterol was calculated by subtracting the sum of HDL-C and triglyceride divided by 5, from total cholesterol, as described by Friedewald et al. (235), provided triglyceride values were <400 mg/dl.

Plasma apo E was determined by enzyme linked immunosorbent assay (ELISA), using a commercially available assay obtained from the Perimmune Corporation (Rockville, MD) (236). In brief, purified monoclonal antibody bound the apo E and apo E containing lipoproteins to the ELISA plate well. Diluted plasma samples (1:400), calibrators, and controls were incubated on microtiter wells for one hour at 37°C. Unbound plasma components were washed off the wells. The immune complex was enlarged after an incubation with a polyclonal anti-apo E horseradish peroxidase (HRP) conjugate for one hour at 37°C. The wells were washed and a chromogenic substrate (tetramethyl-benzidine (TMB)) with hydrogen peroxide was added for 30 min at room temperature. This allowed the HRP enzyme, specifically bound through the immune complex, to react with the TMB to form a blue colored solution. Addition of stop solution turned the solution yellow. The absorbance of each well was determined at a wavelength of 450 nm and was proportional to the concentration of apo E in the sample. The calculated within and between run coefficients of variation (CVs) were less than 10%, respectively.

Plasma apo A-I and apo B were determined with a Spectrum CCX analyzer (Abbot Diagnostics) using commercially available standardized immunoturbidimetric assays from Incstar Corporation (Stillwater, MN). The methods are described in detail by Contois et al. (237,238). In brief, 20 μl of plasma were diluted with 200 μl of sample diluent. The antiserum preparation was also prediluted 20-fold with antibody diluent. The analyzer transferred either 3 μl (for apo A-I) or 12.5 μl (for apo B) of diluted plasma to the cuvette, along with 265 μl each of diluted antiserum. The reactions were monitored at 340 nm and 37°C. Serum blanking was used to account for potential interference by sample turbidity. The calculated within and between run coefficient variations (CVs) were less than 2% and 5%, respectively for apo A-I and less than 3% and 11%, respectively for apo B.

Plasma apo A-IV levels were measured by electroimmunodiffusion technique using the Hydragel apo A-IV kit obtained from Sebia (Sebia, Issy-les Moulineaux, France) as previously described (239). This kit provided agarose gels with incorporated anti apo A-IV monospecific antibodies. Fife μL of saline diluted standards and pure samples were applied into the wells of the gel.

Dispersion of all samples prior to migration for 20 min was allowed in order to obtain a homogenous diffusion. Placement of the gel into the electrophoresis chamber and migration at 40 V, 15 mA for 10 min, followed by 130 V, 15 mA for 110 min. Subsequently, the remaining proteins were absorbed by applying filter paper previously soaked with saline under pressure of 1 kg for 20 min. This procedure was repeated of for another 10 min after having washed the gel with saline. The gel was dried at 80°C, immersed in a staining solution, destained, and dried again. The immunoprecipitated rockets were measured and their heights were proportional to apo A-IV concentrations. The calibration was performed using standard sera calibrated to apolipoprotein AIV. The calculated within and between run coefficient variations (CVs) were less than 8%, respectively.

All assays were run on frozen plasma, stored at -80°C, and thawed only once. No significant differences in fresh versus frozen plasma values for apo E, A-I, A-IV, and apo B were noted.

DNA Isolation

The genomic DNA was isolated from 200 µl EDTA blood using the QIAamp Blood Kit (Qiagen GmbH, Germany). The procedure was comprised of three steps. The first step included lysis of the blood, which was initiated by the addition of the enzyme proteinase K and lysis buffer provided by the manufacturer. The sample was incubated at 70°C for 10 min, and ethanol (99%) was added to stop cell lysis. The second step included placement of the mixture onto a spin column. The subsequent washing and centrifugation procedures ensured the proper purification of the DNA. While the DNA was bound to the spin column filter, the rest was discarded in the filtrate. The last step included the elution of the DNA with distilled water. To increase the yield of the DNA, water was preheated at 70°C.

DNA Concentration and Purity

The DNA concentration and purity were measured with a Photospectrometer DU-600 (Beckman Instruments GmbH, Germany). After diluting the DNA samples with distilled water (1:50), extinction was measured at the wavelength of 260 nm (maximum of absorption for nucleic acids) and 280 nm (maximum of absorption for proteins). Regarding the DNA concentration, 1 OD_{260} was equivalent to either 50 µg/ml dsDNA or 20 µg/ml oligonucleotides. The average DNA concentration of the samples used was 30 ng/µl.

With regard to the purity of the DNA, the relation of OD_{260}/OD_{280} resembles the grade of protein pollution of a nucleic acid solution. The recommended range of 1.7 to 1.9 in a protein free solution was kept.

Genotyping

The genotyping was performed using the polymerase chain reaction (PCR) followed by the determination of restriction fragment length polymorphisms (RFLPs).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a highly specific, reliable and fast method to amplify specific DNA segments. The idea behind it is to copy *in vitro* the identical reduplication of a single-stranded DNA sequence (see Figure 2). The procedure includes three steps. The first step includes heat denaturation that mimics the naturally occurring enzymatic separation of double-stranded DNA into two single strands. In the second step, two oligonucleotide primers frame the selected DNA segment. Each primer is complementary to the flanking sequence of one of the original single strands. They hybridize with their 3´-OH ends to the DNA in a way that the amplification only occurs in the direction of the target sequence. The last step incorporates the extension of the primers with the help of the thermostable "*Thermus aquaticus*" (*Taq*) DNA polymerase. Repetition of these cycles (denaturation, primer annealing, and extension) result in an exponential amplification (2^n) where (n) resembles the number of cycles.

Restriction Fragment Length Polymorphism (RFLP)

The RFLP-method is used in this study to identify different isoforms of a specific polymorphism. The principal behind it is to find a restriction enzyme that creates or omits a cutting site at the location of the gene mutation. Therefore, it is required to know the gene sequence, location and type of mutation to select the proper restriction endonuclease. It also should be taken into consideration that the enzyme does not cut too often within that sequence, as fragments under 30 bp are difficult to identify. The *HUSAR*-system was used for optimal enzyme selection.

Figure 2: Mechanism of the Polymerase Chain Reaction (PCR)
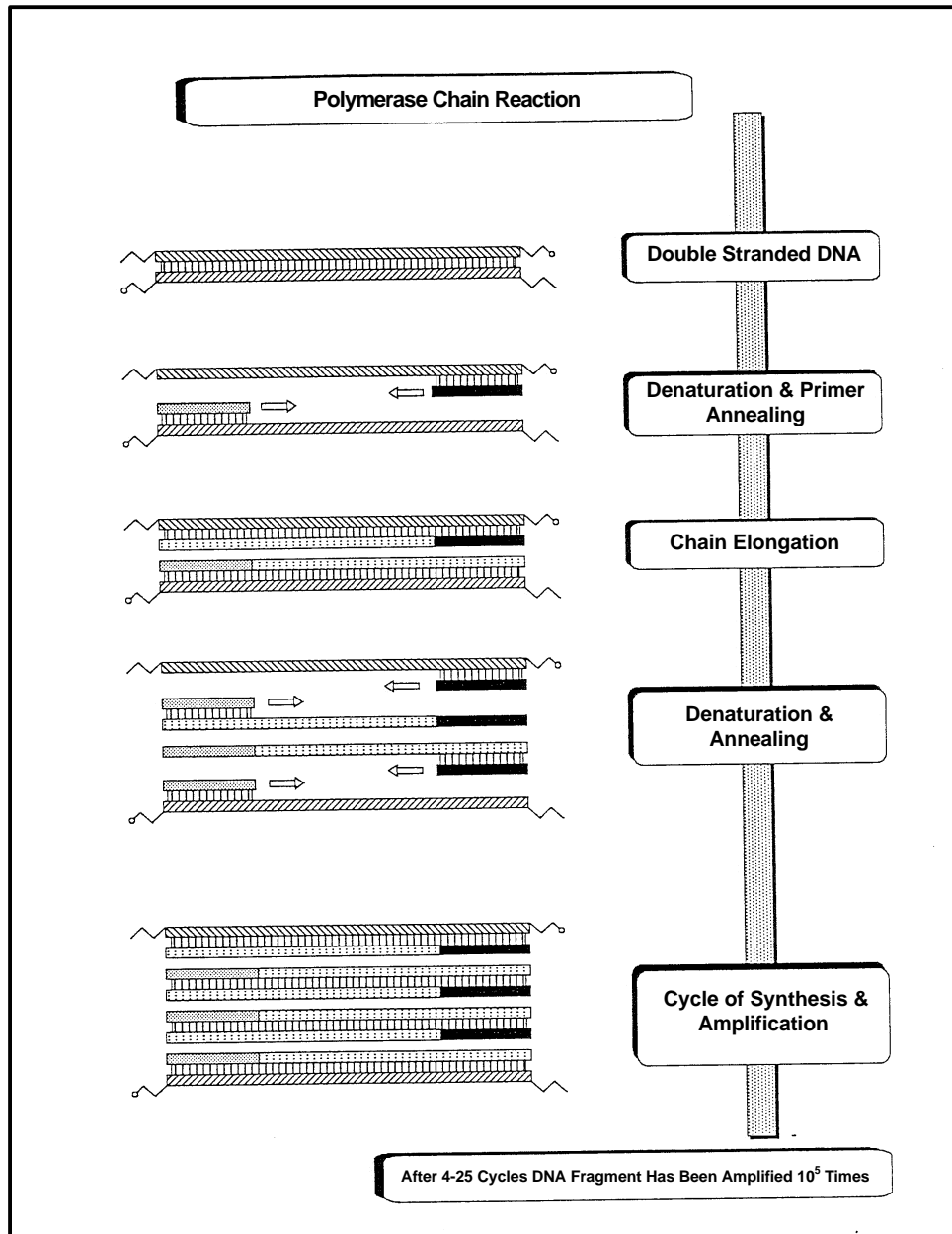
Apolipoprotein E Genotyping

The amplification of the 244 base pair (bp) fragment located in the exon 4 of the apo E gene was done by the polymerase chain reaction (PCR). Primers used were (52):

      F6: 5´-taagcttGGCACGGCTGTCCAAGGA-3´ (sense)

      F4: 5´-acagaattcGCCCCGGCCTGGTACAC-3´ (antisense)

The amplification reaction of 50 µl contained 3 µg genomic DNA, 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 mM MgCl$_2$, 0.3 mM each of dATP, dCTP, dGTP, and dTTP, 0.6 µM of each primer, 10% dimethylsulfoxide (DMSO), and 1.5 U of *Taq* DNA polymerase (Gibco, BRL). The mixture was overlaid with mineral oil and placed into an UNO-Thermoblock Cycler (Biometra, Germany). Amplification protocol consisted of an initial denaturation step at 95°C of 5 min, following 32 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C, and extension at 72°C for 2 min, and a final extension at 72°C for 5 min. Sixteen microliters of the PCR product were digested with 2 µl of restriction endonuclease Cfo I (10 U/µl) and 2 µl of the buffer provided by the manufacturer (Boehringer Mannheim, Germany) at 37°C for 3 hours. The digested DNA fragments were loaded onto a 4% NuSieve/agarose gel (1:3) stained with ethidium bromide and electrophoresed in 1x TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) for 45 min at 100 V. The DNA bands were visualized by UV-light at a wavelength of 322 nm. Pictures were taken with Polaroid film. The marker V (Boehringer Mannheim, Germany) served as a control standard. Figure 3 shows the various apo E genotypes on a polyacrylamide gel, which allowed a higher resolution of the small fragments.

Figure 3: DNA Fragments of Apo E Genotype after PCR and RFLP
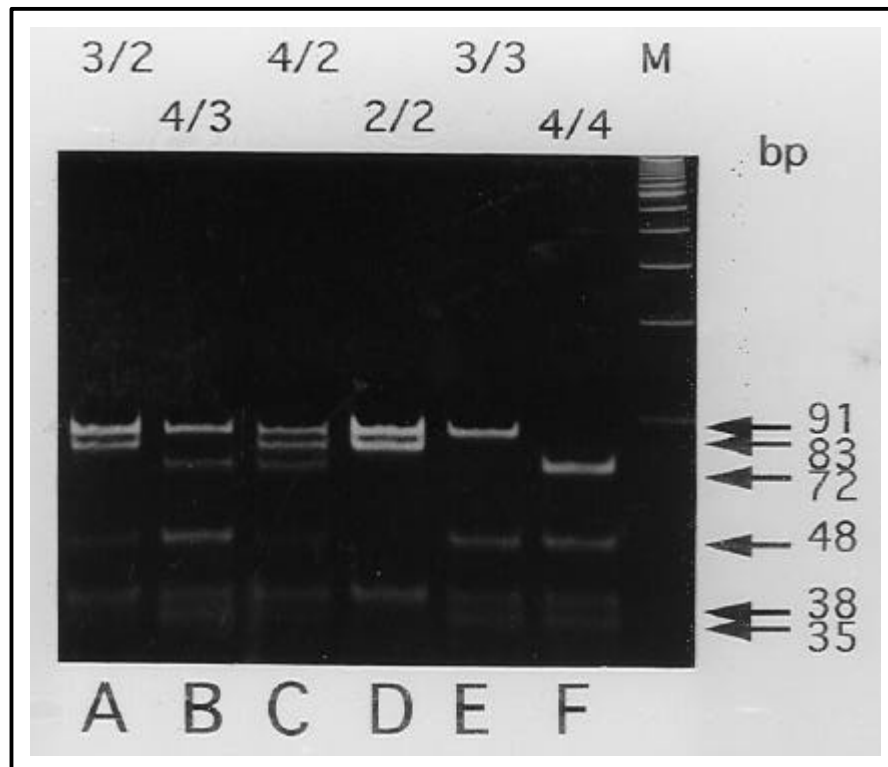
The resulting RFLP fragments are shown in Figure 4.

Figure 4: Apo E Genotyping
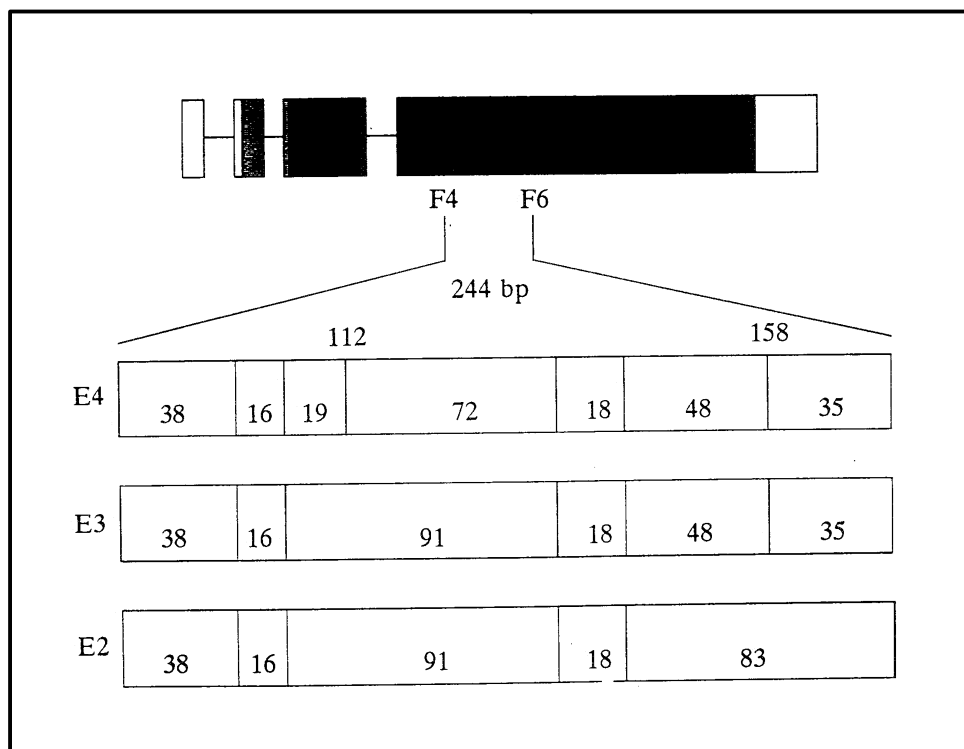
Apolipoprotein A-I Genotyping

The 435 bp apo A-I gene fragment, including the -75 bp polymorphism in the promoter region and the +83/84 bp mutations located in the first intron, was amplified by PCR, followed by simultaneous digestion with the restriction endonuclease MspI. The oligonucleotide pair used was described by Saiki et al. (240):

        P1: 5´-AGGGACAGAGCTGATCCTTGAACTCTTAAG-3´ (sense)
        P2: 5´-TTAGGGGACACCTACCCGTCAGGAAGAGCA-3´ (antisense)

The 50 µl amplification mixture contained 60 ng genomic DNA, 50 mM KCl, 20 mM Tris-HCl, (pH 8.4), 2.5 mM MgCl$_2$, 0.6 µM of each primer, 0.2 mM each dNTP, 10% DMSO, and 1.5 Units of *Taq* DNA polymerase (Gibco, BRL). The amplification protocol was executed by an UNO-Thermoblock Cycler (Biometra, Germany) and consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles of primer annealing and extension at 94°C for 30 sec, 60°C

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for 45 sec, and 72°C for 1 min, and ended with a final extension at 72°C for 5 min. The PCR product was digested with 2.5 units of MspI (Boehringer Mannheim, Germany) for 3 hours at 37°C. The digested DNA fragments were loaded onto a 4% NuSieve/agarose gel (1:3) (FMC, BioProducts) with incorporated ethidium bromide and subjected to electrophoresis for 45 min at 100 V. The bands were visualized by an UV-transilluminator. The marker V (Boehringer Mannheim, Germany) served as a control standard (Figure 5).

Figure 5: DNA Fragments of Apo A-I –75, +83 bp Genotype after PCR and RFLP
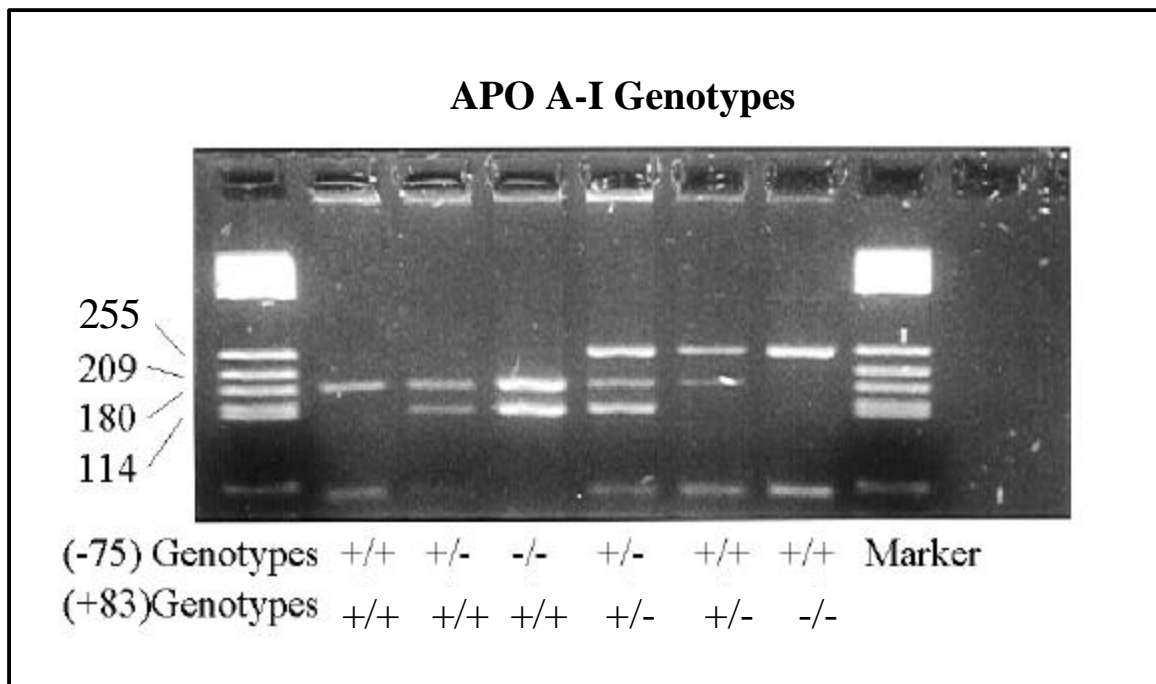
Concerning the promoter polymorphisms at position -75 bp, the presence of the restriction site, representing the more common G-allele, resulted in the fragments 209 bp, 114 bp, 66 bp, and 46 bp. The absence of the restriction site, caused by the G to A substitution, resulted in the fragments 209 bp, 180 bp, and 46 bp. With regard to the polymorphism in the first intron of the A-I gene, the C to T and/or G to A substitutions at positions +83/84 bp abolished the MspI cutting site and resulted a longer 255 bp DNA fragment in individuals having the rare M2- allele. The alleles of the -75 bp and 83 bp genotypes as well as the haplotypes are demonstrated in Figure 6.

Figure 6: Haplotypes of the Apo A-I –75 bp and +83 bp Polymorphisms

Apo A-I Haplotypes

DNA fragments of the apo A-I -75bp and +83bp haplotypes after amplification and digestion with the restriction enzyme MspI.
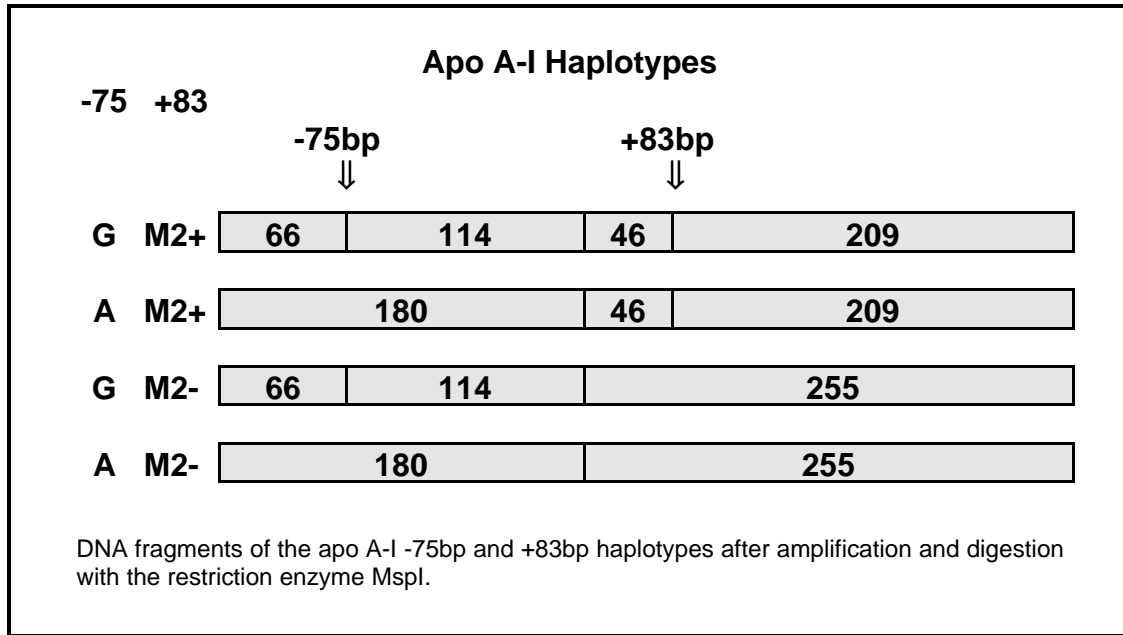
Apolipoprotein A-IV Genotyping

The two different polymorphisms at site 360 bp and 347 bp in the apo A-IV gene were amplified using PCR procedure. Primers used were (241):

F1: 5´-CCTGAGGCACAAGGTCAACTC-3´(sense)

R1: 5´-CACCTGCTCCTGCTA* CTGCTCC-3´ (antisense)

> * In the tailored primer (R1), an A is inserted instead of a G that would be the nucleotide corresponding to the sequence apo A-IV. This leads to the disappearance of the two Fnu4H1 restriction sites present downstream from the point mutation (see Figure 7, cited from Tenkanen et al. (241)).

Figure 7: A-IV Primer Selection

Schematic presentation of the exon 3 of the apoA-IV gene. In the upper part of the diagram the positions of the primers used to amplify part of exon 3 (2285-2411) by the polymerase chain reaction are indicated. In the lower part an enlarged region of exon 3, adjacent to the polymorphic site located at nucleotide 2387, is presented. In the apoA-IV[1] allele the three recognition sites for the Fnu4H1 restriction enzyme (GCNGC) are indicated in boldface letters. In the tailored Primer II the modification, an A is inserted instead of a G, which leads to the abolishment of the two downstream recognition sites is indicated by a square. Thus, after amplification of the apoA-IV[1] allele, there is only one Fnu4H1 restriction site left while the two downstream recognition sites have disappeared (crossed arrows). The product obtained after amplification of the apoA-IV[2] allele using the tailored primer is without recognition sites for the Fnu4H1 enzyme.

The 50 μl reaction mixture contained 270 ng genomic DNA, 1x PCR-buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 0.5 mM each primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 15 mM $(NH_4)SO_4$, 0.1% tween 20, 0.1 mg/ml gelatin, and 1.5 U *Taq* DNA polymerase (Gibco, BRL). The mixture was overlaid with mineral oil and placed into an UNO-Thermoblock Cycler (Biometra, Germany) for amplification. The protocol was as followed: initial denaturation at 95°C for 3 min, then 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified PCR product was divided into two tubes for digestion. To identify the apo A-IV 360 mutation, 25 μl of PCR product were combined with 0.16 units restriction enzyme Ita I (10 U/μl) (Boehringer Mannheim, Germany). For the apo A-IV 347 mutation, the other 25 μl were mixed with 1 unit of the restriction enzyme Hinf I (10 U/μl) (Boehringer Mannheim, Germany). Both tubes were incubated for 4 hours at 37°C. The digested DNA fragments were each loaded onto a 4% NuSieve/agarose gel (1:3), stained with ethidium bromide and electrophoresed for 45 min at 100 V. The DNA bands were

visualized by UV-light. The marker V (Boehringer Mannheim, Germany) served as a control standard (see Figure 8).

Figure 8: DNA Fragments of APO A-IV 360,347 Genotypes after PCR and RFLP
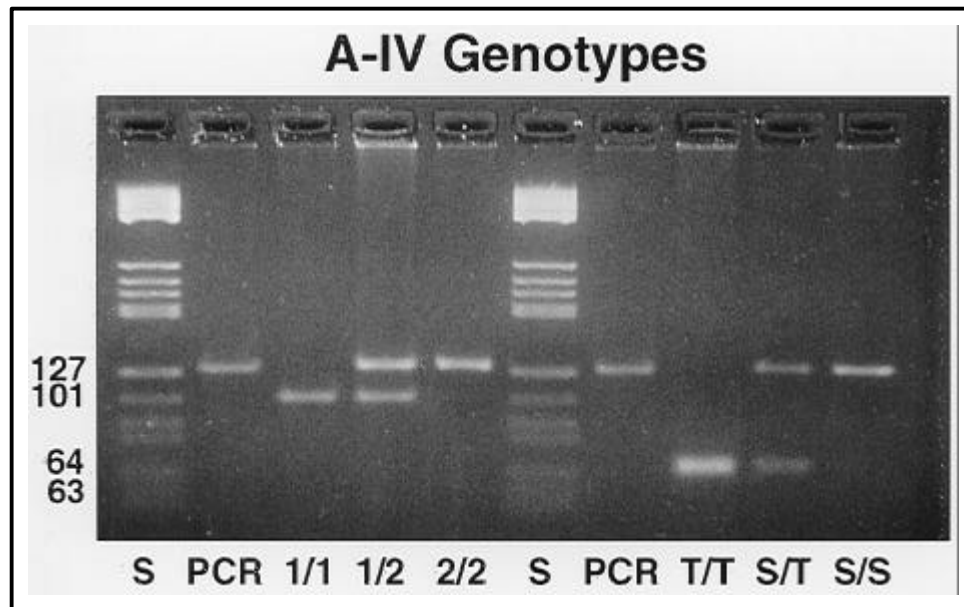
The digestion of the apo A-IV 360 mutation results either in a 127 bp fragment (uncut), which indicates the presence of the more common apo A-IV-1 allele (Gln), or in two 101 bp and 26 bp fragments (cut), which indicates the presence of the apo A-IV-2 allele (His). With regard to the apo A-IV 347 mutation, the two fragments 64 bp and 63 bp (cut) are due to the presence of the more common T-allele, while the127 bp fragment (uncut) is due to the less common S-allele (Figure 9).

Figure 9: Apo A-IV 360 and 347 Genotype RFLP Fragments

Apo A-IV 360

Common 1-Allele

101 bp	26 bp

Rare 2-Allele

127 bp

Apo A-IV 347

Common T-Allele

64 bp	63 bp

Rare S-Allele

127 bp
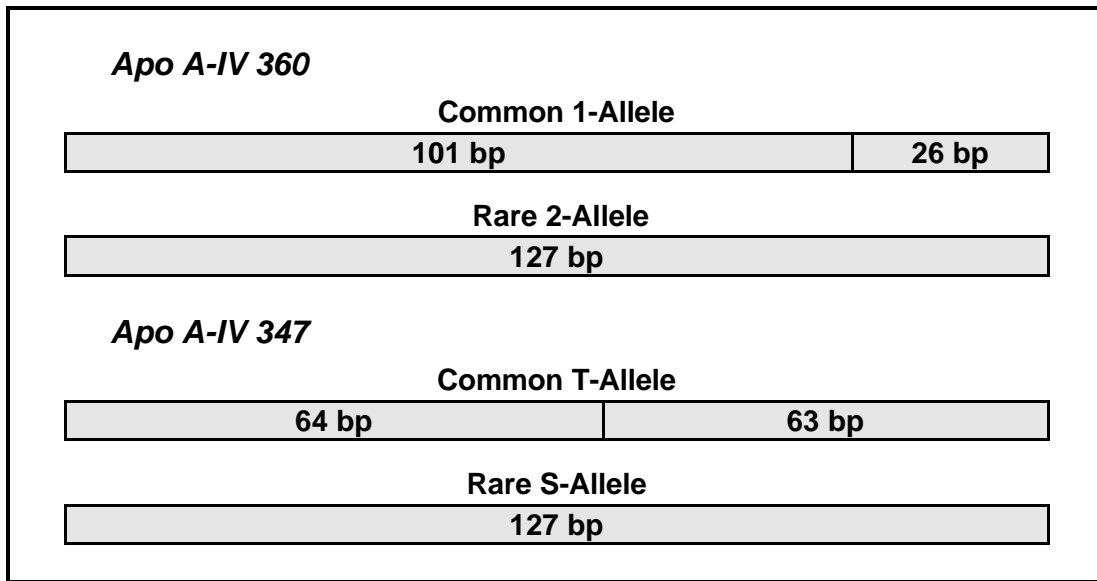
Lipoprotein Lipase Genotyping

The Hind III genotype in intron 8 of the LPL gene was determined by PCR followed by digestion with the restriction endonuclease Hind III. The following oligonucleotides were used for amplification of the 356 bp product (226):

> 5´-GATGCTACCTGGATAATCAAAG-3´ (sense)
> 5´-CTTCAGCTAGACATTGCTAGTGT-3´ (antisense)

The 25µl reaction mixture contained 50 ng genomic DNA, 1x PCR buffer (Gibco, BRL), 0.1 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 µM of each primer and 0.6 U of *Taq* DNA polymerase (Gibco, BRL). Amplification was performed in an UNO-Thermoblock Cycler (Biometra, Germany) and consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec and 57°C for 30 sec, and finished with a final extension at 72°C for 5 min. The amplified product was incubated with 5 U of Hind III (10 U/µl) (Gibco, BRL) overnight at 37°C. The digested DNA fragments were loaded onto a 5% NuSieve/agarose gel (1:3) (FMC BioProducts), electrophoresed, and visualized by UV-light (Figure 10). The marker V (Boehringer Mannheim, Germany) served as a control standard.

Figure 10: DNA Fragments of LPL Hind III Genotype after PCR and RFLP
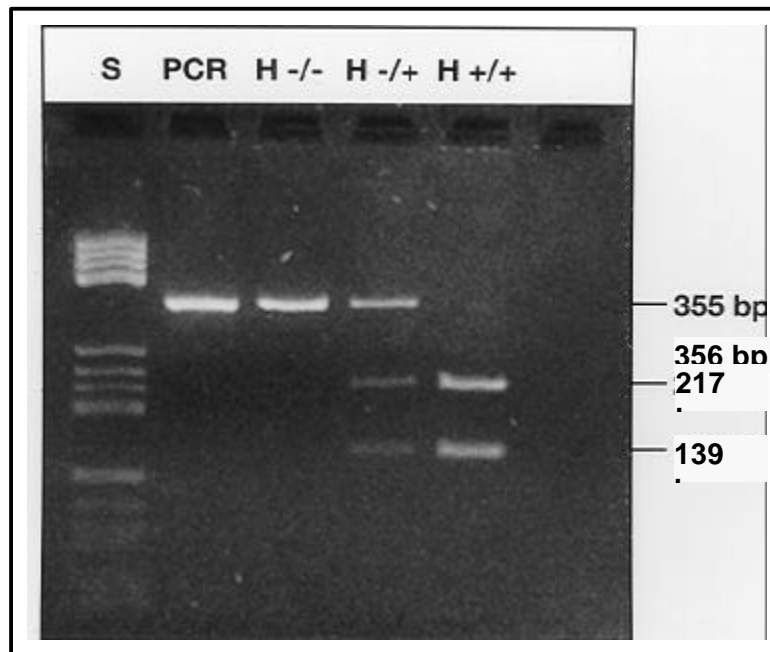
The resulting RFLP fragments were 356 bp (uncut) for the H- allele, or 217 bp and 139 bp (cut) for the H+ allele (Figure 11).

Figure 11: Lipoprotein Lipase Hind III Genotype RFLP Fragments
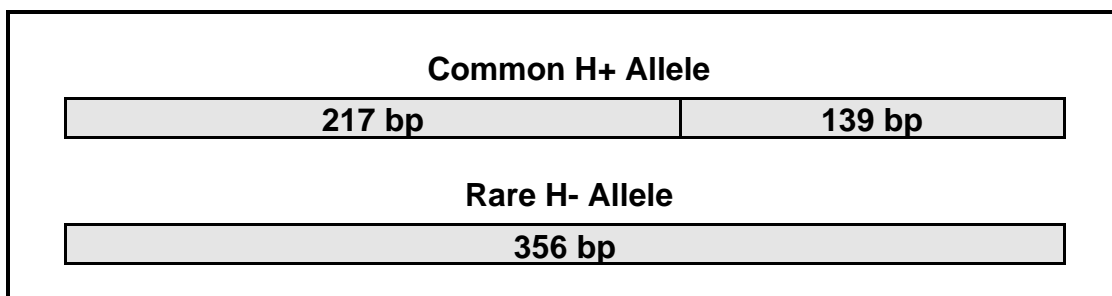
Statistical Analysis

Statistical analysis was performed with the software package SPSS/PC+. The statistical significance was set at $\alpha=0.05$. Allele frequencies for the various polymorphic sites were

estimated by gene counting. Agreement of the genotype frequencies with the Hardy-Weinberg equilibrium expectations was tested using a X2 goodness-of-fit test. Linkage disequilibrium between the apo A-I -75 bp and +83 bp single nucleotide polymorphisms (SNP´s) was also assessed using the Chi square test. Regarding apo E genotype, subjects were grouped into E2+ (E2/2, E2/3), E3/3, and E4+ (E3/4, E4/4). Subjects carrying E2/4 were excluded from the analysis. For the apo A-I -75 bp baseline analyses, subjects carrying the A-allele (G/A and A/A) were combined to increase the power. With regard to the apo A-I +83 bp genotype, the one female carrying the M2-/- genotype was excluded from the analysis due to her unusual lipid profile. The same was true for the one female carrying the rare apo A-IV 360 2/2 genotype. She also was excluded from the analyses.

Distributions of all variables were tested for normality. Data for body mass index (BMI) (computed as weight in kilograms divided by height in meters squared), plasma glucose, HDL-C, and triglyceride were $\log_{10}$ transformed prior to analysis of covariance (ANCOVA) to reduce skewness of the data. Antilogs and unadjusted mean values ± standard deviations (SD) of the lipid traits are presented in all the various tables and figures. Analyses were carried out separately for each polymorphism, as well as for females and males, respectively. Comparisons of all the biochemical parameters between females and males were performed with Student's t-test.

Because environmental aspects can influence plasma glucose, lipid, lipoprotein, and apolipoprotein concentrations, adjustments were made for age in females and age^2 in males, BMI, smoking status, alcohol use, and medications (cholesterol lowering drugs, diabetes medication, hormonal replacements, and thyroid supplementation). ANCOVA was performed to test the null hypothesis that phenotypic variations in these traits were not associated with genetic variation at the candidate gene loci. The continuous variables age and BMI were entered in the general linear model (GLM) as covariates. The dichotomous variables: smoking status, alcohol use, medications, and apo E alleles were included as factors. Apo E alleles (2+, 3/3, 4+) were incorporated in the analyses of apo A-I, apo A-IV, and Hind III, due to their known effects on various lipid traits. In addition, two-way interactions between these factors and the various genotypes have been added to the model. In case of significant effects of genetic variability on lipid traits, one-way ANOVA (Tukey test) or Student's t-test was performed to compare interindividual differences between genotypes.

For the analyses of responsiveness, ANOVA-repeated measurements was performed to test the null hypothesis that differences in fasting glucose, lipid, and lipoprotein response were not associated with genetic variation at the various candidate gene loci.

Results

A. Effects of Apolipoprotein E, A-I, A-IV and Lipoprotein Lipase Genotypes on Baseline Levels of Glucose, Lipid, Lipoprotein, and Apolipoproteins

Subjects

Data on the 361 females and 373 males are presented in Table 6.

Table 6: Study Subjects*			
	Females (n = 361)	**Males (n = 373)**	**Percentage Difference**
Age (years)	56.1 ± 13.4	58.7 ± 12.3 †	+ 4.6%
Body mass index (kg/m^2)	28.5 ± 6.8	30.6 ± 5.5 †	+ 7.4%
Height (cm)	163.9 ± 6.5	177.8 ± 6.6 †	+ 8.5%
Weight (kg)	76.8 ± 20.5	96.3 ± 19.1 †	+ 25.4%
Waist (cm)	99.1 ± 17.9	108.8 ± 14.6 †	+ 9.7%
Cholesterol-lowering medication	42 (5.7%)	77 (10.5%) †	+ 84.2%
Medication for diabetes	21 (2.9%)	53 (7.2%) †	+ 148.3%
Hormonal replacement	133 (18.1%)	1 (0.1%) †	− 99.9%
Thyroid medication	87 (11.9%)	22 (3.0%) †	− 74.8%
Alcohol users (>1 drink/week)	144 (40.0%)	195 (52.1%) †	+ 30.3%
Cigarette smoking (current)	25 (6.9%)	49 (13.1%) †	+ 89.9%

* Mean values ± SD or number of subjects with percentage of total in parentheses.

† Significantly different ($p < 0.05$) from females.

The mean age in females was 56.1 years (ranging from 16 to 81 years) and in males 58.7 years (ranging from 15 to 91 years). As a group, their body mass index was somewhat elevated, with a mean of 28.5 kg/m^2 in the females and 30.6 kg/m^2 in the males. Men had significantly higher body weight at 96.3 kg compared to women at 76.8 kg. With regard to medications, 5.7% of the females and 10.5% of the males were on cholesterol-lowering medication, 2.9% of the females

and 7.2% of the males were on medication for diabetes, 18.1% of the females and 0.1% of the males were receiving hormonal replacement, and 11.9% of the females and 3.0% of the males were taking thyroid supplementation. Menopausal status in females was not assessed. Alcohol use with more than one drink per week was noted in 40.0% of the females and 52.1% of the males, and current cigarette smoking was noted in 6.9% of the females and 13.1% of the males. Significant differences in all of these parameters were noted when males were compared with females.

Data on biochemical parameters are provided in Table 7. Males had significantly ($p<0.05$) higher values for apo E (7.8%), apo A-IV (6.9%), apo B (6.0%), glucose (12.0%) and the TC/HDL cholesterol ratio (26.7%) than did females. They also had significantly lower levels of apo A-I (10.1%), total (3.2%) and HDL cholesterol (23.6%) than females. In addition, triglyceride values were also 21.8% higher in men than in women. With regard to LDL cholesterol, no significant gender difference was noted.

Table 7: Biochemical Parameters*			
	Females (n = 361)	Males (n = 373)	Percentage Difference
Apo A-I	(146.7 ± 21.6)	(131.9 ± 17.4) †	– 10.1%
Apo A-IV	(14.4 ± 4.2)	(15.4 ± 4.9) †	+ 6.9%
Apo E	(10.7 ± 6.8)	(11.5 ± 4.57) †	+ 7.8%
Apo B	(109.3 ± 29.8)	(115.8 ± 31.6) †	+ 6.0%
Total Cholesterol	5.56 ± 1.08 (214.6 ± 41.5)	5.38 ± 1.13 † (207.8 ± 43.8)	– 3.2%
LDL-C	3.15 ± 0.91 (121.5 ± 35.4)	3.21 ± 0.92 (123.8 ± 35.5)	+ 1.9%
HDL-C	1.61 ± 0.39 (62.1 ± 15.2)	1.23 ± 0.32 † (47.4 ± 12.2)	– 23.6%
Triglyceride	1.74 ± 0.90 (154.0 ± 80.0)	2.12 ± 1.25 † (187.2 ± 110.2)	+ 21.8%
TC/HDL ratio	3.63 ± 1.0	4.60 ± 1.30 †	+ 26.7%
Glucose	5.23 ± 2.74 (95.8 ± 39.1)	5.86 ± 2.45 † (107.3 ± 45.6)	+ 12.0%

*   Mean values ± SD, all values, except ratios, are in mmol/L, values in parentheses are in mg/dl, conversions from mg/dl were division by 18.3 for glucose, 38.6 for cholesterol, and 88.5 for triglyceride.

†   Significantly different ($p < 0.05$) than females.

Genotype Frequencies

The genotype distribution and allele frequencies of the apo E polymorphism are presented in Table 8. The frequencies were similar in men and women, with about 60% of the population having the apo E3/3 genotype, and about 20% of the population having the apo E3/4 genotype, and another approximately 12% having the apo E2/3 genotype. The remainder was divided among the rarer apo E4/4, apo E2/4, and apo E2/2 genotypes. The allele frequency for apo E3 was 0.78 in both females and males, for E4 it was 0.16 in females and 0.12 in males, and for apo E2 it was 0.06 in females and 0.10 in males.

Table 8: Apo E Genotype Distributions and Allele Frequencies*

	Females (n = 361)		Males (n = 373)	
E 3/3	215	(59.6%)	234	(62.7%)
E 3/4	89	(24.7%)	67	(18.0%)
E 2/3	42	(11.6%)	50	(13.4%)
E 4/4	9	(2.5%)	6	(1.6%)
E 2/4	6	(1.7%)	13	(3.5%)
E 2/2	0	(0.0%)	3	(0.8%)

* Apo E allele frequencies: in females, ϵ3: 0.78, ϵ4: 0.16, ϵ2: 0.06; in males, ϵ3: 0.78, ϵ4: 0.12, ϵ2: 0.10.

Genotype distributions and allele frequencies of the apo A-I -75 bp and the +83 bp polymorphisms, as well as their haplotypes, are presented in Table 9. With regard to the -75 bp polymorphism in the promoter region, 74.0% of the women and 70.5% of the men were homozygous for the common G-allele, 24.4% of the women and 25.7% of the men were heterozygous, and 1.7% of the women, and 3.8% of the men were homozygous for the rare A-allele. Overall, the frequency for the G-allele was 0.86 in women and 0.83 in men, and for the A-allele it was 0.14 and 0.17 in women and men, respectively. Due to the small sample size of the latter group, G/A and A/A genotypes were combined and compared with the G/G homozygotes.

Table 9: Apolipoprotein A-I -75 bp and +83 bp Genotype Distributions and Allele Frequencies*

Genotype	Females		Males	
A-I -75 bp				
G/G	267	(74.0%)	263	(70.5%)
G/A	88	(24.4%)	96	(25.7%)
A/A	6	(1.7%)	14	(3.8%)
A-I +83 bp				
M2+/+	331	(91.7%)	336	(90.1%)
M2+/-	29	(8.0%)	37	(9.9%)
M2-/-	1	(0.3%)	0	(0.0%)
Haplotypes				
G/G M2+/+	244	(67.6%)	231	(61.9%)
G/A M2+/+	87	(24.1%)	105	(28.2%)
G/G M2+/-	23	(6.4%)	32	(8.6%)
G/A M2+/-	7	(1.9%)	5	(1.3%)

* Allele frequencies for G and A in females were 0.86 and 0.14, and in males, they were 0.83 and 0.17, respectively. Allele frequencies for M2+ and M2- were 0.96 and 0.04 in females and 0.95 and 0.05 in males, respectively.

Concerning the +83 bp polymorphic site in the first intron of the apo A-I gene, 91.7% of the women and 90.1% of the men were homozygous for the common M2+ allele, while 8.0% and 9.9% were heterozygous, respectively. Only one female was homozygous for the rare M2- allele, and she was excluded from the analysis due to her unusual lipid profile: glucose: 4.75 mmol/L (87 mg/dl), apo A-I: 151.0 mg/dl, apo B: 71.0 mg/dl, total cholesterol: 4.09 mmol/L (158 mg/dl), LDL-C: 1.66 mmol/L (64 mg/dl), HDL-C: 1.94 mmol/L (75 mg/dl), and triglyceride: 1.05 mmol/L (93 mg/dl).

Linkage disequilibrium (X^2: 4.359, p=0.037) was detected between the apo A-I -75 bp and +83 bp polymorphisms. With regard to the haplotype distribution, 67.6% of the women and 61.9% of the men had the combination G/G M2+/+, 24.1% and 28.2% of women and men carried G/A M2+/+, 6.4% and 8.6% had G/G M2+/- and 1.9% and 1.3% of women and men carried both rare alleles G/A M2+/-, respectively.

Genotype distributions for the apo A-IV 360 and 347 mutations are shown in Table 10. With regard to the 360 (Gln/His) polymorphism, 83.7% of the women and 86.6% of the men were homozygous for the common apo A-IV-1 allele, 16.1% of the women and 13.4% of the men were heterozygous (A-IV 1/2). Only one female was homozygous for the rare A-IV-2 allele. Her lipid profile was: fasting glucose 4.64 mmol/L (85.0 mg/dl), apo A-I: 109.0 mg/dl, apo A-IV: 11.4 mg/dl, apo B: 59.0 mg/dl, total cholesterol: 3.24 mmol/L (125.0 mg/dl), LDL-C: 1.81 mmol/L (70.0 mg/dl), HDL-C: 1.06 mmol/L (41.0 mg/dl), and triglyceride: 0.78 mmol/L (69.0 mg/dl). She was excluded from the analysis.

Table 10: Apolipoprotein A-IV 360/ 347 Genotype Distribution and Allele Frequencies*

	Females		Males	
A-IV 360				
1/1	302	(83.7%)	323	(86.6%)
1/2	58	(16.1%)	50	(13.4%)
2/2	1	(0.3%)	0	(0.0%)
A-IV 347				
T/T	240	(66.5%)	234	(62.7%)
S/T	107	(29.6%)	119	(31.9%)
S/S	14	(3.9%)	20	(5.4%)

* Allele frequencies for apo A-IV 360 of 1 and 2 in females are 0.92 and 0.08, and in males are 0.93 and 0.07, respectively. Allele frequencies for apo A-IV 347 of T and S in females are 0.81 and 0.19, and in males are 0.79 and 0.21, respectively.

Concerning the A-IV 347 polymorphic site, 66.5% of the women and 62.7% of the men were homozygous for the common threonine (T) allele, 29.6% and 31.9% were heterozygous (T/S), while 3.9% and 5.4% of women and men were homozygous for the rare serine (S) allele, respectively.

Table 11 shows the genotype distribution and allele frequencies of the LPL Hind III polymorphism. In females, 43.8% and in males 50.1% carried the H+/+ wildtype, 44.0% of the females and 42.4% of the males were heterozygous (H+/-), while 12.2% of the females and 7.5% of the males were homozygous for the rare H- allele. Therefore, the allele frequencies of H+ and H- in females were 0.71 and 0.29, and in males 0.66 and 0.34, respectively.

Table 11: Lipoprotein Lipase Hind III Genotype Distributions and Allele Frequencies*

Genotype	Females		Males	
H -/-	26	(7.5%)	43	(12.2%)
H +/-	147	(42.4%)	155	(44.0%)
H +/+	174	(50.1%)	154	(43.8%)

* Allele frequencies for H+ and H- for females were 0.71 and 0.29, and for males, they were 0.66 and 0.34, respectively.

The combined data are consistent with the concept that all genotype distributions and allele frequencies of the various polymorphisms are similar in men and women.

Effect of Apolipoprotein E Genotype on Glucose and Lipid Parameters

Data on biochemical variables by apo E genotype are provided in Table 12. Females and males with the apo E2 allele had 32% and 27% higher apo E values than those with the apo E3/3 genotype (p<0.0001), while subjects with the apo E4 allele had 33% and 18% lower values than the apo E3/3 group (p<0.0001), respectively.

As in many studies with females and males, there were clearly higher total cholesterol levels in subjects with the apo E4 allele than in those with the apo E 3/3 genotype, and lower values were seen in those carrying the apo E2 allele. This was also true for LDL cholesterol. In the case of LDL cholesterol for females, those carrying the apo E2 allele had significantly (p<0.05) lower LDL cholesterol levels, at 2.83 mmol/L, than those carrying the apo E4 allele, with a mean value of 3.31 mmol/L. A similar finding was noted in males, but here those carrying the apo E2 allele had significantly lower LDL cholesterol levels than subjects with the apo E3/3 genotype.

Table 12: Apolipoprotein E Genotype and Biochemical Parameters*

	Females (n = 354)			Males (n = 359)		
	E2+ (n = 42)	E3/3 (n = 213)	E4+ (n = 98)	E2+ (n = 53)	E3/3 (n = 233)	E4+ (n = 73)
Apo E	(14.5 ± 4.4) †‡	(11.0 ± 4.8)	(8.3 ± 3.0) †	(14.3 ± 5.0) †‡	(11.3 ± 4.3)	(9.6 ± 4.0) †
Total Cholesterol	5.16 ± 1.22 ‡ (199.3 ± 47.1)	5.53 ± 1.08 (213.3 ± 41.6)	5.80 ± 0.97 (223.7 ± 37.3)	5.14 ± 1.02 (198.4 ± 39.3)	5.40 ± 1.12 (208.5 ± 43.2)	5.56 ± 1.28 (214.8 ± 49.4)
LDL-C	2.83 ± 1.13 ‡ (109.1 ± 43.4)	3.14 ± 0.88 (121.2 ± 34.0)	3.31 ± 0.88 (127.9 ± 33.9)	2.92 ± 0.86 † (112.6 ± 33.1)	3.26 ± 0.95 (125.7 ± 36.8)	3.33 ± 0.85 (128.5 ± 32.9)
HDL-C	1.57 ± 0.30 (60.5 ± 11.7)	1.58 ± 0.40 (60.9 ± 15.4)	1.71 ± 0.41 § (65.8 ± 15.8)	1.32 ± 0.33 †‡ (50.9 ± 12.7)	1.21 ± 0.32 (46.8 ± 12.4)	1.20 ± 0.29 (46.2 ± 11.1)
Triglyceride	1.68 ± 0.64 (148.5 ± 56.4)	1.75 ± 0.95 (154.6 ± 84.0)	1.71 ± 0.89 (151.2 ± 78.9)	1.97 ± 1.73 (174.6 ± 114.4)	2.10 ± 1.14 (185.6 ± 100.5)	2.29 ± 1.52 (207.7 ± 134.5)
TC/HDL ratio	3.38 ± 0.94	3.69 ± 1.09	3.58 ± 0.98	4.11 ± 1.31 †	4.68 ± 1.32	4.81 ± 1.14
Glucose	5.01 ± 1.18 † (91.6 ± 21.6)	5.45 ± 2.55 (99.8 ± 46.7)	4.87 ± 1.29 † (89.1 ± 23.6)	6.12 ± 2.48 (111.9 ± 45.3)	5.82 ± 2.38 (106.4 ± 43.5)	6.03 ± 3.02 (110.3 ± 55.2)

* Mean values ± SD in mmol/L, values in parentheses are in mg/dl

§ Significantly different (p<0.05) from apo E3 group and apo E4 group before adjustments

† Significantly different (p < 0.05) from apo E3 group

‡ Significantly different (p < 0.05) from apo E4 group

With regard to HDL cholesterol, results have to be distinguished between before and after adjustments were made for age, sex, medications, alcohol, and smoking. Before adjustments, females carrying the apo E4 allele had significantly higher HDL cholesterol levels than females who had the apo E3/3 or E2+ genotypes. No such difference was observed in men. However, after adjustments, significant effects on HDL-C levels appeared in males, while existing effects disappeared in females. Males carrying the apo E2 allele had significantly higher levels of HDL-C compared to men carrying the apo E3/3 or E4+ genotype. With regard to the TC/HDL cholesterol ratio, no significant effects of apo E genotype were observed in females. However, males with the apo E2 allele had significantly lower TC/HDL cholesterol ratios than subjects with the apo E3/3 genotype.

For glucose values, there was a trend clearly observed in women, where subjects with the apo E4 and apo E2 alleles had the lowest glucose values, and subjects with the apo E3 allele had the highest values. In men, no such clear trend with regard to apo E genotype and glucose levels was noted.

Data on statistical association of apo E genotype with biochemical variables, after adjustment for medications, alcohol use, smoking, age, and body mass index, are shown in Table 13.

Table 13: Adjusted Effects of Apolipoprotein E Genotype on Biochemical Parameters (p values)*

	Females (n = 361)	Males (n = 373)
Apo E	0.0001	0.0001
Total Cholesterol	0.021	N.S.
LDL-C	0.032	0.037
HDL-C	N.S.	0.012
TC/HDL ratio	N.S.	0.004
Triglyceride	N.S.	N.S.
Glucose	0.002	N.S.

* Adjustment for medications, alcohol use, smoking, age, and body mass index.

Apo E genotype had a significant effect in females on apo E ($p<0.001$), glucose ($p=0.002$), total cholesterol ($p=0.021$), and LDL cholesterol levels ($p=0.032$). In contrast, in males no significant

effects of apo E genotype were noted for glucose or total cholesterol. However, a significant effect was observed for apo E (p<0.0001), LDL cholesterol (p=0.037), and HDL cholesterol levels (p=0.012), as well as the TC/HDL cholesterol ratio (p=0.004) after these adjustments.

Data comparing the effects of apo E alleles on LDL cholesterol, HDL cholesterol, and triglyceride values in this population was compared with 495 postmenopausal women and 527 men over age 50 participating in cycle 3 of the Framingham Offspring Study are shown in Table 14.

	Females			Males		
	E2+	**E3/3**	**E4+**	**E2+**	**E3/3**	**E4+**
LDL-C						
Current	2.83 (109)	3.14 (121)	3.31 (128)	2.92 (113)	3.26 (126)	3.33 (129)
Framingham	3.39 (131)	3.81 (147)	4.04 (156)	3.58 (138)	3.81 (147)	3.81 (147)
HDL-C						
Current	1.57 (61)	1.58 (61)	1.71 (66)	1.32 (51)	1.21 (47)	1.20 (46)
Framingham	1.48 (57)	1.44 (56)	1.50 (58)	1.14 (44)	1.17 (45)	1.11 (43)
Triglyceride						
Current	1.68 (149)	1.75 (155)	1.71 (151)	1.97 (175)	2.10 (186)	2.29 (208)
Framingham	1.32 (117)	1.48 (131)	1.37 (121)	1.71 (151)	1.71 (151)	1.70 (168)

Table 14: Comparison with Data from the Framingham Offspring Study*

* Comparison with 495 post-menopausal women and 527 men over age 50 participating in cycle 3 of the Framingham Offspring Study. Mean values are in mmol/L, values in parentheses are in mg/dl.

These data indicate that the health conscious subjects enrolling in a lifestyle modification program had lower LDL cholesterol values and higher triglyceride and HDL cholesterol values than those in Framingham (60).

Effect of Apolipoprotein A-I Genotypes on Glucose and Lipid Parameters

Data on biochemical variables according to gender and -75 bp and +83 bp genotypes, as well as their haplotypes, are provided in Tables 15 - 17. The effects after adjustment for age, body mass index, medications, alcohol use, smoking, and apo E alleles are shown in Table 18. With regard to the -75 bp polymorphism, a significant difference between genotype classes was observed in

females. Women carrying the A-allele had significantly higher levels of apo B (p=0.016), total cholesterol (p=0.005), LDL-C (p=0.010), and TC/HDL ratio (p=0.026) compared to G/G homozygotes. In men, however, no significant difference between the apo A-I -75 bp genotype and any of the glucose, lipid, and apolipoprotein parameters was observed.

Table 15: Effects of Apolipoprotein A-I -75 bp Genotype on Biochemical Parameters*

	Females (n = 359)		Males (n = 372)	
	G/G (n = 266)	G/A+A/A (n = 93)	G/G (n = 263)	G/A+A/A (n = 109)
Apo A-I	(146.7 ± 22.1)	(146.4 ± 19.9)	(131.6 ± 17.4)	(133.1 ± 18.0)
Apo B	(107.7 ± 28.4)	(113.8 ± 33.1) †	(115.6 ± 31.6)	(116.4 ± 31.0)
Total Cholesterol	5.50 ± 1.00 (212.5 ± 38.4)	5.72 ± 1.27 † (220.7 ± 48.9)	5.39 ± 1.16 (208.2 ± 44.6)	5.36 ± 1.09 (207.0 ± 42.1)
LDL-C	3.10 ± 0.84 (119.5 ± 32.4)	3.30 ± 1.10 † (127.4 ± 42.6)	3.21 ± 0.92 (123.9 ± 35.5)	3.21 ± 0.93 (123.8 ± 35.7)
HDL-C	1.62 ± 0.40 (62.6 ± 15.3)	1.58 ± 0.39 (61.0 ± 15.4)	1.23 ± 0.33 (47.5 ± 12.7)	1.22 ± 0.29 (47.2 ± 11.0)
Triglyceride	1.70 ± 0.87 (150.4 ± 77.1)	1.86 ± 1.00 (167.4 ± 89.0)	2.11 ± 1.29 (186.8 ± 114.4)	2.13 ± 1.13 (188.1 ± 99.7)
TC/HDL ratio	3.57 ± 0.99	3.81 ± 1.16 †	4.61 ± 1.31	4.59 ± 1.29
Glucose	5.15 ± 1.79 (94.2 ± 32.8)	5.50 ± 2.90 (101.5 ± 54.8)	5.87 ± 2.55 (107.4 ± 46.7)	5.86 ± 2.36 (107.6 ± 44.8)

*   Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

†   Significantly different (p < 0.05) from the G/G genotype

Considering that a lot of women were on hormonal replacement therapy, analysis was carried out excluding women taking those hormones. The significances for total (p=0.006) and LDL cholesterol (p=0.026) remained (data not shown).

Concerning the +83 bp polymorphism in the first intron of the apo A-I gene, women exhibited an association of the rare M2+/- genotype with lower total cholesterol (p=0.036) and glucose (p=0.000) compared to M2+/+ homozygotes (Table 16). However, the association with glucose is mainly due to an interaction with diabetes medication. In men, levels of apo A-I (p=0.002)

were significantly higher in M2+/- carriers when compared to M2+/+ genotypes. Total cholesterol (p=0.046) was also higher in M2+/- heterozygotes than in M2+ homozygotes.

Table 16: Effects of Apolipoprotein A-I +83 bp Genotype on Biochemical Parameters*

	Females (n = 358)		Males (n = 372)	
	M2+/+ (n = 329)	M2+/- (n = 29)	M2+/+ (n = 335)	M2+/- (n = 37)
Apo A-I	(146.5 ± 21.8)	(149.3 ± 17.0)	(131.0 ± 17.3)	(140.1 ± 15.8) †
Apo B	(109.2 ± 112.2)	(112.2 ± 30.0)	(115.4 ± 30.9)	(119.7 ± 37.3)
Total Cholesterol	5.56 ± 1.08 (214.7 ± 41.8)	5.58 ± 1.00 † (213.4 ± 38.6)	5.35 ± 1.08 (206.6 ± 41.6)	5.65 ± 1.56 † (218.3 ± 60.2)
LDL-C	3.16 ± 0.92 (122.0 ± 35.7)	3.02 ± 0.98 (116.8 ± 32.8)	3.20 ± 0.93 (123.7 ± 35.9)	3.24 ± 0.84 (125.0 ± 37.3)
HDL-C	1.61 ± 0.40 (62.0 ± 15.4)	1.65 ± 0.35 (64.0 ± 13.5)	1.22 ± 0.31 (47.2 ± 12.1)	1.27 ± 0.34 (49.1 ± 13.2)
Triglyceride	1.73 ± 0.86 (153.0 ± 76.5)	1.89 ± 1.29 (165.2 ± 113.2)	2.10 ± 1.18 (185.5 ± 104.3)	2.28 ± 1.75 (201.8 ± 115.0)
TC/HDL ratio	3.64 ± 1.04	3.55 ± 1.02	4.60 ± 1.27	4.67 ± 1.54
Glucose	5.23 ± 2.15 (95.7 ± 39.4)	5.33 ± 1.99 † (97.2 ± 35.9)	5.81 ± 2.35 (106.4 ± 43.1)	6.34 ± 3.53 (116.0 ± 64.7)

* Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

† Significantly different (p < 0.05) from M2+/+ genotype

Haplotype analysis presented in Table 17 revealed the following results. Overall, women carrying the rare alleles of both polymorphisms (G/A M2+/-) had the highest levels of apo B, total and LDL cholesterol, triglyceride, TC/HDL ratio, and fasting glucose and the lowest levels of HDL-C compared to the rest of the haplotype combinations. However, only the TC/HDL ratio (p=0.031) reached statistical significance. In men, significant differences were observed for apo A-I (p=0.021) and total cholesterol (p=0.044). G/G M2+/+ and G/A M2+/+ carriers had significantly lower apo A-I levels compared to the G/G M2+/- haplotype. With regard to total cholesterol, men carrying the G/G M2+/+ or the G/A M2+/- combinations had significantly lower concentrations than their G/G M2+/- counterparts.

Table 17: Effects of Apolipoprotein A-I -75 bp and +83 bp Haplotypes on Glucose, Lipid, Lipoprotein, and Apolipoproteins in Females*

	Females (n = 358)			
	G/G M2+/+ (n = 243)	**G/A M2+/+** (n = 86)	**G/G M2+/-** (n = 22)	**G/A M2+/-** (n = 7)
Apo A-I	(146.7 +/- 22.4)	(146.2 +/- 20.0)	(149.3 +/- 16.6)	(149.5 +/- 20.2)
Apo B	(108.1 +/- 28.8)	(112.1 +/- 32.4)	(105.4 +/- 24.3)	(137.3 +/- 37.7)
Total-Cholesterol	5.52 +/- 1.00 (213.0 +/- 38.6)	5.69 +/- 1.28 (219.7 +/- 49.6)	5.43 +/- 0.93 (207.4 +/- 36.8)	6.04 +/- 1.04 (233.1 +/- 40.3)
LDL-C	3.11 +/- 0.89 (120.1 +/- 32.4)	3.29 +/- 1.12 (127.0 +/- 43.3)	2.98 +/- 0.79 (112.8 +/- 31.8)	3.42 +/- 0.90 (132.2 +/- 34.8)
HDL-C	1.61 +/- 0.40 (62.3 +/- 15.4)	1.58 +/- 0.40 (61.1 +/- 15.3)	1.70 +/- 0.36 (66.0 +/- 13.8)	1.49 +/- 0.28 (57.6 +/- 10.7)
Triglyceride	1.70 +/- 0.88 (151.1 +/- 77.7)	1.79 +/- 0.82 (158.2 +/- 73.0)	1.65 +/- 0.82 (143.3 +/- 71.5)	2.68 +/- 2.12 (237.0 +/- 188.0)
TC/HDL ratio	3.59 +/- 1.00	3.77 +/- 1.15	3.33 +/- 0.84 ‡	4.25 +/- 1.29
Glucose	5.15 +/- 1.83 (94.2 +/- 33.4)	5.46 +/- 2.89 (99.9 +/- 52.8)	5.12 +/- 1.43 (93.5 +/- 25.6)	5.97 +/- 3.28 † (109.3 +/- 60.0)

*   Mean values +/- DS, except for TC/HDL ratios, are in mmol/L. Values in parenthesis are in mg/dl.

†   Significantly different ($p < 0.05$) from G/G M2+/+ haplotype

‡   Significantly different ($p < 0.05$) from G/A M2+/+ and G/A M2+/- haplotypes

Table 18: Effects of Apolipoprotein A-I -75 bp and +83 bp Haplotypes on Glucose, Lipid, Lipoprotein, and Apolipoproteins in Males*

	Males (n = 372)			
	G/G M2+/+ (n = 231)	**G/A M2+/+** (n = 104)	**G/G M2+/-** (n = 32)	**G/A M2+/-** (n = 5)
Apo A-I	(130.2 +/- 17.1)	(132.6 +/- 17.8)	(140.0 +/- 16.3) †	(136.4 +/- 15.4)
Apo B	(114.6 +/- 30.8)	(117.2 +/- 31.2)	(122.5 +/- 38.6)	(101.6 +/- 22.1)
Total-cholesterol	5.34 +/- 1.07 (206.0 +/- 41.4)	5.39 +/- 1.09 (208.2 +/- 42.1)	5.80 +/- 1.60 † (224.0 +/- 61.7)	4.70 +/- 0.90 (181.6 +/- 40.3)
LDL-C	3.19 +/- 0.93 (123.2 +/- 36.0)	3.24 +/- 0.93 (124.9 +/- 35.7)	3.34 +/- 0.81 (129.0 +/- 31.2)	2.63 +/- 0.82 (101.4 +/- 31.7)
HDL-C	1.22 +/- 0.32 (47.2 +/- 12.5)	1.22 +/- 0.29 (47.2 +/- 11.1)	1.28 +/- 0.35 (49.7 +/- 13.7)	1.18 +/- 0.25 (45.4 +/- 9.7)
Triglyceride	2.08 +/- 1.19 (184.1 +/- 105.6)	2.13 +/- 1.15 (188.8 +/- 101.6)	2.33 +/- 1.87 (206.4 +/- 165.7)	1.95 +/- 0.54 (172.8 +/- 47.4)
TC/HDL ratio	4.59 +/- 1.27	4.61 +/- 1.29	4.75 +/- 1.58	4.16 +/- 1.25
Glucose	5.80 +/- 2.34 (106.1 +/- 42.8)	5.84 +/- 2.40 (106.9 +/- 43.9)	6.39 +/- 3.78 (116.9 +/- 69.1)	6.04 +/- 1.33 (110.6 +/- 24.4)

* Mean values +/- SD, except for TC/HDL ratios, are in mmol/L. Values in parenthesis are in mg/dl.

† Significant different ($p < 0.05$) from G/G M2+/+ haplotype

Table 19: Adjusted Effects of Apolipoprotein A-I Genotypes and Haplotypes on Biochemical Parameters (p values)*

Genotype	Females	Males
Apo A-I -75 bp	Apo B (p = 0.016)	—
	Total Cholesterol (p = 0.005)	—
	LDL-C (p = 0.018)	—
	TC/HDL ratio (p = 0.026)	—
Apo A-I +83 bp	—	Apo A-I (p = 0.002)
	Total Cholesterol (p = 0.040)	Total cholesterol (p = 0.046)
	Glucose (p = 0.000)	—
Haplotypes	—	Apo A-I (p = 0.021)
	—	Total Cholesterol (p = 0.044)
	TC/HDL ratio (p = 0.031)	—
	Glucose (p = 0.050)	—

* Adjusted for age, body mass index, medications, alcohol use, smoking, and apo E genotype.

Effect of Apolipoprotein A-IV Genotypes on Glucose and Lipid Parameters

In Table 20 data are presented regarding the relationship of the apo A-IV 360 genotype and serum glucose, lipid, and apolipoprotein measurements. The only significant effect noted was that females with the apo A-IV 1/2 genotype had significantly higher glucose levels (11.9%) than subjects with the common apo A-IV 1/1 genotype (Table 21). No such effect on glucose levels was seen in males. With regard to lipid, lipoprotein, and apolipoprotein parameters, in particular apo A-IV and HDL cholesterol levels, no association of the 360 mutation was observed in females or males, respectively.

Table 20: Effects of Apolipoprotein A-IV 360 Genotype on Biochemical Parameters*

	Females (n = 358)		Males (n = 372)	
	1/1 (n = 300)	1/2 (n = 58)	1/1 (n = 322)	1/2 (n = 50)
Apo A-I	(146.9 ± 21.5)	(146.9 ± 20.6)	(132.0 ± 17.0)	(130.5 ± 19.3)
Apo A-IV	(14.4 ± 4.4)	(14.4 ± 3.4)	(15.5 ± 4.8)	(15.8 ± 5.6)
Apo B	(109.4 ± 30.3)	(109.4 ± 26.4)	(115.8 ± 31.6)	(115.6 ± 31.5)
Total Cholesterol	5.56 ± 1.09 (214.6 ± 42.1)	5.60 ± 0.95 (216.3 ± 36.6)	5.39 ± 1.16 (207.5 ± 44.8)	5.35 ± 0.96 (206.6 ± 37.0)
LDL-C	3.15 ± 0.94 (121.5 ± 36.2)	3.17 ± 0.80 (122.4 ± 31.0)	3.22 ± 0.94 (124.1 ± 36.2)	3.16 ± 0.81 (121.9 ± 31.3)
HDL-C	1.61 ± 0.39 (62.0 ± 15.1)	1.64 ± 0.41 (63.2 ± 15.8)	1.23 ± 0.31 (47.6 ± 12.2)	1.19 ± 0.32 (46.1 ± 12.5)
Triglyceride	1.74 ± 0.89 (153.8 ± 78.9)	1.77 ± 0.98 (156.7 ± 86.3)	2.10 ± 1.23 (185.4 ± 108.8)	2.24 ± 1.35 (198.3 ± 119.2)
TC/HDL ratio	3.63 ± 1.05	3.61 ± 1.01	4.58 ± 1.30	4.75 ± 1.28
Glucose	5.14 ± 2.00 (94.0 ± 36.2)	5.76 ± 2.70 † (105.5 ± 49.5)	5.94 ± 2.54 (108.6 ± 46.4)	5.42 ± 2.16 (99.1 ± 39.6)

* Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

† Significantly different from the 1/1 genotype ($p < 0.05$).

Table 21: Adjusted Effects of Apolipoprotein A-IV Genotype on Biochemical Parameters (p values)*

	Females	Males
Apo A-IV 360	Glucose (p = 0.004)	—

* Adjusted for body mass, age, medications, alcohol, smoking, and apo E genotype

In Table 22 data is listed on the effect of the apo A-IV 347 mutation on fasting glucose as well as several other lipid parameters. No significant association was observed in females or males, respectively.

Table 22: Effects of Apolipoprotein A-IV 347 Genotype on Biochemical Parameters*

	Females (n = 359)			Males (n = 372)		
	T/T (n = 238)	S/T (n = 107)	S/S (n = 14)	T/T (n = 233)	S/T (n = 119)	S/S (n = 20)
Apo A-I	(145.8 ± 21.4)	(149.4 ± 20.4)	(144.4 ± 28.3)	(132.8 ± 18.4)	(129.8 ± 15.4)	(132.9 ± 15.2)
Apo A-IV	(14.0 ± 4.1)	(14.9 ± 4.6)	(15.9 ± 3.4)	(15.4 ± 4.4)	(15.3 ± 5.3)	(16.9 ± 8.0)
Apo B	(110.2 ± 30.4)	(109.1 ± 27.8)	(96.3 ± 33.1)	(118.5 ± 33.4)	(109.7 ± 28.4)	(121.4 ± 21.1)
Total Cholesterol	5.58 ± 1.11 (215.3 ± 42.9)	5.55 ± 1.02 (214.4 ± 39.3)	5.29 ± 0.89 (204.2 ± 34.2)	5.39 ± 1.16 (208.0 ± 44.9)	5.31 ± 1.13 (205.0 ± 43.7)	5.75 ± 0.73 (221.9 ± 28.0)
LDL-C	3.16 ± 0.93 (121.9 ± 35.9)	3.14 ± 0.91 (121.4 ± 35.1)	3.04 ± 0.84 (117.5 ± 32.3)	3.17 ± 0.90 (122.6 ± 34.7)	3.22 ± 1.00 (124.5 ± 38.6)	3.48 ± 0.60 (134.2 ± 23.0)
HDL-C	1.60 ± 0.41 (61.6 ± 15.6)	1.63 ± 0.36 (63.0 ± 13.8)	1.67 ± 0.48 (64.3 ± 18.6)	1.24 ± 0.35 (47.8 ± 13.6)	1.21 ± 0.25 (46.6 ± 9.8)	1.20 ± 0.19 (46.5 ± 7.2)
Triglyceride	1.78 ± 0.96 (157.9 ± 85.0)	1.70 ± 0.79 (150.8 ± 69.5)	1.27 ± 0.67 (112.6 ± 54.1)	2.19 ± 1.38 (194.2 ± 122.4)	1.92 ± 0.92 (170.2 ± 81.3)	2.33 ± 1.15 (205.9 ± 101.9)
TC/HDL ratio	3.68 ± 1.08	3.54 ± 0.93	3.43 ± 1.18	4.60 ± 1.35	4.56 ± 1.23	4.90 ± 1.04
Glucose	5.09 ± 1.56 (93.2 ± 28.6)	5.66 ± 3.10 (103.6 ± 56.7)	4.50 ± 0.66 (82.4 ± 12.0)	5.82 ± 2.36 (106.5 ± 43.2)	5.99 ± 2.77 (109.6 ± 50.6)	5.68 ± 2.40 (104.0 ± 44.0)

* Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

Effect of Lipoprotein Lipase Genotype on Glucose and Lipid Parameters

Data on biochemical variables by gender and LPL Hind III genotype are listed in Table 24. The effects of the genetic variation after adjustments are shown in Table 23. Concerning plasma glucose levels, no significant difference between genotype classes was observed in females and males. With regard to total cholesterol, females showed a significant gene-dosage effect of the Hind III genotype (p = 0.039). Women being homozygous for the H- allele had the lowest levels, women being heterozygous (H+/-) had intermediate levels, and women carrying both H+ alleles had the highest total cholesterol levels. The same gene-dosage effect was observed in women with regard to LDL cholesterol. While women having the H-/- genotype had the lowest LDL levels, at 2.87 mmol/L, those having the H+/+ genotype had the highest levels, at 3.27 mmol/L (p=0.004). In the male population, no significant difference between the Hind III genotype and total cholesterol or LDL cholesterol was found. The same was true for triglyceride levels. However, with regard to HDL-C, there was a significant association, with H-/- homozygote men having significantly higher HDL-C levels, at 1.34 mmol/L, compared to their H+/+ counterparts, at 1.17 mmol/L (p=0.003). No such association was found in women.

Table 23: Adjusted Effects of Lipoprotein Lipase Hind III Genotype on Biochemical Parameters*

	Females	Males
Total Cholesterol	p = 0.039	—
LDL-C	p = 0.004	—
HDL-C	—	p = 0.003

* Adjusted for age, body mass index, medications, alcohol use, smoking, and apo E genotype.

Table 24: Effects of Lipoprotein Lipase Hind III Genotype on Biochemical Parameters*

	Females (n = 346)			Males (n = 351)		
	H +/+ (n = 174)	H +/- (n = 146)	H -/- (n = 26)	H +/+ (n = 154)	H +/- (n = 154)	H -/- (n = 43)
Total Cholesterol	5.73 ± 1.11 (221.3 ± 42.7)	5.39 ± 1.03 † (208.2 ± 39.6)	5.27 ± 1.03 (203.6 ± 39.6)	5.29 ± 1.06 (204.1 ± 41.8)	5.44 ± 1.13 (209.8 ± 43.7)	5.54 ± 1.47 (213.7 ± 56.6)
LDL-C	3.27 ± 0.95 (126.2 ± 36.8)	3.04 ± 0.89 † (117.5 ± 34.3)	2.87 ± 0.78 † (110.8 ± 30.1)	3.12 ± 0.92 (120.3 ± 35.6)	3.29 ± 0.96 (126.8 ± 37.2)	3.23 ± 0.80 (124.7 ± 30.8)
HDL-C	1.63 ± 0.40 (62.9 ± 15.6)	1.57 ± 0.40 (60.6 ± 15.5)	1.67 ± 0.31 (64.3 ± 15.1)	1.17 ± 0.25 (45.1 ± 9.7)	1.25 ± 0.31 † (48.2 ± 12.0)	1.34 ± 0.45 † (51.8 ± 17.5)
Triglyceride	1.80 ± 0.98 (159.5 ± 87.0)	1.70 ± 0.86 (150.8 ± 76.2)	1.63 ± 0.69 (143.9 ± 60.9)	2.29 ± 1.41 (202.4 ± 125.1)	2.02 ± 1.07 (178.7 ± 94.3)	1.91 ± 1.33 (168.6 ± 117.9)
TC/HDL ratio	3.72 ± 1.10	3.60 ± 1.01	3.24 ± 0.72	4.70 ± 1.26	4.56 ± 1.35	4.43 ± 1.33
Glucose	5.19 ± 2.26 (94.9 ± 41.3)	5.27 ± 1.89 (96.4 ± 34.5)	5.21 ± 2.43 (95.3 ± 44.4)	5.71 ± 2.28 (104.5 ± 41.8)	5.80 ± 2.22 (106.1 ± 40.7)	6.45 ± 3.51 (118.1 ± 64.2)

* Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

† Significantly different ($p < 0.05$) from the H+/+ group

B. **Effects of Apolipoprotein E, A-I, A-IV, and Lipoprotein Lipase Genotypes on Glucose, Lipid, and Lipoprotein Response to Lifestyle Intervention**

Subjects

Results of the lifestyle intervention on biochemical parameters and body weight are presented in Table 25.

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Table 25: Effects of Lifestyle Intervention on Biochemical Parameters*

</div>

	Females (n = 355)			Males (n = 360)		
	Pre	**Post**	**Percent Change**	**Pre**	**Post**	**Percent Change**
Glucose	5.23 ± 2.14 (95.8 ± 39.1)	5.00 ± 1.45 † (91.6 ± 26.6)	− 1.9%	5.86 ± 2.45 (107.3 ± 45.6)	5.37 ± 1.79 † (98.2 ± 32.8)	− 1.8%
Total Cholesterol	5.56 ± 1.08 (214.6 ± 41.5)	4.97 ± 1.00 † (191.8 ± 38.8)	− 10.4%	5.38 ± 1.13 (207.8 ± 43.8)	4.73 ± 0.99 † (182.6 ± 38.4)	− 11.6%
LDL-C	3.15 ± 0.91 (121.5 ± 35.4)	2.86 ± 0.89 † (110.5 ± 34.2)	− 12.9%	3.21 ± 0.92 (123.8 ± 35.5)	2.89 ± 0.91 † (111.6 ± 35.1)	− 8.8%
HDL-C	1.61 ± 0.39 (62.1 ± 15.2)	1.45 ± 0.35 † (56.1 ± 13.4)	− 8.9%	1.23 ± 0.32 (47.4 ± 12.2)	1.12 ± 0.25 † (43.1 ± 9.5)	− 7.4%
Triglyceride	1.74 ± 0.90 (154.0 ± 80.0)	1.41 ± 0.62 † (125.2 ± 54.9)	− 12.9%	2.12 ± 1.25 (187.2 ± 110.2)	1.55 ± 0.67 † (137.5 ± 59.7)	− 15.4%
TC/HDL ratio	3.63 ± 1.04	3.59 ± 1.06	− 0.5%	4.60 ± 1.30	4.50 ± 2.39	− 1.1%
Weight (kg)	76.8 ± 20.5	73.2 ± 21.6 †	− 2.2%	96.3 ± 19.1	94.0 ± 17.7 †	− 2.7%

*   Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl.

† Significantly different from baseline (p ≤ 0.001)

While this was a two-week intervention program and the body weight data reflect the total two-week intervention, blood samples were only drawn eight days into the intervention, so they reflect approximately one week of intervention rather than two. It was our understanding that blood would also be sampled after either two or three weeks of lifestyle intervention. However, this was not done, except in isolated cases, because of procedure changes at the Pritikin Longevity Center. In females, a significant reduction in serum glucose of 1.9% was noted. In addition, total

cholesterol and LDL-C were reduced by 10.4% and 8.4%, while HDL-C and triglycerides were also reduced by 8.9% and 12.9%, respectively. The total cholesterol TC/HDL cholesterol ratio was not significantly altered, with a reduction of 0.5%; however, body weight, like all of the other parameters, was reduced significantly, by 2.2%. This represented a mean weight reduction from 76.8 kg to 73.2 kg over the two-week period in these women, which represents a 3.6 kg, or 8 lb. weight loss. This is a considerable weight loss in a fairly short period of time. Very similar effects were noted in the males. Serum glucose levels were reduced by 1.8%, total and LDL cholesterol levels were reduced by 11.6% and 8.8% respectively, (all $p<0.001$), HDL-C and triglyceride levels were reduced by 7.4% and 15.7% respectively. In males the TC/HDL ratio was reduced by 1.1%, which was not statistically significant. Similar to the females, the males had a significant reduction in body weight of 2.7%, going from 96.3 to 93.0 kg, which represented a mean 3.3 kg weight loss, or approximately 7 pounds. It is important to stress that body weight changes were observed over a two-week period, whereas alteration in other parameters, reflect changes over a one-week period.

In order to ascertain what the effects on response would have been, had the subjects been sampled at the two-week point, we independently analyzed a subset of 31 individuals (9 females and 22 males) who participated in the three-week program and had blood samples drawn at baseline, eight days, and at 15 days of the intervention.

The data on the subset of 31 individuals are listed in Table 26. The mean age for this group was 57.5 years, with an elevated body mass index of 31.9 kg/m^2 and a weight of 95.1 kg. Their average waist circumference was also increased at 116.0 cm. With regard to medication, 12.9% were on cholesterol-lowering medication, 12.9% on medication for diabetes, and 12.9% on thyroid medication. In the entire sample, 19.4% were on hormonal replacement, which was all due to 6 females (66.6%). Alcohol use with more than one drink per week was noted in 35.5%, and current cigarette smoking was noted in 12.9%.

Data on their biochemical parameters are presented in Table 27. In this subset there was no significant change in glucose, but total cholesterol and LDL cholesterol were reduced significantly by 12.8% and 13.5% in these 31 subjects. Similarly, HDL-C was reduced by 9.2% and triglycerides were reduced by 13.0%. Moreover, the TC/HDL ratio was reduced by 3.2%. These data are consistent with the view that some additional benefit is observed in the second week of the intervention, especially with regard to LDL cholesterol reduction.

Table 26: Subset of 31 Study Subjects*

	All Subjects (n = 31)
Age (years)	57.5 ± 9.2
Body mass index (kg/m^2)	31.9 ± 6.5
Height (cm)	171.7 ± 11.0
Weight (kg)	95.1 ± 26.3
Waist (cm)	116 ± 17.2
Cholesterol-lowering medication	4 (12.9%)
Medication for diabetes	4 (12.9%)
Hormonal replacement	6 (19.4%)
Thyroid medication	4 (12.9%)
Alcohol users (>1 drink/week)	11 (35.5%)
Cigarette smoking (current)	4 (12.9%)

* Mean values ± SD, or total numbers of subjects with percentage in parentheses

Table 27: Effects of Intervention on Biochemical Parameters at 1 and 2 Weeks in 31 Subjects*

	Pre	Post I	Post II	% Difference Pre to Week 1	% Difference Pre to Week 2
Glucose	5.62 ± 2.0 (102.8 ± 36.3)	5.46 ± 1.62 (100.0 ± 29.6)	5.69 ± 2.56 (104.2 ± 46.8)	⁻ 2.8%	+ 1.0%
Total Cholesterol	5.67 ± 1.39 (218.9 ± 53.5)	5.10 ± 1.39 † (196.7 ± 53.5)	4.87 ± 1.26 † (187.8 ± 48.6)	⁻ 9.8%	⁻ 12.8%
LDL-C	3.41 ± 1.13 (131.7 ± 43.6)	3.14 ± 1.19 † (121.3 ± 46.1)	2.86 ± 1.00 † (110.4 ± 38.6)	⁻ 7.9%	⁻ 13.5% †
HDL-C	1.31 ± 0.40 (50.6 ± 15.6)	1.13 ± 0.35 † (43.6 ± 13.5)	1.16 ± 0.29 † (44.9 ± 11.1)	⁻ 10.9%	⁻ 9.2% †
Triglyceride	2.18 ± 1.24 (193.0 ± 109.3)	1.66 ± 0.83 † (146.5 ± 73.6)	1.74 ± 0.96 † (154.4 ± 84.8)	⁻ 17.7%	⁻ 13.0% †
TC/HDL ratio	4.68 ± 1.65	5.80 ± 7.09	4.43 ± 1.92	+ 29.8%	⁻ 3.2%

*   Mean values ± SD, except for ratios, are in mmol/L, values in parentheses are in mg/dl; data from 31 subjects, 22 males and 9 females, blood drawn at 8 and 15 days of intervention.

†   Significantly different ($p < 0.05$) from baseline or pre value.

Apolipoprotein E Genotype and Response

Data on the effect of the apo E genotype on fasting glucose, lipid and lipoprotein levels in response to the dietary and lifestyle intervention is presented in Table 28. Apo E genotype had no significant effect on total cholesterol, HDL-C, or triglyceride lowering in either men or women. Regarding LDL cholesterol response, there was a trend in males, with carriers of the E2+ allele having the highest (-10.5%), E3/3 genotypes having intermediate (-8.8%), and carriers of the E4+ allele having the lowest (-6.8%) response, but the difference did not reach statistical significance. However, a statistically significant effect of apo E genotype in males was observed on glucose lowering. The 53 subjects who carried the apo E2 allele had a 10.6% reduction in glucose, as compared to the 233 subjects with the normal apo E3 genotype, who had a +0.8% change in glucose, in other words, no net effect. These data suggest that males carrying the apo E2 allele may be more responsive, with regard to glucose lowering. No such affect was observed in females.

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Table 28: Effects of Apolipoprotein E Genotype in Response to Intervention*

Apo E Genotype	Females (n = 353)			Males (n = 359)		
	E2+ (n = 42)	E3/3 (n = 213)	E4+ (n = 98)	E2+ (n = 53)	E3/3 (n = 233)	E4+ (n = 73)
Glucose	+ 0.7%	− 2.9%	− 0.6%	− 10.6% †	+ 0.8%	− 3.7%
Total Cholesterol	− 11.8%	− 9.8%	− 11.0%	− 12.9%	− 11.6%	− 10.1%
LDL-C	− 12.0%	− 7.5%	− 8.8%	− 10.5%	− 8.8%	− 6.8%
HDL-C	− 9.6%	− 8.1%	− 10.4%	− 7.4%	− 7.3%	− 8.4%
Triglyceride	− 10.3%	− 11.5%	− 16.5%	− 12.4%	− 15.7%	− 15.4%

*   Mean percent changes from baseline value

†   Significantly different (p < 0.05) from the E3/3 genotype after adjustment for age, body mass index, medications, alcohol use, and cigarette smoking.

Apolipoprotein A-I Genotypes and Response

Data on the effects of the apo A-I -75 bp genotype are shown in Table 29. With this genotype no effects on reductions in glucose, total cholesterol, LDL-C, HDL-C, or triglyceride were noted in either females or males in this study. However, it is worth mentioning that a trend was noted for LDL-C, with A/A homozygote females and males having a greater reduction in LDL-C (14.3% and 15.3%) as compared to their G/G homozygote counterparts (8.3% and 9.0%), respectively.

Table 29: Effects of Apolipoprotein A-I −75 bp Genotype on Response to Intervention*

Apo A-I −75 bp Genotype	Females (n = 359)			Males (n = 372)		
	G/G (n = 266)	G/A (n = 87)	A/A (n = 6)	G/G (n = 263)	G/A (n = 95)	A/A (n = 14)
Glucose	−1.6%	−3.0%	−0.9%	−1.5%	−1.8%	−6.8%
Total Cholesterol	−10.6%	−9.4%	−13.8%	−11.6%	−11.0%	−15.1%
LDL-C	−8.3%	−8.2%	−14.3%	−9.0%	−7.7%	−15.3%
HDL-C	−9.4%	−6.8%	−15.6%	−7.1%	−8.3%	−7.7%
Triglyceride	−12.9%	−13.8%	+0.7%	−14.6%	−18.4%	−20.0%

* Mean percent changes from baseline value

Data on the effects of the apo A-I +83 bp genotype are shown in Table 30. With this genotype, no effect on reductions in glucose, total cholesterol, LDL-C, HDL-C, or triglycerides were noted in either females or males.

Table 30: Effects of Apolipoprotein A-I +83 bp Genotype on Response to Intervention*

Apo A-I +83 bp Genotype	Females (n = 358)		Males (n = 372)	
	M2 +/+ (n = 329)	M2 +/- (n = 29)	M2 +/+ (n = 335)	M2 +/- (n = 37)
Glucose	−1.6%	−5.2%	−1.2%	−4.0%
Total Cholesterol	−10.5%	−9.0%	−11.7%	−10.9%
LDL-C	−8.4%	−7.6%	−8.8%	−8.9%
HDL-C	−9.0%	−7.5%	−7.5%	−6.8%
Triglyceride	−13.1%	−10.3%	−16.1%	−12.3%

* Mean percent changes from baseline value

Apolipoprotein A-IV Genotypes and Response

Data on the effects of the apolipoprotein A-IV 360 genotype in response to the intervention are shown in Table 31, for both females and males. No significant effects on any of the biochemical variables in response to lifestyle were noted. However, looking at the gender difference, it is worth mentioning that 1/2 heterozygote females had a greater HDL-C response (-10.6%) compared to 1/2 heterozygote males (-6.0%). In addition, the same group of women had about half the reduction of triglyceride levels (-10.5%) compared to their male counterparts (-21.2%).

Table 31: Effects of Apolipoprotein A-IV 360 Genotype on Response to Intervention*

Apo A-IV 360 Genotype	Females (n = 358)		Males (n = 372)	
	1/1 (n = 300)	1/2 (n = 58)	1/1 (n = 322)	1/2 (n = 50)
Glucose	– 1.8%	– 2.6%	– 1.7%	– 2.6%
Total Cholesterol	– 10.2%	– 11.5%	– 11.8%	– 10.8%
LDL-C	– 8.2%	– 9.5%	– 9.2%	– 6.6%
HDL-C	– 8.5%	– 10.6%	– 7.6%	– 6.0%
Triglyceride	– 13.3%	– 10.5%	– 14.8%	– 21.2%

* Mean percent changes from baseline value

With regard to the effects of the apolipoprotein A-IV 347 genotype, which are listed in Table 32, no significant association of this genotype on responsiveness to lifestyle intervention was noted, except for reductions in HDL-C in women. In this study, the mean reductions in women with the T/T genotype was 8.6%, in women with the T/S genotype it was 8.4%, while in 14 women with the S/S genotype, the reduction was almost twice as high at 16.7%. At the same time, women with the S/S genotype had by far the lowest triglyceride response (-4.5%) compared to women with the T/T genotype (-13.5%), but the difference did not reach statistical significance. No such effect was observed in males.

Table 32: Effects of Apolipoprotein A-IV 347 Genotype on Response to Intervention*

Apo A-IV 347 Genotype	Females (n = 359)			Males (n = 372)		
	T/T (n = 238)	T/S (n = 107)	S/S (n = 14)	T/T (n = 233)	T/S (n = 119)	S/S (n = 20)
Glucose	− 1.2%	− 4.1%	+ 3.8%	− 0.4%	− 3.5%	− 8.0%
Total Cholesterol	− 10.5%	− 9.6%	− 13.5%	− 12.1%	−10.2%	− 14.1%
LDL-C	− 8.6%	− 7.5%	− 12.0%	− 9.3%	− 7.4%	− 12.3%
HDL-C	− 8.6%	− 8.4%	− 16.7% †	− 8.3%	− 6.1%	− 5.0%
Triglyceride	− 13.5%	− 12.7%	− 4.5%	− 15.8%	− 14.5%	− 22.1%

* Mean percent changes from baseline value

† Significantly different ($p < 0.05$) from the T/T genotype after adjustment for age, body mass index, medications, alcohol use, and cigarette smoking.

Lipoprotein Lipase Genotype and Response

Data on the effects of the LPL Hind III genotype are presented in Table 33.

Table 33: Effects of Lipoprotein Lipase (Hind III) Genotype on Response to Intervention*

Lipoprotein Lipase Genotype	Females (n = 346)			Males (n = 351)		
	H +/+ (n = 174)	H +/− (n = 146)	H −/− (n = 26)	H +/+ (n = 154)	H +/− (n = 154)	H −/− (n = 43)
Glucose	− 0.2%	− 0.9%	− 2.9%	− 3.5%	− 6.2%	− 3.3%
Total Cholesterol	− 9.4%	− 10.6%	− 10.4%	− 10.3%	− 11.4%	− 12.6%
LDL-C	− 12.1%	− 8.4%	− 7.8%	− 9.5%	− 9.3%	− 9.2%
HDL-C	− 5.6%	− 9.0% †	− 9.3%	− 10.3%	− 7.0%	− 6.8%
Triglyceride	− 10.8%	− 12.8%	− 13.0%	− 7.3%	− 16.7%	− 16.7%

* Mean percent changes from baseline value

† Significantly different ($p < 0.05$) from the H+/+ genotype after adjustment for age, body mass index, medications, alcohol use, and cigarette smoking.

No effects of variation at this restriction site on the response or reduction of any of the biochemical parameters were noted, either in females or males, except for reductions in HDL cholesterol. Here, females who were heterozygous for the rare H- allele (H+/-) had a 9.0% reduction versus a 5.6% reduction in those women carrying the wildtype (H+/+). Female subjects homozygous for the H- allele (H-/-) also showed a 9.3% reduction in HDL-C, however, the difference was not statistically significant, probably due to the small sample size of this group. In males, only a trend was seen for triglyceride response. Men carrying the rare H- allele had greater reduction (-16.7%) compared to men carrying the H+/+ genotype (-7.3%), but the difference did not reach statistical significance due to the large variability of triglyceride levels.

It should be noted, however, that these findings must be interpreted with caution, since with multiple comparisons, the possibility of finding associations by chance are fairly high, especially with small sample sizes, as in this case.

C. Effects of Diet and Exercise on Glucose, and Plasma Lipoproteins: Results of Long-Term Follow up

Subjects

Information on 202 study subjects is provided in Table 34. The mean age of these subjects was approximately 61 years. The mean body mass index in the women was 27.2 kg/m^2, and in the men 29.9 kg/m^2, with mean weights being 71.9 kg and 95.1 kg, respectively. The waist circumference was substantial, in women being 97.8 cm, and in men being 107.8 cm. Approximately 20% of both women and men were on cholesterol-lowering medication, another 6% of both genders were on medication for diabetes. None of the men, but 36.8% of the women, were on hormonal replacement, specifically estrogen alone or estrogen plus progesterone in post-menopausal women. However, menopause status in those women was not assessed. Another 13.6% of the women, and 9.4% of men were on thyroid medication, and 40.4% of the women and over half of the men had more than one drink per week, in terms of alcohol intake. Cigarette smoking was fairly uncommon, with only 4.3% of the women and 9.5% of the men being current smokers.

Table 34: Study Subjects*		
	Men (n = 107)	Women (n = 95)
Age (years)	61.3 ± 10.6	60.6 ± 9.8
Body mass index (kg/m^2)	29.9 ± 5.2	27.2 ± 4.9
Weight (kg)	95.1 ± 18.6	71.9 ± 14.2
Waist (cm)	107.8 ± 14.6	97.8 ± 14.6
Cholesterol-lowering medication	22 (20.6%)	19 (20.0%)
Medication for diabetes	6 (5.6%)	6 (6.3%)
Hormonal replacement	0 (0.0%)	35 (36.8%)
Thyroid medication	10 (9.4%)	30 (31.6%)
Alcohol users (>1 drink/week)	57 (53.8%)	35 (40.4%)
Cigarette smoking (current)	10 (9.5%)	4 (4.3%)

* Mean values ± SD or number with percentage of total in parentheses.

With regard to the effects of lifestyle intervention program on biochemical parameters in men, data are listed in Table 35. After eight days of intervention, glucose levels decreased by 10.7% (p=0.036) and total and LDL cholesterol were reduced by 8.6% and 7.2%, respectively (p≤0.001). Triglyceride reductions were even greater, at 27.1% (p≤0.001). As has often been observed with such diets, HDL-C levels were also decreased by this intervention by 4.3% (p=0.006). When subjects returned to the program for an evaluation and a second lifestyle modification, their values were compared at this second baseline time point with the first baseline, prior to any intervention. In men, glucose levels were 1.6% lower. Total cholesterol levels were 5.2% higher (p=0.007), and LDL-C levels were 6.2% higher (p=0.013). In addition, HDL-C levels were 6.9% higher (p<0.001), while triglyceride levels were 2.3% lower. The post-intervention results obtained on the eighth day of the second intervention again revealed that the program resulted in significant changes in glucose, total and LDL cholesterol, and triglyceride, whether this was compared to baseline values for baseline 1 or versus baseline 2. Only percent changes and statistics are provided for comparison with baseline 1. These results are consistent with the concept that this intervention does not provide lasting effects in men on lowering levels of glucose, total and LDL cholesterol, and triglyceride.

Table 35: Effects of Intervention on Biochemical Parameters in Men* (n = 107)

	Baseline 1	Post 1	Baseline 2	Post 2
Glucose	5.63 ± 2.60	5.03 ± 1.15 † (− 10.7%)	5.54 ± 1.87 (− 1.6%)	5.18 ± 1.43 † (− 8.0%)
Total Cholesterol	4.98 ± 1.03	4.55 ± 0.93 (− 8.6%)	5.24 ± 1.14 † (+ 5.2%)	4.65 ± 0.94 † (− 6.6%)
LDL-C	2.92 ± 0.85	2.71 ± 0.80 † (− 7.2%)	3.10 ± 0.96 † (+ 6.2%)	2.81 ± 0.90 (− 3.8%)
HDL-C	1.16 ± 0.28	1.11 ± 0.29 † (− 4.3%)	1.24 ± 0.33 † (+ 6.9%)	1.13 ± 0.27 (− 2.6%)
Triglyceride	2.14 ± 1.67	1.56 ± 0.72 † (− 27.1%)	2.09 ± 1.26 (− 2.3%)	1.52 ± 0.64 † (− 29.0%)

* Mean values ± SD in mmol/L, conversions from mg/dl were division by 38.6 for cholesterol, 88.5 for triglyceride, and 18.3 for glucose; percent values are percentage change in mean value from baseline 1.

† Significantly different (p < 0.05) from baseline value.

We see the same pattern in the women with intervention resulting in significant reductions in all biochemical parameters, except for glucose, which only was reduced by 2.5% (Table 36). Total

cholesterol was reduced by the intervention by 11.4% (p≤0.001), triglycerides were decreased by 21.1% (p≤0.001), LDL-C was lowered by 11.1%, and HDL-C was lowered by 7.7%, both p<0.001. Therefore, just over a week of intervention had a fairly significant effect on lowering all of the lipid parameters. Despite this, when subjects returned on average approximately two years later, no significant differences were observed with regard to any of the parameters, in terms of lowering, when one compared the second baseline values with the first baseline values. The only exception was HDL-C, which was 7.1% higher (p<0.001). The modest glucose reduction that was noted was in fact retained, but this was only 2.3%. Total cholesterol was essentially unchanged, with values that were 2.0% higher, triglyceride levels that were 9.8% lower than the first baseline, and LDL-C values that were also unchanged, being only 0.9% higher than the first baseline.

Table 36: Effects of Intervention on Biochemical Parameters in Women* (n = 95)

	Baseline 1	Post 1	Baseline 2	Post 2
Glucose	5.22 ± 1.74	5.09 ± 1.52 (− 2.5%)	5.10 ± 1.83 (− 2.3%)	5.00 ± 1.59 (− 4.2%)
Total Cholesterol	5.61 ± 0.95	4.97 ± 0.96 † (− 11.4%)	5.72 ± 0.94 (+ 2.0%)	5.03 ± 0.94 † (− 10.3%)
LDL-C	3.23 ± 0.88	2.87 ± 0.86 † (− 11.1%)	3.26 ± 0.85 (+ 0.9%)	2.88 ± 0.88 † (− 10.8%)
HDL-C	1.56 ± 0.41	1.44 ± 0.40 † (− 7.7%)	1.67 ± 0.38 † (+ 7.1%)	1.48 ± 0.33 † (− 5.1%)
Triglyceride	1.94 ± 1.51	1.53 ± 0.66 † (− 21.1%)	1.75 ± 0.94 (−9.8 %)	1.42 ± 0.59 † (− 26.8%)

* Mean values ± SD in mmol/L, conversions from mg/dl were divisor by 38.6 for cholesterol, 88.5 for triglyceride, and 18.3 for glucose; percent values are percentage change in mean value from baseline 1.

† Significantly different (p < 0.05) from baseline value.

Not surprisingly, the second intervention, after eight days provided the same results as the first one, with significant reductions in total, LDL, and HDL cholesterol as well as triglyceride. However, as for men, these data are consistent with the concept that this intensive lifestyle intervention resulted in no lasting effects on these biochemical parameters related to heart disease risk.

Discussion

A. Effects of Apolipoprotein E, A-I, A-IV, and Lipoprotein Lipase Genotypes on Baseline Levels of Glucose, Lipid, Lipoprotein, and Apolipoproteins

Subjects

It has been a well-known fact that significant differences in biochemical parameters exist between females and males. This study clearly demonstrated that data analysis should be carried out in a gender specific way. Anthropometric measurements as well as all the various biochemical parameters, except for LDL-C, were significantly different in men and women. This result is consistent with previous observations (242) regarding a significant gender difference, especially for HDL-C and Apo A-I. Females had significantly higher levels of both traits compared to males. The reason for this difference appears to be due to a significantly higher production rate of apo A-I in females, presumably due to hormonal influences. Estrogen administration has been shown to increase the production rates of HDL-C and apo A-I (243,244). In addition, Seishima et al. (245) reported that both apo A-I expression and metabolism are affected by endogenous sex hormones. More recently, studies have indicated that the thyroid hormone triiodothyronine (T3) is another potent mediator of expression of various genes (246,247). In our study population, it was interesting to notice that a relatively large percentage of females were either on sex hormones and/or thyroid medication. This fact may partly explain the higher levels of apo A-I and HDL-C as well. In addition, differences in environmental background, lifestyle, and dietary intake are also reasons for such a gender effect.

Furthermore, a gene-gender interaction was observed, with women being affected differently by various genetic variations as compared to men. Those differences, however, will further be discussed in each of the following sections.

Apolipoprotein E

Apo E genotype frequencies in this population of middle-aged and elderly men and women, were similar to those reported for other Caucasian populations (41,49,57,59). These results were also

consistent with previous apo E phenotype data as determined by IEF by other groups as well as by us (49,54-60).

In this study population significant allele effects of the apo E genetic variability on plasma lipoproteins and apoproteins were observed. Similar to others studies (248-251), our data clearly indicated that apo E genotype has a significant effect on LDL cholesterol in both men and women, such that subjects with the apo E2 allele had the lowest LDL cholesterol levels, those with the apo E3/3 genotype had intermediate levels, and subjects carrying the apo E4 allele had the highest LDL cholesterol levels (Figure 12).

Figure 12
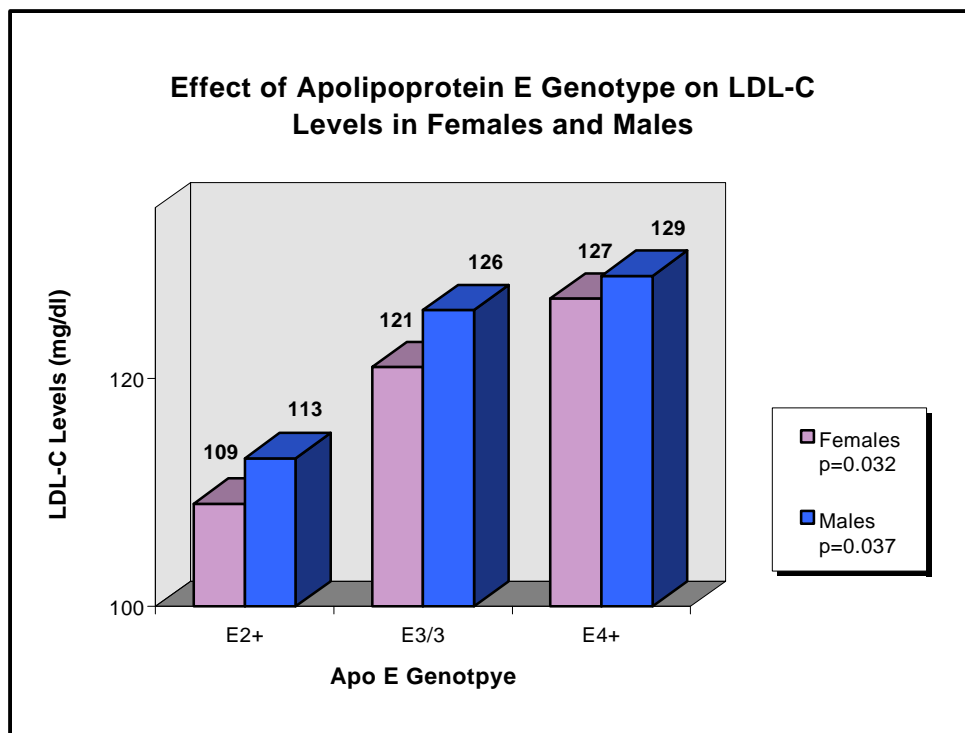
In this scenario it did appear that the apo E2 allele caused a greater reduction in LDL cholesterol as compared to the E3/3 genotype than the apo E4 allele did in raising LDL cholesterol. The effect of the apo E isoforms on LDL concentrations is thought to be mediated by the LDL-receptor (49,248,251,252). In individuals homozygous for the apo E2 allele, low LDL cholesterol levels have been reported to be associated with both a decreased conversion of VLDL to LDL and an accelerated catabolism of LDL particles (252-255). This latter effect is mediated by two mechanisms: [1] the upregulation of the LDL-receptor due to the lower intracellular

cholesterol availability associated with the deficient uptake of remnants and [2] the increased availability of the hepatic LDL-receptors due to the lack of competition for binding by chylomicron and VLDL remnants.

The physiological effects of apo E4 are felt to be opposite to those of apo E2. In individuals carrying the E4 allele, more cholesterol from apo E-containing lipoproteins is internalized by the hepatocytes. This increase in intracellular cholesterol levels is compensated for by a down-regulation of the amount of LDL-receptors. Furthermore, a more efficient conversion of VLDL remnants to LDL cholesterol has been observed, resulting in a higher LDL production rate. All these metabolic effects are responsible for the increase in plasma LDL cholesterol.

Whereas the apo E allele effect on serum cholesterol levels is almost constant in most populations, controversial results concerning triglyceride levels are found in the literature. Since triglycerides vary widely among and within individuals (66,265), this variability could mask a clear apo E genotype effect. Dallongeville et al. (58) as well as other studies (61,63,65,66,69) documented a significant association between apo E2 and E4 and higher triglyceride levels compared to E3/3 homozygotes. In our study, however, no such association in either gender was observed. This is in agreement with a previous meta-analysis of Davignon et al. (49) combining results of 7 studies. In this large data set they could not detect a relationship between the apo E genotype and triglyceride concentrations. Similar results were published not only in the Framingham Offspring population (60), but also in several other populations (59,67,257-259).

With regard to HDL cholesterol, we observed a significant effect in men, with men carrying the apo E3/3 genotype and the apo E4 allele having lower HDL cholesterol levels, as compared to men carrying the apo E2 allele. This result is somewhat different compared to previous reports. While several investigators failed to show an association between the apo E gene locus and HDL cholesterol levels (60,65,66,258,260-263), others did report a similar positive effect of the apo E2 allele in a Finnish population (67) as well as in students from 11 European countries (61). Hegele et al. (259) and Kamboh et al. (59) could only detect an association in the female population of the Hutterite Brethren and non-Hispanic Whites, respectively. In their studies, women carrying the E2 allele had higher levels and those carrying the E4 allele had lower HDL-C levels compared to the E3/3 wildtype. No such effects were noted in men. Some investigators have reported that the apo E2 allele is associated with increased HDL-apo E (264) or HDL_2 cholesterol levels (59,257). The mechanism of this relation still remains to be clarified. Recently, in vivo (265) and in vitro (266) studies have shown that apo E plays an important role in the regulation of cholesterol ester transfer protein (CETP). Assuming that CETP activity is affected by the apo E

polymorphism, the interrelation between apo E genotype and HDL concentrations might be reasonable. Another possible explanation may be the involvement of apo E in the extracellular efflux of cholesterol (267). If the efflux rate varies with the apo E genotype, it might have a corresponding effect on HDL cholesterol levels.

Not surprisingly, men with the apo E2 allele had significantly lower TC/HDL ratios than other apo E genotype groups. This is in accordance with the findings of investigators suggesting that in the population at large the E4 allele exerts a deleterious effect and, to a lesser extent, the E2 allele exerts a protective effect on risk for CHD (63,261,268-271).

Several studies have shown significant associations between plasma apo E levels and apo E isoforms (61,63,66,24,248,263) and genotypes (272). In agreement with those studies, we noted a striking effect of the apo E genotype on plasma apo E levels in both men and women, those with the apo E2 allele had the highest levels, and those with the apo E4 allele had the lowest levels. These differences were highly significant (p<0.0001), and indicate that the apo E locus is a major factor controlling apo E levels in plasma (272) (Figure 13).

Figure 13

Effect of Apolipoprotein E Genotype on Apo E Levels in Females and Males
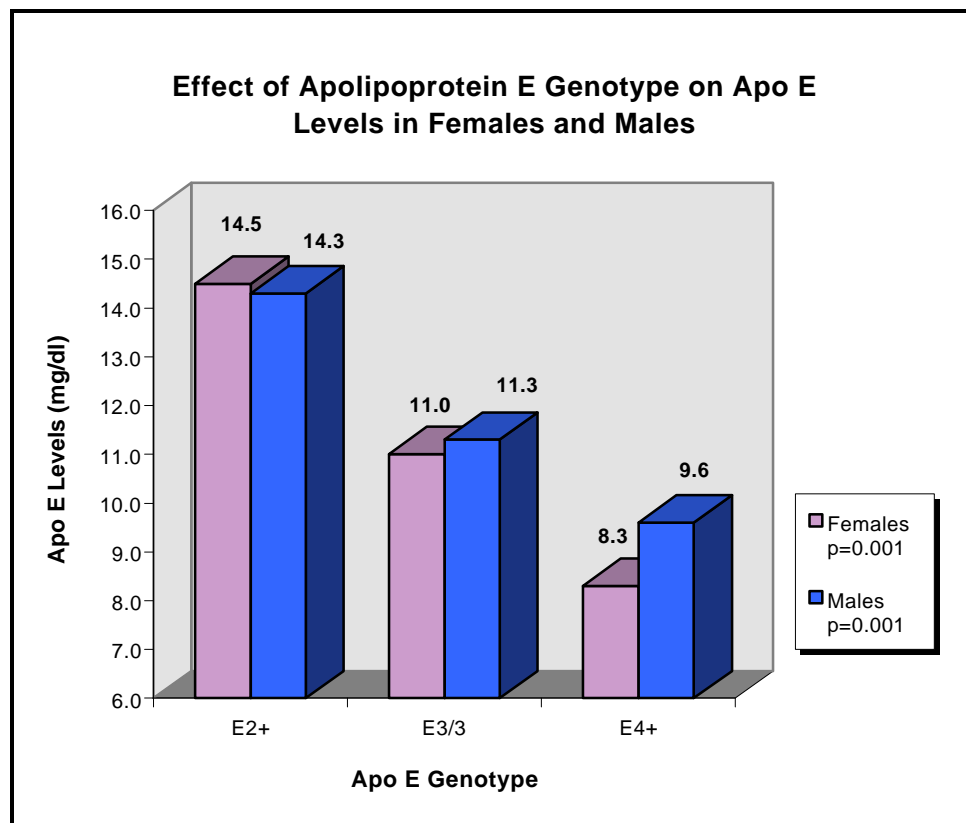
These data are consistent with the concept that apo E2 is catabolized more slowly than apo E3 and apo E4, as has been previously reported (273-275). Moreover, it is known that apo E is essential for the catabolism of triglyceride-rich lipoproteins since in its absence (familial apo E deficiency), the clearance of TRL apoB-100 and apoB-48 is markedly delayed, resulting in triglyceride-rich lipoprotein remnant accumulation (276).

Furthermore, a significant relationship was detected between apo E genotype and glucose levels in females only, after adjusting for the effects of medication, alcohol use, smoking, age, and body mass index. Women carrying the apo E2 and apo E4 alleles had lower glucose levels than those with the apo E3/3 genotype. These results confirm recent data reported by Kataoka et al. (69) who found the same association between fasting glucose and apo E genotype in diabetic American Indian women. E3/3 homozygotes had higher fasting glucose levels compared to women carrying the E2 and E4 alleles. Kamboh et al. (59), however, failed to show such an effect in Hispanic and non-White Hispanic men and women, respectively. How glucose could be related to apo E genotype remains to be determined.

Our overall data are consistent with the concept that the apo E genotype has a major effect in determining plasma apo E and lipid levels. The apo E2 allele is associated with increases in apo E levels in both men and women, decreases in LDL cholesterol in both men and women, and decreases in glucose in women. In contrast, the apo E4 allele is associated with decreases in the apo E levels and increases in LDL cholesterol in both men and women, and decreases in glucose levels in women only, as compared to subjects with the common apo E3/3 genotype.

Apolipoprotein A-I

High plasma HDL cholesterol and apo A-I levels have been found to be protective against CAD (104,105). Therefore, it is of public interest to investigate the environmental and genetic factors determining those levels. The apo A-I gene itself and in particular the promoter region with its sequences implicated in the transcriptional control, is a prime candidate for such investigation. Mutations in this region could affect transcription rates, and thus alter hepatic and/or intestinal synthesis and secretion rates, which could affect levels of apo A-I and HDL cholesterol. The aim of the present study was to examine the association of polymorphisms at position -75 bp and +83 bp of the apo A-I gene on various lipid, lipoprotein, and apolipoprotein traits in a large population, and in a gender specific way.

Conflicting reports as to the significance of the G to A mutation at -75 bp in the promoter region of the apo A-I gene on plasma on HDL-C and apo A-I levels have been accumulating in the literature (see Table 1). The earliest study by Pagani et al. (132) documented that the A-allele was over-represented in Italian women in the highest decile of HDL levels, but not in Italian men in the corresponding decile. Furthermore, Xu et al. (138) reported that the G/A mutation was associated with higher HDL-C and apo A-I levels in Italian boys, but not in girls. Similar effects of the A-allele on HDL-C and/or Apo A-I have been reported in males from England (131), Belgium (137), and Finland (136). In two of those studies (134,140), however, this effect of the A-allele on apo A-I levels was restricted to non-smoking men only. The investigators did not detect such an effect in women or male smokers.

In contrast, our study, carried out in 734 men and women, did not show significant associations of the A-allele with higher HDL-C or apo A-I. This is in agreement with some recent results, in which no effect of the -75 bp polymorphism was evident (143-147,150). Barre et al. (143) studied 22 Caucasian nuclear families and found no significant difference between individuals with the A-allele and their G/G siblings. Our own previous study showed no difference in allele frequencies in males and females with HDL values below the 25th or above the 75th percentile of the population distribution (147). Furthermore, Minnich et al. (135), who described higher levels of HDL-C and A-I in heterozygous French females, concluded that there was no direct effect of the A-allele, but that a bimodal distribution of HDL-C, a missing gene-dosage effect, and segregation of hyperalphalipoproteinemia with a subset of the A-allele family members could be the cause of the association.

In general, however, the G/A variation is believed to have a direct effect on levels of HDL-C and apo A-I. Sastry et al. (277) described in in vitro studies, that DNA segments, located between -2052 and -192 bp and between -256 and -41 bp upstream from the transcription start site of the human apo A-I gene, are necessary for maximal levels of apo A-I expression in the intestine and liver, respectively. Furthermore, different cis- (277-279) and trans-acting factors (280) involved in the expression of the apo A-I gene have been identified. DNase I footprinting experiments have identified four protein binding domains between -220 and +17 bp, including one at -128 to -77 bp, which is in close proximity to the -75 bp polymorphic site (281). Therefore, it is hypothesized that the base substitution from G to A modifies the affinity for transcription factors binding to this region and thereby affects the synthesis or secretion of apo A-I (134). Indeed, Angotti et al. (282) noted that the presence of the A-allele increased the basal transcriptional efficiency of the apo A-I promoter due to a lower affinity of a 90 kDa binding factor. These authors suggest that the G to A substitution in the promoter region results in a more efficient

expression and ultimately in higher apo A-I levels in individuals carrying the A-allele than the G-allele. In contrast, Smith et al. (283) measured the promoter activity of the two different alleles in vitro by linkage to a chloramphenicol acetyltransferase (CAT) reporter gene followed by transfection into the HepG2 human hepatoma cell line. These investigators noted a 68% reduction in promoter activity of the A-allele compared to the G-allele, which in vivo accounted for the significantly lower apo A-I production rate (by 11%) in G/A heterozygotes. In spite of this apparent effect of the -75 bp polymorphism on the apo A-I production rate, they did not observe an effect on levels of HDL-C or apo A-I. Additional in vitro studies (284) failed to show different promoter activities from the G or A constructs when tested under basal conditions or after stimulation with steroids or retinoic acid. Therefore, they suggest that the G/A polymorphism does not directly affect the transcriptional efficiency of the apo A-I gene, supporting the findings from this and previous studies (143-147,150).

We found in women a significant association between the A-allele and higher levels of apo B, total and LDL cholesterol, as well as the TC/HDL ratio compared to G/G homozygotes (Figure 14).

Figure 14
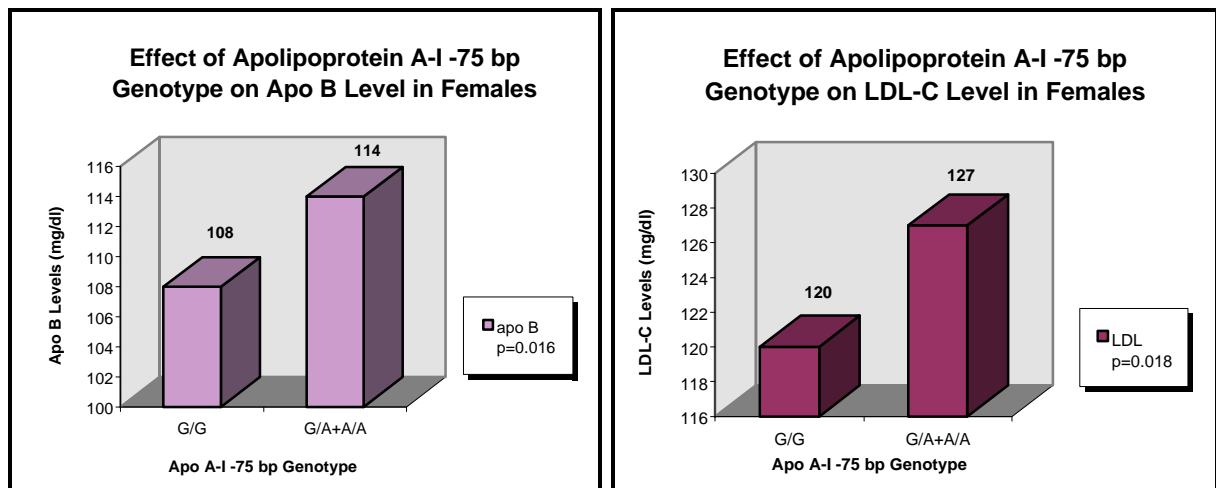
These results are unexpected considering that several studies assigned a beneficial effect to the A-allele. However, looking at more recent studies, similar results have been documented in the literature, but in a smaller number of subjects. Xu et al. (138) described that the G/A substitution was associated with higher plasma levels of total cholesterol, LDL-C, and apo B in 111 Italian boys, but not in girls. More recently, Mata et al. (150) observed in 69 subjects carrying the A-

allele a higher plasma cholesterol, LDL-C and triglyceride than in those with the G/G genotype. Somewhat related results were published by Matsunaga et al. (285) who documented in healthy Japanese students carrying the G/A genotype significantly lower plasma concentrations of apo A-I than G/G homozygotes. In CAD patients, Wang et al. (146) reported a higher likelihood of having one or more significantly diseased vessels (>50% luminal obstruction) in homozygotes for the -75 bp substitution compared to G/G homozygotes.

The physiological explanations that could reconcile the controversial findings described above remain elusive. It has been proposed that the G to A exchange could be a functional mutation and therefore have a direct effect on the phenotypes examined. Alternatively, it has been proposed that this polymorphism maybe a marker in linkage disequilibrium with one or more functional mutations either in close proximity or at a nearby gene. In this study, we found linkage disequilibrium between the -75 bp and the +83 bp polymorphisms. No subject carrying A/A and the rare M2- allele was found, which confirms previous findings (142) and indicates that the +83 bp mutation is linked to the G-allele only. It is interesting to note that in females the presence of the rare M2- allele appeared to potentiate the already existing negative effect of the A-allele. While women carrying the G/A M2+/+ haplotype already showed higher levels of apo B, total and LDL cholesterol compared to their G/G M2+/+ counterparts, women with the additional rare allele of the +83 bp mutation (G/A M2+/-) exhibited an even greater increase in all those levels. However, differences did not reach statistical significance, probably due to the small sample size.

Another strong linkage disequilibrium between the A-allele (G/A substitution) and the X2-allele (XmnI RFLP) has been described (137,286). The XmnI polymorphism is also located in the 5´ flanking region of the apo A-I gene and the X2-allele has been reported to be associated with higher plasma levels of total and LDL cholesterol in healthy men (287). In addition, the X2-allele is found to be associated with a higher frequency in individuals with combined hyperlipidemia (286).

Another possible explanation is the linkage with the SstI polymorphism located in the 3´uncoding region of the apo C-III gene. Apo C-III is an inhibitor of lipoprotein lipase and is involved in the metabolism of triglyceride-rich lipoproteins. Thus, genetic variations affecting plasma apo C-III levels may be responsible for the effect on plasma LDL-C levels through the metabolism of VLDL. In Germans, a significant association between the S1S2 genotype and higher serum cholesterol levels has been described (288). Moreover, Lopez-Miranda et al. (289) observed that the interaction between the SstI and the -75 bp polymorphism had an additive effect on changes in total cholesterol, LDL-C, and apo B induced by diet. Furthermore, Dallinga-Thie et al. (290,291)

found linkage disequilibrium between all three of those polymorphisms (G/A substitution, XmnI RFLP, and SstI polymorphism). They reported associations between higher frequencies of rare alleles (A, X2, S2) and elevated levels of plasma cholesterol, triglycerides, LDL-C, apo B, and apo C-III. Their quantitative sib-pair analysis (290) revealed linkage between the rare A-allele of the -75 bp polymorphism and LDL-C levels. Therefore, it could be hypothesized that the differences in plasma LDL-C levels in subjects with different apo A-I -75 bp genotypes are mediated through an effect on plasma apo C-III levels.

As previously reported (see Table 1), we found gender-related differences for the associations between the A-allele and some of the lipid traits examined. Some studies only observed an effect of the A-allele in men (134,137,138,140), whereas others found this association only in females (132,139,135). Some potential explanations for this gender differences may be found in interactions of environmental and life-style factors with the different genotypes. It is known that factors such as age, gender, body mass index, diet, alcohol consumption, smoking, physical exercise, and exogenous hormones as well as lipid lowering drugs (115-117,292) play a major role in the variance of glucose and lipid traits. In a population-based study, Moll et al. (120) calculated that those factors might account as much as 20% of the variance of the apo A-I levels. In this study, all these factors were present. Even though, we considered these in the statistical analysis, there still is a chance that such factors could have influenced the results. Furthermore, multivariate analyses were carried out, which could result in some significant associations being observed due to chance.

Additional analyses were carried out, excluding women that were taking sex hormones. However, genotype associations with total cholesterol and LDL-C levels still remained significant (data not shown).

Regarding the +83 bp polymorphism located in the first intron of the apo A-I gene very few studies have been reported and, like those examining the -75 G/A mutations, they show conflicting results (141,142,146,148) (see Table 2). Wang et al. (141) observed in 243 healthy Caucasians a significant effect of the rare M2- allele on HDL-C, with M2-/+ adults and children having higher levels compared to M2+/+ subjects. Moreover, they also detected linkage disequilibrium between the -75 bp promoter and the +83 bp polymorphisms. Their joint effect on HDL-C levels was also significant and individuals with rare alleles at both sites (A-allele and M2-allele) had the highest HDL-C levels. This is somewhat in agreement with our results, in which males carrying both rare alleles had the highest apo A-I levels. However, these data, especially the results on the linkage disequilibrium, have to be evaluated with care as only a small number of

subjects were in the various groups. Surprisingly, a second study by Wang et al. (146), screening 644 CAD patients, revealed opposite results. Patients carrying the rare +83 bp allele were not associated with higher levels of HDL-C or A-I. Instead, they were even more likely to have increased severity of CAD as well as a positive family history of CAD. Kamboh et al. (142), on the other hand, demonstrated in males non-smokers with the M2+/- genotype higher levels of apo A-I compared to the M2+/+ wildtype. In women, they noted a significant difference in HDL-C levels, with M2+/- heterozygotes having higher levels than M2+/+ homozygotes. In the most recent study (148) investigating 69 subjects with heterozygous familial hypercholesterolemia, no association with altered lipids or with dietary response to an NCEP Step I diet was detected.

Our own results revealed a strong association of the +83 bp polymorphism on apo A-I in men, shown in Figure 15.

Figure 15

Effect of Apolipoprotein A-I +83 bp Genotype on Apo A-I Level in Males
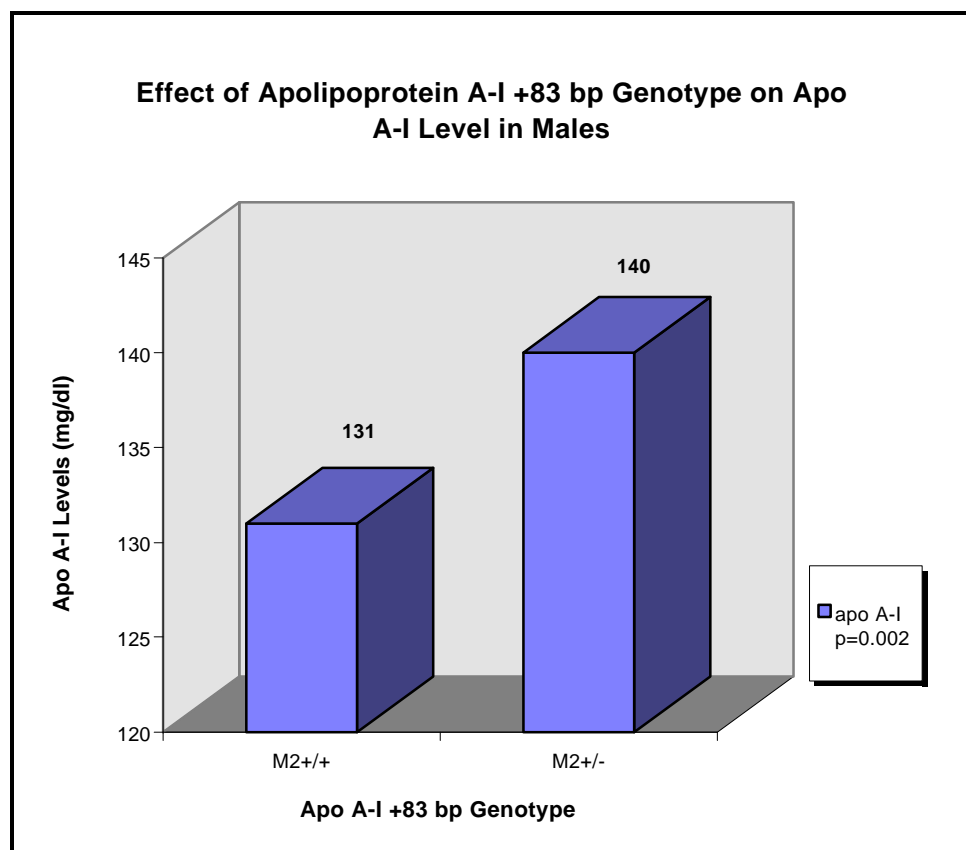
Heterozygotes (M2+/-) had significantly higher apo A-I levels than M2+ homozygotes. Furthermore, males showed a weak significance of the rare M2- allele with higher total cholesterol levels. In females, the M2- allele noted a similar weak effect on total cholesterol, but in the opposite direction. Women carrying the rare allele had lower levels of total cholesterol than their M2+/+ homozygous counterparts.

The metabolic explanation, however, can only be speculative. The +83/84 bp mutation/s located in the first intron of the apo A-I gene might have an effect on mRNA stability, with the T and/or A substitutions being more stable than the wildtype. Alternatively, as suggested for the -75 G/A polymorphism, the +83 bp polymorphism maybe in linkage disequilibrium with another functional polymorphism. Another possible explanation is the methylation hypothesis. Schemer et al. (293) reported that the methylation pattern of the 5′ region of the apo A-I gene was negatively correlated with the extent of its expression. While a hypomethylated 5′ region is found in tissues expressing the gene (liver), heavy methylation is found in non-expressing tissues (kidney). Therefore, it is believed that the T and/or A mutations at +83 bp may lead to an enhanced demethylation causing more cells to express the apo A-I gene. However, it still remains to be determined if hormone levels affect this process and therefore explain the gender differences observed.

In conclusion, our data is consistent with the concept that the apo A-I -75 bp does not affect levels of HDL cholesterol and apo A-I in males or females. Furthermore, our data does not support the hypothesis that the rare A-allele of the -75 bp mutation has a beneficial effect since higher levels of apo B, total cholesterol, and LDL-C and an TC/HDL ratio was noted in females carrying the A-allele compared with G/G homozygotes. With regard to the +83 bp mutation, the rare M2- allele seems to have an effect on apo A-I levels with M2+/- heterozygotes having higher levels than their M2+/+ counterparts. The linkage disequilibrium, found between the two polymorphisms, seems to exhibit a potentiating effect in women. Women carrying both rare alleles (G/A M2+/-) had the highest levels of apo B, total and LDL cholesterol, and TC/HDL ratio, and the lowest levels of HDL-C compared to any other haplotype. In men, on the other hand, the addition of the rare M2- allele exhibited a positive effect, due to a greater increase in apo A-I and HDL-C levels compared to men without the +83 bp mutation.

Apolipoprotein A-IV

For a number of years, special interest has been focused on apo A-IV, because of its potential role in the reverse cholesterol transport and triglyceride-rich lipoprotein metabolism. In this study, we

investigated the effects of two different apo A-IV gene polymorphisms on fasting glucose, lipid, lipoprotein, and apolipoprotein levels in a large population. One of the polymorphisms is located at position 360 and is due to substitution of His for Gln and the other one is located at codon 347 and is caused by an amino acid exchange of Thr to Ser in the mature protein. Using polymerase chain reaction (PCR) and restriction isotyping, instead of two-dimensional electrophoresis or isoelectric focusing, ensured an unambiguous typing of the known isoforms and prevented mimicking of other isoforms with the same charge properties. Additionally, it is a faster and less expensive way of analyzing these two polymorphisms on a large data set. The frequencies of the various alleles, however, are comparable to the ones analyzed by phenotyping (see Table 3).

With regard to the apo A-IV 360 polymorphism previous metabolic and physiological experiments revealed considerable differences between the two most common isoforms apo A-IV-1 and apo A-IV-2. Weinberg et al. (294) reported that the apo A-IV-2 isoprotein has more alpha-helical structure, higher binding affinity for phospholipids, and greater ability to activate LCAT compared to the apo A-IV-1 isoprotein. In addition, apo A-IV-2 has been observed to be a more potent activator of lipoprotein lipase than apo A-IV-1 (188). Rader et al. (295) found that the fractional catabolic rate of the apo A-IV-2 is slower than that of the apo A-IV-1 wildtype. All these structural and functional differences provided support for the concept that apo A-IV-1 and apo A-IV-2 may have different *in vivo* metabolisms, which may affect plasma levels of lipids, lipoproteins, and apo A-IV levels in a differential manner.

Population studies, however, have revealed mixed and even contradictory results (see Table 3). Some reports have documented significant associations between the apo A-IV 1/2 genotype and higher HDL cholesterol (185,186,296,297) and lower triglyceride (186) levels while others have reported that subjects carrying the 2-allele had significantly lower concentrations of HDL, HDL_2, and HDL_3 cholesterol (187) and higher triglyceride levels (298). Furthermore, reports have been published showing that subjects with the A-IV 1/2 genotype had significantly lower levels of Lp (a) (197,299), higher levels of Lp A-I, apo A-IV, higher plasma activity of LCAT, and lower activity of CETP than their apo A-IV 1/1 counterparts (193). In contrast, Carrejo et al. (195) examined the apo A-IV 360 gene mutation in 119 subjects with high cholesterol and high triglyceride levels in whom carotid wall thickness had been assessed. In these subjects, no effect of this mutation was noted on apo A-I, apo C-III, apo A-IV levels, neither on HDL-C and other lipid variables. Their results as well as our own and several others (191,192,194,196,198,300-302) are consistent with the concept that genetic variations within the apo A-IV gene at codon 360 are not significant factors when it comes to variation within apolipoproteins or HDL-C levels. Although our sample size, at 734, is substantially greater than most of the other studies, we could

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not detect associations between the apo A-IV 360 polymorphism and any of the lipid traits in both sexes. Even de Knijff et al. (192), screening 1393 Dutch men as well as the EARS study I (301) and II (302) investigating students from 11 European countries, did not observe any significant effect of the 360 polymorphism on various lipid phenotypes. However, in the EARS studies, they did not differentiate between males and females, which we feel is essential due to the different lipid profiles, and therefore, their different susceptibility to gene variation and cardiovascular disease.

With regard to the apo A-IV 347 threonine (T) to serine (S) mutation very few studies have been published, and the literature and their results are controversial as well (Table 4). While some reports detected an association of the rare S-allele with lower concentrations of apo B (197), total (298) and LDL cholesterol (197,298), others noted significantly increased levels of apo B (302,303), total (302) and LDL cholesterol (303), and triglyceride (302) next to elevated BMI and waist/hip ratios (302) in subjects carrying the S-allele. This study, however, investigating more than 700 subjects, is in confirmation with the third group of studies, which did not observe any effect of the 347 polymorphism on apolipoprotein, lipid, and lipoprotein traits (195,300,304,305). However, it is difficult to compare these results due to differences in study population with regard to ethnic background, age, gender selection, and number of subjects.

Another point of interest was to investigate whether genetic variation at the apo A-IV gene locus may be responsible for variability on apo A-IV levels. Previous reports documented decreased levels in patients with malabsorption syndrome, chronic pancreatitis, and in patients receiving total parenteral nutrition (306-308), while high levels of plasma apo A-IV levels have been observed in patients with renal failure (309) and in those with hypertriglyceridemia (310,311).

Genetics also seem to play a role, as an apo A-IV gene deletion, described by Ordovas et al. (312), resulted in low levels of apo A-IV. In addition, v Eckardstein et al. (193) reported in German males that the apo A-IV 360 mutation had an effect on A-IV levels with 1/2 heterozygotes having higher levels than their 1/1 homozygous counterparts. Several other investigators (186,195,296,301) including ourselves, however, did not observe such an effect, for either of the two apo A-IV (360, 347) polymorphisms. Therefore, the data indicate that, even though these mutations alter the protein sequence, they do not seem to be responsible for any variability in plasma apo A-IV levels.

In contrast, the glucose findings are of note. Data in our study suggest that genetic variation at the apo A-IV locus affects glucose levels in females. Women carrying the apo A-IV 1/2 genotype had significantly higher fasting glucose levels compared to 1/1 homozygotes.

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Figure 16

Effect of Apo A-IV 360 Genotype on Glucose Levels in Females
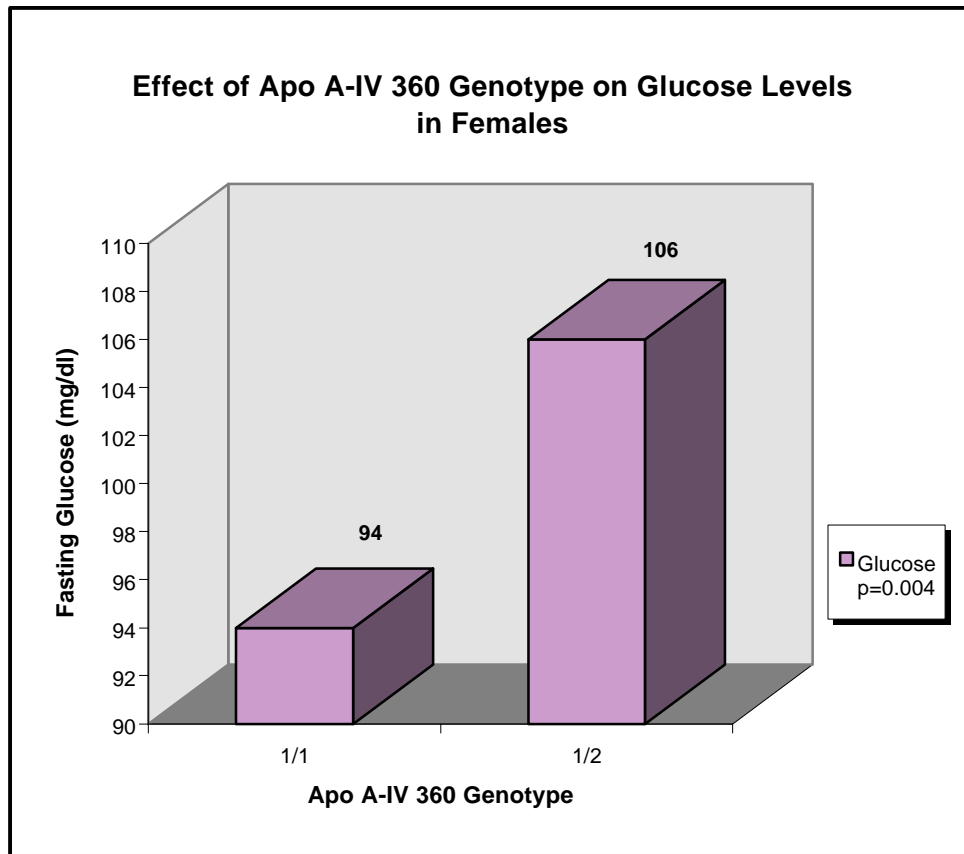
Similar results have been previously reported by Visvikis et al. (189). They documented in 158 nuclear healthy families an association of the rare apo A-IV-2 isoform with higher plasma glucose levels. Kamboh et al. (190), on the other hand, noted an effect of the apo A-IV-2 allele on higher fasting insulin levels in normoglycemic and non-insulin-dependent diabetes mellitus (NIDDM) males. Verges et al. (296) reported that the potential protective lipid profile found in their control subjects associated with the apo A-IV 1/2 genotype was erased in NIDDM patients. However, the physiological explanation behind these phenomena still needs to be elucidated. Further research in this area appears to be warranted to find out whether the apo A-IV 360 polymorphism affects glucose levels directly or indirectly through hormones such as insulin.

In rats, Uchida et al. (313) demonstrated that the apo A-IV production in hepatocytes was regulated by several hormones including insulin. Incubation of these cells with insulin resulted in an inhibition of the apo A-IV production in a dose-dependent matter. Furthermore, there is evidence in rats, that insulin deficiency stimulates the production of VLDL-type triglyceride-rich particles by the intestine, and causes hypertrophy of the gut, both of which can result in increased

intestinal apo A-IV secretion (314). In humans, Attia et al. (315) found higher levels of apo A-IV in young IDDM patients compared to healthy controls, and noted a close relationship with a strong glycemic control, as evidenced by strong positive correlation between apo A-IV and HbA1c. Similar results were published by Verges et al. (296) who detected higher apo A-IV levels in NIDDM patients as compared to normoglycemic controls. Their view was that the hypertriglyceridemia observed in diabetes is likely to promote the increase of plasma apo A-IV rather than the opposite.

Another possibility for the apo A-IV 360 effect on glucose levels could be due to either linkage disequilibrium between this apo A-IV protein polymorphism and another closely linked marker or an interaction between the apo A-IV type and another unlinked apolipoprotein gene product (316).

In conclusion, our data are consistent with the concept that genetic variation at codon 360 and 347 within the general population, do not have a direct physiological influence on plasma cholesterol, triglyceride, LDL or HDL cholesterol levels, the ratio of TC/ HDL cholesterol or apo A-IV levels, but do affect glucose levels in women.


Lipoprotein Lipase

Lipoprotein lipase is known to be the rate-limiting enzyme in triglyceride catabolism. In this study we investigated the lipoprotein lipase Hind III genotype-phenotype interactions. In addition, by recruiting a large number of people, we were able to effectively focus on a possible gene-gender interaction.

The genotype distribution of the Hind III polymorphism in this study was not significantly different from the Hardy-Weinberg equilibrium and the overall allele frequencies were similar to previously published studies in Caucasians (208,222,223,228,229,231,232).

In this study, we could demonstrate a significant effect of the LPL Hind III polymorphism on the variation of plasma concentrations of total cholesterol in women. The H+ allele was associated with significantly higher levels of total cholesterol. Females carrying the wildtype (H+/+) had significantly higher total cholesterol levels compared to females being homozygotes for the H- allele (H-/-). Similar effect on LDL cholesterol was observed in females, with subjects carrying the H+/+ genotype having the highest and those carrying the H-/- genotype having the lowest levels (Figure 17). In males, however, no significant effect was observed for total and LDL cholesterol.

Figure 17
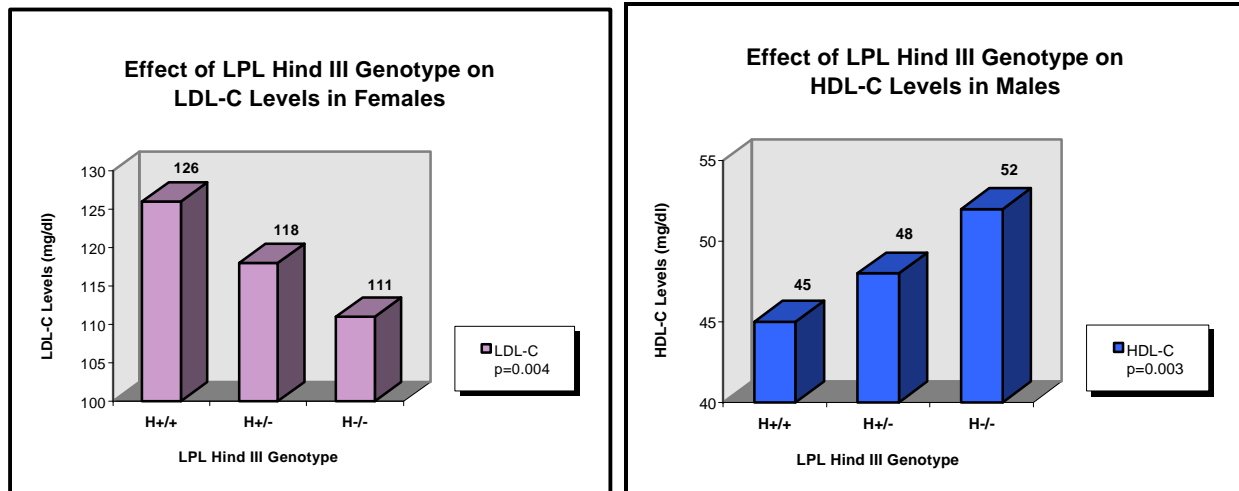
The majority of previous studies did not find significant associations between the LPL Hind III genotype and total or LDL cholesterol (see Table 5). However, Mattu et al. (226) reported similar associations of the Hind III H+ allele with total and LDL cholesterol as well as the same gene-dosage effect with H+ having the highest and H- having the lowest lipid levels. Their study was carried out in a small group containing men only. In contrast, Heinzmann et al. (208) found a significant association of the H+ allele with total cholesterol in 189 US Caucasians, but in the opposite direction. Individuals carrying the H- allele had higher total cholesterol levels compared to homozygotes carrying the H+ allele. With regard to LDL-C, the same trend as total cholesterol was documented, but did not reach statistical significance.

Elevated plasma LDL-C levels have been accepted as a major risk factor for coronary artery disease (CAD). Our result of a positive relationship between the H+ allele and total and LDL cholesterol may now provide an explanation for previously reported associations of the H+ allele and CAD (223,226,317,318). Thorn et al. (223) reported in Caucasians with severe CAD a significantly higher frequency of the H+ allele compared to healthy controls. Furthermore, Chen et al. (317) indicated in their study a significant effect of the Hind III site on carotid artery atherosclerosis in white males. Individuals with the H+/+ genotype not only had a significantly higher average carotid wall thickness, but the H+/+ genotype was also associated with hypertriglyceridemia and hypercholesterolemia compared to the other two genotypes. In addition, patients with type 2 diabetes mellitus and the H+/+ genotype were reported to have the highest prevalence of CHD (90%) compared with the H+/- (55.4%) and H-/- (54.6%) genotypes, respectively (319).

The metabolic mechanism responsible for the association of the Hind III polymorphism with LDL-C levels is still not completely understood. It has been suggested that this polymorphic site has an indirect effect on the LPL activity, with the H+ allele having a lower and the rare H- allele having a higher LPL activity, respectively. Since the Hind III polymorphism is located in intron 8 of the LPL gene, it does not affect the sequence or the gene product. It is believed that this polymorphism is in linkage disequilibrium with one or more regions within or in close proximity to the LPL gene, affecting LPL activity and/or the clearance rate of triglyceride-rich lipoproteins and their remnants. In fact, a recent report by Humphries et al. (320) provides strong evidence for significant linkage disequilibrium between the Hind III site and the LPL S447X mutation. Recently, it has been convincingly shown that the S447X mutation results in increased LPL activity (321). Therefore, individuals with the Hind III site and the LPL wildtype would be expected to have lower LPL activity. Our results can also be compared with patients, who are heterozygous for familial lipoprotein lipase deficiency, and characterized by a 50% reduced LPL activity. Brunzell (322) as well as Miesenboeck et al. (323) detected in some of these patients abnormalities across the lipoprotein density spectrum including an increase in VLDL or intermediate density lipoproteins (IDL) and small dense LDL particles. Although, this increase in LDL is not typical, it might be due to the combined measurement of IDL and small dense LDL in the total LDL fraction (322). Furthermore, this alteration in lipoprotein compositions might cause a reduced affinity of those particles to the LDL-receptor, which could explain the higher LDL levels. As suggested before (320) also nonenzymatic effects of LPL, such as its bridging function, may be more efficient in individuals with the S447 stop mutation.

Concerning HDL-C and triglyceride levels, the results of previous studies are very controversial. While several investigators demonstrated a relationship between the LPL Hind III polymorphism and HDL cholesterol and/or triglyceride levels, several others could not verify such an effect (see Table 5). Our findings agree with results of the former group. We could demonstrate a significant association with the Hind III genotype and HDL-C levels, but in males only (Figure 17). Those carrying the H-/- genotype had significantly higher HDL-C levels compared with their H+/+ counterparts. At the same time, H-/- men had a trend of lower triglyceride values than men carrying the H+/+ wildtype. This is in agreement with the concept that the polymorphism is somehow associated with the LPL activity by finding an inverse relationship between HDL-C and triglyceride levels. Our findings confirm previous results (225) suggesting that the H- allele is associated with a higher LPL activity and the H+ allele is associated with a lower activity.

In terms of the gene-gender effect, a difference with regard to the LPL Hind III polymorphism and various lipid traits was noted. The female population showed significant associations between the Hind III genotypes and total as well as LDL cholesterol, while men showed a significant effect on HDL cholesterol. The reason might be due to differences in hormonal status or other environmental and lifestyle factors. Further research is warranted to elucidate the gene-gender difference.

In addition, it is important to mention, that the data in the publication (see Attachment) was analyzed differently than in the thesis. Instead of using baseline data, we evaluated data after eight days, to ensure a stabilization period for all the subjects. As a result, the association between the Hind III polymorphism and HDL-C in men was abolished. In females, however, the effect of total and LDL cholesterol became even more significant.

B. **Effects of Apolipoprotein E, A-I, A-IV, and Lipoprotein Lipase Genotypes on Glucose, Lipid, and Lipoprotein Response to Lifestyle Intervention**
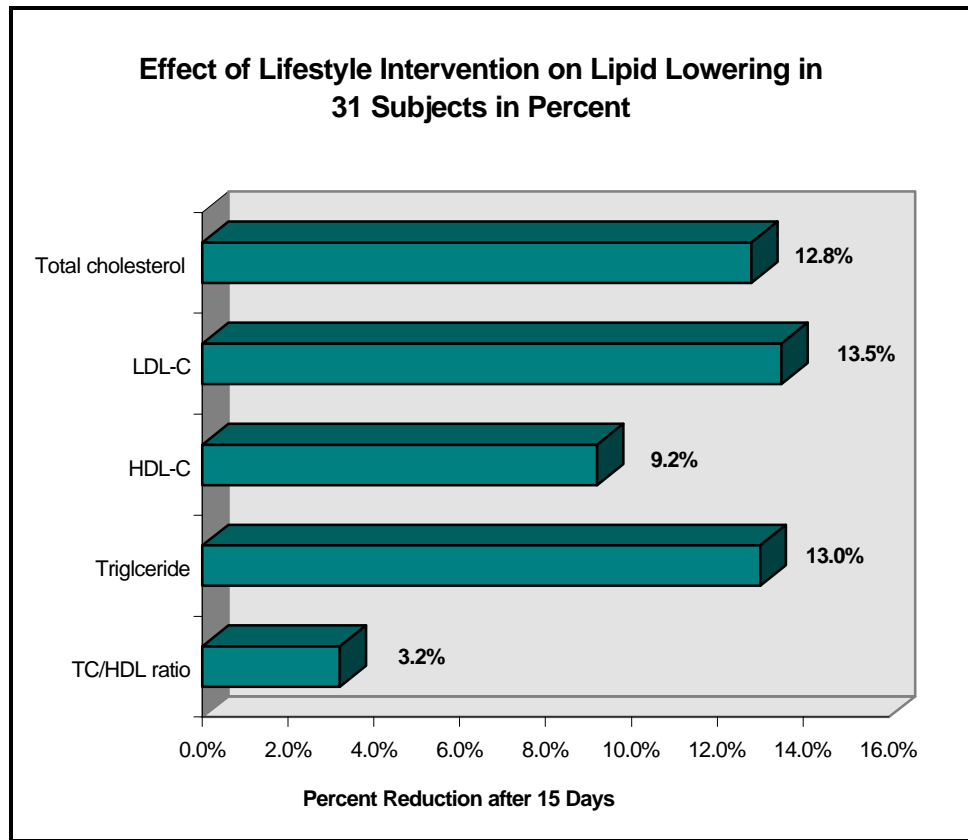
Subjects

Lifestyle intervention with diet and exercise had a significant effect on lowering glucose and lipid parameters in females and males. The reduction of each phenotype in percent is demonstrated in Figure 18.

Figure 18
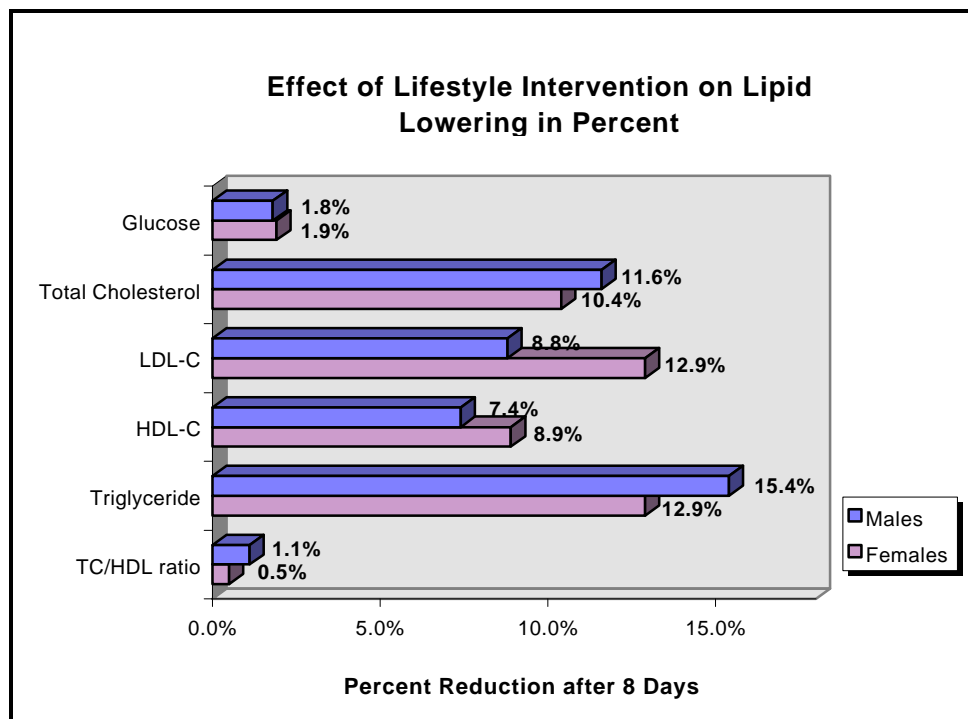
These results indicate that the intensive program at the Pritikin Longevity Center is very effective in lowering cholesterol levels even in a short period of time. To ascertain what the changes would have been, had participants been sampled at the two-week time point, a subset of 31 subjects that participated in the 3 week program was screened (Figure 19).

Figure 19

Effect of Lifestyle Intervention on Lipid Lowering in 31 Subjects in Percent

As expected, greater reductions were achieved by the 31 subjects versus participants of the eight day intervention program. However, comparing our data with results previously published on over 4000 participants (13) who took part in the same intervention program, but were sampled after 3 weeks, reductions reported for total and LDL cholesterol were significantly greater at 23% each as compared to our results at 12.8% and 13.5%, respectively. One of the reasons for the greater reduction is the fact, that subjects participated one week longer in the intervention program compared to our 31 subjects. The other reason may be the difference in baseline levels of total and LDL cholesterol. Their baseline values for total cholesterol compared to ours were significantly greater at 5.99 mmol/L versus 5.38 mmol/L in males and 6.15 mmol/L versus 5.56 mmol/L in females. The same was true for LDL-C levels, with males, participating in their study, having higher baseline levels at 3.90 mmol/L versus 3.21 mmol/L, and females having higher levels at 3.97 mmol/L versus 3.15 mmol/L, respectively.

Overall, the lifestyle intervention program seems to be effective in lowering various lipid parameters and therefore, reduce the risk for coronary heart disease.

The question whether genetic variation at apolipoprotein and lipoprotein lipase gene loci is responsible for differences in cholesterol lowering response to diet and exercise is discussed in the following chapters.
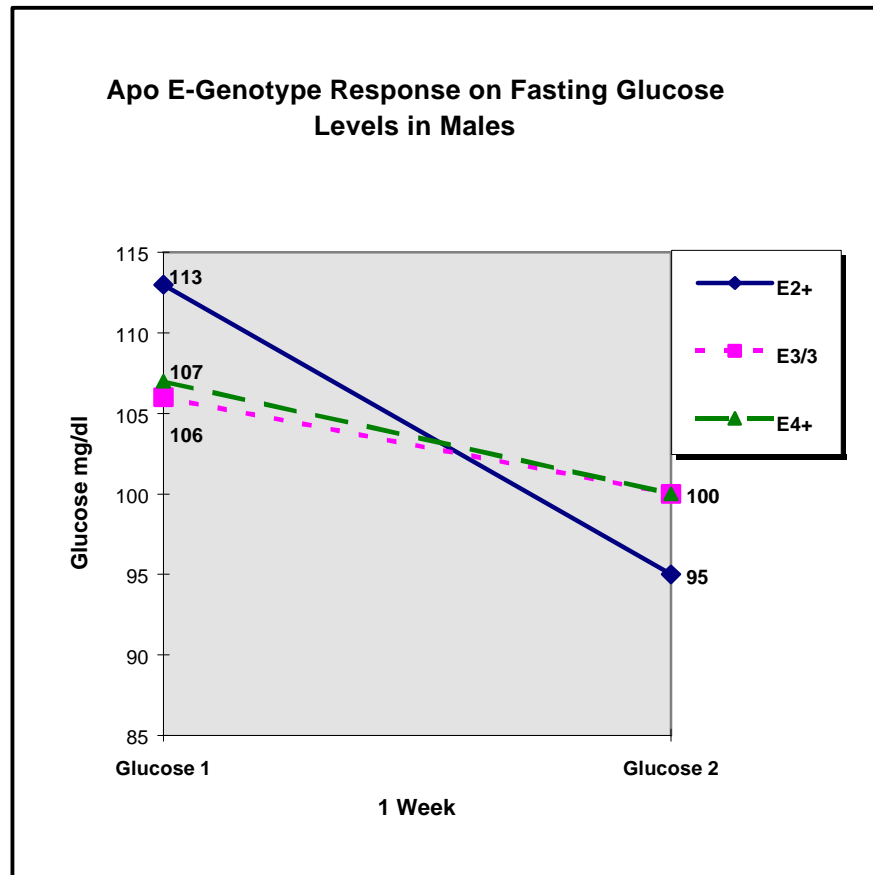
Apolipoprotein E Response

Several investigators (20,22,324-326) have previously documented that there is a significant variability in response to restriction of dietary saturated fat and cholesterol for reductions in various lipid parameters. Moreover, others (12,79,80,86-88,90,92,94) have detected that some of the variability in this response, which is very considerable, may be related to genetic variation at the apo E gene locus, specifically due to the apo E4 allele, where subjects carrying the apo E4 allele due to an arginine for cysteine substitution at amino acid 112 were more responsive to diet than subjects without this allele (12,79,80,86,87,94,98). Conversely, Ordovas et al. (327) reported that male subjects heterozygous for the apo E2 allele due to an amino acid substitution of cysteine for arginine at residue 158 are more responsive to HMG CoA reductase inhibitors than other subjects. Similar results were published in non-insulin-dependent diabetics (NIDDM) (90). Diabetic subjects with the E2 allele had the greatest response, whereas those with the E4 allele had the least response after consumption of a "therapeutic diet". In this study, we found for the first time in men carrying the E2 allele a greater response in fasting glucose levels compared to men carrying the E3/3 genotype or the E4 allele (Figure 20).

As shown in a previous chapter, we presented data demonstrating a significant effect of the apo E genotype on baseline glucose levels, with females with the E2+ and E4+ allele having lower levels of fasting glucose compared to the E3/3 wildtype. The metabolic explanation for the relationship between apo E and glucose levels as well as response still needs to be elucidated.

Several other studies (76,82-85,87,93,96,100), on the other hand, could not find an association between the apo E genotypes and lipid traits in response to dietary manipulation, which is in agreement with our results presented in this study. We did not observe these effects in lipid phenotypes, possibly because this was not just an intervention in which saturated fat and cholesterol were reduced, but also a situation where subjects were encouraged to exercise, they could restrict their calories, they lost weight, and it was also a diet high in fiber and complex carbohydrate. Moreover, in contrast to some previous studies (12,84,87,94,326), it was not strictly controlled, where the subjects were given all of their food on a metabolic ward and body weight was maintained. In this study, these were voluntary participants interested in reducing their heart disease risk, who paid to participate in this residential program. Nevertheless, it does

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represent a large number of subjects who participated in a residential setting in the same diet, exercise and lifestyle modification program.

Figure 20

Apo E-Genotype Response on Fasting Glucose Levels in Males

Apolipoprotein A-I Response

With regard to the apolipoprotein A-I mutation at -75bp, which is located in the promoter region of apo A-I gene, the literature has been inconclusive as to whether or not it can affect transcription of the apo A-I gene (282-284). The hypothesis here is that, mutations at this gene locus would be most likely to affect response to HDL cholesterol due to the fact that apo A-I is a major protein constituent of HDL; however, no significant effects were noted in this regard in this study. A previous study (144) in males showed that those carrying the rare A-allele had a significant increase in total and LDL cholesterol after a fat-rich meal (40%) compared with their

G/G counterparts. A more recent study (150) reported that changing from a saturated fatty acid (SFA) to a polyunsaturated fatty acid (PUFA) diet resulted in a significant greater decrease of total and LDL cholesterol in women carrying the G/A genotype than in G/G homozygotes. Responsiveness to a low fat and low cholesterol diet (136,148), on the other hand, demonstrated that the difference in lipid response between the -75 bp (G/A) genotypes was not statistically significant. This is in agreement with our data presented in this study on a larger population. We did not observe an effect of the (G/A) mutation in either gender. It should, however, be mentioned that the number of subjects, especially for the rarer genotypes, was relatively small, despite the fact that we studied 734 subjects in total.

With regard to the apolipoprotein A-I genotype at +83 bp, which is located in the first intronic region of the apo A-I gene, only very little is known. So far, one previous study (148) on 69 familial hypercholesterolemic patients reported no effect of this mutation on any lipid traits in response to an NCEP-I diet. This is in agreement with our results. We also failed to find an effect on various lipid phenotypes, especially on HDL cholesterol lowering, which was our primary hypothesis. However, more research regarding this polymorphism is necessary.

Overall, our data would indicate that neither of those two polymorphisms of the apo A-I gene has an effect on the response to dietary modifications.

### *Apolipoprotein A-IV Response*

We also examined the effects of the apolipoprotein A-IV 360 genotype. This is due to a specific mutation in which glutamine replaces histidine at residue 360 in the apo A-IV gene locus. Our data indicate no significant effect of this polymorphism in response to diet, specifically LDL cholesterol lowering. Similar results were observed using an HMG CoA reductase inhibitor (pravastatin) instead of diet (327). In contrast, other investigators (198,326,238), have documented that genetic variability at this locus in the heterozygous state, namely the presence of this mutation, results in reduced responsiveness of LDL-C levels to dietary cholesterol and saturated fat modulation. For instance, Mata et al. (328) found in males that the apo A-IV phenotype modulates the LDL cholesterol lowering response to a diet meeting NCEP Step I criteria. Men carrying the apo A-IV-2 isoform had significantly less reduction (7%) than apo A-IV 1/1 homozygotes (16%). Similar results were shown by McCombs et al. (198). They placed a small group of Caucasians on a high cholesterol diet and found that the apo A-IV-2 allele attenuates a hypercholesterolemic response. While the apo A-IV 1/1 group noted an increase of LDL-C of 19 mg/dl, the apo A-IV 1/2 group only noted one of 1 mg/dl. Since one of the studies

only restricted dietary cholesterol, presumably this mutation can affect the response to dietary cholesterol. Contrary, others (199) reported a greater responsiveness of HDL-C and A-I levels in subjects carrying the 2-allele after having switched from a SFA to an NCEP-I diet. However, compared to all the previous studies, we provided data on a significantly larger number of subjects, but we did not observe any effect of the apo A-IV 360 mutation on lipid response in this study, possibly because so many interventions were carried out at the same time.

The apolipoprotein A-IV 347 mutation, which is a substitution of threonine (T) for serine (S) at residue 347, is a fairly common mutation in the general population. The few previous studies on a limited amount on subjects revealed mixed results. Jansen et al. (200) documented in 41 males that carriers of the rare apo A-IV 347 S-allele had greater decrease in total cholesterol, LDL-C, and apo B when they were switched from a SFA diet to a NCEP-I diet. In contrast, when those men were switched again from the NCEP-I diet to a diet rich in monounsaturated fatty acids (MUFA), they showed a greater increase in total cholesterol and apo B compared to the 347 T/T wildtype. In addition, Ostos et al. (201) recently reported in 50 males subjected to an vitamin A fat load, that those with the S-allele had lower postprandial response in total triglyceride (TG), large triglyceride rich (TRL) TG, and small TRL-TG, and a higher response in large TRL apo A-IV and apo B-100 levels than in subjects homozygous for the T-allele. This would also indicate an involvement of this polymorphism in the variability of LDL cholesterol response after consumption of a high saturated fat diet. However, one has to consider that all those studies were performed on a limited number of subjects as well as in males only.

In contrast, data of this study, including more than 700 subjects, revealed no significant effect of this mutation on dietary response, except in females where subjects homozygous for the mutation (S/S) had a significantly greater reduction in HDL cholesterol at 16.7%, versus 8.6% in females homozygous for the T-allele (Figure 21).

Figure 21

**Apo A-IV 347 Genotype Response on HDL-C Levels in Women**
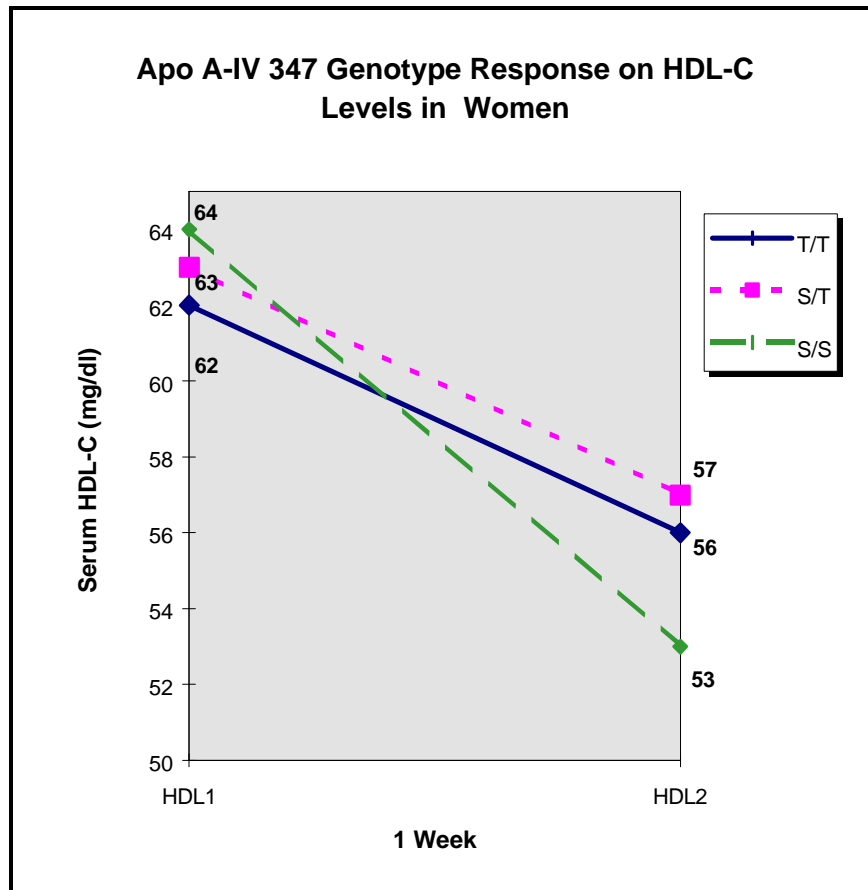
These results, however, must be interpreted with extreme caution, because when one carries out multiple analyses, as we did in this study, one can find associations by chance. It should be noted, however, that our a priori hypothesis was that apolipoprotein A-IV mutations would be most likely to affect HDL cholesterol response, because apolipoprotein A-IV is a protein constituent not only of triglyceride-rich lipoproteins, but also HDL.

Lipoprotein Lipase Response

Lipoprotein lipase is the major enzyme responsible for triglyceride hydrolysis. Patients with a decreased lipoprotein lipase activity due to various mutations in the lipoprotein lipase gene can develop marked hypertriglyceridemia and an increased risk of pancreatitis. Our hypothesis was that alterations in the lipoprotein lipase genotype due to the Hind III locus might affect responsiveness with regard to triglyceride lowering. Indeed, subjects heterozygous or

homozygous for the rare Hind III restriction site showed a greater responsiveness. Males heterozygous for this mutation (H+/-) had a 16.7% reduction in triglycerides versus the males without the mutation (H+/+) who had only a 7.3% reduction. However, because of the wide variability in triglyceride response, these reductions did not achieve statistical significance, either in men or in women. However, HDL cholesterol reductions of 9.0% in heterozygous females (H+/-) were noted to be significantly greater than the reduction of the 5.6% seen in women homozygous for the common H+ allele (Figure 22).

Figure 22
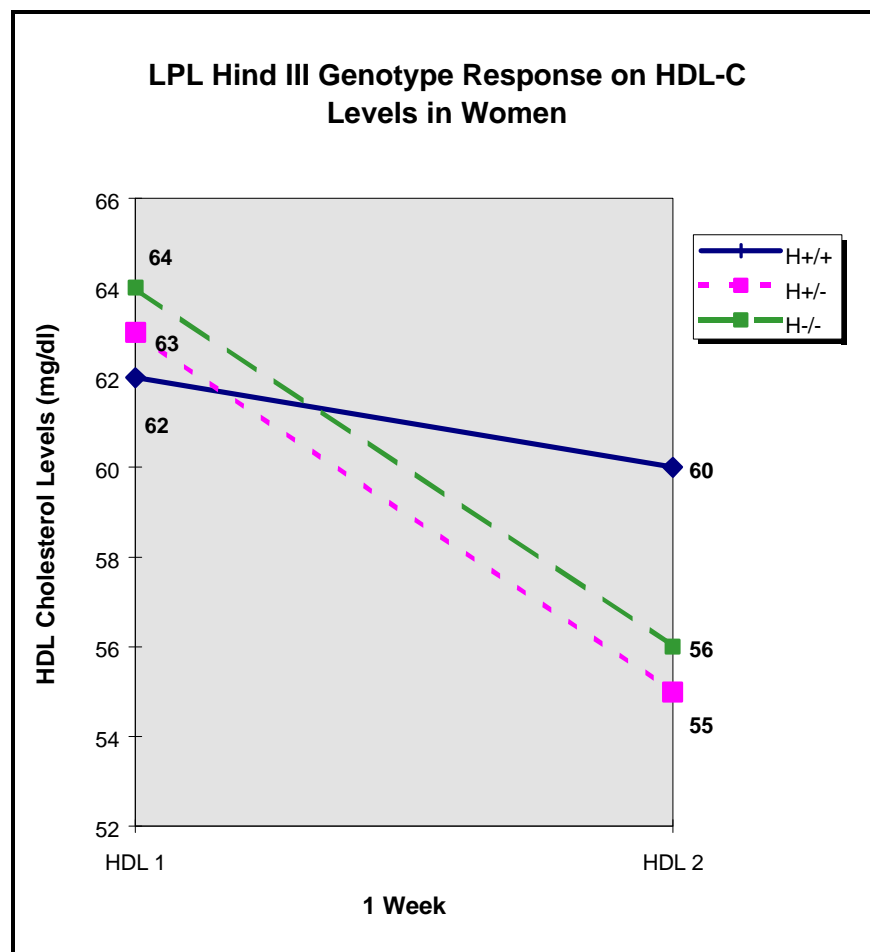
As the effect of LPL on HDL particles is indirect via the transfer of surface components from triglyceride-rich lipoproteins to the HDL fraction, it is likely that the variation in HDL response to diet is due to the genetic variation at the Hind III gene locus. A previous study (233) also documented an effect of this polymorphism on response. Switching from a SFA to a PUFA diet,

individuals with the H+/+ genotype had significantly smaller change in total cholesterol (9.3%) than those with the rare H- allele (14.4%). Contrary, another study (234) presented in subjects carrying the H+/+ genotype a greater reduction of VLDL triglyceride and apo B levels than carriers of the H- allele in response to a 25% restriction in energy intake. Therefore, to clarify whether or not this LPL polymorphism has an effect and on which lipoproteins, further research is necessary.

While dietary intervention and exercise are the cornerstones of therapy for heart disease risk reduction in order to favorably modify both plasma lipoproteins, glucose, and blood pressure, and induce changes in body weight, it is well known that there is striking variability in response to such diets and such lifestyle intervention (12). It should, however, be stated that another major factor in regard to responsiveness may be related to compliance, especially in an ad libitum setting, where body weight changes considerably from one individual to another, and where subjects are allowed to eat as much or as little as they want. On the other hand, it is virtually impossible to do metabolic ward studies on over 700 individuals, to assess the genetic response. In this intervention, we see that despite the fact that it was a two-week intervention, the effects on plasma lipoproteins, by the nature of the program, were only assessed at one week. Even so, the one-week assessment did indicate significant reductions in total and LDL cholesterol. However, we were not able to clearly relate any of the apolipoprotein genotypes or lipoprotein lipase genotype to this responsiveness. We did note that apo E genotype clearly affected responsiveness to glucose in men, and this is an observation that deserves further follow-up, both in population studies as well as in carefully controlled metabolic ward studies, to determine how apo E genotype might affect response with regard to glucose lowering. It is known that subjects with the apo E2 allele have impaired uptake of chylomicron remnants by the liver, and this might make them more sensitive to restriction of dietary calories and fat, so that less fuel would be taken up by the liver, and consequently it might affect glucose homeostasis.

In conclusion, the over-all data indicate that this lifestyle intervention program can have a significant effect in modulating heart disease risk factors by reducing lipid and lipoprotein levels, and that within the context of such a broad-based intervention, genetic variability at the various gene loci investigated in this study, plays only a modest role.

C. Effects of a Lifestyle Intervention on Plasma Levels of Glucose, Lipid, and Lipoproteins: A Long-Term Follow-up

After having demonstrated that this diet and exercise program had a significant effect on short-term response, it was of interest to investigate the long-term effect as well. Therefore, data were obtained from a subset of 202 females and males. Those subjects participated in the program at the Pritikin Longevity Center on two different occasions, with a mean time span of 1.7 years between visits. Concerning the short-term response, similar results as described for the whole group were found in this subset of 95 females and 107 males. Each intervention resulted in a significant reduction of all lipid parameters in females and males, respectively (Figure 23).

Figure 23
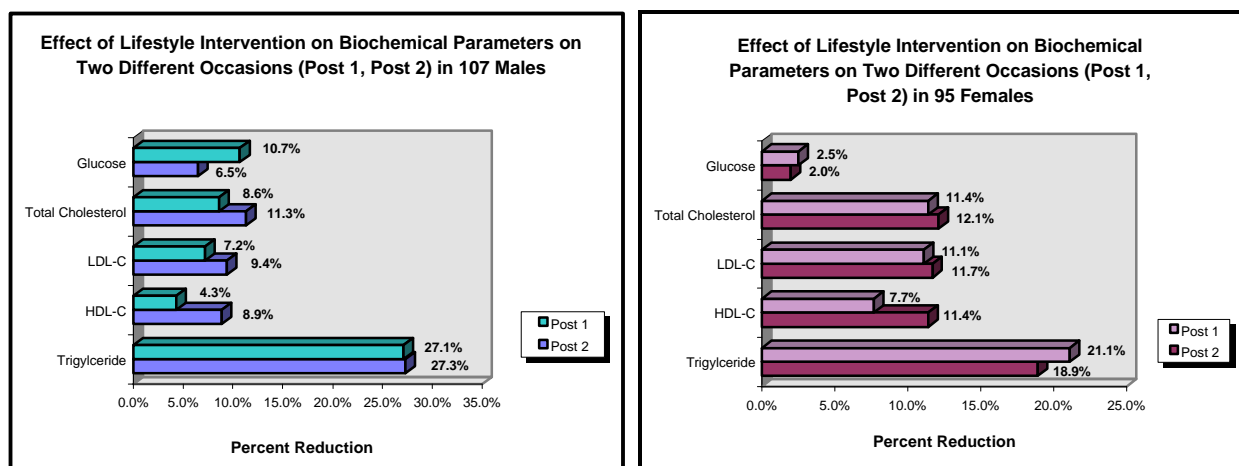
These data are consistent with the concept that an intensive and controlled diet and exercise program can be very effective in lowering lipid levels, which has been discussed in detail in the previous chapter.

Regarding the long-term response, however, no lasting effect was noted in females and males. Baseline levels at return (Baseline 2) were similar to baseline levels at the initial visit (Baseline 1), as shown in Figures 24 and 25.
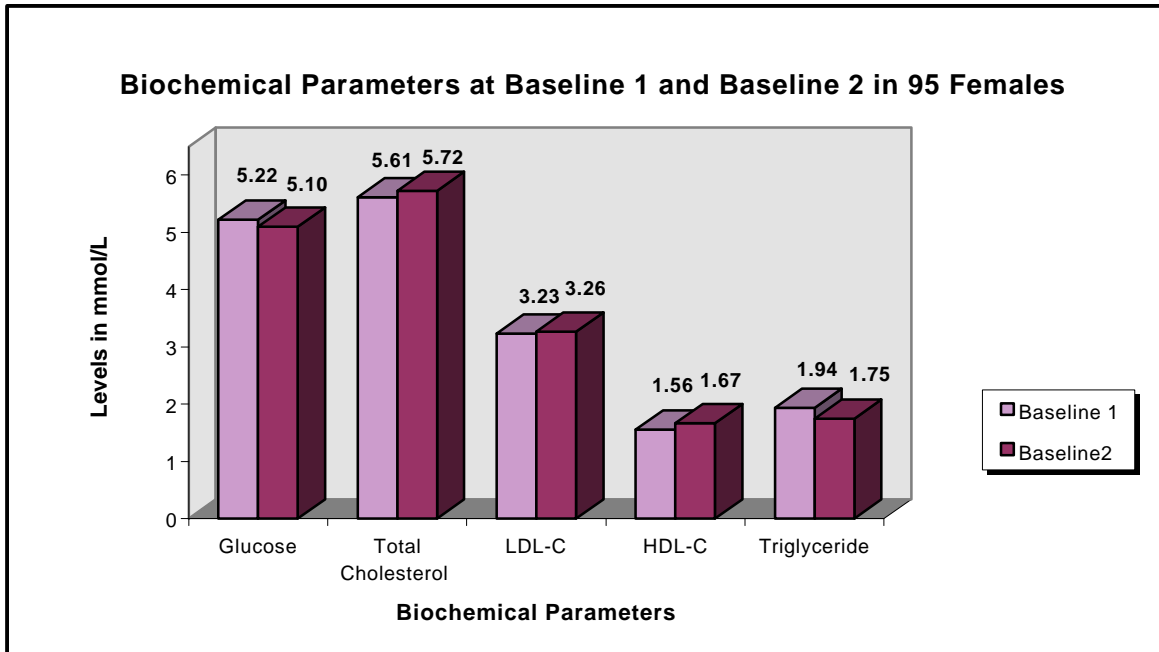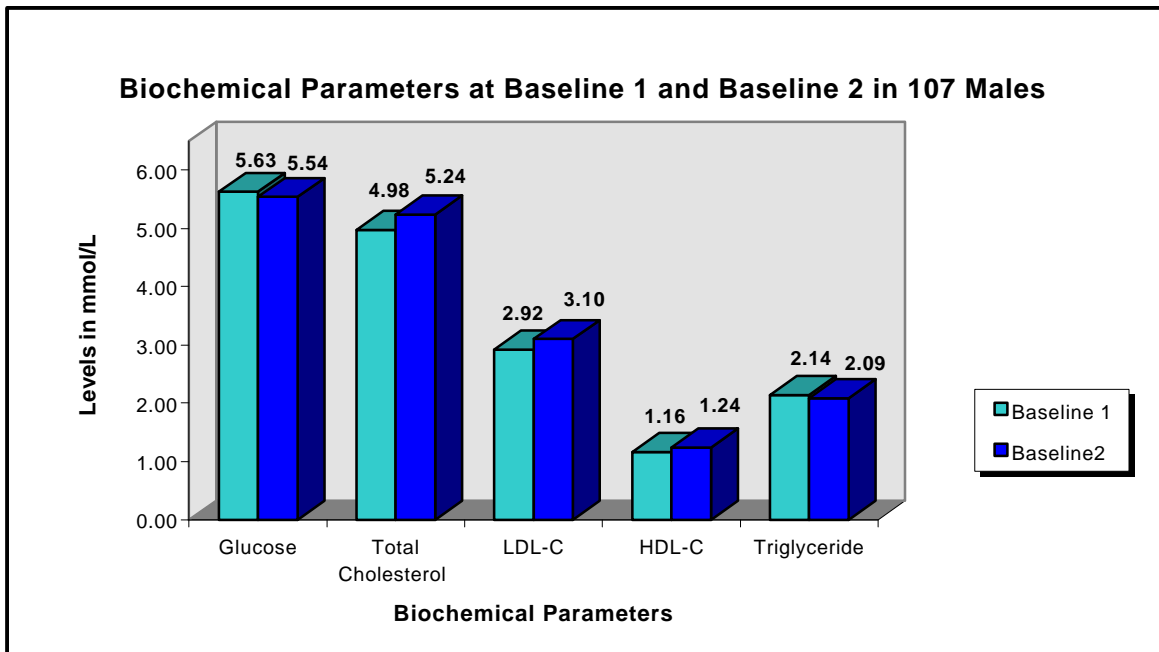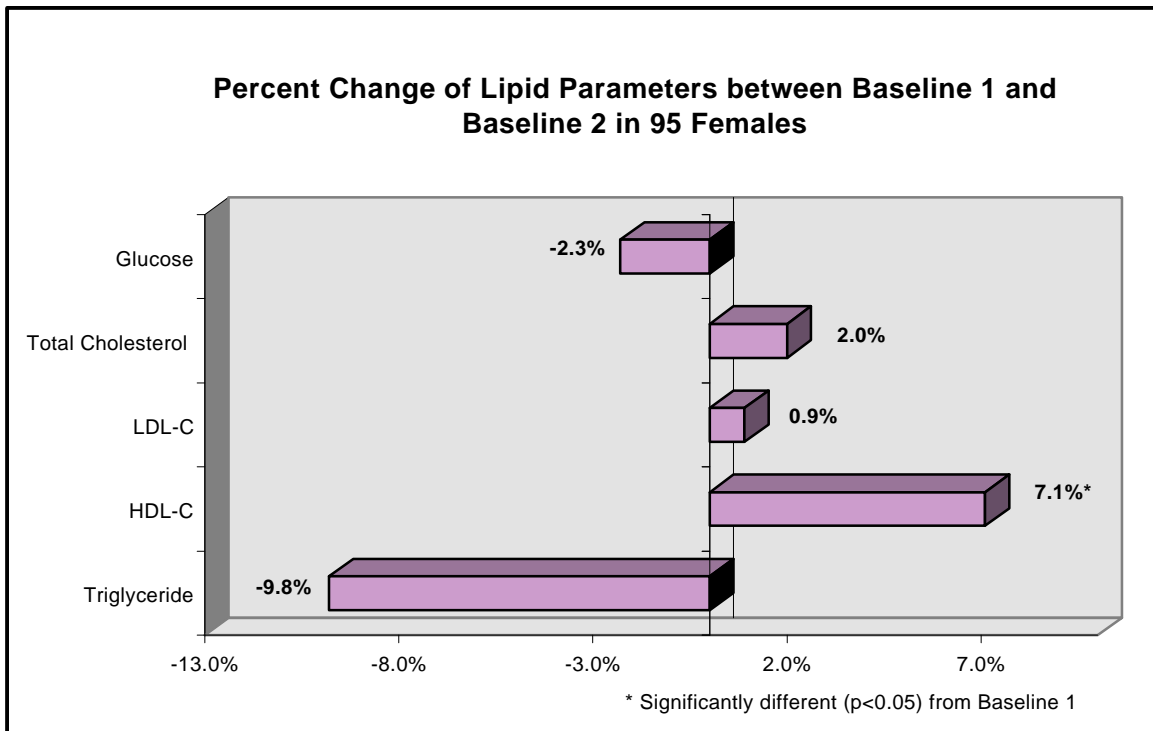
Figure 24

Biochemical Parameters at Baseline 1 and Baseline 2 in 95 Females

Figure 25

Biochemical Parameters at Baseline 1 and Baseline 2 in 107 Males

In females, we noted a trend of higher total and LDL cholesterol levels, and lower glucose and triglyceride levels at baseline 2 compared to baseline 1. Levels of HDL-C, however, were significantly increased at time point 2 (Figure 26).

Figure 26

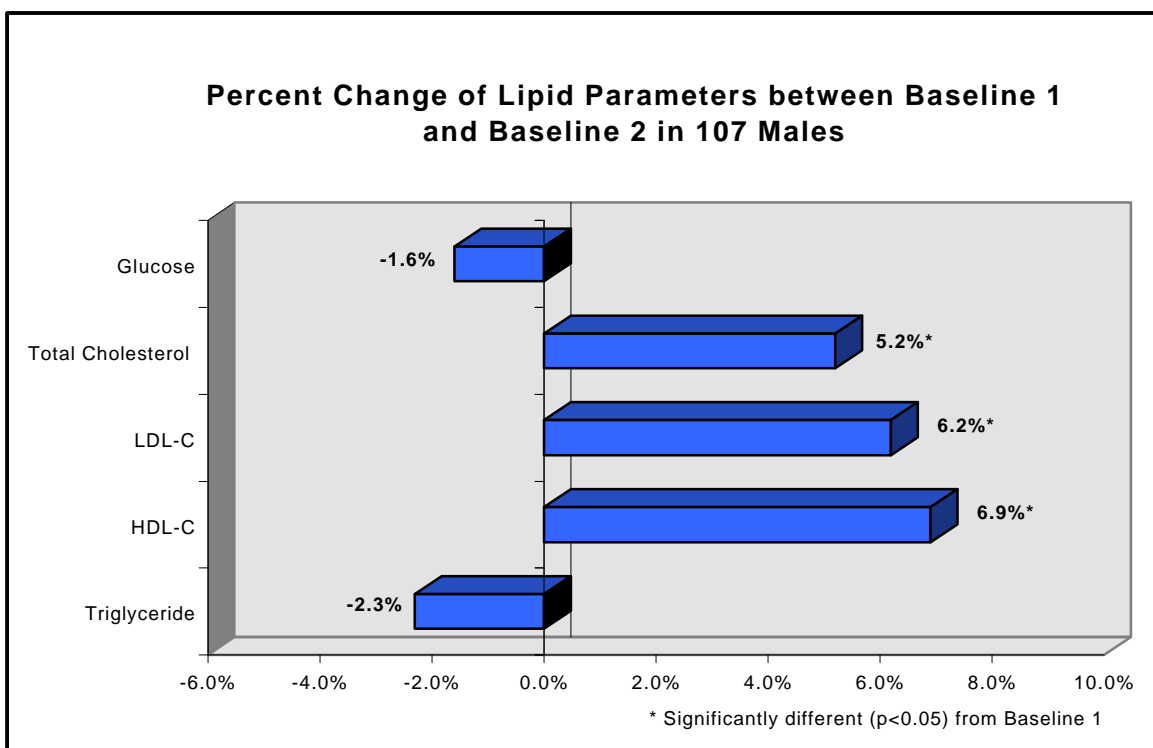Percent Change of Lipid Parameters between Baseline 1 and Baseline 2 in 95 Females

In males, comparison of baseline 1 with baseline 2 revealed that levels of total, LDL-C, and HDL-C were significantly higher, and levels of glucose and triglyceride were non significantly lower (Figure 27). These results indicate that in these subjects, the benefits achieved in the short-term, were not sustained.

Similar results were published by several investigators (329-333). Bae et al. (329) detected in 120 men and women with hypercholesterolemia, that modest reductions achieved after 6 weeks of a Step I diet intervention, did not result in further reductions after 18 weeks. Rather, there was a trend to return or even exceed baseline levels. Furthermore, Henkin et al. (331) noted in 73 subjects who received intensive education on the Step I diet during an initial 12 week period, that levels of plasma cholesterol (19%), HDL-C (8%), LDL-C (16%), and VLDL (66%) had significantly increased after a 6 months follow-up. Another study (334) did not find an effect of either Step I or Step II intervention on LDL-C lowering in men and women, unless subjects engaged in aerobic exercise in addition to the diet. Even meta-analyses, carried out by different investigators (332,333) documented negative effects. Ramsey et al. (332) presented data on 16 controlled trials of six months to ten years duration. Their conclusion indicated that the response of the Step I diet was too small to have any value in the clinical management of lowering serum cholesterol levels in adults. Similar results were recently published by Tang et al. (333). They

demonstrated in an overview of 19 controlled trials, including 28 comparisons, that in free-living subjects only modestly reductions of cholesterol concentrations were observed. Subjects following the Step I lowered cholesterol concentrations by about 3%, and an additional 3% were achieved with diets that are more intensive.

Figure 27

Percent Change of Lipid Parameters between Baseline 1 and Baseline 2 in 107 Males

Parameter	Value
Glucose	-1.6%
Total Cholesterol	5.2%*
LDL-C	6.2%*
HDL-C	6.9%*
Triglyceride	-2.3%

Axis: -6.0%, -4.0%, -2.0%, 0.0%, 2.0%, 4.0%, 6.0%, 8.0%, 10.0%

* Significantly different (p<0.05) from Baseline 1

There may be multiple reasons for the lack of long-term compliance. In general, factors such as biological nonresponsiveness or inadequate knowledge and understanding of the dietary principles can be a possible explanation. Moreover, pre-existing restricted diets as well as subject fatigue to the prudent diet may be another cause. In addition, accuracy of the food questionnaires filled out by the subjects may have an influence on results. One of the major reasons, however, may be the lack of continuous support and dietary advice from medical practitioners and dietitians.

Concerning our study, other explanations may apply for the failure of long-term response. Participants in this study were not recruited into a long-term intervention trial. The majority of subjects spent their vacation time and money to attend the program. Their primary goal may have been to enjoy themselves, while improving their health at the same time. Thus, they may have not been as eager to change their lifestyle long-term. Furthermore, subjects resided at the center and

had all day to focus on their diet, body, and mind, while there. To implement and pursue such intensive lifestyle modifications in the free-living state, however, may be unrealistic. The diet changes alone consisted of drastic reductions of daily fat to less than 10% and daily cholesterol to less than 100 mg. Therefore, even small trends to return to more usual fat and cholesterol intakes would result in increased lipid parameters. In addition, to maintain the intensity of the exercise program every day may be difficult to fit into a working schedule. Another reason may have been that subjects were not supported or supervised by physicians and dietitians once they left the center. Thus, motivation and compliance to continue these changes may have declined after a period. There is also the possibility that only those subjects returned to the center who were not able to adhere to the lifestyle changes in the free-living state.

In contrast to those negative results, effective long-term diet intervention studies have been documented in the literature as well (13,335-340). Barnard (13), for instance, demonstrated in an earlier study on 29 subjects, attending the same Pritikin program, that levels of total and LDL-C after a 12 months follow-up were still significantly lower compared to baseline levels before intervention. Other investigators published various long-term responses on lipids, ranging from moderate changes of 3-6% in young children (337), to 5-7% in subjects from the UK (339), to 15% in men adhering to a Mediterranean diet (338), to 37% reduction in 333 heart disease patients (336). In addition, a recent meta-analysis by Yu-Poth et al. (341) included 37 dietary intervention studies in free-living subjects ranging between 3 weeks and 4 years duration. They described an effective lipid response to the Step I and II diets. The Step I diet was reported to lower total and LDL-C, triglycerides, and TC/HDL-C ratio by 10%, 12%, 8%, and 10%, respectively. The Step II diet reduced those levels by 13%, 16%, 8%, and 7%, respectively. HDL-C levels, however, were also reduced by 7% after the Step II diet. In addition, they documented an even greater reduction of total and LDL-C without a decrease in HDL-C, when subjects were exercising at the same time. This beneficial effect of exercise on HDL-C levels, which was mentioned before by Stefanick et al. (334), was mainly due to weight loss. Thus, their recommendations for effective intervention programs include diet modifications, exercise, and weight control to achieve maximal CHD risk reduction.

Overall, it has to be mentioned that all those meta-analyses need careful interpretation, as the studies included differed greatly in their design, population, gender, type of diet, duration and intensity of intervention, as well as in cholesterol response. Further research is warranted to study the effects of diet and exercise interventions in a large population in the free-living state, but in a well supported and controlled environment.

In conclusion, it appears that the long-term success of cholesterol lowering therapies aimed to reduce CHD risk depends on the administration of an intensive lifestyle modification program including, in addition to diet, exercise and weight loss. Even more important for this goal is the continuous support and advice of practitioners and dietitians to monitor and motivate participants. Therefore, my suggestion would be to support people that are at high CHD risk, in a group setting on a community basis. An ideal setting would be a community center which includes exercise facilities, and is operated by a team of physicians, nutritionists, dietitians, nurses, psychologists, social workers, and exercise physiologists.

Conclusions

The following conclusions can be drawn from this study of 734 females and males who served as subjects for this research and who also entered into the Pritikin Longevity Center dietary and lifestyle program.

Conclusion # 1: Genetic variation at the various gene loci had different effects on baseline glucose and lipid levels in females and males. The same was true regarding the gene-gender interaction and response to lifestyle intervention. However, the general reduction of lipids in response to dietary and lifestyle intervention was not significantly different between genders.

Conclusion # 2: Apolipoprotein E genotype had a significant effect on plasma apo E, total cholesterol and LDL cholesterol levels and in females on HDL cholesterol levels, as well as plasma glucose. Subjects carrying the apo E4 allele had the lowest plasma apo E levels and the highest LDL cholesterol levels, while subjects carrying the apo E2 allele had the lowest LDL cholesterol levels and the highest levels of apo E. This was true for both men and women.

Conclusion # 3: Apolipoprotein A-I -75 bp genotype was observed to have a significant effect on levels of apo B, total and LDL cholesterol in women. Females carrying the rare A-allele had higher levels than women carrying the G/G genotype. The apo A-I +83 bp genotype was noted to have significant effects on total cholesterol and apo A-I levels in men, such that the presence of the rare M2- allele was associated with higher levels of apo A-I in the plasma than the more common M2+ allele. We also detected linkage disequilibrium between those two polymorphic gene loci.

Conclusion # 4: Apolipoprotein A-IV 360 genotype had no significant effect on any biochemical parameters, except for glucose levels in females. Women with the 2-allele had significantly higher fasting glucose levels compared to women with the 1/1 wildtype. Therefore, these data indicate some association of apolipoprotein A-IV genotype and glucose metabolism in females.

Conclusion # 5: Lipoprotein Lipase Hind III polymorphism was noted to have a significant effect on total and LDL cholesterol in females, and a significant effect on HDL cholesterol in males. In females, the presence of rare H- allele resulted in decreased levels of total and LDL cholesterol, especially when it was represented in the homozygous state. This was not observed in men. In

men, however, the presence of the rare H- allele was associated with an increase in HDL cholesterol levels.

Conclusion # 6: The effects of the lifestyle intervention program, i.e. restriction of total fat, saturated fat, and cholesterol, coupled with an exercise program in this residential Center, resulted in significant decreases in total cholesterol, LDL-C, HDL-C, triglycerides and body weight in both men and women over an eight day period. No significant effects of genotype were noted on the response of these variables, except that the presence of the apo E2 allele in men appeared to cause an enhanced glucose lowering, the presence of the apo A-IV 347 rare S-allele in the homozygous state resulted in excess in HDL cholesterol lowering in females, and the presence of the rare H-allele of lipoprotein lipase Hind III, either in the heterozygous or homozygous state, appeared to result in excess HDL cholesterol lowering in females. Moreover, when effects of this lifestyle intervention were examined comparing the eight day data with the fourteen day data, additional reductions were observed from the end of week one to the end of week two. By the end of week two, substantial greater reduction of HDL cholesterol was observed.

Conclusion # 7: This intervention does not appear to result in any lasting benefit when the subjects are in the free-living state and then return to the Center for another intervention program, because at their base line levels for men, the LDL cholesterol levels were in fact approximately 6% higher than at the first baseline for men, and virtually unchanged for women. These data are consistent with the concept that this intervention results in no lasting change, certainly for LDL cholesterol. A better intervention for people would be to incorporate lifestyle modification into the free-living state, possibly as part of a group approach, where people get regularly supported and monitored on a community basis.

Limitations

A major limitation of this study:

1. Taking blood samples at the eight day point is less than optimal. Our original plan was to have received samples from two or three week points, where response to the lifestyle intervention would have been greater. As part of this protocol, however, only samples were obtained at the eight day point for the vast majority of subjects, because patients were discharged prior to the blood drawing day on the two week program. This represents a significant limitation in our ability to determine whether the genotypes really had an effect on responsiveness.

2. This study was not just a change in dietary content, but also resulted in decreased caloric intake and body weight. In addition, there was an exercise component, so that this intervention was not a pure dietary modification under a controlled iso weight situation, which other studies have done in the past. On the other hand, the advantage of this approach is that we could look at large numbers of subjects, who all went through the exact same lifestyle modification program and all the food was provided by the Center.

3. Another concern is that a large number of subjects had medical conditions, which are normally excluded from studies. Even though we adjusted for all those factors in the analyses, they still may have affected our results. However, including these subjects may be a more realistic approach to study these effects in a middle-aged and elderly population.

4. The last concern is regarding our conclusion about long-term effects of this diet and lifestyle intervention. It is possible, that subjects who returned for a second intervention program may have been those individuals who were no longer complying with the program.

Summary

Aims of the Study

The first aim of this study was to investigate the effects of apolipoprotein (apo) E, A-I, A-IV, and lipoprotein lipase genotypes on various plasma apolipoprotein levels as well as on serum total, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, and glucose values in 734 middle-aged and elderly female and male subjects. The second aim was to study the effect of a short-term lifestyle intervention program, including diet and exercise, on glucose and lipid response as well as the results of a long term follow-up.

Methods

Polymerase chain reaction (PCR) and restriction isotyping were used to examine the presence of restriction fragment length polymorphisms (RFLP´s) at the various gene loci.

Results

The allele frequencies for all polymorphisms investigated were similar to those reported in other studies for Caucasians. After adjustment for age, body-mass index, medications, alcohol use, and smoking, apo E genotype was noted to have significant effects on apo E, total cholesterol, LDL cholesterol, and glucose levels in females, and on apo E, LDL cholesterol, and HDL cholesterol levels, as well as on the total cholesterol (TC)/ HDL ratio in males. Female and male subjects with the apo E4 allele had significantly ($p < 0.05$) lower plasma apo E (25 and 15%) and higher LDL cholesterol levels (5 and 2%), while those with the apo E2 allele had significantly ($p < 0.05$) higher apo E (32 and 27%) and lower LDL cholesterol levels (10 and 10%) than the apo E3/3 group. Moreover, female apo E4 carriers had significantly ($p < 0.05$) lower glucose values (11%) than the apo E3/3 group. With regard to response, we observed for the first time, that the apo E genotype had a significant effect on glucose levels in males, with those carrying E2 allele having a greater response (-10.6%) compared to apo E3/3 (+0.8%) men, and those carrying the E4+ (-3.7%) allele. These data are consistent with the concept that, in addition to the well known effects of apo E genotype on LDL-C values, this locus plays a significant role in modulating plasma apo E levels and plasma glucose response to behavioral intervention.

Concerning the apo A-I -75 bp polymorphism in the promoter region and +83 bp polymorphism in the first intron of the apo A-I gene, significant associations were found for the -75 bp mutation in females only. Women carrying the rare A-allele had significantly ($p < 0.05$) higher levels of apo B (5%), total (4%) and LDL cholesterol (6%), as well as a higher TC/HDL ratio (6%) compared to women carrying the G/G genotype. For the recently detected +83 bp mutation, we found a significant raising effect ($p = 0.002$) of the rare M2- allele on apo A-I in men, and a weaker raising

effect (p<0.05) on total cholesterol in men and women, respectively. Furthermore, a linkage disequilibrium (p=0.037) was found between the two polymorphisms. Males carrying the G/G M2+/- haplotype had the highest apo A-I levels (p=0.021) compared to all the other haplotypes. Therefore, these data indicate an association between the -75 bp allele and factors regulating LDL-C metabolism. However, no interactions between these apo A-I genotypes and HDL cholesterol levels, as well as lipid response to diet and exercise were observed.

Concerning the apo A-IV 360 and 347 polymorphisms, no significant effects on any lipid traits were noted in females and males. However, in females, a significant association (p=0.004) was observed with glucose levels, with women carrying the rare 2-allele having higher levels (11%) compared to the 1/1 genotype. In response to lifestyle intervention, females with the apo A-IV 347 S/S genotype had significantly greater reduction in HDL-C (16.7%) versus females homozygous for the common T-allele (8.6%). These results indicate that the apo A-IV 360 and 347 mutations have some effect on lipid metabolism in these subjects.

The lipoprotein lipase Hind III genotype was noted to have a significant effect (p<0.05) on total and LDL cholesterol in women and on HDL cholesterol levels in men. Women being homozygous for the rare H- allele had significantly lower levels of total and LDL cholesterol (4% and 4%) than women being H+/+ homozygotes. In men, those carrying the H- allele had significantly (p=0.003) higher HDL-C levels compared to the H+/+ genotype. However, with regard to the lifestyle intervention, females heterozygous for the H- allele had significantly greater reductions in HDL-C (-9.0%) compared to females homozygous for the H+ allele (-5.6%). Therefore, this data suggest that in females, the rare H- allele has a cholesterol lowering, and therefore potentially cardioprotective effect, which seems to be offset by HDL cholesterol lowering during diet and exercise intervention.

A long-term follow-up was carried out on a subset of 202 females and males who attended the program at two different occasions. The analysis of these data revealed no long lasting effect of the short-term intervention program. When subjects returned to the Pritikin center after a mean time span of 1.7 years, their baseline lipid levels at visit 2 were similar or even slightly exceeded the levels at baseline 1. These data shows that it is difficult for free-living subjects to implement and comply long-term with the intensive lifestyle changes carried out short term under carefully monitored conditions.

Conclusion

These results show that some of the candidate gene loci examined have a major impact on lipid and glucose levels, whereas others play a minor role. Concerning lipid lowering response to diet and exercise, the gene loci investigated were not significantly associated with individual variability in response in this particular experimental design. Our data shows that diet and exercise should remain the cornerstone for the reduction of CHD risk factors. The challenge is how to achieve long-term compliance to successful short-term intervention programs.

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Erklärung

Hiermit erkläre ich, Ilona Larson, dass ich die vorliegende Arbeit selbständig, lediglich mit den angegebenen Hilfsmitteln unter der Verwendung der angegebenen Literatur angefertigt habe.

Portland, September 1999