Polymorphisms at the 5'-end of the apolipoprotein AI gene and severity of coronary artery disease.

X L Wang, … , R M McCredie, D E Wilcken

J Clin Invest. 1996;98(2):372-377. https://doi.org/10.1172/JCI118802.

Research Article

Elevated HDL-cholesterol (C) and apo AI are associated with decreased coronary artery disease (CAD) risk. We determined distributions of two MspI polymorphisms of the apo AI gene, associated in other studies with increased HDL-C, among 644 patients aged < or = 65 years in relation to circulating lipids and CAD severity assessed angiographically. The rare allele distributions at both sites were in Hardy-Weinberg equilibrium in these patients but the base changes were not associated with HDL-C and apo AI levels. However, patients homozygous for the -75 bp substitution were more likely to have one or more significantly diseased vessels (> 50% luminal obstruction)(OR: 4.75, 95%CI: 1.10- 20.46) as also were patients with the rare +83 bp alleles (OR: 2.56, 95%CI: 1.13-5.81). While there was an additive effect of the two polymorphisms to have severe CAD (OR: 6.33, 95%CI: 1.33-30.02), the polymorphism at +83 bp remained significant in predicting CAD severity after adjusting for other variables in a logistic regression analysis (OR: 2.95, 95%CI: 1.26-6.90), which was also strongly associated with the positive family CAD history (P = 0.009). We conclude that patients with these base changes in this Australian coronary population do not have increased HDL-C and apo AI levels but do have more severe CAD.

Polymorphisms at the 5′-End of the Apolipoprotein AI Gene and Severity of Coronary Artery Disease

X.L. Wang, S.-X. Liu, R.M. McCredie, and D.E.L. Wilcken
Department of Cardiovascular Medicine, University of New South Wales, Prince Henry/Prince of Wales Hospitals, Sydney, Australia

Abstract

Elevated HDL-cholesterol (C) and apo AI are associated with decreased coronary artery disease (CAD) risk. We determined distributions of two MspI polymorphisms of the apo AI gene, associated in other studies with increased HDL-C, among 644 patients aged ≤ 65 years in relation to circulating lipids and CAD severity assessed angiographically. The rare allele distributions at both sites were in Hardy-Weinberg equilibrium in these patients but the base changes were not associated with HDL-C and apo AI levels. However, patients homozygous for the −75 bp substitution were more likely to have one or more significantly diseased vessels (> 50% luminal obstruction)(OR: 4.75, 95%CI: 1.10–20.46) as also were patients with the rare +83 bp alleles (OR: 2.56, 95%CI: 1.13–5.81). While there was an additive effect of the two polymorphisms to have severe CAD (OR: 6.33, 95%CI: 1.33–30.02), the polymorphism at +83 bp remained significant in predicting CAD severity after adjusting for other variables in a logistic regression analysis (OR: 2.95, 95%CI: 1.26–6.90), which was also strongly associated with the positive family CAD history ($P = 0.009$). We conclude that patients with these base changes in this Australian coronary population do not have increased HDL-C and apo AI levels but do have more severe CAD. (*J. Clin. Invest.* 1996. 98:372–377.) Key words: apolioproteins • coronary disease • angiography • Apo AI DNA polymorphism • cardiovascular risk factor

Introduction

Many epidemiological studies have shown that elevated levels of high-density-lipoprotein cholesterol (HDL-C) and its principal carrier protein apolipoprotein (apo) AI are associated with reduced occurrence and severity of coronary artery disease (CAD)(1–6). Apo AI is mainly associated with HDL particles but circulating free apo AI is also present in low concentration (7, 8). The protective effect of HDL or apo AI is thought to be mediated mainly through the promotion of cholesterol efflux from peripheral cells. However, recent studies indicate that both HDL and apo AI may have antioxidant, anti-thrombotic and anti-inflammatory properties which could have important anti-atherogenic effects as reviewed by Forte and McCall (3).

Both environmental and genetic factors influence HDL-C and apo AI levels. Among the environmental variables, cigarette smoking, dietary fat content, body mass index (BMI) and exercise are the most important (9, 10). Genetic studies have identified polymorphisms and mutations in the apo AI gene and in other genes which are associated with either increased or decreased HDL-C and apo AI levels (1, 5, 11, 12). Among healthy subjects, increased transcription of the apo AI gene and higher circulating levels of apo AI and HDL-C associated with a G→A substitution at −75 bp have been documented in several studies (12-17) although not confirmed in two (18, 19). The association between the base substitution at −75 bp and HDL-C levels are also reported to be influenced by gender (12, 13) and smoking (12, 20). More recently, we have shown that C→T and/or G→A transitions at +83 bp and/or +84 bp of the apo AI gene are also associated with increased HDL-C levels (21, 22). Whilst the frequency in a healthy population of the +83 bp substitution was less common (rare allele frequency: 0.041) than that at −75 bp (rare allele frequency: 0.221), the base substitution at +83 bp was associated with a much greater elevation in circulating HDL-C level (22). Both base substitutions result in loss of MspI restriction sites so that both polymorphic markers can be detected with a single PCR and a single MspI digestion (22).

Since these base changes are associated with increased HDL-C levels, it is logical to suppose that individuals with the base change(s) are likely to have a reduced risk of developing CAD. We explored this hypothesis in the present study by investigating the relationship between these polymorphisms in the apo AI gene and the occurrence and severity of CAD in Australian Caucasian patients assessed by coronary angiography.

Methods

The patients. We studied Caucasian patients aged 65 years or less, both men and women, consecutively referred to the Eastern Heart Clinic at Prince Henry Hospital for coronary angiography over a 20-m period in 1994 and 1995. We excluded only patients shown to have significant left main disease (> 50% luminal obstruction) because it was difficult to categorize this small proportion of the total (5%) within the classification system we used (see below). A written consent was obtained from every patient after a full explanation of the study which was approved by the Ethics Committee of the University of New South Wales.

A 4-ml venous blood sample was drawn into an EDTA sample tube from patients before the angiogram after at least a 6-h fast. The blood sample was centrifuged within two hours and plasma and cellular components stored separately at −70°C in aliquot until analysis.

Determination of polymorphisms in the apo AI gene. DNA was extracted from these frozen whole blood samples by the salting out

Address correspondence to Professor David Wilcken, Department of Cardiovascular Medicine, Clinical Sciences Building, Prince Henry Hospital, Little Bay, NSW 2036, Australia. FAX: 2-382-5755; Phone: 2-382-5026.

Received for publication 11 March 1996 and accepted in revised form 3 May 1996.

1. *Abbreviations used in this paper:* BMI, body-mass-index; CAD, coronary artery disease; HDL-C, HDL-cholesterol; Lp(a), Lipoprotein(a); OR, odds ratio.

J. Clin. Invest.

0021-9738/96/07/0372/06 $2.00
Volume 98, Number 2, July 1996, 372–377

method (23). The following primers were used to amplify a 433-bp fragment 5′-end of the apo AI gene (14):

P1: 5′-AGGGACAGAGCTGATCCTTGAACTCTTAAG-3'

P2: 5′-TTAGGGGACACCTAGCCCTCAGGAAGAGCA-3'.

The PCR was performed in a volume of 25 μl containing ~ 100 ng genomic DNA. The amount of [Mg^{2+}], dNTP (Promega, Madison, WI) and *Taq* polymerase (Advanced Biotechnology, Surrey, UK) used in each reaction were 1.5 mM, 25 μM, and 1 unit, respectively. The thermal cycles (OmniGene Temperature Cycler, Hybaid Ltd, Middlesex, UK) started with 95°C for 5 min and were followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. A total volume of 10 μl containing 20 units of MspI (Boehringer Mannheim, Germany) diluted in manufacturer recommended buffer was added directly to the PCR product and incubated at 37°C overnight. The digested products were visualized on 8% polyacrylamide gel with silver staining.

There are three MspI restriction sites within the region amplified by the PCR and these are located at −75, +37, and +83 bp. Both the G to A substitution at −75 bp and the C to T and/or G to A substitutions at +83 bp sites result in the loss of the sites which are detected simultaneously by a single MspI digestion after the single PCR. The genotypes for the base change at −75 bp were previously referred to as GG, GA and AA (14) in accordance with usual practice. In order to avoid possible confusion with the base change at +83 bp we have used different symbols for the polymorphism at −75 bp with the previous ones enclosed in brackets. The putative genotypes at −75 bp are therefore M1+/+ (GG) for the presence of the MspI site in both alleles, M1−/− (AA) for the absence of the site in both alleles, and M1+/− (GA) for a heterozygote. The putative genotypes at +83 bp are M2+/+, M2+/−, and M2−/− in which "+" indicates the presence and "−" the absence of the *Msp* restriction site.

Biochemical analysis. Total cholesterol, HDL-C and triglyceride levels were measured in the patients by the hospital's clinical chemistry department using standard enzymatic methods. The LDL-cholesterol levels were calculated using the Friedewald formula. Levels of apo AI, apo B, Lp(a) were measured using ELISA methods developed in our laboratory (24–26).

Documentaton of CAD severity. The severity of coronary artery disease was determined by the number of significantly stenosed coronary arteries as follows. The angiograms were assessed by two cardiologists before the availability of genotyping results. Each angiogram was classified as revealing either no coronary lesion with > 50% luminal stenosis or as having one, two, or three major epicardial coronary arteries with > 50% luminal obstructions. Those without significantly diseased vessels were further subgrouped into those with angiographically normal coronary arteries and those with lesions of 50% or less luminal stenosis. We also used the Green Lane coronary scoring system which provides a numerical value for lesion severity and takes account of the amount of myocardium supplied by an affected vessel; the maximal score is 15.

Documentation of other medical conditions. The relevant history was obtained for each patient using a questionnaire with standardized choices of answers to be ticked during the interview. We recorded the presence or absence (yes/no) of a history of myocardial infarction, hypertension requiring treatment, diabetes, and angina pectoris. The presence or absence of CAD among first degree relatives (parents and siblings) and the age of first onset were recorded for a quantitative assessment of family history of premature CAD. We recorded the presence and severity of angina according to whether each patient was experiencing no angina, stable angina, or unstable angina before and during the current hospitalization. All those classified as having unstable angina had an increase in pain frequency as well as rest pain. The life-time smoking dose in pack years was recorded as described previously (27), and patients were also divided into non-smokers, current smokers and ex-smokers.

Statistical analysis. We determined whether or not the distributions of genotypes were in Hardy-Weinberg equilibrium by χ^2 analysis as described by Emery (28). The frequencies of the alleles and genotypes among different subgroups were compared by χ^2 test. A logistic regression analysis was also used to evaluate genotypic effects on CAD severity while other independent variables were controlled and the odds ratios (OR) were calculated.

Results

Genetic characteristics of the patients. The demographic information for the 644 patients is shown in Table I. As expected, men tended to have more unfavorable lipoprotein variables and smoking habits relevant to CAD risk.

Genotype distributions at both sites were in Hardy-Weinberg equilibrium and there was a strong linkage disequilibrium between the two polymorphisms (χ^2 = 74.45, df = 2, P = 0.000001). There were 25 patients with M1−/− (AA) genotypes. For the M2 genotype there were 47 heterozygotes and only 2 M2−/− homozygotes and these were grouped together in the subsequent statistical analysis. The rare allele frequencies at −75 and +83 bp of the apo AI gene were not statistically different between males ("M1-": 0.195, "M2-": 0.037) and females ("M1-": 0.161, "M2-": 0.043).

apo AI polymorphisms and lipoprotein variables in the patient population. Using ANOVA we compared the relationships between apo AI polymorphisms and levels of lipoproteins, apolipoproteins and other relevant variables. There were no consistent associations between the apo AI polymorphisms and any of the lipoproteins, or apolipoproteins, or the BMI or waist/hip ratio. In particular, the "M1-" (A) and "M2-" alleles were not associated with increased HDL-C and apo AI levels as would have been expected. Since there are many confounding factors affecting relationships with lipid metabolism in this CAD population, including lipid lowering and other drug usage, increased BMI, cigarette smoking, and genetic factors relevant to coronary disease, we further analyzed the relationship between the polymorphisms and levels of apo AI and HDL-C among patients who were non-smokers, not receiving lipid lowering drugs and did not have significantly diseased vessels. There were no consistent relationships with HDL-C or apo AI levels in this small number of patients (n = 74) among whom

Table I. Demographic Characteristics of Patients in the Study (Mean±SEM)

	Males	Females
N	467	177
Age (yr)	55.7±0.3	57.0±0.5*
BMI (kg/m^2)	28.2±0.2	28.1±0.4
TC (mmol/liter)	5.42±0.04	5.55±0.08
Triglyceride (mmol/liter)	2.10±0.05	1.71±0.07***
HDL-C (mmol/liter)	1.00±0.01	1.29±0.03***
LDL-C (mmol/liter)	3.43±0.04	3.45±0.08
TC/HDL-C	5.25±0.08	4.59±0.11***
Apo AI (grams/liter)	0.94±0.01	1.12±0.03***
Apo B (grams/liter)	1.01±0.02	0.97±0.02
Log_eLp(a) (mg/liter)	4.96±0.07	5.15±0.11
Coronary scores	5.68±0.19	3.26±0.33***
Smoking dose (packyrs)	27.5±1.4	14.3±1.8***

*$P < 0.05$, ***$P < 0.001$ by Student t test. Values are presented as mean ±SEM.

Table II. The Apo AI Polymorphism at −75 bp and the Number of Significantly Diseased Vessels (> 50% Luminal Obstruction)

	Number of significantly diseased vessels (> 50% luminal obstruction)				
	0	1	2	3	Total
Males					
M1+/+	62 (67.4%)	98 (70.5%)	68 (60.7%)	77 (62.1%)	305
M1+/−	30 (32.6%)	35 (25.2%)	39 (34.8%)	39 (31.5%)	143
M1−/−	0 (0%)	6 (4.3%)	5 (4.5%)	8 (6.5%)	19
"M1-" allele	0.163	0.169	0.219	0.223	0.195
Females					
M1+/+	63 (70.0%)	26 (68.4%)	19 (73.1%)	18 (78.3%)	126
M1+/−	25 (27.8%)	11 (28.9%)	5 (19.2%)	4 (17.4%)	45
M1−/−	2 (2.2%)	1 (2.6%)	2 (7.7%)	1 (4.3%)	6
"M1-" allele	0.162	0.171	0.173	0.13	0.161
Total					
M1+/+	125 (68.7%)	124 (70.1%)	87 (63.0%)	95 (64.5%)	431
M1+/−	55 (30.2%)	46 (26.0%)	44 (31.9%)	43 (29.7%)	188
M1−/−	2 (1.1%)	7 (4.0%)	7 (5.1%)	9 (6.1%)	25
"M1-" allele	0.162	0.170	0.210	0.208	0.186

The observed numbers of cases and the column precentages (in brackets) are presented. There was a trend for an association between the polymorphism at −75 bp and the number of significantly diseased vessels in males. Comparing all individual groups, the Pearson χ^2 for male, female and total patients were respectively 12.40 (df = 6, $P = 0.053$), 3.53 (df = 6, $P = 0.74$), 9.04 (df = 6, $P = 0.172$). The Mantel-Haenszel test for linear association is $\chi^2 = 3.6$ (df = 1, $P = 0.057$) for males; $\chi^2 = 1.23$ (df = 1, $P = 0.267$) for females; and $\chi^2 = 3.36$ (df = 1, $P = 0.067$) for the total. The results for the combined analysis are statistically significant ($P < 0.01$)—see text.

there were 4 with the +83 bp rare allele, 21 M1+/− heterozygotes and 1 M1−/− homozygote.

CAD severity and apo AI polymorphisms. Among the patients we assessed for possible CAD there were 58 of the 467 men and 71 of the 177 women who were found to have angiographically normal coronary arteries. In male patients, the rare allele frequency of the *Msp*I1 polymorphism ("M1-") tended to be higher ($\chi^2 = 5.76$, df = 2, $P = 0.056$) among those with angiographically diseased arteries (0.195) than in those with normal arteries (0.181). However, the difference was highly significant in the male patients for the rare allele of the MspI2 polymorphism ("M2-") in those with (0.042) than those without (0.00) diseased arteries ($\chi^2 = 9.41$, df = 1, $P = 0.002$). But the findings were not the same in the female patients in whom there were no differences in the rare allele frequencies for both polymorphisms between those with and without diseased arteries.

To evaluate a possible association between the apo AI polymorphisms and the severity of CAD, patients were subgrouped into those with 0, 1, 2, and 3 significantly diseased (> 50% luminal obstruction) coronary arteries. By a simple χ^2 test we found that the apo AI MspI1 polymorphism tended to associate the number of significantly diseased vessels either individually (Table II) or when patients with 1 or more significantly diseased vessels were combined as a single group. The frequency of M1−/− (AA) homozygotes and the rare allele "M1-" frequency increased linearly with the number of significantly diseased vessels (Table II). The odds ratios (95% CI) for M1−/− (AA) homozygotes to have 3, 2, and 1 significantly diseased vessels compared with those with M1+/+ (GG) genotypes were respectively 5.9 (1.25–27.9), 5.0 (1.02–24.8), and 3.52 (0.71–17.3). This was also true for M1−/− (AA) patients with 1 or more significantly diseased vessels (OR: 4.75, 95% CI: 1.10–20.46). The same relationships were also observed among the 467 male patients when they were analyzed separately. However this was not found for the smaller number ($n = 177$) of female patients (Table II). It should be noted that none of the 92 male patients without significantly diseased vessels was a M1−/− homozygote by 8 (42.1%) of the 19 M1−/− homozygotes had triple vessel disease (Table II).

A similar relationship was also observed for that between the number of significantly diseased vessels and the MspI2 polymorphism (Table III). There was an increased likelihood for patients with the "M2-" allele to have severe CAD. The "M2-" allele frequencies increased with the increase in the number of significantly diseased vessels (Table III). The odds ratios (95% CI) for these patients to have 3, 2, and 1 significantly diseased vessels were respectively 3.46 (1.4–8.53), 1.95 (0.72–5.27) and 2.15 (0.84–5.43). This association was also statistically significant when patients with 1 or more significantly diseased vessels were combined as a single group. The odds ratio for "M2-" patients to have 1 or more significantly diseased vessels was 2.56 (95% CI: 1.13–5.81). However, as observed for the MspI1 polymorphism this relationship was only significant among male patients ($\chi^2 = 10.29$, df = 3, $P = 0.016$). The odds ratio for M2+/− male patients to have triple vessel disease was 6.67 (95% CI: 1.49–29.7). Although there was a similar trend among the females it was not statistically significant (Table III).

In a logistic regression analysis, the MspI2 polymorphism remained an independent predictor for patients having one or more significantly diseased vessels ($P = 0.008$) when sex, hypertension, diabetes, MspI1 polymorphism, age, BMI, lifetime smoking dose, levels of total cholesterol, HDL-C, triglyceride, LDL-C, apo AI, apo B, and Lp(a) were entered into the model as independent variables. The odds ratio for M2+/− having 1 or more significantly diseased vessels was 2.95 (95% CI: 1.26–6.90) after adjusting for these independent predictors.

Table III. The Apo AI Polymorphism at +83 bp and the Number of Significantly Diseased Vessels (> 50% Luminal Obstruction)

	Number of significantly diseased vessels (> 50% luminal obstruction)				
	0	1	2	3	Total
Males					
M2+/+	90 (97.8%)	129 (92.8%)	106 (94.6%)	108 (87.1%)	433
M2+/−	2 (2.2%)	10 (7.2%)	6 (5.4%)	16 (12.9%)	34
"M2-" allele	0.011	0.036	0.027	0.065	0.037
Females					
M2+/+	85 (94.4%)	34 (89.5%)	22 (84.6%)	21 (91.3%)	162
M2+/−	5 (5.6%)	4 (10.5%)	4 (15.4%)	2 (8.7%)	15
"M2-" allele	0.039	0.053	0.077	0.044	0.043
Total					
M2+/+	175 (96.2%)	163 (92.1%)	128 (92.8%)	129 (87.8%)	595
M2+/−	7 (3.8%)	14 (7.9%)	10 (7.2%)	18 (12.2%)	49
"M2-" allele	0.025	0.040	0.036	0.061	0.038

The observed numbers of cases and the column percentages (in brackets) are presented. There was a significant association between the polymorphism at +83 bp and the number of significantly diseased vessels in males. Comparing all individual groups, the Pearson χ^2 for males, females and all patients were respectively 10.29, (df = 3, $P = 0.016$), 2.79 (df = 3, $P = 0.424$), 8.40 (df = 3, $P = 0.038$). The Mantel-Haenszel test for a linear association is $\chi^2 = 7.35$ (df = 1, $P = 0.007$) for males; $\chi^2 = 1.27$ (df = 1, $P = 0.259$) for females; and $\chi^2 = 7.06$ (df = 1, $P = 0.008$) for all patients. The results for the combined analysis are statistically significant ($P < 0.01$) —see text.

The MspI1 polymorphism however was not independently associated with CAD severity.

Although in this logistic model there was no interaction between MspI1 and MspI2 polymorphisms in relation to CAD severity, there was an additive effect for patients having triple vessel disease (Table IV). When compared to patients with wild type alleles at both sites (genotypes: M1+/+ and M2+/+) the odds ratios (95%CI) for triple vessel disease patients with rare alleles for both sites (genotypes: M1+/− or M1−/− and M2+/−), those with rare alleles only at +84 bp (genotypes: M1+/+ and M2+/−) and those with rare alleles only at −75 bp (genotypes: M1+/− or M1−/− and M2+/+) were respectively 6.33 (1.33–30.02), 2.53 (0.82–7.82) and 1.12 (0.69–1.82). A similar trend was also observed for the risk of having one or more significantly diseased vessels.

Positive family history of premature CAD and apo AI polymorphisms. Although there was no difference in the frequency of M1−/− (AA) genotypes among patients who had 0 (3.8%), 1 (3.3%), and 2 (3.7%) first degree relatives with a history of premature CAD—defined as onset aged ≤ 65 years—patients who had three or more first degree relatives with premature CAD history tended to have a higher frequency of M1−/− (AA) homozygotes (8.2%). This was reflected in the "M1-" allele frequencies (0.173, 0.198, 0.153, and 0.267 respectively for 0, 1, 2, and 3 or more first degree relatives with CAD). The association was statistically significant only for male patients. The odds ratios for male M1−/− (AA) homozygotes to have three or more first degree relatives with CAD histories compared to these without a positive family history was 5.52 (95%CI: 1.48–20.57). The frequencies of the "M1-" alleles were 0.184, 0.204, 0.144, and 0.358 respectively for male patients having 0, 1, 2, and 3 or more first degree relatives with CAD history ($\chi^2 = 15.34$, df = 6, $P = 0.018$). This relationship was not found in the female patients. There was no M1−/− (AA) homozygote among the 22 female patients who had 3 or more first degree relatives with a premature CAD history.

The MspI2 polymorphism also showed a strong association with a positive family CAD history ($\chi^2 = 15.22$, df = 5, $P = 0.009$). Patients with "M2-" alleles had the highest chance of having three or more first degree relatives with a positive CAD history (OR: 4.94, 95%CI: 2.07–11.80). This relationship remained significant in both men (OR: 4.15, 95%CI: 1.33–13.00) and women (OR: 4.87, 95%CI: 1.18–20.15).

Medical conditions and apo AI polymorphisms. Whilst we found no relationship between the MspI1 polymorphism and the severity of angina as graded by no angina, stable angina and an unstable angina, the MspI2 polymorphism showed a significant association with the presence of unstable angina. The odds ratio for patients of "M2-" allele to have unstable angina was 2.31 (95% CI:1.02–5.26) compared to those with no angina. The relationship was just statistically significant for male patients (OR: 2.79, 95%CI: 1.1–7.8) but not for the female patients (OR: 1.39, 95% CI:0.35–5.48).

Apo AI polymorphisms at both sites were not associated with a past history of myocardial infarction, of hypertension requiring treatment or of diabetes, age, or BMI.

Discussion

Our findings are precisely the opposite of those we had predicted and it is therefore of critical importance to consider the level of confidence that we can place on the accuracy of our results. First there can be no doubt that the genotype distributions at both sites are in strong linkage disequilibrium, and also that they are in Hardy-Weinberg equilibrium indicating that they are normally distributed in this coronary population. Whereas one might have supposed that the allele frequencies would be lower in the coronary population if these genotypes conferred a degree of protection, i.e. associated with an elevated HDL-C, the allele frequencies were not statistically different from the frequencies we have found in a healthy population (22). That the genotypes were not associated with elevated HDL-C and apo AI is perhaps not surprising consid-

Table IV. The Association between the Presence of the apo AI Polymorphisms and the Number of Significantly Diseased Vessels

	Apo AI polymorphisms at −75 bp and +83 bp			
	M1+/+		M1+/−, M1−/−	
CAD severity*	M2+/+	M2+/−	M2+/+	M2+/−
0	121 (30.3%)	5 (15.6%)	55 (28.1%)	2 (11/8%)
1	113 (28.3%)	11 (34.4%)	50 (25.5%)	3 (17.7%)
2	80 (20%)	7 (21.9%)	48 (24.5%)	3 (17.7%)
3	86 (21.5%)	9 (28.1%)	43 (21.9%)	9 (52.9%)
Total	400	32	196	17

*CAD severity is defined as the number of significantly diseased vessels (> 50% luminal obstruction). The observed numbers of cases and the column percentages (in brackets) are presented. M1 is the polymorphism at −75 bp and M2 is the polymorphism at +83 bp. The "+" represents the presence and "−" the absence of MspI restriction site. We grouped patients of M1+/− and M1−/− together in this analysis due to the small number of the M1−/− patients ($n = 25$). The presence of both "M1-" and "M2-" alleles is associated with more severe CAD ($P < 0.01$).

ering the multifactorial aetiology of CAD and many environmental influences tending to lower HDL and apo AI in a coronary population (1-6). But the very significant, and unexpected, association between both genotypes and CAD severity was the principal finding of the study.

These were strong associations when comparisons were made between those patients who had no or mild coronary disease demonstrated at angiography and those individually with one, two, or three significantly stenosed coronary arteries; it was also apparent when all patients with significant disease (> 50% luminal obstruction) were analyzed as a single group. It is relevant too that the significant associations were confined in both cases to the male patients although there were similar trends in the smaller number of females in the series. In the logistic regression analysis the MspI2 polymorphism emerged as having the stronger association independent of all the other variables we included in the model. However, there was an additive effect in that having both genotypes increased the likelihood of having more severe CAD. There is therefore an internal consistency in the findings. This is amplified by the associations found between the alleles of both genotypes and a strongly positive family history of premature coronary disease. It was relevant too that this relationship was only found in the male patients for the MspI1 polymorphism but in both male and female patients for the MspI2 polymorphism consistent with it being the more powerful predictor. This was also reflected in the association between unstable angina and the MspI2 polymorphism.

Our study, of course, provides no information about mechanisms and at this stage they must remain a matter for conjecture. But the mechanisms must relate to functional changes associated with the polymorphisms and with the implication that these changes have different effects in health and disease. Both base changes are at regulatory hot spots (29–32). The base change at −75 bp (MspI1 polymorphism) is known to be associated with altered apo AI transcription (16,33,34) and it is possible that the base change at +83 bp (MspI2 polymorphism) also alters either transcription or translation although this has not yet been established (22). One could postulate that their association is with altered apo AI gene expression such that there is altered susceptibility to the effects of environmental influences. This then could be an example of genetically determined predisposition to environmentally induce cardiovascular risk. However, it will require an appropriate experimental model capable of detecting the effects of different environmental influences to determine whether or not these polymorphisms are responsible for functional changes or are simply markers for them.

In conclusion, base changes at −75 and +83 bp of the apo AI gene are not associated with increased HDL-C and apo AI levels in this representative group of Australian Caucasian CAD patients. Patients with the base changes are far more likely to have severe CAD, and the presence of base changes at both sites is associated with additional risk. Since the MspI1 and MspI2 polymorphisms were in strong linkage disequilibrium and can be determined in a single PCR, it would be of value to analyze their haplotypes for CAD risk assessment in patients. Whilst mechanisms remain to be determined our findings demonstrate that genotype-phenotype relationships observed in a healthy population may not be readily applicable to a diseased population.

Acknowledgments

We wish to thank Ms. Lily Fenech, Ms. Shelly Brown, Mr. Steven Brouwer, Dr. C-J Ma, Dr. Greg Cranny, and all nurses in the Eastern Heart Clinic for their assistance in clinical data collection; Ms. Ah Siew Sim, Ms. Renee Badenhop, and Dr. Jun Wang for their laboratory assistance; and Ms. Joanne Kessey for the data entry. We are also most grateful to the cardiologists in the Department for allowing us to study their patients.

This work was supported by grants from National Health and Medical Research Council of Australia.

References

1. Assmann, G., A. von Eckardstein, and H. Funke. 1993. High density lipoproteins, reverse transport of cholesterol, and coronary artery disease. Insights from mutations. [Review]. *Circulation.* 87(suppl III): III28–34.

2. Badimon, J.J., V. Fuster, and L. Badimon. 1992. Role of high density lipoproteins in the regression of atherosclerosis. *Circulation.* 86(suppl III): III86–94.

3. Forte, T.M., and M.R. McCall. 1994. The role of apolipoprotein A-I-containing lipoproteins in atherosclerosis. *Curr. Opinio. Lipidiol.* 5:354–364.

4. Stampfer, M.J., F.M. Sacks, S. Salvini, W.C. Willett, and C.H. Hennekens. 1991. A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. *N. Engl. J. Med.* 25:373–381.

5. Steinmetz, J., E. Boerwinkle, R. Gueguen, S. Visvikis, J. Henny, and G. Siest. 1992. Multivariate genetic analysis of high density lipoprotein. *Atherosclerosis.* 92:219–227.

6. Tribble, D.L., and R.M. Krauss. 1993. HDL and coronary artery disease [Review]. *Adv. Int. Med.* 38:1–29.

7. Asztalos, B.F., and P.S. Roheim. 1995. Presence and formation of 'free apolipoprotein A-I-like' particles in human plasma. *Arteriosclerosis, Thrombosis & Vascular Biol.* 15:1419–1423.

8. Forte, T.M., R. Goth-Goldstein, R.W. Nordhausen, and M.R. McCall. 1993. Apolipoprotein A-I-cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. *J. Lipid Res.* 34:317–324.

9. Wilson, P.W., K.M. Anderson, T. Harris, W.B. Kannel, and W.P. Castelli. 1994. Determinants of change in total cholesterol and HDL-C with age: the Framingham Study. *J. Gerontology.* 49:M252–257.

10. Breslow, J.L., S. Eisenberg, and E.A. Brinton. 1993. Metabolic determinants of low HDL-C levels. [Review] *Ann. NY Acad. Sci.* 676:157–162.

11. Blangero, J., and S. Williams-Blangero, and M.C. Mahaney. 1993. Multivariate genetic analysis of apo AI concentration and HDL subfractions: evidence for major locus pleiotropy. *Genet. Epidemiol.* 10:617–622.

12. Humphries, S., V. Gudnason, H. Paul-Hayase, N. Saha, and M. Rosse-

neu. 1993. Identification of common genetic polymorphisms that determine plasma levels of apo AI and HDL-C. *In* Human Apolipoprotein Mutants III: Diagnosis and Treatment. C.R. Sirtori, G. Franceschini, B.H. Brewer, Jr. Springer-Verlag, Berlin. 247–256.

13. Pagani, F., G.A. Giudici, F.E. Baralle, and C. Vergani. 1992. Association of a polymorphism in the apo AI gene promoter with hyperalphalipoproteinemia. *Eur. J. Epidemiol.* 8(suppl 1): 54–58.

14. Paul-Hayase, H., M. Rosseneu, D. Robinson, J.P. van Biervliet, J.P. Deslypere, and S.E. Humphries. 1992. Polymorphisms in the apolipoprotein (apo) AI-CIII-AIV gene cluster: detection of genetic variation determining plasma apo AI, apo CII and apo AIV concentrations. *Hum. Genet.* 88:439–446.

15. Talmud, P.J., S. Ye, S.E. Humphries, and EARS Group. 1994. Polymorphism in the promoter region of the apolipoprotein AI gene associated with differences in apolipoprotein AI levels: the European atherosclerosis research study. *Genet. Epidemiol.* 11:265–280.

16. Tuteja, R., N. Tuteja, C. Melo, G. Casari, and F.E. Baralle. 1992. Transcription efficiency of human apolipoprotein A-I promoter varies with naturally occurring A to G transition. *FEBS Lett.* 304:98–101.

17. Xu, C-F., M.N. Nanjee, J. Savill, P.J. Talmud, F. Angelico, M. del Ben, R. Antonini, B. Mazzarella, N. Miller, and S.E. Humphries. 1990. Variation at the apolipoprotein (apo) AI-CIII-AIV gene cluster and apo B gene loci is associated with lipoprotein and apolipoprotein levels in Italian children. *Am. J. Hum. Genet.* 47:429–439.

18. Barre, D.E., R. Guerra, R. Verstraete, Z. Wang, S. M. Grundy, and J.C. Cohen. 1994. Genetic analysis of a polymorphism in the human apolipoprotein AI gene promoter: effect on plasma HDL-cholesterol levels. *J. Lipid Res.* 35: 1292–1296.

19. Lopez-Miranda, J., J.M. Ordovas, A. Espino, C. Marin, J. Salas, F. Lopez-Segura, J. Jimenez-Pereperez, and F. Perez-Jimenez. 1994. Influence of mutation in human apolipoprotein A-I gene promoter on plasma LDL cholesterol response to dietary fat. *Lancet.* 343:1246–1249.

20. Sigurdsson, G. Jr., V. Gudnason, G. Sigurdsson, and S.E. Humphries. 1992. Interaction between a polymorphism of the apo A-I promoter region and smoking determines plasma levels of HDL and APO A-I. *Artherioscler. Thromb.* 12:1017-1022.

21. Wang, X.L., R. Badenhop, K. Humphrey, and D.E.L. Wilcken. 1995. C to T and G to A transitions are responsible for loss of a MspI restriction site in the 5′-region of the human apolipoprotein AI gene. *Hum. Genet.* 95:473–475.

22. Wang, X.L., R. Badenhop, K. Humphrey, and D.E.L. Wilcken. 1996. New MspI polymorphism at +83 bp of the human apolipoprotein AI gene: association with increased circulating high density lipoprotein cholesterol levels. *Genet. Epidemiol.* 13:1–10.

23. Miller, S.A., D.D. Dykes, and H.F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16:1215.

24. Wang, X.L., N.P.B. Dudman, B.L. Blades, and D.E.L. Wilcken. 1989. Changes in the immunoreactivity of apo AI during storage. *Clin. Chim. Acta.* 179:285–294.

25. Wang, X.L., N.P.B Dudman, and D.E.L. Wilcken. 1989. Enzyme-linked immunosorbent assay of apolipoprotein B in blood spotted onto filter paper, suitable for neonatal screening. *Clin. Chem.* 35:1000–1004.

26. Wang, X.L., D.E.L. Wilcken, and N.P.B. Dudman. 1992. An indirect sandwich ELISA for serum and dried blood spot Lp(a) measurements, comparison to a commercial RIA. *Clin. Chim. Acta.* 207:73–86.

27. Wang, X.L., C. Tam, R.M. McCredie, and D.E.L. Wilcken. 1994. Determinants of severity of coronary artery disease in Australian men and women. *Circulation.* 89:1974–1981.

28. Emery, A.E.H. 1976. Hardy-Weinberg equilibrium the estimation of gene frequencies. *In* Methodology in Medical Genetics. An Introduction to Statistical Methods. A.E.H. Emery, editor. Churchill Livingstone, Edinburgh. 3–9.

29. Papazafiri, P., K. Ogami, D.P. Ramji, A. Nicosia, P. Monaci, C. Cladaras, and V.I. Zannis. 1991. Promoter elements and factors involved in hepatic transcription of the human apo AI gene positive and negative regulators bind to overlapping sites. *J. Biol. Chem.* 266:5790–5797.

30. Shemer, R., A. Walsh, S. Eisenberg, J.L. Breslow, and A. Razin. 1990. Tissue-specific methylation patterns and wxpression of the human apolipoprotein AI gene. *J. Biol. Chem.* 265:1010–1015.

31. Sonenberg, N. 1994. mRNA translation: influence of the 5′ and 3' untranslated regions. *Curr. Opin. Genet. & Dev.* 4:310–315.

32. Falcone, D., and D.W. Andrews. 1991. Both the 5′ untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation in vitro. *Mol. Cell Biol.* 11:2656–2664.

33. Angotti, E., E. Mele, F. Costanzo, and E.V. Avvedimento. 1994. A polymorphism (G-A transition) in the −78 position of the apolipoprotein AI promoter increases transcription efficiency. *J. Biol. Chem.* 269:17371–17374.

34. Smith, J.D., E.A. Brinton, and J.L. Breslow. 1992 Polymorphism in the human apolipoprotein AI gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro. *J. Clin. Invest.* 89:1796–1800.

35. Smith, J.D., E.A. Brinton, and J.L. Breslow. 1992. Polymorphism in the human apolipoprotein AI gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro. *J. Clin. Invest.* 89:1796–1800.