# Functional polymorphism in *ABCA1* influences age of symptom onset in coronary artery disease patients

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ATP-binding-cassette-transporter-A1 (ABCA1) plays a pivotal role in intracellular cholesterol removal, exerting a protective effect against atherosclerosis. ABCA1 gene severe mutations underlie Tangier disease, a rare Mendelian disorder that can lead to premature coronary artery disease (CAD), with age of CAD onset being two decades earlier in mutant homozygotes and one decade earlier in heterozygotes than in mutation noncarriers. It is unknown whether common polymorphisms in ABCA1 could influence age of symptom onset of CAD in the general population. We examined common promoter and non-synonymous coding polymorphisms in relation to age of symptom onset in a group of CAD patients (n = 1164), and also carried out in vitro assays to test effects of the promoter variations on ABCA1 promoter transcriptional activity and effects of the coding variations on ABCA1 function in mediating cellular cholesterol efflux. Age of symptom onset was found to be associated with the promoter -407G>C polymorphism, being 2.82 years higher in C allele homozygotes than in G allele homozygotes and intermediate in heterozygotes (61.54, 59.79 and 58.72 years, respectively; P = 0.002). In agreement, patients carrying ABCA1 haplotypes containing the -407C allele had higher age of symptom onset. Patients of the G/G or G/C genotype of the -407G>C polymorphism had significant coronary artery stenosis (>75%) at a younger age than those of the C/C genotype (P = 0.003). Reporter gene assays showed that ABCA1 haplotypes bearing the -407C allele had higher promoter activity than haplotypes with the -407G allele. Functional analyses of the coding polymorphisms showed an effect of the V825I substitution on ABCA1 function, with the 825I variant having higher activity in mediating cholesterol efflux than the wild-type (825V). A trend towards higher symptom onset age in 825I allele carriers was observed. The data indicate an influence of common ABCA1 functional polymorphisms on age of symptom onset in CAD patients.

## INTRODUCTION

ATP-binding-cassette-transporter-A1 (ABCA1) mediates transport of intracellular cholesterol and phospholipids

across cell membranes where these lipid molecules are removed from cells by apolipoprotein AI and other apolipoproteins of nascent high-density lipoprotein (HDL), although the mechanisms by which ABCA1 mediates lipid export is still

\*To whom correspondence should be addressed at: Clinical Pharmacology, William Harvey Research Institute, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, UK. Tel: +44 2078823425; Fax: +44 2078823408; Email: s.ye@qmul.ac.uk \*The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/ licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. incompletely understood (1,2). This process plays an important role in maintaining cellular cholesterol homeostasis and exerts a protective effect against atherosclerosis (1-3).

Loss-of-function mutations in the *ABCA1* gene cause Tangier disease, a rare genetic disorder characterized by near absence of HDL and accumulation of lipids within cells in various tissues including the blood vessel wall (4–6). *ABCA1* mutation carriers have markedly higher incidence of coronary artery disease (CAD) compared with non-carriers (7). In addition, in families of Tangier disease patients, onset of CAD is significantly earlier in mutation carriers than in non-carriers (8,9). Increased incidence of early onset CAD in *ABCA1* mutation carriers is likely attributed to the accumulation of lipid-laden macrophage foam cells in the vascular wall, which would promote development and progression of atherosclerosis (1–3).

Common single nucleotide polymorphisms (SNPs) in *ABCA1* have been associated with susceptibility to CAD in the general population (10–16). In this study, we investigated whether common promoter and non-synonymous coding SNPs in *ABCA1* have an influence on age of symptom onset in CAD patients. In addition, we tested whether the promoter SNPs had an influence on *ABCA1* promoter transcriptional activity and whether the coding SNPs had an effect on ABCA1 function in mediating cellular cholesterol efflux.

## RESULTS

The ABCA1 gene had been re-sequenced using DNA samples from individuals of European ancestry in previous studies by other researchers (14.15), which identified a number of SNPs. In this study, we focused on common SNPs in the promoter and common non-synonymous SNPs in the coding region, using the conventional definition of common SNPs as those which have a minor allele frequency >0.05 (17). We genotyped a group of British European CAD patients (n = 1164, demographic and clinical characteristics summar-)ized in Table 1) for the SNPs depicted in Figure 1, which had previously been shown to have a minor allele frequency >0.05 in Europeans (10–15). The observed genotype distributions of all SNPs were consistent with Hardy-Weinberg equilibrium, and the allele frequencies were similar to those reported in other population samples of European ancestry (10-15). As in other studies (14,15), we detected strong linkage disequilibrium (LD) among the promoter SNPs, substantial LD among some of the coding SNPs, but little LD between promoter SNPs and coding SNPs (Table 2). Therefore, separate haplotype analyses were carried out for the promoter SNPs and the coding SNPs, as in other studies (14,15).

# *ABCA1* single nucleotide polymorphisms and age of symptom onset in coronary artery disease patients

Mean age of symptom onset was 2.82 years higher in patients who were homozygous for the C allele of the *ABCA1* promoter -407G>C SNP than in those who were homozygous for the -407G allele, and intermediate in heterozygotes [mean (SD) = 61.54 (9.72), 59.79 (9.59) and 58.72 (9.99) years for the C/C, C/G and G/G genotypes, respectively; P = 0.002, Table 3]. The association remained significant after adjusting Table 1. Characteristics of subjects

	Mean (SD) or %
Age of symptom onset (years)	59.77 (9.83)
Male gender	76.3%
Current and previous smokers	74.6%
Body mass index (kg/m <sup>2</sup> )	27.54 (4.27)
Cholesterol (mmol/L)	5.11 (1.02)
HDL cholesterol (mmol/L)	1.25 (0.31)
Triglycerides (mmol/L)	1.86 (1.22)
Hypertension	45.0%
Type 1 diabetes	3.1%
Type 2 diabetes	10.2%
Family history of CAD	48.5%

Data shown are mean (SD) for continuous variables or % for categorical variables.

for gender, smoking, body mass index, cholesterol, triglycerides, lipid-lowering treatment, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD (P = 0.004). Gender ratio, percentage of smokers, body mass index, cholesterol and triglyceride levels, hypertension, type 2 diabetes mellitus and family history of CAD did not significantly differ among the different -407G>C genotype groups. The percentage of type 1 diabetics was found to be higher among subjects of the C/C genotype (6.3%) than among those of the G/C or G/G genotype (2.4 and 2.2%, respectively) (P = 0.01). The association between -407G>C and age of CAD onset remained significant after excluding subjects with type 1 diabetes (n = 36) and adjusting for the other variables mentioned above (P = 0.003).

Some weaker relationships between CAD symptom onset age and -278G>C (P = 0.007), -565C>T (P = 0.01), -940T>G (P = 0.03) and -1395C>T (P = 0.04) were observed (Table 3), however, they failed to reach significance after multiple testing correction with the use of the SNPSpD method (18) which indicated that a significance threshold of P < 0.0042 is required taking into account the number of SNPs examined and LD between the SNPs.

No significant association was detected between age of onset and the other promoter SNPs or the coding region SNPs examined, although it was noted that onset age in 825I/825I homozygotes was 2.71 years higher than that in 825V/825I heterozygotes and 3.76 years higher than that in 825V/825V homozygotes [mean (SD) = 63.46 (9.07), 60.75 (9.52) and 59.70 (9.86) years, respectively; P > 0.05; Table 3] and that onset age in 883M/883M homozygotes was 3.02 years higher than that in 883I/883I homozygotes and 1.74 years higher than that in 883I/883I homozygotes [mean age (SD) = 62.12 (6.36), 59.10 (9.65) and 60.38 (9.66) years, respectively; P > 0.05; Table 3].

# ABCA1 haplotypes and age of symptom onset in coronary artery disease patients

There were six major haplotypes (each having a frequency >0.05) deriving from the 15 promoter SNPs studied, i.e.  $T_{-1801}$  A\_{-1652}G\_{-1506}T\_{-1395}G\_{-1252}C\_{-1217}Ins\_{-1034}T\_{-940}G\_{-803}C\_{-565}G\_{-407}C\_{-302} G\_{278}C\_{-99}C\_{-14}, C\_{-1801}G\_{-1652}G\_{-1506}C\_{-1395}A\_{-1252}C\_{-1217}Ins\_{-1034}



Figure 1. Schematic diagram of the locations of SNPs studied in the ABCA1 gene. Drawing not to scale.

 $T_{-940}G_{-803}C_{-565}G_{-407}C_{-302}G_{-278}G_{-99}C_{-14}, C_{-1801}A_{-1652}C_{-1506}C_{-1395}$  $G_{-1252}C_{-1217}Del_{-1034}G_{-940}G_{-803}T_{-565}C_{-407}T_{-302}C_{-278}G_{-99}T_{-14}$ C-1801G-1652G-1506C-1395G-1252C-1217Ins-1034G-940G-803T-565  $C_{-407}C_{-302}C_{-278}G_{-99}T_{-14}, \quad C_{-1801}G_{-1652}G_{-1506}C_{-1395}G_{-1252}T_{-1217}$  $Ins_{-1034}G_{-940}G_{-803}T_{-565}C_{-407}C_{-302}C_{-278}G_{-99}C_{-14}, \quad T_{-1801}A_{-1652}$ G-1506T-1395G-1252C-1217Ins-1034T-940A-803C-565G-407C-302G-278 G<sub>-99</sub>C<sub>-14</sub>. These haplotypes could be tagged by a set of five SNPs: -803G>A, -407G>C, -302C>T, -99G>C and -14C>T, identified using the SNPtagger program (19). A haplotype analysis of these five tagging SNPs in relation to age of CAD symptom onset showed that the G<sub>-803</sub>-C<sub>-407</sub>-C<sub>-302</sub>-G<sub>-99</sub>-C<sub>-14</sub> haplotype was associated with higher onset age (P = 0.003, Table 4), and the association remained significant (P = 0.002) after adjusting for gender, body mass index, smoking, cholesterol, triglycerides, lipid-lowering treatment, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD. G-803-C-407-C-302-G<sub>-99</sub>-C<sub>-14</sub> haplotype carriers have slightly higher percentages of type 1 diabetic subjects (8.8% in subjects with two copies, 3.6% in those with one copy, 2.3% in those with zero copy, of the  $G_{-803}$ - $C_{-407}$ - $C_{-302}$ - $G_{-99}$ - $C_{-14}$  haplotype, P = 0.057). The association between the  $\mathrm{G}_{\text{-}803}\text{-}\mathrm{C}_{\text{-}407}\text{-}\mathrm{C}_{\text{-}302}\text{-}\mathrm{G}_{\text{-}99}\text{-}\mathrm{C}_{\text{-}14}$  haplotype and increased age of CAD onset remained significant (P = 0.002) after excluding type 1 diabetic subjects. There was no difference among the different haplotypes in gender ratio, percentage of smokers, body mass index, cholesterol levels, triglyceride levels, hypertension, type 2 diabetes mellitus and family history of CAD. A weaker relationship between increased CAD onset age and another haplotype, i.e.  $G_{-803}$ - $C_{-407}$ - $T_{-302}$ - $G_{-99}$ - $T_{-14}$ , was also observed (P = 0.006 after adjusting for the covariates mentioned above, Table 4).

A previous study showed that the *ABCA1* promoter region up to nucleotide position -580 is essential in regulating *ABCA1* transcription (20). An analysis of haplotypes derived from the SNPs in this region (i.e. -565C>T, -407G>C, -302C>T, -278G>C, -99G>C and -14C>T) showed that there were five major haplotypes (each with a frequency of >0.05, Table 4), and that the T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> haplotype was associated with higher age of CAD onset (P = 0.002, Table 4). The association remained significant after adjustment for gender, body mass index, smoking, cholesterol, triglycerides, lipid-lowering treatment, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD (P = 0.003). A weaker relationship between the T<sub>-565</sub>-C<sub>-407</sub>-T<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-T<sub>-14</sub> and increased CAD onset age was also detected (P = 0.01 after adjusting for covariates, Table 4).

# ABCA1 single nucleotide polymorphisms and coronary stenosis

Patients of the G/G or G/C genotype of the -407G>C SNP had significant coronary stenosis (>75%) at a younger age

than those of the C/C genotype (P = 0.003; Fig. 2A). A haplotype analysis of the promoter-tagging SNPs described above showed that compared with patients of the G<sub>-803</sub>-C<sub>-407</sub>-C<sub>-302</sub>-G<sub>-99</sub>-C<sub>-14</sub> or G<sub>-803</sub>-C<sub>-407</sub>-T<sub>-302</sub>-G<sub>-99</sub>-T<sub>-14</sub> haplotype, those of other haplotypes had significant coronary stenosis at a younger age (P = 0.02; Fig. 2B). Similarly, a haplotype analysis of the six proximal promoter SNPs showed that compared with patients of the T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> or T<sub>-565</sub>-C<sub>-407</sub>-T<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-T<sub>-14</sub> haplotype, those of other haplotypes had significant coronary stenosis at a younger age (P = 0.01; Fig. 2C).

# *ABCA1* single nucleotide polymorphisms and plasma high-density lipoprotein level

825I/825I homozygotes had higher mean HDL level than 825V/825I heterozygotes who in turn had higher mean HDL level than 825V/825V homozygotes [mean (SD) = 1.54 (0.07), 1.34 (0.31) and 1.23 (0.30) mmol/l, respectively; P = 0.01; Table 5]. Comparing different genotypes of the I883M polymorphism, 883M/883M homozygotes and 883I/ 883M heterozygotes had higher mean HDL level than 883I/ 883I homozygotes [mean (SD) = 1.34 (0.34), 1.39 (0.36) and 1.20 (0.27) mmol/l, respectively; P = 0.0004; Table 5]. The relationships were still observed after adjusting for age. gender, smoking, body mass index, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD (P = 0.048) for V825I and P = 0.001 for I883M). Gender ratio, percentage of smokers, body mass index, total cholesterol and triglyceride levels, hypertension, type 1 and type 2 diabetes and family history of CAD did not significantly differ among the different genotypes of the V825I and I883M SNPs. No significant association was observed between HDL level and the other SNPs studied.

#### ABCA1 promoter activity assays

To investigate whether there were differences in promoter activity between the different ABCA1 promoter haplotypes, transient transfection and luciferase reporter assays were carried out. The experiments showed that compared with the most common haplotype C-565-G-407-C-302-G-278-G-99-C-14, the T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> haplotype had approximately 2-fold higher promoter activity, and the T-565-C-407-C-302-C-278-G-99-T-14 approximately 1.2-fold higher promoter activity (Fig. 3A). The promoter activity of all ABCA1 haplotypes examined was significantly increased transfected cells were treated when the with 22(R)-hydroxycholesterol and 9-cis-retinoic acid which had been shown to upregulate *ABCA1* transcription (21-23)(Fig. 3B). An increase in promoter activity of all the

Table 2. Coefficie	nts $(D')$ o	f pair-wise	linkage d	isequilibriu	ım between	ABCAI S	NPs											
	-1801	- 1652	-1506	-1395	-1252	-1217	-1034	- 940	- 803	-565	-407	-302	-278	- 99	-14	R219K	V825I	I883M
-1652A>G	$-0.97^{*}$																	
-1506G>C	$-0.97^{*}$	$-0.86^{*}$																
-1395C>T	$0.94^{*}$	$-0.94^{*}$	$-0.91^{*}$															
-1252G > A	$-0.90^{*}$	$0.89^*$	$-0.64^{\dagger}$	$-0.95^{*}$														
-1217C > T	$-1.00^{*}$	$0.96^{*}$	$-0.94^{*}$	$-0.97^{*}$	$-0.91^{\dagger}$													
-1034ATins/del	$-0.91^{*}$	$-0.90^{*}$	$0.89^{*}$	$-0.94^{*}$	$-0.80^{*}$	$-0.94^{*}$												
-940T>G	$-0.95^{*}$	$0.40^{*}$	$0.90^{*}$	$-0.95^{*}$	$-0.79^{*}$	$0.96^{*}$	$0.84^{*}$											
-803G > A	$0.93^{*}$	$-0.91^{*}$	$-0.95^{*}$	$0.96^{*}$	$-0.83^{\dagger}$	$-1.00^{*}$	$-0.95^{*}$	$-0.98^{*}$										
-565C>T	$-0.95^{*}$	$-0.42^{*}$	$0.71^{*}$	$-0.97^{*}$	$-0.81^{*}$	$1.00^{*}$	$0.74^{*}$	$0.92^{*}$	$-0.98^{*}$									
-407G>C	$-0.87^{*}$	$0.39^{*}$	$0.71^{*}$	$-0.86^{*}$	$-0.74^{*}$	$0.88^{*}$	$0.73^{*}$	$0.86^{*}$	$-0.95^{*}$	$0.92^{*}$								
-302C>T	$-0.85^{*}$	$-0.90^{*}$	$0.87^{*}$	$-0.88^{*}$	$-0.77^{*}$	$-0.87^{*}$	$0.90^{*}$	$0.84^{*}$	$-0.89^{*}$	$0.94^{*}$	$0.98^{*}$							
-278G>C	$-0.94^{*}$	0.41*	$0.72^{*}$	$-0.94^{*}$	$-0.83^{*}$	0.96	$0.77^{*}$	$0.93^{*}$	$-0.98^{*}$	$0.99^*$	$0.91^{*}$	$0.92^{*}$						
-99G>C	$0.79^{*}$	$-0.81^{*}$	$-0.84^{*}$	$0.82^{*}$	$-0.96^{*}$	$-0.88^{*}$	$-0.80^{*}$	$-0.82^{*}$	$-0.96^{*}$	$-0.85^{*}$	$-0.75^{*}$	$-0.88^{*}$	$-0.85^{*}$					
-14C>T	$-0.93^{*}$	$0.10^{\dagger}$	$0.78^{*}$	$-0.94^{*}$	$-0.81^{*}$	$-0.89^{*}$	$0.77^{*}$	$0.91^*$	$-1.00^{*}$	$0.98^{*}$	$0.90^{*}$	$0.84^{*}$	$0.98^{*}$	$-0.86^{*}$				
R219K	$0.14^{\dagger}$	$-0.17^{\dagger}$	$-0.18^{\ddagger}$	$0.14^{\dagger}$	$-0.62^{*}$	$0.01^{\ddagger}$	$-0.16^{\ddagger}$	$0.00^{\ddagger}$	$0.09^{\ddagger}$	$-0.11^{\dagger}$	$0.10^{\ddagger}$	$-0.17^{\ddagger}$	$0.11^{\ddagger}$	$0.04^{\ddagger}$	$0.05^{\ddagger}$			
V825I	$-0.03^{\ddagger}$	$-0.39^{\dagger}$	$0.14^{\dagger}$	$0.19^{\ddagger}$	$-0.35^{\ddagger}$	$-0.62^{\ddagger}$	$0.17^{\dagger}$	$-0.06^{\ddagger}$	$0.02^{\ddagger}$	$0.12^{\ddagger}$	$0.15^{\ddagger}$	$0.03^{\ddagger}$	$-0.12^{\ddagger}$	$0.09^{\ddagger}$	$0.04^{\ddagger}$	$-0.88^{*}$		
I883M	$0.08^{\ddagger}$	$-0.38^{\dagger}$	$0.09^{\dagger}$	$0.05^{\ddagger}$	$-0.24^{\ddagger}$	$-0.18^{\ddagger}$	$0.11^{\dagger}$	$0.02^{\ddagger}$	$-0.05^{\ddagger}$	$0.04^{\ddagger}$	$-0.04^{\ddagger}$	$0.04^{\ddagger}$	$0.02^{\ddagger}$	$-0.12^{\ddagger}$	$0.01^{\ddagger}$	$0.25^{*}$	$0.81^{*}$	
R1587K	$0.00^{\ddagger}$	$0.03^{\ddagger}$	$-0.04^{\ddagger}$	$-0.05^{\ddagger}$	$0.06^{\ddagger}$	$0.00^{\ddagger}$	$0.01^{\ddagger}$	$-0.04^{\ddagger}$	$-0.08^{\ddagger}$	$-0.07^{\ddagger}$	$-0.08^{\ddagger}$	$-0.08^{\dagger}$	$-0.06^{\ddagger}$	$0.01^{\ddagger}$	$-0.03^{\ddagger}$	$0.14^{\dagger}$	$-0.27^{\ddagger}$	$-0.01^{\ddagger}$
$^{*}P < 0.001.$																		
$^{\dagger}P < 0.05.$																		
1 / 0.00.																		

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ABCA1 haplotypes was also observed when transfected cells were treated with 8-bromoadenosine-3',5'-cyclic monophosphate (8-br-cAMP) which had also been demonstrated to increase *ABCA1* expression (Fig. 3C) (21). In 22(R)-hydroxy-cholesterol and 9-*cis*-retinoic acid treated cells and in 8-br-cAMP treated cells, the promoter activity of T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> was over 2-fold higher, T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-T<sub>-14</sub> over 1.5-fold higher and T<sub>-565</sub>-C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-T<sub>-14</sub> approximately 1.5-fold higher, than the most common haplotype C<sub>-565</sub>-G<sub>-407</sub>-C<sub>-302</sub>-G<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> (Fig. 3B and C).

# Functional analysis of *ABCA1* coding single nucleotide polymorphisms

We also investigated whether any of the common nonsynonymous coding SNPs studied had an effect on the activity of ABCA1 in facilitating cholesterol efflux, by transfecting cells with plasmids expressing the different ABCA1 alleles and then determining the rates of cholesterol efflux from the cells. The assays showed that cells expressing the 825I variant had higher rate of apoAI-mediated cholesterol efflux than cells expressing the wild-type (825V) ABCA1 (P = 0.003; Fig. 4). There was also a trend towards increased rate of apoAI-mediated cholesterol efflux in cells expressing the 883M variant compared with cells expressing the wild-type (883I). Cells expressing the 219K or 1587K variants had similar rates of apoAI-mediated cholesterol efflux to those expressing the wild-type (219R and 1587R) (Fig. 4). As expected, compared with the rate of cholesterol efflux in cells transfected with the plasmid expressing the wild-type ABCA1, the rates of cholesterol efflux were significantly lower in untransfected cells and in cells transfected with the plasmid expressing the ABCA1 (V1704D and L1379F) mutant which had previously been shown to result in complete loss of ABCA1 function (24) (*P* < 0.001; Fig. 4).

## DISCUSSION

In this study of a large group of CAD patients, we found an association between age of symptom onset and the ABCA1 promoter -407G>C SNP. Age of symptom onset was 2.82 years higher in -407C allele homozygotes than in -407G allele homozygotes, and intermediate in heterozygotes, suggesting an allele-dose effect. The influence of this common SNP on age of symptom onset is moderate, compared with the effects of rare, severe loss-of-function ABCA1 mutations that cause Tangier disease and that can lead to CAD two decades earlier in homozygotes and one decade earlier in heterozygotes (8,9). However, the high frequency of the -407G>C SNP (the C/C and G/C genotypes were 20 and 48%, respectively, in our patient group) makes it a potentially important genetic factor in many CAD patients in the population. In agreement with results of the individual SNP analyses, haplotype analyses showed that patients carrying common promoter haplotypes bearing the -407C allele had higher age of symptom onset. In addition, we found that -407G allele homozygotes had significant coronary artery

 Table 3. Age (years) of CAD symptom onset according to ABCA1 genotypes

Polymorphism	Genotype	Mean (SD), n	P-value	Polymorphism	Genotype	Mean (SD), n	P-value
	C/C	60.48 (9.83), 353			C/C	58.95 (9.92), 318	
-1801C>T (rs2487046)	C/T	59.58 (9.74), 463	0.09	-565C>T (rs2422493)	C/T	59.84 (9.59), 496	0.01
	T/T	59.03 (9.68), 168			T/T	61.13 (10.1), 214	
	A/A	59.45 (9.96), 389			G/G	58.72 (9.99), 296	
-1652A>G (rs10124728)	A/G	59.59 (9.62), 488	0.29	-407G>C (rs2246293)	G/C	59.79 (9.59), 458	0.002
	G/G	60.74 (10.0), 119			C/C	61.54 (9.72), 191	
	G/G	59.51 (9.74), 609			C/C	59.45 (9.82), 619	
-1506G>C (rs2487047)	G/C	60.26 (9.84), 343	0.12	-302C>T (rs2246298)	C/T	60.38 (9.86), 276	0.15
	C/C	61.54 (10.41), 41			T/T	60.90 (10.5), 33	
	C/C	60.52 (9.90), 357			G/G	58.94 (9.82), 313	
-1395C>T (rs2487048)	C/T	59.67 (9.66), 462	0.04	-278G>C (rs1800976)	G/C	59.70 (9.67), 463	0.007
	T/T	58.72 (9.79), 180			C/C	61.39 (10.3), 204	
	G/G	59.94 (9.79), 708			G/G	60.41 (9.90), 505	
-1252G > A	G/A	59.80 (9.88), 180	0.57	-99G>C (rs2740483)	G/C	59.41 (9.68), 363	0.12
	A/A	57.80 (8.82), 15			C/C	59.18 (9.35), 78	
	C/C	59.47 (9.82), 769			C/C	59.80 (9.69), 460	
-1217C>T (rs10991420)	C/T	61.11 (9.48), 209	0.05	-14C>T (rs1800977)	C/T	59.74 (9.80), 401	0.54
	T/T	59.82 (11.39), 11			T/T	60.68 (10.5), 113	
	AT/AT	59.63 (9.71), 621			R/R	60.07 (9.94), 503	
-1034ATins/del (rs34669957)	AT/ -	60.14 (9.73), 350	0.37	R219K (rs2230806)	R/K	59.82 (9.84), 392	0.52
	_/_	60.49 (11.13), 43			K/K	59.33 (8.81), 78	
	T/T	59.11 (9.51), 273			V/V	59.70 (9.86), 866	
-940T>G (rs2980083)	T/G	59.85 (9.68), 430	0.03	V825I (rs28587567)	V/I	60.75 (9.52), 100	0.24
	G/G	61.07 (9.86), 200			I/I	63.46 (9.07), 4	
	G/G	59.83 (9.74), 812			I/I	60.38 (9.66), 644	
-803G>A (rs10991419)	G/A	59.73 (9.91), 192	0.79	I883M (rs4149313)	I/M	59.10 (9.65), 217	0.23
	A/A	58.94 (10.85), 14			M/M	62.12 (6.36), 19	
					R/R	60.23 (9.95), 496	
				R1587K (rs2230808)	R/K	58.90 (9.50), 301	0.30
					K/K	60.93 (9.12), 41	

stenosis at a younger age than heterozygotes who in turn had significant coronary stenosis at a younger age than -407Callele homozygotes. In vitro assays showed that the -407Callele-bearing promoter haplotypes had higher promoter activity than the promoter haplotypes with the -407Gallele. The association of the -407C allele with higher age of symptom onset in CAD patients and a delay in developing significant coronary stenosis together with the higher promoter activity of the -407C allele suggest a protective effect of this allele potentially through increased ABCA1 expression. In this study, we also found weak relationships of age of symptom onset with the -278G>C, -565C>T, -940T>G and -1395C>T SNPs which are in strong LD with the -407G>C SNP, but the relationship of the -278G>C, -565C>T, -940T>G and -1395C>T SNPs with age of symptom onset did not reach statistical significance after correction for multiple testing.

We noted that age of symptom onset in 825I/825I homozygotes was 2.71 years higher than that in 825V/825I heterozygotes and 3.76 years higher than that in 825V/825V homozygotes and that symptom onset in 883M/883M homozygotes was 3.02 years later than that in 883I/883I homozygotes, although the differences were not statistically significant. In addition, we observed a relationship of the 825I and 883M alleles with higher HDL levels. The 825I allele has previously been reported to be associated with higher plasma HDL level in an investigation of over 9000 individuals from the Danish general population (15), and the 883M allele has been associated with higher plasma HDL level in studies of European Americans (25), African Americans (25), Japanese (26) and Inuit Canadians (10). The V825I and I883M SNPs were found to be in strong LD in our sample and other European populations (14,15). In the *in vitro* assays, we found that the rate of cholesterol efflux in cells expressing the 825I variant was higher than in cells expressing the ABCA1 wild-type (825V), indicating a potential effect of V825I on ABCA1 function in facilitating cellular cholesterol efflux, which could potentially explain its association with HDL level. The assays also showed a higher rate of cholesterol efflux in cells expressing the wild-type (883I), although the difference did not reach statistical significance.

The R1587K SNP was also found to be associated with plasma HDL levels in the study of the Danish population (15) mentioned above and in a study in Dutch men (11), with the 1587K allele associating with lower HDL levels. In addition, a study of individuals from Scotland and Northern Ireland showed that the 1587K allele was associated with lower plasma levels of apoAI, the major apolipoprotein in HDL (14). In the present study, we observed a lower mean HDL level in 1587K allele homozygotes but it was not statistically significant. In the *in vitro* assays, there was no significant difference in cholesterol efflux between cells expressing the 1587K variant and the wild-type ABCA1 (1587R). It is plausible that the relationship of this SNP with plasma HDL level might have arisen from its LD with other SNPs such as V825I. In the studies mentioned above (10,11,15,26), no

Haplotypes	Haplotype frequency	Mean (95% CI) onset age per haplotype	Inferred mean (95% CI) onset age in homozygotes	P-value <sup>a</sup>	<i>P</i> -value <sup>b</sup>	<i>P</i> -value <sup>c</sup>
Promoter-tagging SNPs G.803-G407-C.302-C.99-C.14	0.250	29.23 (28.51–29.95)	58.46 (57.02–59.90)	Reference	Reference	Reference
G-803-G-407-C-302-G-99-C-14	0.178	29.06 (27.62–30.50)		0.80	0.97	0.97
A-803-U-407-U-302-U-99-U-14 G-803-U-407-U-300-G-80-T_14	0.106 0.134	29./6 (28.39–31.13) 29.87 (28.89–30.85)	59.74 (57.78–61.70) 59.74	0.38	0.20	0.68
G_803-C_407-T_302-G_99-T_14	0.168	30.85(29.88 - 31.82)	61.70(59.76-63.64)	0.01	0.006	0.007
G <sub>-803</sub> -C <sub>407</sub> -C <sub>-302</sub> -G <sub>-99</sub> -C <sub>-14</sub> Global haplotypic association	0.114	31.60 (30.41–32.80)	63.20 (60.82–65.60)	0.003 0.01	0.002 0.006	0.002 0.005
Proximal promoter SNPs						
C-565-G-407-C-302-G-278-G-99-C-14	0.277	29.14(28.44 - 29.84)	58.28 (56.88-59.68)	Reference	Reference	Reference
C-565-G-407-C-302-G-278-C-99-C-14	0.251	29.56(28.16 - 30.96)	59.12 (56.32-61.92)	0.50	0.82	0.71
T <sub>-565</sub> -C <sub>-407</sub> -C <sub>-302</sub> -C <sub>-278</sub> -G <sub>-99</sub> -T <sub>-14</sub>	0.131	30.02(29.01 - 31.03)	60.04(58.02 - 62.06)	0.25	0.59	0.62
T <sub>-565</sub> -C <sub>-407</sub> -T <sub>-302</sub> -C <sub>-278</sub> -G <sub>-99</sub> -T <sub>-14</sub>	0.167	30.89(30.07 - 31.71)	61.78 (60.14–63.42)	0.009	0.01	0.010
T <sub>-565</sub> -C <sub>-407</sub> -C <sub>-302</sub> -C <sub>-278</sub> -G <sub>-99</sub> -C <sub>-14</sub>	0.105	31.69(30.47 - 32.91)	63.38(60.94 - 65.82)	0.002	0.003	0.002
Global haplotypic association				0.006	0.006	0.004

Table 4. Age (years) of CAD symptom onset versus ABCA1 promoter haplotypes

cholesterol efflux. In this study we detected an association between the -407G>C SNP and age of symptom onset in the CAD patients without an association between this SNP with plasma HDL level. This could potentially be due to inadequate statistical power in the test of genotypic effects on HDL levels, as data on HDL level were available only for 276 subjects who were not on lipid-lowering medication. A power calculation suggested that with this sample size and the -407G>CSNP minor allele frequency being 0.44, the minimal effect size detectable in this study was 0.033 mmol/l in HDL level with 0.80 power and at  $\alpha = 0.0042$ , and was 0.025 mmol/l at  $\alpha = 0.05$ . Thus, if the -407G > C SNP has a smaller effect than the abovementioned minimal effect size, it would be unlikely to be detected in this study. An alternative explanation is that ABCA1 genotypes can influence CAD phenotypes without displaying a significant effect on plasma HDL levels. Several previous studies have encountered a situation where ABCA1 SNPs are associated with CAD in the absence of an association with plasma HDL level (11-13). It has been suggested that subtle changes in cellular cholesterol efflux in the vascular wall could have an impact on atherogenesis, without an apparent effect on plasma HDL level (11,13). In contrast with the traditional 'reverse cholesterol transport' theory in which HDL is thought to originate from peripheral tissues and subsequently transferred to the liver (27), recent studies have revealed that the major source of plasma HDL is actually the liver (28-32), and that cholesterol efflux from macrophages accounts for only a very small portion of the total plasma HDL but nevertheless is very important with regard to the development of atherosclerosis (28,33,34). It has been demonstrated that the functional effects of SNPs in the promoter of the interleukin-1B gene are dependent on

<sup>b</sup>Adjusting for gender, body mass index, smoking, cholesterol, triglycerides, lipid-lowering treatment, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD. <sup>c</sup>Excluding subjects with type 1 diabetes (n = 36) and adjusting for the other covariates mentioned above.

haplotype context, i.e. a particular allele of a SNP can reduce promoter activity in the context of one haplotype but increase promoter activity in the context of another haplotype (35). Such functional interactions between promoter SNPs have also been detected in the genes for interleukin-6 and cholesteryl ester transfer protein (36,37). We previously observed that in the C<sub>-407</sub>-C<sub>-302</sub>-C<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> haplotype background, the -565T allele (i.e.  $T_{-565}$ - $C_{-407}$ - $C_{-302}$ - $C_{-278}$ - $G_{-99}$ - $C_{-14}$ ) had a lower promoter activity than the -565C allele (i.e.  $C_{-565}$ - $C_{-407}$ - $C_{-302}$ - $C_{-278}$ - $G_{-99}$ - $C_{-14}$ ) (16). We subsequently found that in the G-407-C-302-G-278-G-99-C-14 haplotype background, the -565T allele (i.e. T<sub>-565</sub>-G<sub>-407</sub>-C<sub>-302</sub>-G<sub>-278</sub>- $G_{-99}$ - $C_{-14}$ ) had higher promoter activity than the -565Callele (i.e. C<sub>-565</sub>-G<sub>-407</sub>-C<sub>-302</sub>-G<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub>). This suggests that the effect of the ABCA1 - 565C > T SNP is also dependent on the context of the other ABCA1 promoter SNPs. The most relevant comparison, however, would be the T-565-C-407-C-302-C-278-G-99-C-14 haplotype versus the C<sub>-565</sub>-G<sub>-407</sub>-C<sub>-302</sub>-G<sub>-278</sub>-G<sub>-99</sub>-C<sub>-14</sub> haplotype as shown in Figure 1, since these two haplotypes have high frequencies (0.277 and 0.105, respectively, in this study) whereas the C-565-C-407-C-302-C-278-G-99-C-14 and T-565-G-407-C-302-G-278-G<sub>-99</sub>-C<sub>-14</sub> are extremely rare (frequencies 0.0007 and 0.001,

association was detected between the R219K SNP and plasma level of HDL. In line with these studies, we found neither an association between this SNP and plasma HDL level nor an effect of this SNP on ABCA1 function in mediating cellular



Figure 2. Cumulative hazards of significant (>75%) stenosis in at least one coronary artery over age according to -402G>C genotypes (A), promoter-tagging SNPs haplotypes (carriage of  $G_{-803}$ - $C_{-407}$ - $T_{-302}$ - $G_{-99}$ - $T_{-14}$  or  $G_{-803}$ - $C_{-407}$ - $C_{-302}$ - $G_{-99}$ - $C_{-14}$ ) (B) and proximal promoter SNPs haplotypes (carriage of  $T_{-565}$ - $C_{-407}$ - $T_{-302}$ - $G_{-278}$ - $G_{-99}$ - $C_{-14}$ ) (C).

Table 5. Plasma HDL levels (mmol/L) according to ABCA1 genotypes

Polymorphism	Genotype	Mean (SD), n	P-value	Polymorphism	Genotype	Mean (SD), n	P-value
	C/C	1.26 (0.31), 93			C/C	1.25 (0.32), 81	
-1801C>T (rs2487046)	C/T	1.23 (0.31), 122	0.88	-565C>T (rs2422493)	C/T	1.24 (0.30), 137	0.78
	T/T	1.29 (0.33), 40			T/T	1.23 (0.28), 54	
	A/A	1.22 (0.29), 99			G/G	1.24 (0.32), 76	
-1652A>G (rs10124728)	A/G	1.25 (0.31), 131	0.37	-407G>C (rs2246293)	G/C	1.23 (0.30), 123	0.80
	G/G	1.29 (0.35), 30			C/C	1.24 (0.27), 52	
	G/G	1.26 (0.33), 156			C/C	1.26 (0.32), 165	
-1506G>C (rs2487047)	G/C	1.21 (0.28), 90	0.47	-302C>T (rs2246298)	C/T	1.21 (0.27), 68	0.67
	C/C	1.24 (0.29), 14			T/T	1.27 (0.26), 13	
	C/C	1.26 (0.31), 96			G/G	1.25 (0.32), 80	
-1395C>T (rs2487048)	C/T	1.22 (0.29), 115	0.83	-278G>C (rs1800976)	G/C	1.24 (0.30), 126	0.79
	T/T	1.26 (0.32), 45			C/C	1.23 (0.28), 51	
	G/G	1.23 (0.29), 193			G/G	1.25 (0.29), 139	
-1252G>A	G/A	1.27 (0.29), 44	0.13	-99G>C (rs2740483)	G/C	1.23 (0.31), 93	0.83
	A/A	1.27 (0.34), 7			C/C	1.30 (0.28), 21	
	C/C	1.25 (0.32), 202			C/C	1.25 (0.31), 126	
-1217C>T (rs10991420)	C/T	1.23 (0.28), 56	0.64	-14C>T (rs1800977)	C/T	1.25 (0.32), 99	0.59
	T/T	0.97 (-), 1			T/T	1.20 (0.23), 30	
	AT/AT	1.27 (0.33), 157			R/R	1.26 (0.30), 129	
-1034ATins/del (rs34669957)	AT/ -	1.21 (0.26), 89	0.36	R219K (rs2230806)	R/K	1.23 (0.30), 104	0.58
	-/-	1.24 (0.29), 14			K/K	1.25 (0.34), 18	
	T/T	1.24 (0.32), 70			V/V	1.23 (0.30), 232	
-940T>G (rs2980083)	T/G	1.24 (0.31), 117	0.77	V825I (rs28587567)	V/I	1.34 (0.31), 26	0.01
	G/G	1.22 (0.29), 53			I/I	1.54 (0.07), 3	
	G/G	1.26 (0.31), 208			I/I	1.20 (0.27), 172	
-803G>A (rs10991419)	G/A	1.20 (0.28), 51	0.40	I883M (rs4149313)	I/M	1.39 (0.36), 56	0.0004
	A/A	1.32 (0.47), 4			M/M	1.34 (0.34), 9	
					R/R	1.27 (0.31), 142	
				R1587K (rs2230808)	R/K	1.24 (0.30), 84	0.39
					K/K	1.18 (0.20), 9	

respectively). The abovementioned findings in several different genes (interleukin-1B, interleukin-6, cholesteryl ester transfer protein and *ABCA1*) indicate that it is important to examine haplotypes rather than an individual SNP in functional analyses of promoter SNPs.

contribute to inter-individual differences in CAD predisposition and progression in the general population.

## MATERIALS AND METHODS

#### Subjects

In summary, the results of our study indicate an influence of common functional polymorphism in the *ABCA1* gene on age of symptom onset in CAD patients. The data provide new evidence supporting the notion that genetic variations in *ABCA1* 

We studied a group (n = 1164) of British European patients with angiographically confirmed CAD, recruited from



**Figure 3.** Relative promoter activity of different *ABCA1* haplotypes in unstimulated cells (**A**), cells treated with 22(R)-hydroxycholesterol and 9-*cis*-retinoic acid (**B**) and cells treated with 8-bromoadenosine-3',5'-cyclic monophosphate (**C**). Note. different scales shown in the three charts. \*\*P < 0.01 compared with the wild-type haplotype C-G-C-G-G-C; \*P < 0.05 compared with the wild-type haplotype C-G-C-G-G-C.

consecutive patients undertaking diagnostic or interventional angiography in Southampton General Hospital from May 1999 to March 2002, as previously described (38). The main demographic and clinical characteristics of the patients are summarized in Table 1. Age of symptom onset refers to the age of first episode of angina pectoris or myocardial infarction. Data on plasma HDL level were available for 276 subjects who were not on lipid-lowering medication. The study was approved by the Local Ethics Committee and all subjects gave written consent.

# Single nucleotide polymorphism selection and determination of genotypes

The subjects of this study were genotyped for 15 common SNPs in the proximal promoter region, i.e. -1801C>T (rs2487046), -1652A>G (rs10124728), -1506G>C (rs2487047), -1395C>T (rs2487048), -1252G>A (rs number unavailable), -1217C>T (rs10991420), -1034A Tins/del (rs34669957),

-940T>G (rs2980083), -803G>A (rs10991419), -565C>T (rs2422493), -407G>C (rs2246293), -302C>T (rs2246298), -278G>C (rs1800976), -99G>C (rs2740483) and -14C>T (rs1800977), respectively, and four common non-synonymous SNPs, i.e. R219K (rs2230806), V825I (rs28587567), I883M (rs4149313) and R1587K (rs2230808) (Fig. 1). These SNPs had previously been shown to have a minor allele frequency >0.05 in Europeans (10–15). The genotyping methods and PCR primers are summarized in Supplementary Material, Table S1.

#### Promoter activity assays

Transient transfection and reporter assays were performed to investigate whether there were differences in promoter activity among the different *ABCA1* haplotypes derived from the six proximal promoter SNPs. For each haplotype, the corresponding *ABCA1* promoter (from position -588 bp to +21 bp relative to the transcriptional start site) was generated by PCR using genomic DNA as template and then inserted into a



Figure 4. Results of apoAI-mediated cholesterol efflux assay of ABCA1 coding SNPs. Asterisk indicates P = 0.003 comparing the rate of apoAI-mediated cholesterol efflux in cells transfected with the plasmid expressing the 8251 variant with that in cells transfected with the plasmid expressing the wild-type ABCA1 (825V). 825I + 833M refers to cells co-transfected with the 825I and 883M plasmids. K.O. refers to an ABCA1 (V1704D and L1379F) mutant which had previously been shown to result in complete loss of ABCA1 function (24).

plasmid (pGL3-basic vector, Promega) containing a firefly luciferase reporter gene. All constructs were verified by DNA sequencing. RAW264.7 cells were transfected with each of the above constructs with the use of FuGENE 6 transfection reagent (Roche Diagnostics). A plasmid (pRL-TK, Promega) containing a renilla luciferase gene under the control of a thymidine kinase promoter, was co-transferred into the cells to serve as a reference for transfection efficiency. Transfected cells were untreated or treated with 22(R)-hydroxycholesterol (20 µM) and 9-cis-retinoic acid (10 µM) for 24 h or treated with 8-bromoadenosine 3',5'-cyclic monophosphate (0.3 mM) for 24 h. At 36 h after transfection, the cells were lysed, and the activities of firefly luciferase and renilla luciferase in the lysates were measured with the use of a dual-luciferase assay kit (Promega). ABCA1 gene promoter activity was determined according to the ratio of firefly luciferase activity to renilla luciferase activity. Three independent experiments were performed. In each experiment, transfection and luciferase assays were carried out in duplicate for each construct.

#### Cholesterol efflux assays

Using a previously constructed plasmid expressing full-length human wild-type *ABCA1* cDNA (24) as a template, plasmids expressing the *ABCA1* 219K, 825I, 883M and 1587K variants, respectively, were generated with the use of the site-directed mutagenesis method described by Scott *et al.* (39). All plasmid constructs were verified by DNA sequencing. Cultured COS-7 cells were transfected with each of the above plasmids or a plasmid expressing an *ABCA1* mutant (V1704D and L1379F) described in Albrecht *et al.* (24). Transfection was carried out with the use of FuGENE 6 transfection reagent (Roche Diagnostics). At 24 h posttransfection, transfection efficiency was determined by fluorescence microscopy and fluorescence-activated cell sorting. Cholesterol efflux assays were performed using a method described by Gelissen et al. (40) with minor modifications. In brief, transfected cells were incubated with [<sup>3</sup>H] cholesterol (Moravek Biochemicals, USA) for 48 h, washed and equilibrated for 18 h in serum-free medium. The cells were then incubated in efflux medium with or without  $20 \,\mu g/ml$ apolipoprotein AI (apoAI, Sigma) as an acceptor. Six hours later, efflux media were removed and cells were washed and then dissolved in 0.1 M NaOH solution. Radioactivity (disintegrations per minute) was measured in the medium and in the cell extract. Three independent experiments were performed, in each of which transfection and cholesterol efflux assav were carried out in triplicate for each construct. The rate of cholesterol efflux was calculated using the following formula: radioactivity in medium/(radioactivity in medium + radioactivity in cell extract). The values (apoA1-mediated efflux minus mean of unstimulated cells) for cells transfected to express the 219K, 825I, 883M or 1587K variant or co-express 825I and 883M were compared with the mean of values (apoA1-mediated efflux minus mean of unstimulated cells) for cells transfected with the plasmid expressing ABCA1 wild-type.

#### Statistical analyses

Allele and genotype frequencies were calculated by gene counting.  $\chi^2$  test with 1 degree of freedom and  $\alpha = 0.05$  level was used to examine whether the observed genotype distributions deviated from Hardy–Weinberg equilibrium. LD coefficient (*D'*), haplotypes and haplotype frequencies were determined with the use of the THESIAS and PHASE (version 2.1) programs (41–43).

The SNPSpD method by Nyholt (18) was used to calculate the significance threshold required in this study to keep type I error rate at 5% accounting for the number of SNPs tested and LD between the SNPs, which gave a significant threshold of 0.0042. Single SNP association analyses of age of CAD symptom onset and plasma HDL level were performed by linear regression using an additive genetic model at each SNP. Using the stepwise regression procedure, the relationship between genotype and age of CAD onset was adjusted for gender, smoking, body mass index, cholesterol, triglycerides, lipid-lowering treatment, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD. Using the same procedure, the relationship of genotypes with HDL level was adjusted for age, gender, smoking, body mass index, hypertension, type 1 diabetes, type 2 diabetes and family history of CAD. Minimum effect sizes detectable in this study were calculated *post hoc* by approximating the linear model by a score test for which the non-centrality parameter is available (44). Haplotype-tagging SNPs were identified with the use of the SNPtagger computer program developed by Ke and Cardon (19). Haplotype effects on age of CAD symptom onset and plasma HDL level were tested with the use of the THESIAS program (41). The Kaplan-Meier procedure with Breslow test was used to examine whether there was a relationship between the presence of significant coronary stenosis (>75% stenosis in at least one coronary artery) and age at the time of coronary angiography and whether this relationship was influenced by *ABCA1* genotypes and haplotypes.

In the luciferase assays and cholesterol efflux assays, differences in luciferase activity between haplotypes and in cholesterol efflux rate between cells transfected with different constructs were assessed by ANOVA and *t*-test.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None.

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