Circulation Research Peer Review Plus
Manuscript PDF

APOLIPOPROTEIN AIV GENE VARIANT S347 IS ASSOCIATED WITH INCREASED RISK OF

Submission Type: Original Contribution

APOLIPOPROTEIN AIV GENE VARIANT S347 IS ASSOCIATED WITH INCREASED RISK OF CORONARY HEART DISEASE AND LOWER APOLIPOPROTEIN AIV PLASMA CONCENTRATIONS

Wai-man R Wong[1*], Emma Hawe[1*], Lai K Li[1], George J Miller[2], Viviane Nicaud[3], Len A. Pennacchio[4], Steve E Humphries[1] and Philippa J Talmud[1]

[1]Division of Cardiovascular Genetics, Department of Medicine, British Heart Foundation Laboratories, Rayne Building, Royal Free and University College Medical School, 5 University St, London WC1E 6JJ, United Kingdom .

[2] MRC Cardiovascular Research Group, Wolfson Institute of Preventive Medicine, Charterhouse Square, London EC1M 6BQ.

[3] INSERM U525, Faculte de Medecine Pitie-Salpetriere, 91 Boulevard de l' Hopital, 75634 Paris cedex 13, FRANCE

[4] Genome Sciences Department, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

*These two authors contributed equally to the paper.

Corresponding author: Dr Philippa J Talmud,

Division of Cardiovascular Genetics, British Heart Foundation Laboratories, Department of Medicine, Rayne Building, Royal Free and University College Medical School, 5 University St, London WC1E 6JJ, United Kingdom

Tel: 44 – 20-7679-6968 Fax: 44-2-7679-6212. Email: p.talmud@ucl.ac.uk

Short title: *APOA4* T347S: CHD risk and apoAIV levels.

Subject codes: [135] Risk factors, [89] Genetics of cardiovascular disease, [90] Lipid and lipoprotein metabolism

Key words: apolipoprotein AIV, CHD risk, genetic polymorphism, haplotype analysis.

Details of authors

Waiman R Wong

Email: mun_jay@hotmail.com

Fax +44 207 679 6212 Tel: +44 207 679 6337

Emma Hawe

Email: rmhaemh@ucl.ac.uk

Fax +44 207 679 6212 Tel: +44 207 679 6964

Lai K Li

Email: rmhalkl@ucl.ac.uk

Fax +44 207 679 6212 Tel: +44 207 679 6977

George J Miller

Email: G.Miller@mds.qmw.ac.uk

Fax +44 207 882 6262 Tel: +44 207 882 6249

Viviane Nicaud

Email: viviane.nicaud@chups.jussieu.fr

Fax +33 1 40 77 97 28 Tel :+ 33 1 40 77 96 93

Len A. Pennacchio

Email: LAPennacchio@lbl.gov

Fax: (510) 486-4229 Tel: (510) 486-7498

Steve E Humphries

Email: rmhaseh@ucl.ac.uk

Fax +44 207 679 6212 Tel: +44 207 679 6962

Philippa J Talmud

Email:p.talmud@ucl.ac.uk

Fax +44 207 679 6212 Tel: +44 207 679 6968

ABSTRACT

The impact of common variants in the apolipoprotein gene cluster (*APOC3-A4-A5)* on prospective CHD risk was examined in healthy UK men. Of the 2808 men followed over nine years, 187 had a clinically defined CHD event. Examination of 9 single nucleotide polymorphisms (SNPs) in this group revealed that homozygotes for *APOA4* S347 had significantly increased risk of CHD [Hazard ratio (HR) of 2.07 (95%CI 1.04-4.12)] while men homozygous for *APOC3* 1100T were protected (HR 0.28 (95%CI 0.09-0.87)). In stepwise multiple regression analysis, after entering all the variants and adjusting for established risk factors *APOA4* T347S alone remained in the model. Using nine-SNP haplotype analysis, highest risk-estimate haplotypes carried *APOA4* S347 and rare alleles of the two flanking intergenic markers. The protective effect of *APOC3*1100T could be explained by negative linkage disequilibrium with these alleles. To determine the association of *APOA4* T347S with apoAIV levels, the relationship was examined in over 1600 healthy young European men and women. S347 homozygotes had significantly lower apoAIV plasma levels (13.48 $\pm$ 0.60mg/dl) compared to carriers of the T347 allele (14.85 $\pm$ 0.12 mg/dl) (p=0.025). These results demonstrate that genetic variation in and around *APOA4*, independent of effects of TG, is associated with risk of CHD and apoAIV levels, supporting an anti-atherogenic role for apoAIV.

Word count 211

Total word count 5163

INTRODUCTION

The relationship between raised plasma triglycerides (TG) and coronary heart disease (CHD) risk has been confirmed by meta-analysis [1], identifying TG as an independent CHD risk factor. Apolipoproteins (apo) play a central role in lipid metabolism and the cluster of apolipoprotein genes on chromosome 11q23 (*APOC3-A4-A5)* has been identified as a locus involved in TG determination, with variants in *APOC3* [2, 3] and *APOA5* [4-6]and less consistently *APOA4*[3], influencing TG levels[5]. ApoCIII levels strongly correlate with TG levels [7,8], suggesting a major role in the catabolism of TG rich lipoproteins (TGRL). Animal studies have identified that apoCIII acts as an inhibitor of the lipoprotein lipase-mediated hydrolysis of TGRL [9] and further perturbs TG metabolism by the displacement of apoE, the major ligand for TGRL clearance, from lipoprotein particles [10]. ApoAIV has been suggested to play a role in reverse cholesterol transport as an activator of lecithin cholesterol acyl transferase (LCAT) [11], and may influence lipid absorption and chylomicron assembly [12]. The exact role of apoAV is not known but apoAV levels are inversely correlated to TG levels and while *APOA5* transgenic mice have a 65% reduction in TG levels, *Apoav* knockout mice have 4 times higher TG levels than control litter-mates [4].

To ascertain whether these reported associations of variants in the gene cluster with TG levels were independent of each other or merely reflected the strong linkage disequilibrium (LD) across the region, a recent study examined 9 single nucleotide polymorphisms (SNPs) spanning the cluster (three in and around *APOA5*, two in the *APOA4* gene, and four in and flanking *APOC3)* using haplotype analysis in the Northwick Park Heart Study II (NPHSII), a prospective study of over 3000 healthy middle-aged UK men [5]. CHD risk was not examined at that time. Although in univariate analysis several SNPs were associated with differences in TG levels,

haplotype analysis identified that the determinant SNPs were *APOA5* S19W and *APOC3 –482C>T* and these effects were statistically independent. An association of the *APOA4* T347S rare allele with lower TG levels could be explained by LD with common alleles at those sites [5].

Additional studies have provided some evidence of an association between apoCIII levels and CHD risk. In both the Monitored Atherosclerosis Regression Study (MARS) and Cholesterol Lowering Atherosclerosis Study (CLAS) the ratio of apoCIII between TG-containing particles and HDL served as a risk predictor [13, 14]. Conversely apoAIV has been suggested to be risk-protective [15] while to date there is no information about the relationship of apoAV and CHD risk.

The purpose of the current study was to evaluate, in a prospective study of CHD in healthy middle-aged men, the effect of variants within the *APOC3-A4-A5* gene cluster on risk of CHD and to examine whether this could be explained by the genotype effects on TG levels [5]. Our finding that the major risk-associated gene was *APOA4* led us to examine *APOA4* genotypic effects on plasma levels of apoAIV, which were available in a study of young healthy men and women.

MATERIALS AND METHODS

Study populations

Northwick Park Heart study (NPHSII)

NPHSII is a large prospective study of healthy middle-aged (50-61 years) men drawn from 9 general medical practices throughout the UK. Of the initial cohort of 3052 men, 2808 DNA samples were available. The study has been ongoing for nine years and men were followed-up annually for lipid and clotting factor measures. Full details of anthropometric and biochemical measurements and other aspects of the study are well documented elsewhere[16-18]. CHD events taken as end-points were fatal (sudden or not) and non-fatal MI (n=134), based on WHO criteria [19], plus coronary artery surgery (n=33) and silent MI on the follow-up ECG (n=20) (in which case the time to event was assumed to have been mid-way between the baseline and follow-up records). Ethical approval was obtained from the USA National Institutes of Health, who partially funded the study, and from the local ethical committee in the UK.

European Atherosclerosis Research Study (EARS)

Male and female students aged 18-26 years were recruited from 14 universities from 11 European countries (Austria, Belgium, Denmark, Finland, France, Germany, Italy, Spain, Sweden, Switzerland and the United Kingdom) [20]. 'Cases', were defined as those whose fathers had documented acute myocardial infarction (MI) before the age of 55 years. Two age and sex matched controls were recruited by random selection from the same university population.A detailed description of lifestyle and quantitative trait measurements as well as additional protocols have been previously

described [20]. Serum apoAIV concentrations were measured by sandwich ELISA assay in Bruges [21].

DNA genotyping

Genotyping of the nine SNPs in this study have been previously described. This includes the *APOA5* S19W, -1131T>C and *APOA4-A5* intergenic SNPs [4,5]; *APOA4* Q360H and T347S [22] and *APOC3* SNPs -2845T>G, -482C>T, 1100C>T and 3238C>G [23].

Statistical analysis:

For NPHSII log-transformations were used for data which were not normally distributed (body mass index (BMI), apoB, systolic blood pressure and TG). One way analysis of variance was used to assess differences between the continuous baseline characteristics for those with and without CHD, using either the raw values or log transformed values, as appropriate and for categorical variables a chi-squared test. Survival analysis with respect to genotypes was carried out using Cox's proportional hazards model, thus allowing for varying follow-up intervals and censoring due to competing events. For this, 'failure time' was taken as the time to the first CHD event. The significance of the parameters in the Cox model was assessed using the Likelihood ratio (LR) Test. 95% Confidence Intervals (CI) for the estimates were calculated from the standard errors assuming a normal distribution. All results were exponentiated and are presented as hazard ratios (HR) with their corresponding 95% CI. All survival analyses were adjusted for age differences in the baseline hazard by practice were permitted. Stepwise multiple regression analysis was performed entering all the variants and correcting for established risk factors and then apoAI.

HDL levels could not be included since these were determined on plasma samples drawn at year 6 and therefore events prior to this would be ignored. Haplotypes were estimated using PHASE [24] and its use fully described [5]. The effects of haplotype on risk were compared by calculating the proportion of events for each haplotype.

For EARS allele frequencies were estimated by gene counting. Hardy-Weinberg (H-W) equilibrium was tested by chi-square analysis in subgroups of cases and controls from each region. The association of *APOA4 T347S* and apoAIV levels was analysed by ANOVA adjusted for case-control status, age, sex and region. A p-value of <0.05 was considered to be statistically significant.

RESULTS

To evaluate the effect of variants within the *APOC3-A4-A5* gene cluster on CHD risk, the association of the nine SNPs in the cluster with CHD risk was examined in the prospective NPHSII. The positions of these *APOC3-A4-A5* variants are presented in Figure 1a. Compared to men who remained free of CHD (n= 2621) men who had an event (n=187) were statistically significantly older, had a greater BMI and higher systolic and diastolic blood pressures, were more likely to be smokers and had higher TG, total and low density lipoprotein density lipoprotein (LDL)–cholesterol and apoB levels and lower high density lipoprotein (HDL)-C and apoAI levels (Table 1).

Of the three genes *APOA4* has the greatest effect on CHD risk in NPHSII

The risk of a coronary event was assessed as the hazard ratio (HR) for each of the variants (Table 2) and compared to a HR ratio of 1 for men homozygous for the common allele. Of the *APOC3* variants, 1100CT and TT showed a significant (protective) effect on risk [HR 0.65 (95%CI 0.56-0.92) and 0.28 (95%CI 0.09-0.87), respectively]. For *APOA4* only the 347SS showed a significant effect on risk (HR 2.04 (95%CI 1.02-4.05). Neither *APOA5* –1131T>C nor the S19W variants had a significant impact on risk, while the *APOA4-A5* intergenic T>C showed a borderline statistically significant effect on risk, with CC men having a HR 1.59 (95%CI 0.99-2.56). Using stepwise regression analysis, after adjustment for age, cholesterol, TG and stratification by medical practice, *APOA4* T347S alone remained in the model. Compared to TT homozygotes, TS heterozygotes had a HR of 1.2 (95%CI 0.93-1.79) and SS homozygotes had a HR of 2.07 (95%CI 1.04-4.12) demonstrating a codominant effect on CHD risk independent of TG level. To test if this effect was modulated by apoAI levels, apoAI was added to the regression model. The hazard ratio remained essentially the same (SS men: HR 2.08 (95% 0.96-4.5); TS men HR

1.2 (95%CI 0.82- 1.67) but was no longer statistically significant presumably because of loss of power as apoAI levels were only available for 1911 men with T347S genotype.

APOA4 S347 carriers have reduced survival rates

Considering the HRs, Q360H showed no statistically significant effect on risk, although men homozygous for the H360 allele had a HR of 3.27 (95%CI 0.45, 23.69). Since there is strong negative allelic association (D'-0. 91 ($p<0.0005$) between the two *APOA4* variants [5], we determined whether the survival rate of T347S was independent of Q360H by examining only those men homozygous for the Q360 (Figure 2). This clearly shows the lower survival rate in men homozygous for the S347 allele (HR 2.08 (95%CI 1.04, 4.18) compared to men homozygous for T347 with heterozygous men showing intermediate survival (HR 1.40 (95%CI 0.98, 2.02) (p-value overall =0.05).

APOA4 S347-carrying haplotypes and high CHD event rates.

To assess the overall effect of the 9 variants on risk, haplotype association with risk was examined. Nineteen haplotypes which occurred in more than 10 individuals with at least 1 recorded CHD events, representing 88% of the sample, were studied. The proportion of CHD events for each haplotype was calculated and ranked according to the proportion of risk (Figure 1b). A comparison was made with the TG-associated haplotypes ranked in the same order (Figure 1c) [5]. Of the five high-risk haplotypes, haplotypes 1, 3 and 5 (representing 17.5% of the sample) all carry the *APOA4* S347 in combination with the intergenic *APOA4-A5* C and/or *APOC3* -2845G and/or *APOC3* –482T alleles. Haplotype 2 and 4 (only found in 0.7% and 0.8% of the sample,

respectively) were defined by *APOC3*-2845G and *APOA5* W19, respectively, on the wildtype background.

It is clear that the ranking by proportion of events and by TG did not correspond (Figure 1). Haplotypes 1, 3 and 5 were associated with TGs below or around the sample mean of 1.80mmol/L (1.71mmol/L, 1.79mmol/L and 1.82mmol/L, respectively) while haplotypes 2 and 4, defined by *APOC3* –2854G and *APOA5* W19, respectively, were associated with TG levels of 1.67mmol/L and 2.16mmol/L, respectively (Figure 1c). The wildtype haplotype (haplotype 6), representing 36% of the sample, was associated with an event rate of 8.1%, significantly higher than the mean event rate of 6.7% (p=0.04) (Figure 1b), yet men who carried this haplotype had mean TG levels of 1.75mmol/L, i.e. below the sample mean. Since each individual will have two haplotypes, if one was wildtype, the second could be risk-raising, risk-lowering or risk-neutral. Thus the overall risk-effect associated with the wildtype haplotype would depend on the haplotype frequencies of these other haplotypes. To analyse this in more detail, we estimated the risk associated with the 2 common risk-raising haplotypes 1 and 3, characterised by *APOA4* S347, intergenic *APOA4/A5* C and/or *APOC3* –2845G, considering only those men who had, in addition, a wildtype haplotype (haplotype 6), and compared this to the risk of all other haplotypes combined with the wildtype haplotype (Table 3). The proportion of events for men carrying haplotypes 1/6 and 3/6 was 14.3% compared to 7.2% for the haplotype 6/all other haplotypes pooled (p=0.02). Thus this analysis provided information on the major risk-raising haplotypes and confirmed that the haplotype effects on risk were not acting through effects on TG.

***APOA4* T347S is associated with plasma apoAIV levels in EARS**

To determine the relationship between *APOA4* T347S and apoAIV levels, we examined subjects in EARS, since plasma apoAIV levels were not available for NPHSII. EARS is a multicentre study recruited from 5 regions of Europe. The frequencies of the T347s in EARS within the different regions is shown in the Appendix (Table 1). All genotype distributions were in H-W equilibrium. There was significant evidence for allele frequency heterogeneity amongst regions (p=0.04) with the 'middle region' having the highest S347 allele frequency (see appendix). There was no significant heterogeneity of frequency of T347S between cases and controls, thus for all subsequent analyses cases and controls were considered together. Considering the effect of genotype on apoAIV levels, there was no significant heterogeneity of the genotype effect between cases and controls, across regions or between sexes, therefore the effect of T347S on apoAIV levels is presented in the sample as a whole (Figure 3). Individuals homozygous for the S347 had significantly lower apoAIV plasma levels (13.48 $\pm$ 0.60 mg/dl) than those carrying the T374 allele (14.85 $\pm$ 0.12 mg/dl) (p=0.025), which when further adjusted for possible confounders (BMI and physical activity) remained significant (p=0.019). There was no statistically significant effect of this genotype on any lipid variable, BMI or WHR (data not shown).

DISCUSSION

This study shows that of the nine SNPs within the *APOC3-A4-A5* cluster on chromosome 11p23, the *APOA4* T347S alone was associated with a significant and independent effect on risk of CHD in healthy UK men. Men homozygous for the S347 had a 2-fold risk compared to T347 homozygotes. Although in univariate analysis there was significant evidence that the *APOC3* 1100C>T was risk-protective, considering the simultaneous effects of all the variants *APOA4* T347S alone remained statistically significant and independent of established risk factors such as BMI, smoking, blood pressure, age, cholesterol, TG and apoAI levels. While the multiple regression analysis and the Kaplan-Meier plot provided statistical examination of the results, haplotype analysis suggested possible genetic interpretations.

We previously considered the effect of the *APOC3-A4-A5* gene cluster on plasma TG using haplotype analysis derived from these 9 SNPs [5]. In the current analysis, the association of these same haplotypes with risk of CHD events was examined. Comparing the ranking of the haplotypes it was clear that those associated with the greatest CHD risk were not the same as those associated with the highest TG levels. Three haplotypes, representing 17% of the study sample, associated with amongst the highest proportion of events (11.9%, 9.5% and 9.1%, respectively), were defined by *APOA4* S347 in combination with flanking markers, *APOC3* –2854G in the intergenic region between *APOA4* -*APOC3* and/or the intergenic *APOA4-APOA5*C. These SNPs are in tight positive LD [5] and this strongly implicates *APOA4* as a gene involved in CHD risk determination.

These results raise three questions. Firstly, in view of the effects of *APOA5* S19W and *APOC3* –482C>T as the two main determinants of TG in the cluster [5], what is their effect on risk. Secondly, does the *APOA4* S347 association with risk explain the well documented *APOC3* SstI (3238C>G) association with risk and finally, and what is the mechanism of the effect of *APOA4* T347S on risk?

APOA5 W19 homozygotes have 52% higher TG levels than S19 homozygotes [5]and the *APOA5* W19-carrying haplotype, on a wildtype background ranked as the 4th highest risk-associated haplotype. However, in univariate analysis W19S did not have a statistically significant effect on risk but only 21 men carried this haplotype. The *APOC3* –482C>T rare allele showed a TG-raising effect when interaction with smoking was considered and compared to non-smoking CC homozygotes, -482T homozygous men who smoked had 28% higher TG levels [23] but –482C>T showed no statistically significant effect on risk in the univariate analysis. *APOC3* –482T-carrying haplotypes, for the most part, were associated with low risk and the study did not have the power to consider the effect of smoking on CHD risk in conjunction with genotype. Thus, at the population level both *APOA5* S19 and *APOC3* –482T are acting as polygenic determinants of TG, with environmental interactions, and while the *APOA5* S19 variant may have a small effect on risk, *APOC3* –482T does not appear to be a risk-determining allele.

Considering the relationship to *APOC3* SstI (3238C>G) and risk, the *APOC3* SstI variant has frequently been associated with raised TG, and in case-control studies with increased risk by frequency comparisons [25]. However there has been no meta-analysis to provide a robust risk estimate. In NPHSII *APOC3* 3238G had a highly

statistically significant effect on TG in univariate analysis, but in multivariate analysis this effect on TG could be explained by the strong positive LD with *APOC3* –482T [23]. Considering the univariate risk analysis *APOC3* 3238C>G had no significant effect and 3238G-carrying haplotypes were associated with lower risk. Our results suggest that effects of *APOC3* 3238C>G on TG and risk are the result of strong LD with the other SNPs in *APOC3* and the cluster and do not support a high CHD risk-association with this site.

Finally, an anti-atherogenic role for apoAIV has been suggested by two CHD case-control studies, showing that apoAIV levels were significantly lower in CHD cases compared to controls [15]. Mice over expressing apoAIV support this hypothesis since *Apoe-/-* mice transgenic for *APOA4* in the liver [26] or mouse *Apoa4* [27] showed protection from diet-induced atherosclerotic lesions. To investigate whether this *APOA4* T347S effect on CHD risk may be related to genotypic effects on plasma apoAIV levels, we examined the association in the EARS where apoAIV concentrations were available. We did not see a significant 'case-control' difference in apoAIV levels but since these are all healthy young subjects, and EARS is an offspring study, any risk effect in the parents would have been diluted in the offspring [28]. We did, however, find a statistically significant effect of T347S on plasma apoAIV levels in the group, S347 homozygosity being associated with a statistically significantly lower plasma apoAIV level compared to T347 homozygosity. The effect of apoAIV-1 (Q360) and -2 (H360) phenotypes on apoAIV levels had been examined in EARS, with no statistically significant association with apoAIV levels [29].

Taken together, the association of S347 with increased CHD risk and lower apoAIV levels suggest that variation in *APOA4* may be affecting risk *directly*. Whether this is due to the amino acid change at 347 is unclear. T347 is conserved in higher mammals and the residue is located at the end of a stretch of 13 amino acids in a helical confirmation in the C-terminus. The hydrophilicity profile of the S347 variant suggests it may have marginally greater hydrophilicity compared to T347 resulting in a reduced affinity for phospholipid surfaces, and structural predictions suggest that S347 could lengthen the adjacent coil region and decrease the propensity for β-sheet. [30]. However apoAIV-S347 has not been characterised and these predictions still remain to be confirmed. Haplotye analysis, however, suggests that S347 might not be functional. Haplotype 19, which carries S347 on a wildtype background, is associated with low risk, but this haplotype is rare and the estimate of risk is not robust. In addition to S347 the three high risk-associated haplotypes all carry the *APOA4* flanking markers, suggesting that the functional variant, with or without an effect of T347S, may be altering the level of expression of the cluster. Enhancers of both liver and intestinal expression which co-regulate *APOAI-C3-A4* have been mapped to this intergenic region [31] and position -2845 is very close to an enhancing element which is responsive to the nuclear factor HNF4 (mapped between –2893 and -2920). Thus altered levels of expression due to this variant site could result in altered levels of apoAIV. Since the *APOA4* genotypic effect on risk was independent of effects on lipids, it could result from the potential of apoAIV to act as an antioxidant. ApoAIV has been shown to have antioxidant activity *in vitro* [32] and *Apoe* deficient mice, over expressing *APOA4*, have reduced oxidative markers [33].

Support for a differential role of *APOA1, C3, A4* and *A5* on TG levels and risk comes from *APOA1-C3-A4* transgenic mice. These mice developed severe hypertriglyceridemia which correlated to *APOC3* overexpression, but when crossed with the *Apoe-/-* mouse, they showed a 61% reduction in atherogenesis [34] which appeared to be due to the overexpression of apoAI and/or apoAIV. In NPHSII the relative risk of *APOA4* S347 was not reduced by adjustment for apoAI, supporting an effect of this allele on risk, independent of any effects of apoAI levels. Thus our data support a multifunctional role for the *APOC3-A4-A5* cluster with *APOC3* and *APOA5* affecting TG levels, and an anti-atherogenic role for apoAIV that is independent of effects on lipids. This suggests a potential therapeutic role for apoAIV in CHD.

ACKNOWLEDGEMENTS

On behalf of the EARS Group.WRW, EH, LKL, SEH and PJT are all supported by the British Heart Foundation (RG2000/ 015). EARS I was funded by European Community concerted Action MRH4 COMAC Epidemiology. NPHSII was supported by the British Medical Research Council, the USA National Institute of Health (grant NHLBI 33014) and Du Pont Pharma, Wilmington, USA. The following general practices collaborated in the study: The Surgery, Aston Clinton; Upper Gordon Road, Camberley; The Health Centre, Carnoustie; Whittington Moor Surgery, Chesterfield; The Market Place Surgery, Halesworth; The Health Centre, Harefield; Potterells Medical Centre, North Mymms; Rosemary Medical Centre, Parkstone, Poole; The Health Centre, St Andrews. Mortality data in NPHSII were supplied by the office of National Statistics.

FIGURE LEGENDS

Figure 1

a) Map of the *APOC3-A4-A5* gene cluster showing the position of the 9 variants studied.

b) Ranking of the haplotypes derived from the 9 SNPs according to the proportion of events given. The number of events to non-events appears above each bar. The mean proportion of events for the sample is represented by a horizontal line. The common alleles are shaded.

c) Triglyceride levels associated with the haplotypes derived from the 9 SNPs, ranked in the same order as the proportion of events. The number of men with each haplotype is given above. The mean TG level for the sample is represented by a horizontal line.

Figure 2

Survival functions of CHD events in NPHSII by *APOA4* T347S genotype.

Graph of the estimated survivor functions from the Cox proportional hazard model stratified by T347S genotype in those men who were homozygous for the Q360 allele.

Figure 3 Mean apoAIV levels according to *APOA4* T347S genotype in EARS.

Mean values and SEM are given for TT (n=1058), TS (n=501) and SS (n=52).

Reference

1. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk.*1996;3:213-219.

2. Waterworth DM, Ribalta J, Nicaud V, Dallongeville J, Humphries SE, Talmud P. ApoCIII gene variants modulate postprandial response to both glucose and fat tolerance tests. *Circulation.*1999;99:1872-1877.

3. Groenendijk M, Cantor RM, de Bruin TW, Dallinga-Thie GM. The apoAI-CIII-AIV gene cluster. *Atherosclerosis.*2001;157:1-11.

4. Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox DR, Fruchart JC, Krauss RM, Rubin EM. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. *Science.*2001;294:169-173.

5. Talmud PJ, Hawe E, Martin S, Olivier M, Miller GJ, Rubin EM, Pennacchio LA, Humphries SE. Relative contribution of variation within the APOC3-A4-A5 gene cluster in determining plasma triglycerides. *Hum Mol Gen.*2002;11:3039-3046.

6. Pennacchio LA, Olivier M, Hubacek J, Krauss RM, Rubin EM, Cohen JC. Two Independent Apolipoprotein A5 Haplotypes Influence Human Plasma Triglyceride Levels. *Hum Mol Gen.*2002;11:3031-3038.

7. Shoulders CC, Harry PJ, Lagrost L, White SE, Shah NF, North JD, Gilligan M, Gambert P, Ball MJ. Variation at the apo AI/CIII/AIV gene complex is

associated with elevated plasma levels of apo CIII. *Atherosclerosis.*1991;87:239-247.

8. Bainton D, Miller NE, Bolton CH, Yarnell JW, Sweetnam PM, Baker IA, Lewis B, Elwood PC. Plasma triglyceride and high density lipoprotein cholesterol as predictors of ischaemic heart disease in British men: the Caerphilly and Speedwell Collaborative Heart Disease Studies. *Br Heart J.*1992;68:60-66.

9. Shachter NS, Hayek T, Leff T, Smith JD, Rosenberg DW, Walsh A, Ramakrishnan R, Goldberg IJ, Ginsberg HN, Breslow JL. Overexpression of apolipoprotein CII causes hypertriglyceridemia in transgenic mice. *J Clin Invest.*1994;93:1683-1690.

10. Aalto Setala K, Fisher EA, Chen X, Chajek Shaul T, Hayek T, Zechner R, Walsh A, Ramakrishnan R, Ginsberg HN, Breslow JL. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. *J Clin Invest.*1992;90:1889-1900.

11. Steinmetz A, Utermann G. Activation of lecithin cholesterol acyltransferase by human apolipoprotein A-IV. *J Biol Chem.*19852258-2264.

12. Weinberg RB. Apolipoprotein A-IV polymorphisms and diet-gene interactions. *Curr Opin Lipidol.*2002;13:125-134.

13. Hodis HN, Mack WJ. Triglyceride-rich lipoproteins and the progression of coronary artery disease. *Curr Opin Lipidol.*1995;6:209-214.

14. Blankenhorn DH, Alaupovic P, Wickham E, Chin HP, Azen SP. Prediction of angiographic change in native human coronary arteries and aortocoronary bypass grafts. Lipid and nonlipid factors. *Circulation.*1990;81:470-476.

15. Kronenberg F, Stuhlinger M, Trenkwalder E, Geethanjali FS, Pachinger O, von Eckardstein A, Dieplinger H. Low apolipoprotein A-IV plasma concentrations in men with coronary artery disease. *J Am Coll Cardiol.*2000;36:751-757.

16. Miller GJ, Bauer KA, Barzegar S, Cooper JA, Rosenberg RD. Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease. *Thromb Haemost.*1996;75:767-771.

17. Humphries SE, Talmud PJ, Hawe E, Bolla M, Day IN, Miller GJ. Apolipoprotein E4 and coronary heart disease in middle-aged men who smoke: a prospective study. *Lancet.*2001;358:115-119.

18. Talmud PJ, .Hawe E, Robertson K, Miller GJ, .Miller NE, Humphries SE. Genetic and environmental determinants of plasma high density lipoprotein cholesterol and apolipoprotein AI concentrations in healthy middle-aged men. *Ann Hum Genet.*2002;66:111-124.

19.World Health Organization Regional Office for Europe and Myocardial infarction Community register. Public Health in Europe. 5. 1976. Copenhagen, WHO.

20. The EARS group. The European Atherosclerosis Research Study (EARS): design and objectives. *Int J Epidemiol.*1994;23:465-471.

21. Rosseneu M, Michiels G, de Keersgieter W, Bury J, De Slypere JP, Dieplinger H, Utermann G. Quantification of human apolipoprotein A-IV by "sandwich"-type enzyme- linked immunosorbent assay. *Clin Chem.*1988;34:739-743.

22. Fisher RM, Burke H, Nicaud V, Ehnholm C, Humphries SE, on behalf of the EARS Group. Effect of variation in the apo A-IV gene on body mass index and fasting and postprandial lipids in the European Atherosclerosis Research Study II. *J Lipid Res.*1999;40:287-294.

23. Waterworth DM, Talmud PJ, Bujac SR, Fisher RM, Miller GJ, Humphries SE. The contribution of apoCIII gene variants to the determination of triglyceride levels and interaction with smoking in middle-aged men. *Arterioscler Thromb Vasc Biol.*2000;20:2663-2669.

24. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet.*2001;68:978-989.

25. Talmud PJ, Humphries SE. Apolipoprotein C-III gene variation and dyslipidaemia. *Curr Opin Lipidol.*1997;8:154-158.

26. Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, Steinmetz A, Denefle P. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science.*1996;273:966-968.

27. Cohen RD, Castellani LW, Qiao JH, Van Lenten BJ, Lusis AJ, Reue K. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein A-IV. *J Clin Invest.*1997;99:1906-1916.

28. Tiret L, Nicaud V, Ehnholm C, Havekes L, Menzel HJ, Ducimetiere P, Cambien F. Inference of the strength of genotype-disease association from studies comparing offspring with and without parental history of disease. *Ann Hum Genet.*1993;57 (Pt 2):141-149.

29. Ehnholm C, Tenkanen H, de Knijff P, Havekes L, Rosseneu M, Menzel HJ, Tiret L. Genetic polymorphism of apolipoprotein A-IV in five different regions of Europe. Relations to plasma lipoproteins and to history of myocardial infarction: the EARS study. European Atherosclerosis Research Study. *Atherosclerosis.*1994;107:229-238.

30. Lohse P, Kindt MR, Rader DJ, Brewer HB, Jr. Three genetic variants of human plasma apolipoprotein A-IV. apoA-IV- 1(Thr347----Ser), apoA-IV-0(Lys167----Glu,Gln360----His), and apoA-IV- 3(Glu165----Lys). *J Biol Chem.*1991;266:13513-13518.

31. Vergnes L, Taniguchi T, Omori K, Zakin MM, Ochoa A. The apolipoprotein A-I/C-III/A-IV gene cluster: ApoC-III and ApoA-IV expression is regulated by two common enhancers. *Biochim Biophys Acta.*1997;1348:299-310.

32. Qin X, Swertfeger DK, Zheng S, Hui DY, Tso P. Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. *Am J Physiol.*1998;274:H1836-H1840.

33. Ostos MA, Conconi M, Vergnes L, Baroukh N, Ribalta J, Girona J, Caillaud JM, Ochoa A, Zakin MM. Antioxidative and antiatherosclerotic effects of human apolipoprotein A- IV in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.*2001;21:1023-1028.

34. Vergnes L, Baroukh N, Ostos MA, Castro G, Duverger N, Nanjee MN, Najib J, Fruchart JC, Miller NE, Zakin MM, Ochoa A. Expression of human apolipoprotein A-I/C-III/A-IV gene cluster in mice induces hyperlipidemia but reduces atherogenesis. *Arterioscler Thromb Vasc Biol.*2000;20:2267-2274.

35. Walldius G, Jungner I, Holme I, Aastveit AH, Kolar W, Steiner E. High apolipoprotein B, low apolipoprotein A-I, and improvement in the prediction of fatal myocardial infarction (AMORIS study): a prospective study. *Lancet.*2001;358:2026-2033.

Table 1 The mean baseline characteristics and (SD) of the men in NPHSII considering those genotyped for variants in the cluster who had a CHD event or not.

	No CHD event	CHD event	
Trait	n=2621	n=187	p-value
Age (years)	56.01 (3.42)	56.67 (3.62)	0.01
Body mass index (kg/m^2)*	26.19 (3.37)	26.96 (3.42)	<0.0005
Current Smoking[3]	27.43%	38.50%	0.001
Diastolic blood pressure (mmHg) *	83.58 (11.21)	86.69 (11.95)	<0.0005
Systolic blood pressure (mmHg) *	136.64 (18.60)	142.65 (20.16)	<0.00005
Cholesterol (mmol/L)	5.71 (1.01)	6.10 (1.04)	<0.00005
Triglyceride (mmol/L) †	1.78 (0.93)	2.11 (1.14)	<0.00005
ApoB (mg/dl) †	0.86 (0.24)	0.92 (0.23)	0.0007
ApoAI mg/dl)	1.64 (0.32)	1.58 (0.27)	0.02
LDL-C (mmol/L) ‡	3.06 (1.01)	3.41 (0.97)	<0.00005
HDL-C (mmol/L) ‡	1.71 (0.61)	1.53 (0.56)	<0.0005

* geometric means presented with approximate standard deviations

† given are the anti log of the log transformed mean and standard deviations are approximated

‡ Calculated according to [35].

Table 2. Univariate hazard ratios (187 coronary events out of 2808 individuals) for the 9 *APOC3-A4-A5* variants.

All compared to common allele homozygotes with a Hazard Ratio set at 1(unless otherwise stated).

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

*Adjusted for age and practice

†Adjusted for age and practice and triglyceride levels

Table 3 Proportion of events associated with haplotypes in those individuals who carried the wildtype haplotype.

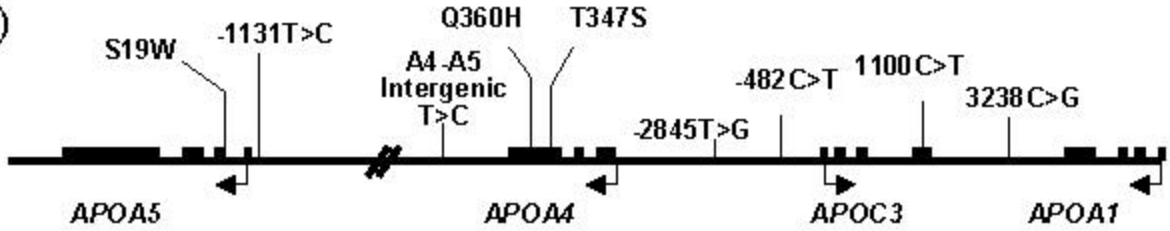
Comparing of haplotypes 1 and 3 (defined by a combination of the rare alleles of *APOA4* S347, *APOA4-A5* intergenic C and *APOC3* –2845G) to all other haplotypes.

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

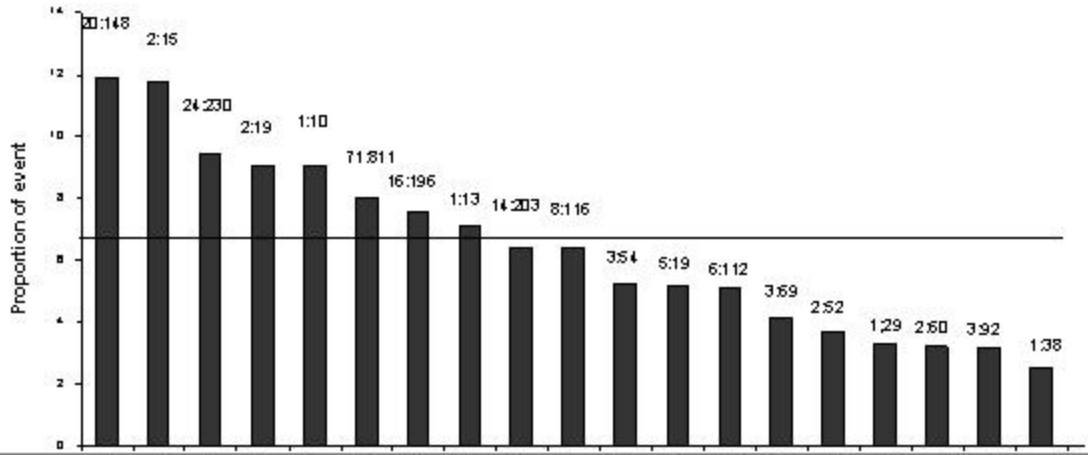
Difference between 2 haplotype groups p=0.02

Figure 1

a)



b)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
APOA5 S19W	S	S	S	W	S	S	S	S	S	S	S	S	W	S	S	S	S	S	S
APOA5 -1131T>C	T	T	T	T	T	T	T	C	T	T	T	C	T	T	T	T	T	T	T
A4-A5 T>C	C	T	C	T	T	T	C	T	T	T	C	T	T	T	C	C	T	C	T
APOA4 Q360H	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
APOA4 T347S	S	T	S	T	S	T	T	T	T	T	T	T	T	T	T	T	T	T	S
APOC3 -2845 T>G	G	G	G	T	G	T	T	T	T	T	G	G	T	G	T	T	G	G	T
APOC3 -482C>T	C	C	T	C	T	C	C	C	C	T	T	T	C	T	C	T	T	C	C
APOC3 1100C>T	C	C	C	C	C	C	C	C	C	T	C	T	T	T	T	C	T	C	C
APOC3 3238C>G	C	C	C	C	C	C	C	C	C	C	C	G	C	C	C	C	G	C	C

c)

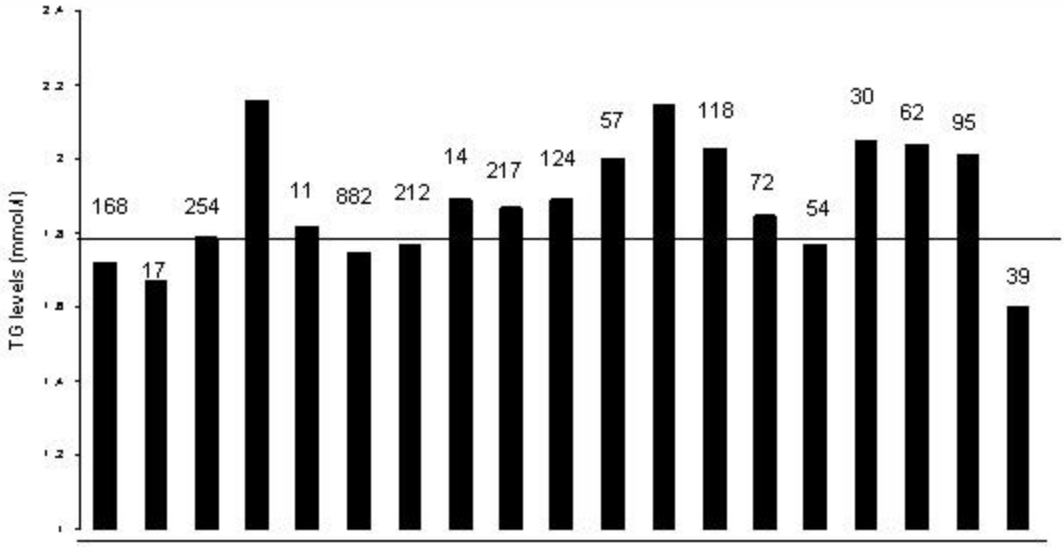
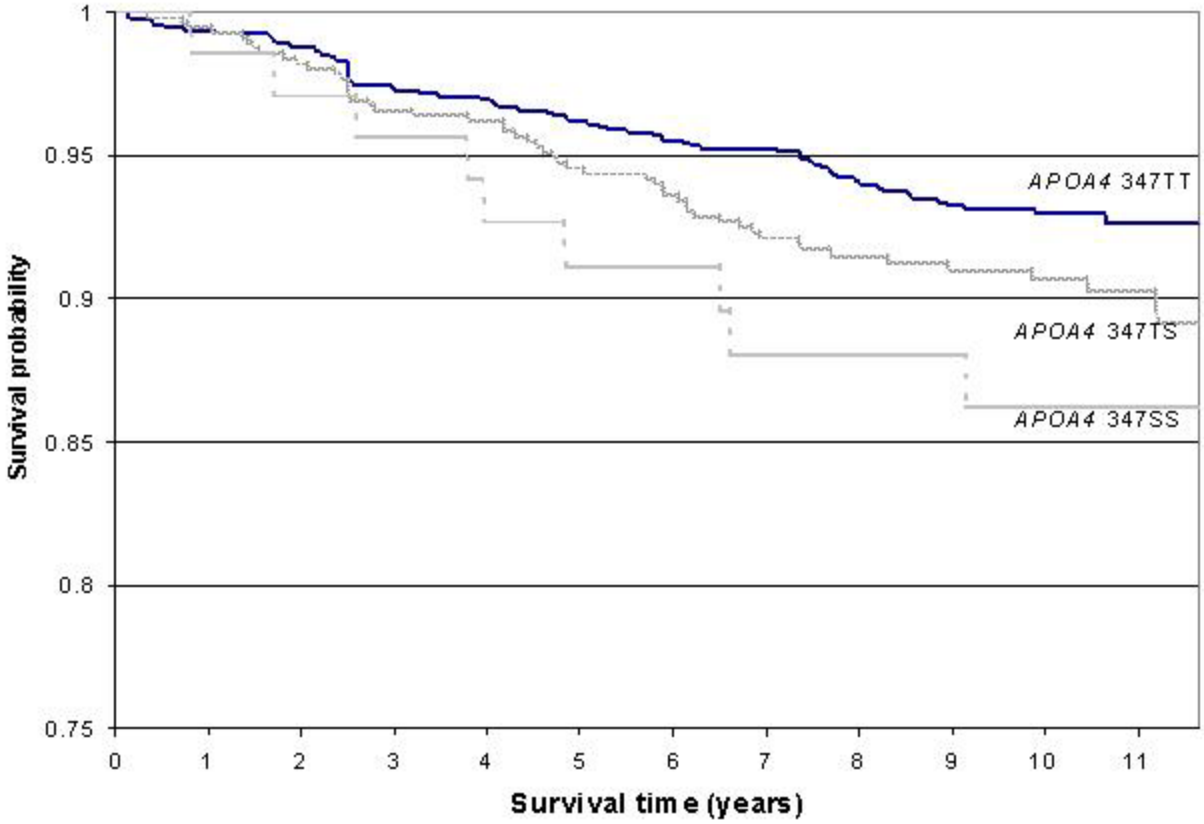


Figure 2


1
0.95
0.9
0.85
0.8
0.75
Survival probability
0
1
2
3
4
5
6
7
8
9
10
11
Survival time (years)
APOA4 347TT
APOA4 347TS
APOA4 347SS

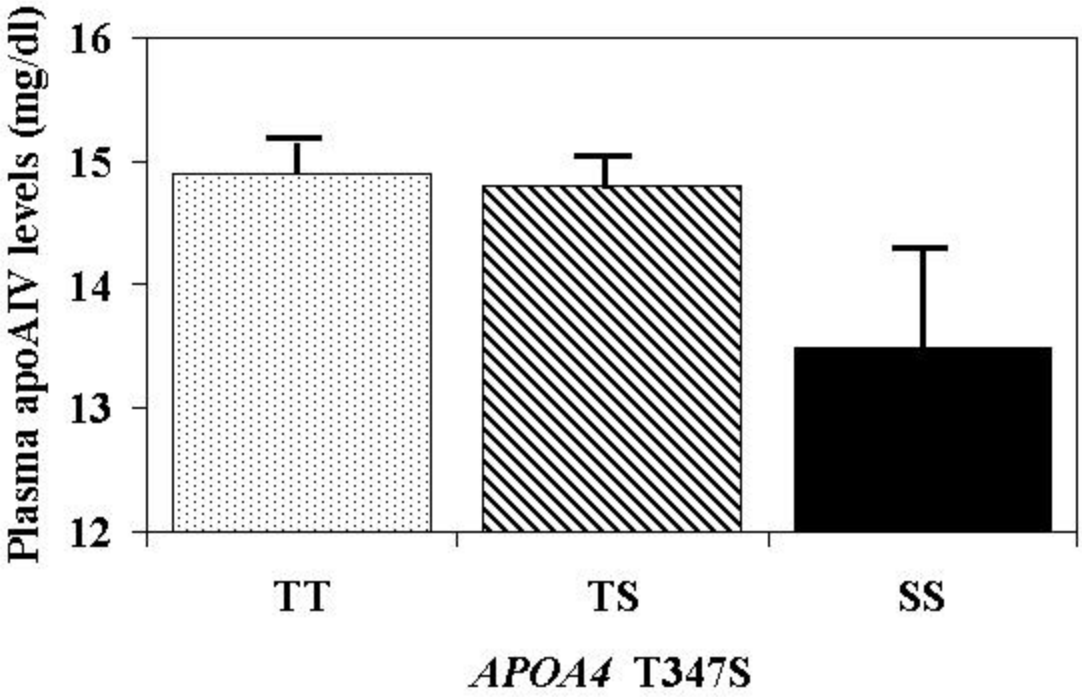



Figure 3