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## Apolipoprotein AIV Gene Variant S347 Is Associated With Increased Risk of Coronary Heart Disease and Lower Plasma Apolipoprotein AIV Levels

Wai-man R. Wong,\* Emma Hawe,\* Lai K. Li, George J. Miller, Viviane Nicaud, Len A. Pennacchio, Steve E. Humphries, Philippa J. Talmud

**Abstract**—The impact of common variants in the apolipoprotein gene cluster (*APOC3-A4-A5*) on prospective coronary heart disease (CHD) risk was examined in healthy UK men. Of the 2808 men followed over 9 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 to 4.12)], whereas men homozygous for *APOC3* 1100T were protected [HR 0.28 (95% CI 0.09 to 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 all nine SNPs, the 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 1600 healthy young European men and women. S347 homozygotes had significantly lower apoAIV plasma levels ( $13.64 \pm 0.59$  mg/dL) compared with carriers of the T347 allele ( $14.90 \pm 0.12$  mg/dL) ( $P=0.035$ ). These results demonstrate that genetic variation in and around *APOA4*, independent of the effects of triglyceride, is associated with risk of CHD and apoAIV levels, supporting an antiatherogenic role for apoAIV. (*Circ Res.* 2003;92:969-975.)

**Key Words:** apolipoprotein AIV ■ coronary heart disease risk ■ genetic polymorphism ■ haplotype analysis

The relationship between raised plasma triglycerides (TGs) and coronary heart disease (CHD) risk has been confirmed by meta-analysis,<sup>1</sup> 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 TG-determination locus, with variants in *APOC3*<sup>2,3</sup> and *APOA5*,<sup>4,5</sup> and less consistently *APOA4*,<sup>3</sup> influencing TG levels.<sup>6</sup> ApoCIII levels strongly correlate with TG levels,<sup>7,8</sup> 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<sup>9</sup> and further perturbs TG metabolism by the displacement of apoE, the major ligand for TGRL clearance, from lipoprotein particles.<sup>10</sup> ApoAIV has been suggested to play a role in reverse cholesterol transport as an activator of lecithin cholesterol acyl transferase (LCAT)<sup>11</sup> and may influence lipid absorption and chylomicron assembly.<sup>12</sup> The exact role of apoAV is not known but apoAV levels are inversely correlated to TG levels, and

whereas *APOA5* transgenic mice have a 65% reduction in TG levels, *Apoa5* knockout mice have 4 times higher TG levels than control littermates.<sup>4</sup>

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.<sup>6</sup> 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* to 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.<sup>6</sup>

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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.<sup>13,14</sup> Conversely, apoAIV has been suggested to be risk-protective,<sup>15</sup> whereas to date there is no information about the relationship of apoAV and CHD risk.

The purpose of the present 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.<sup>6</sup> 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 to 61 years) men drawn from 9 general medical practices (family doctors) throughout the UK. Of the initial cohort of 3052 men, 2808 DNA samples were available. The study has been ongoing for 9 years, and men 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.<sup>16–18</sup> Lipid measures were taken nonfasting, the exact instruction for clinic attendance and the possible implications of nonfasting measures are presented elsewhere.<sup>6</sup> CHD events taken as end-points were fatal (sudden or not) and nonfatal MI (n=134), based on WHO criteria,<sup>19</sup> plus coronary artery surgery (n=33) and silent MI on the follow-up ECG (n=20) (in which case the time to event was assumed to have been midway between the baseline and follow-up records). Ethical approval was obtained from the USA National Institutes of Health, who partly funded the study, and from the local ethical committee in the UK.

#### *European Atherosclerosis Research Study (EARS)*

Male and female students aged 18 to 26 years were recruited from 14 universities from 11 European countries (Austria, Belgium, Denmark, Finland, France, Germany, Italy, Spain, Sweden, Switzerland, and the United Kingdom).<sup>20</sup> "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.<sup>20</sup> Serum apoAIV concentrations were measured by sandwich ELISA assay in Bruges.<sup>21</sup>

### 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<sup>4,6</sup>; *APOA4* Q360H and T347S<sup>22</sup>; and *APOC3* SNPs -2845T>G, -482C>T, 1100C>T, and 3238C>G.<sup>23</sup>

### Statistical Analysis

For NPHSII, log-transformations were used for data that 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 performed 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. Ninety-five percent 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 (HRs) with their corresponding 95% CI. All survival analyses were adjusted for age and general medical practice. 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 because these were determined on plasma samples drawn at year 6, and therefore, events before this would be ignored. Haplotypes were estimated using PHASE<sup>24</sup> and its use fully described in Talmud et al.<sup>6</sup> The effects of haplotype on risk were compared by calculating the proportion of events for each haplotype.

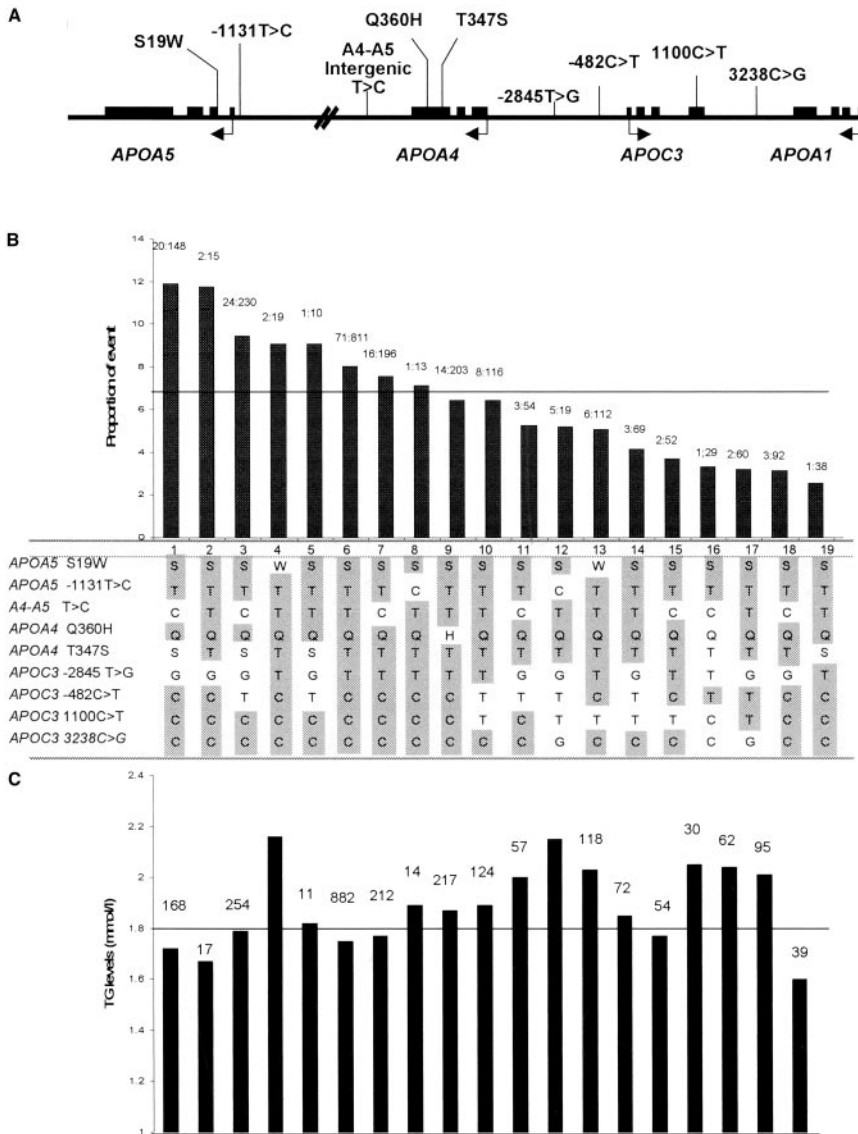
For EARS, allele frequencies were estimated by gene counting. Hardy-Weinberg (H-W) equilibrium was tested by chi-square analysis in subgroups of cases and controls from each region. The association of *APOA4* T347S and apoAIV levels was analyzed by ANOVA adjusted for case-control status, age, sex and region, BMI, physical activity, TG, HDL, and contraception. A value of  $P<0.05$  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 with 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 in the Cluster Studied, *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 with 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 to 0.92) and 0.28 (95%CI 0.09 to 0.87), respectively]. For *APOA4*, only homozygosity for the 347S allele showed a significant effect on risk [HR 2.04 (95%CI 1.02 to 4.05)]. Neither *APOA5* -1131T>C nor the S19W variants had a significant impact on risk, whereas the *APOA4-A5* intergenic T>C showed a borderline statistically significant effect on risk, with CC men having a HR 1.59 (95%CI 0.99 to 2.56). Using stepwise regression analysis, after adjustment for age, cholesterol, TG, and stratification by general medical practice, *APOA4* T347S alone remained in the model. Compared with TT homozygotes, TS heterozygotes had a HR of 1.2 (95%CI 0.93 to 1.79) and SS homozygotes had a HR of 2.07 (95%CI 1.04 to 4.12), demonstrating a codominant effect on CHD risk independent of TG level. To test if apoAI levels modulated this effect, apoAI was added to the regression model. The hazard ratio remained essentially the same [TS men, HR 1.2 (95%CI 0.82 to 1.67); SS men, HR 2.08 (95% 0.96 to 4.5)] but was no longer statistically significant because of loss of power as



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

apoAIV 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). Because there is strong negative allelic association ( $D' = 0.91$ ;  $P < 0.0005$ ) between the two *APOA4* variants,<sup>6</sup> 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 with men homozygous for T347 with heterozygous men showing intermediate survival [HR 1.40 (95%CI 0.98, 2.02)] (overall  $P = 0.05$ ).

***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 that occurred in more than 10 individuals with at least 1 recorded CHD event, representing 88% of the sample, were studied. The proportion of CHD events for each haplotype was calculated and ranked according to the proportion of risk (Figure 1B). A comparison was made with the TG-associated haplotypes ranked in the same order (Figure 1C).<sup>6</sup> Of the 5 high-risk haplotypes, haplotypes 1, 3, and 5 (representing 17.5% of the sample) all carried the *APOA4* S347 in combination with the intergenic *APOA4-A5* C and/or *APOC3* -2845G and/or *APOC3* -482T alleles. Haplotypes 2 and 4 (only found in 0.7% and 0.8% of the sample, respectively) were defined by *APOC3* -2845G and *APOA5* W19, respectively, on the wild-type background.

It is clear that the ranking by proportion of events and by TG did not correspond (Figures 1B and 1C). Haplotypes 1, 3, and 5 were associated with TGs below or around the sample mean of 1.80 mmol/L (1.71, 1.79, and 1.82 mmol/L, respectively), whereas haplotypes 2 and 4, defined by *APOC3* -2845G and *APOA5* W19, respectively, were associated with TG levels of 1.67 and 2.16 mmol/L, respectively (Figure 1C). The common haplotype (haplotype 6), representing 36%

**TABLE 1. 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**

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

\*Geometric means presented with approximate standard deviations.

†Given are the anti-log of the log-transformed mean, and standard deviations are approximated.

‡Calculated according to Walldius et al.<sup>39</sup>

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 1.75 mmol/L, ie, below the sample mean.

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

### ***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, because plasma apoAIV levels were not available for NPHSII and genotypic effects were expected to be the same. EARS is a multicenter study of healthy young men and women, recruited from 5 regions of Europe. The frequencies of the T347s in EARS within the different regions are shown in the online data supplement available at <http://www.circresaha.org>. All genotype distributions were in H-W equilibrium. There was significant evidence for allele frequency heterogeneity among regions ( $P=0.04$ ) with the "middle" region having the highest S347 allele frequency (see online data supplement). 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, because there was no significant heterogeneity of the genotype effect between cases and controls, across regions, or between sexes, the effect of T347S on apoAIV levels is presented in the sample as a whole (Figure 3). After correcting for age, gender, case:control status, BMI, physical activity, TG, HDL, and contraception, individuals homozygous for the S347 had significantly lower apoAIV plasma levels ( $13.68\pm 0.59$  mg/dL) than those carrying the T374 allele ( $14.90\pm 0.12$  mg/dL) ( $P=0.035$ ). There was no statistically significant effect of this genotype on any lipid variable, BMI, or WHR (data not shown).

### **Discussion**

The 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, independent effect on risk of CHD in healthy UK men. This is the first prospective study examining the association of *APOA4* variants and CHD risk. Men homozygous for the S347 had a 2-fold risk compared with 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. Thus, it is becoming very clear that when examining the association of SNPs where there is strong LD across the gene or cluster, it is potentially misleading to use single SNPs, and using haplotypes is the best analytical approach. Our results strongly suggest that the apparent protective effect of the *APOC3* 1100C>T merely reflects the strong LD across the cluster and does not suggest a protective role for *APOC3* per se. Although the multiple regression analysis and the Kaplan-Meier plot provided statistical examination of the results, haplotype analysis suggested possible genetic interpretations.

**TABLE 2. Univariate Hazard Ratios (187 Coronary Events out of 2808 Individuals) for the 9 APOC3-A4-A5 Variants**

	Hazard Ratio (95% CI)*	Hazard Ratio (95% CI)†
<i>APOA5</i> -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 <i>APOA4-A5</i> 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)
<i>APOA5</i> 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)
<i>APOA4</i> 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)
<i>APOA4</i> 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)
<i>APOC3</i> 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)
<i>APOC3</i> 3238C>G		
CG+GG	0.72 (0.45, 1.15)	0.70 (0.44, 1.12)
<i>APOC3</i> -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)
<i>APOC3</i> -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)

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

\*Adjusted for age and practice.

†Adjusted for age and practice and triglyceride levels.

We previously considered the effect of the *APOC3-A4-A5* gene cluster on plasma TG using haplotype analysis derived from these 9 SNPs.<sup>6</sup> 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 among the highest proportion of events (11.9%, 9.5%, and 9.1%, respectively), were defined by *APOA4* S347 in combination with flanking markers *APOC3* -2854G in the intergenic region between *APOA4* -*APOC3* and/or the intergenic *APOA4*-*APOA5C*. These SNPs are in tight positive LD,<sup>6</sup> and this strongly implicates *APOA4* as a gene involved in CHD risk determination. Very few studies have examined the association of *APOA4* T347S with CHD risk. A small case:control study from Finland<sup>25</sup> found no difference in frequency of the T347S in the 180 cases compared with 73 controls, but was underpowered to do so, whereas in a Japanese study the T347S rare allele frequency was too low to give a risk estimate.<sup>26</sup>

Our results raise three questions. First, in view of the effects of *APOA5* S19W and *APOC3* -482C>T as the two main determinants of TG in the cluster,<sup>6</sup> 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, what is the mechanism for the association of *APOA4* T347S with CHD risk?

### ***APOA5* S19W and *APOC3* -482C>T and CHD Risk**

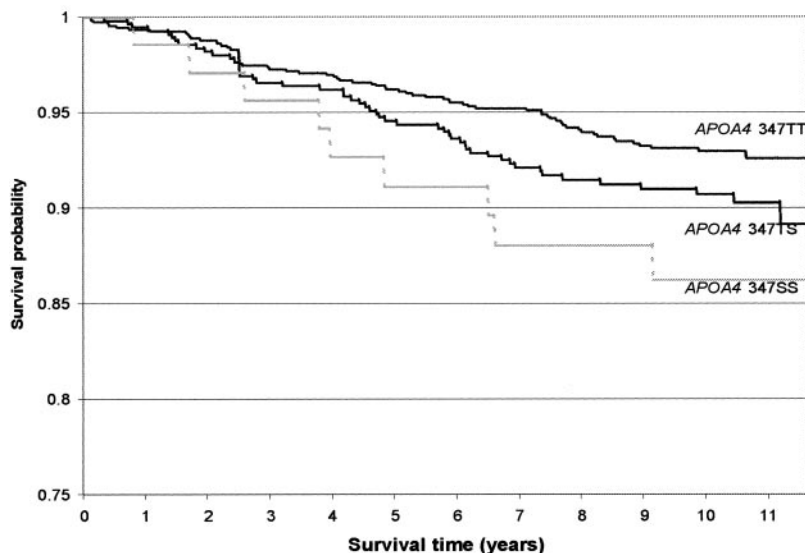
*APOA5* W19 homozygotes have 52% higher TG levels than S19 homozygotes,<sup>6</sup> and the *APOA5* W19-carrying haplotype, on a common allele background, ranked as the 4th highest risk-associated haplotype. However, in univariate analysis, S19W did not have a statistically significant effect on risk. This could be due to the fact that the rare allele frequency of S19W is low and 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 with nonsmoking CC homozygotes, -482T homozygous men who smoked had 28% higher TG levels,<sup>23</sup> 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.

### ***APOC3* SstI (3238C>G) and CHD Risk**

Considering the relationship of *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.<sup>27</sup> 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.<sup>23</sup> A recent study examined *APOC3* -455T>C, 1100C>T, and the 3238C>G with respect to TG levels, apoCIII levels, and CHD risk. All three variants influenced TG levels, the -455C, and 3238G were associated with higher apoCIII levels, but only the -445C, in the insulin responsive element, was associated with increased risk of CAD (OR 2.5 95%CI 1.51, 4.18).<sup>28</sup> However, due to the strong LD across the cluster, our study suggests that without haplotype analysis of *APOC3-A4-A5* variants, their relative contribution cannot be delineated.

### **ApoAIV and CHD Risk**

Finally, an antiatherogenic role for apoAIV has been suggested by three CHD case:control studies that showed that apoAIV levels were significantly lower in CHD cases compared with controls.<sup>15,29</sup> Mice overexpressing apoAIV support this hypothesis because *ApoE*<sup>-/-</sup> mice transgenic for human *APOA4* in the liver<sup>30</sup> or mouse *Apoa4*<sup>31</sup> 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 subjects where apoAIV concentrations were available. We did not see a significant case-control difference in apoAIV levels, but because these are all healthy young subjects and EARS is an



**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.

offspring study, any risk effect in the parents would have been diluted in the offspring.<sup>32</sup> We did, however, find that S347 homozygosity was associated with a statistically significantly lower plasma apoAIV level compared with T347 carriers. We would anticipate that this genotypic effect would be the same in all Caucasians, but none of the two previous studies examining this relationship found significant association.<sup>33,34</sup> In part, this may be because they have been underpowered, and for example, the study by Larson et al,<sup>34</sup> in 743 men and women, had only 23% power at  $P=0.05$  to detect an association based on the genotypic levels we report.

Taken together, the association of S347 with increased CHD risk and lower apoAIV levels suggests that variation in *APOA4* may be affecting risk directly, and in fact, threonine at residue 347 is conserved in higher mammals. However, whether the amino acid change at 347 is functional, remains to be resolved. This is not entirely clear from the haplotype analysis, and for example, haplotype 19, which carries S347 on a background with all the common alleles, is associated with low risk. However, this haplotype is rare and the estimate of risk is not robust.

In addition to *APOA4* 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 that coregulate *APOA1-C3-A4* have been mapped to the *APOC3-A4* intergenic region,<sup>35</sup> and position -2845 is very close to an enhancing element that is responsive to the nuclear factor

**TABLE 3. Proportion of Events Associated With Haplotypes in Those Individuals Who Carried the Wild-Type Haplotype**

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

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.

Difference between 2 haplotype groups;  $P=0.02$ .

HNF4 (mapped between -2893 and -2920). Thus, altered levels of expression due to this variant site could result in altered levels of apoAIV.

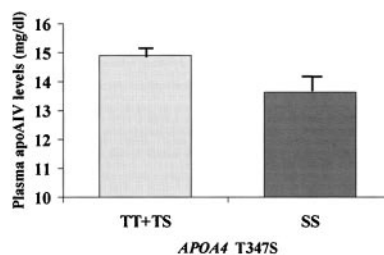
### ApoAIV as an Antioxidant

Because 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,<sup>36</sup> and *ApoE*-deficient mice overexpressing *APOA4* have reduced oxidative markers.<sup>37</sup>

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 that correlated to *APOC3* overexpression, but when crossed with the *ApoE*<sup>-/-</sup> mouse, they showed a 61% reduction in atherogenesis,<sup>38</sup> 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 independent 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 antiatherogenic role for apoAIV that is independent of effects on lipids. This suggests a potential therapeutic role for apoAIV in CHD.

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**Figure 3.** Mean apoAIV levels according to *APOA4* T347S genotype in EARS subjects, adjusted for case-control status, age, sex and region, BMI, physical activity, TG, HDL, and contraception. Mean values and SEM are given for TT+TS ( $n=1559$ ) and SS ( $n=52$ ).

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Online Table 1. Distribution of the *APOA4* T347S in the cases and controls in the 5 regions of Europe participating in the in EARS study

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

There was no significant departure from Hardy-Weinberg equilibrium. Case/control differences in allele frequency, adjusted for region:  $p=0.14$ . Difference between regions, adjusted for status:  $p=0.04$

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