The T Allele of the Hepatic Lipase Promoter Variant C-480T Is Associated With Increased Fasting Lipids and HDL and Increased Preprandial and Postprandial LpCIII:B : European Atherosclerosis Research Study (EARS) II

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The *T* Allele of the Hepatic Lipase Promoter Variant C−480T Is Associated With Increased Fasting Lipids and HDL and Increased Preprandial and Postprandial LpCIII:B

European Atherosclerosis Research Study (EARS) II

Hans Jansen, Grace Chu, Christian Ehnholm, Jean Dallongeville, Viviane Nicaud, Philippa J. Talmud, for the EARS Group

Abstract—The common C−480T transition in the hepatic lipase (HL) promoter has been shown to be associated with lower HL activity and increased high density lipoprotein (HDL) cholesterol. We examined the frequency and lipid associations of this HL polymorphism in 385 healthy, young (18- to 28-year-old) men whose fathers had had a premature myocardial infarction (designated cases) and 405 age-matched controls. These individuals were participants in the European Atherosclerosis Research Study II postprandial trial, who had been recruited from 11 European countries in 4 regions (the Baltic; United Kingdom; and central and southern Europe). Overall, the frequency of the *T* allele was 0.207 in controls and 0.244 in cases ($P=0.08$). The *T* allele was associated with higher fasting plasma total cholesterol ($P<0.01$), triglycerides ($P<0.01$), and HDL cholesterol ($P<0.01$). The strongest association was found with apolipoprotein (apo) A-I concentration, which was 10% higher in individuals homozygous for the *T* allele compared with those homozygous for the *C* allele ($P<0.001$). This polymorphism had no effect on the rise in plasma triglyceride levels after a fatty meal. However, before and after the fat load was ingested, levels of particles containing both apoC-III and apoB (LpC-III:B) were higher in carriers of the *T* allele, with homozygotes having 23% and 27% higher levels preprandially and postprandially, respectively, than those homozygous for the *C* allele ($P<0.05$). Thus, our results demonstrate that the C−480T polymorphism in the HL promoter is associated with alterations in plasma lipids and lipoproteins and the accumulation of atherogenic LpC-III:B particles. **(*Arterioscler Thromb Vasc Biol.* 1999;19:303-308.)**

Key Words: hepatic lipase ■ gene promoter ■ polymorphisms ■ postprandial ■ lipoproteins

Hepatic lipase (HL) plays a role in the metabolism of several lipoproteins, thereby affecting plasma lipid levels.[1,2] The plasma HL activity measured after heparin injection is inversely correlated with HDL cholesterol levels[3] and postprandial lipoproteins.[4] High HL activity is also associated with the occurrence of small, dense LDL particles.[5,6] Several mechanisms may contribute to these associations. HL hydrolyzes phospholipids and triglycerides in LDL directly or after transfer to HDL, thus lowering LDL size. HDL phospholipid and triglyceride are preferred substrates for HL,[7] and hydrolysis of phospholipid and triglyceride in HDL may induce cholesterol (ester) efflux to the lipase-containing tissues.[8–11] During this process HDL is converted to smaller particles, which can again take up cholesterol from peripheral tissues.[8,12] In this way, HL is not only a determinant of HDL cholesterol levels but also may be an important element in reverse cholesterol transport. In humans, HL activity is also correlated with postprandial lipid levels and may affect chylomicron remnant clearance.[4,13] Inhibition of HL activity in rats by specific antibodies leads to impairment of chylomicron (remnant) clearance.[14] The influence of HL on postprandial lipids may be direct or indirect. Hydrolysis of chylomicron remnant phospholipids by HL leads to the "unmasking" of apoE, thereby enhancing the binding of these particles to apoE-binding receptors.[15] In addition, HL may act as a ligand for chylomicron remnants by binding the lipoproteins to receptors on the liver.[16–18]

Altogether, HL is an important enzyme with multiple functions affecting the metabolism, composition, and concentration of several lipoproteins. A deficiency of HL often leads to a mild phenotype with elevation of HDL and/or an increase in LDL concentration and buoyancy.[13,19,20] In conjunction with other hyperlipidemia-causing genes, HL deficiency influences plasma lipoprotein levels more strongly, with tri-

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glyceride enrichment of lipoprotein fractions with a $d>1.006$ g/mL, the presence of β-VLDL, and impaired metabolism of postprandial triglyceride-rich lipoproteins.[19] Thus, because of the multiple effects of HL on lipoprotein metabolism, genetic variation in HL expression may influence atherosclerotic risk.

Several variant sites have been identified in the HL gene, but only a few lead to decreased postheparin plasma HL activity.[21–23] Recently, low HL activity was found to be associated with a common C-to-T base substitution in the HL promoter.[24–27] This transition is at position −480 based on the sequence published by Cai et al[28] and at position −514 based on the sequence of Ameis and coworkers.[29] The −514 C-to-T polymorphism was recently shown to be in complete linkage disequilibrium with 3 other polymorphisms in the HL promoter (−250 G to A, −710 T to C, and −763 A to G).[30] To date no effects of any of these substitutions on HL promoter activity in vitro have been reported. In addition to low HL activity, the *T* allele was found to be associated with an increased HDL cholesterol content in males in the Netherlands, Finland, and the United States.[25,31,30] In a Dutch sample of normolipidemic men with coronary artery disease (CAD), the frequency of the *T* allele was higher than in nonsymptomatic controls.[25] In contrast, in a Finnish study of subjects with a family history of myocardial infarction, the occurrence of the *T* allele was the same as in healthy subjects.[31] We carried out our investigation on subjects taking part in the European Atherosclerosis Research Study (EARS) II, a postprandial trial of young, male, university students from 11 European countries (L. Tiret et al, unpublished observations, 1998) whose fathers had documented CAD (designated as cases) and age-matched controls, thus enabling us to examine populations of different European regions. Moreover, because HL deficiency may result in impaired postprandial lipoprotein metabolism,[19] we studied whether the HL polymorphism influenced the lipid response to an oral fat load.

Methods

Subjects

Subjects were participants of EARS II, which was performed in 1993. Four hundred seven male students between the ages of 18 and 28 years whose fathers had proven myocardial infarction before the age of 55 (cases) were recruited from 14 university student populations from 11 European countries. One age-matched control was recruited by random selection from the same university populations (n=415). Tallinn, Estonia (cases/controls, 32/36) and Helsinki (32/33) and Oulu (23/23), Finland, were designated Baltic; Glasgow (31/31), Belfast (33/33), and Bristol (22/23) were designated United Kingdom; Aarhus, Denmark (30/30), Hamburg, Germany (32/32), Ghent, Belgium (32/32), and Zurich, Switzerland (36/36), were designated central Europe; and Lisbon, Portugal (18/18), Reus, Spain (30/33), Naples, Italy (30/30), and Athens, Greece (26/25), were designated southern Europe. The subjects were presumed to have been born in the country where they were studying. Details of lifestyle, smoking habits, alcohol consumption, medication, physical exercise, a personal and family history, and physiological measurements, were established using standardized questionnaires and protocols (Table 1).

Postprandial Tests

At the first visit each participant underwent a standard 75-g oral glucose tolerance test (OGTT) after a 12-hour overnight fast. Venous blood was withdrawn at 0, 30, 60, 90, and 120 minutes for the determination of glucose and insulin concentrations.

TABLE 1. Anthropometric and Lifestyle Characteristics in Cases and Controls

	Cases (n=385)	Controls (n=405)	*P*
Age, y	22.8 (0.1)	22.8 (0.1)	NS
Alcohol consumption, mL/d	20.1 (1.1)	18.0 (1.0)	NS
Smokers, %	26.6 (2.3)	25.0 (2.2)	NS
Physical activity, %			
Minimal	10.2 (1.6)	8.2 (1.4)	NS
Moderate	75.3 (2.2)	76.4 (2.1)	NS
Heavy	14.5 (1.8)	15.4 (1.8)	NS
Body mass index, kg/m^2	23.4 (0.1)	23.3 (0.1)	NS
Waist-hip ratio	0.849 (0.002)	0.851 (0.002)	NS
Triglycerides, mmol/L	1.00 (0.02)	0.96 (0.02)	NS
Total cholesterol, mmol/L	4.54 (0.04)	4.30 (0.04)	<0.001
HDL cholesterol, mmol/L	1.19 (0.01)	1.19 (0.01)	NS
LDL cholesterol, mmol/L	2.90 (0.04)	2.69 (0.04)	<0.001
ApoA-I, mg/dL	99.9 (0.9)	100.3 (0.8)	NS
ApoB, mg/dL	73.6 (0.9)	69.1 (0.8)	<0.001
ApoE, mg/dL	2.90 (0.04)	2.78 (0.04)	NS

Values are mean and (SD).

One week later the oral fat tolerance test was performed. The standard meal had an energy content of 1493 kcal (6186 kJ) consisting of 21.6 g protein, 65.5 g fat (of which 41.64 g was saturated), 56.2 g carbohydrate, and 416.6 mg cholesterol. The drink was made from 1 can of cream (Nestlé double cream), sugar (sucrose), and milk protein (Marvel). Blood samples were withdrawn at 0, 2, 3, 4, and 6 hours for the determination of triglyceride concentration. Levels of particles containing apoC-III and apoB (LpC-III:B) were measured at 0 and 4 hours.

Lipid and Lipoprotein Particle Measures

All fasting lipids and apolipoproteins were measured at baseline of the OGTT, and fasting triglycerides, insulin, and glucose were remeasured at baseline of the OGTT. LpC-III:B's were measured by a noncompetitive (sandwich) ELISA as described previously.[32] In brief, polystyrene microtiter plates were coated with affinity-purified polyclonal antibodies to human apoC-III (1 mg/mL). Duplicate plasma samples were diluted 1:1000, 1:2000, and 1:5000 with 100 mmol/L PBS containing 1% albumin. The samples were added to the wells along with the standards and controls and incubated for 2 hours at 37°C. After incubation, the plates were washed 4 times with PBS, and a rabbit polyclonal apoB antibody conjugated to peroxidase was added. The plates were incubated for 2 hours at 37°C and then washed. Color development was performed for 30 minutes by addition of a peroxidase substrate (*o*-phenylenediamine dichloride, Sigma Chemical Co). The plates were read at 492 nm on an automated microplate reader model EL340 (Bio-Tek Instruments). A pool of 400 different plasma samples was used as a secondary standard. This pool was calibrated using immunopurified LpC-III:B. The apoB concentration of the particles was determined by nephelometry using the BNA system and apoB standards (Behringwerke). The values are expressed as the amount of apoB associated with apoC-III.

DNA Extraction and Genotyping

DNA was extracted by the salting-out method.[33] The DNA was stored diluted in 96-well Beckman plates,[34] divided into aliquots into 96-well Omnigene plates, and dried. The polymerase chain reaction (PCR) mixture consisted of 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 0.1 g/L gelatin (Sigma), 0.2 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 0.02% W-1 (GIBCO), 0.1 U of *Taq* polymerase (Gibco BRL)/10 mL PCR, and 0.2 nmol/L of each of the PCR primers described by Jansen et al[25] for the C−480T variant.

TABLE 2. Genotype and Allele Frequencies of the C−480T Polymorphism of the HL Gene

	No.	*T* Allele Carriers, %	*T* Allele Frequency
All			
Cases	385	43.6	0.244
Controls	405	38.0	0.207
Baltic states			
Cases	85	45.9	0.241
Controls	90	47.8	0.261
UK			
Cases	76	40.8	0.237
Controls	85	35.3	0.200
Central Europe			
Cases	122	46.7	0.266
Controls	125	40.0	0.212
Southern Europe			
Cases	102	40.2	0.226
Controls	105	29.5	0.162

The PCR products were subjected to restriction enzyme analysis by digestion with 2 U of the restriction endonuclease *Nla*III (NEB) per 7 mL of the PCR sample at 37°C for 4 hours and in the buffer recommended by the manufacturer. The samples were then loaded on to an ethidium bromide–prestained microplate array diagonal gel electrophoresis gel as described,[34] electrophoresed for 1 hour and 20 minutes, and marked for the genotypes.

Statistical Analysis

The data were analyzed using the SAS statistical software package (SAS Institute Inc). Observed numbers of each genotype were compared with those expected for Hardy-Weinberg equilibrium in cases and controls from the 4 regions by using the χ^2 test. Allele frequencies were compared between cases and controls by the Mantel-Haenszel test after adjustment for region. Statistical significance for this and all other tests was taken to be $P<0.05$. Triglycerides and LpC-III:B were logarithmically transformed before the tests to remove positive skewness. For postprandial analyses of triglycerides, 2 parameters were calculated: the area under the curve (AUC, in mmol-h/L) above the fasting concentration, calculated by the trapezoidal rule, and the peak (mmol/L), calculated as the highest value minus the fasting value. The C−480T genotype was tested after assuming additive effects of alleles in a codominant model. For lipid analysis, data for cases and controls were pooled because no significant interaction between case/control status and the HL −480 variant could be detected. The means were adjusted for status, age, and center.

Results

Study Population

The study population consisted of 790 subjects, of whom 385 were cases and 405 were controls. The cases and controls did not significantly differ in anthropometric or lifestyle characteristics. Total cholesterol, LDL cholesterol, and apoB were higher in cases than in controls (Table 1). The presence of the C−480T polymorphism was determined in all subjects. Overall, the frequency of the −480T allele was 0.207 in controls and 0.244 in cases ($P=0.08$). The allele frequencies were in Hardy-Weinberg equilibrium in all 4 regions and are presented in Table 2.

TABLE 3. Plasma Lipids and Apolipoproteins According to the C−480T HL Promoter Polymorphism

	Genotype			
	CC	*CT*	*TT*	Test*
Cases:controls	217:251	148:140	20:14	
Triglyceride, mmol/L				
Cases	0.99 (0.03)	1.02 (0.04)	1.02 (0.10)	<0.05
Controls	0.93 (0.03)	0.99 (0.04)	1.13 (0.11)	
Cholesterol, mmol/L				
Cases	4.47 (0.06)	4.62 (0.07)	4.56 (0.18)	<0.01
Controls	4.23 (0.05)	4.40 (0.07)	4.88 (0.22)	
LDL cholesterol, mmol/L				
Cases	2.88 (0.05)	2.96 (0.06)	2.84 (0.16)	NS
Controls	2.65 (0.05)	2.73 (0.06)	2.89 (0.20)	
HDL cholesterol, mmol/L				
Cases	1.16 (0.02)	1.21 (0.02)	1.27 (0.05)	0.01
Controls	1.16 (0.02)	1.22 (0.02)	1.25 (0.08)	
ApoA-I, mg/dL				
Cases	97.9 (1.1)	101.8 (1.4)	108.1 (3.8)	<0.001
Controls	98.0 (1.1)	103.6 (1.4)	108.5 (4.5)	
ApoB, mg/dL				
Cases	72.5 (1.1)	75.5 (1.4)	71.8 (3.7)	<0.05
Controls	67.8 (1.1)	70.8 (1.4)	74.8 (4.4)	
ApoE, mg/dL				
Cases	2.87 (0.06)	2.92 (0.07)	3.04 (0.19)	NS
Controls	2.74 (0.05)	2.82 (0.07)	3.11 (0.22)	

*The *P* values refer to the levels of significance in the different variables after pooling the data for cases and controls.

Relation to Plasma Lipids, Lipoproteins, and Apolipoproteins

The association of the rare −480*T* allele with plasma lipid and lipoprotein levels in cases and controls is shown in Table 3. There was no significant interaction between case/control status and genotype, so cases and controls were pooled to acquire sufficient numbers for *T*/*T* carriers. In the pooled data, the −480*T* allele was associated with higher fasting plasma total cholesterol, triglyceride, and HDL cholesterol. These results conform to a codominant model, because the homozygous *T*/*T* carriers had the highest values followed by the *C*/*T* carriers, and the lowest values were found in the *C*/*C* homozygotes. LDL cholesterol did not differ among the different genotypes. Although in carriers of the *T* allele both plasma triglyceride and HDL were increased, the inverse correlation between fasting triglyceride level and HDL cholesterol concentration was significantly stronger in the −480*T* homozygotes than in heterozygotes or −480*C* homozygotes (correlation coefficients of −0.65, −0.26, and −0.32, respectively; $P<0.05$). The strongest association of the C−480T polymorphism was with apoA-I. In *T*/*T* carriers the apoA-I concentration was 10% higher than in −480*C*/*C* homozygotes ($P<0.001$). ApoB was slightly higher in *T* allele carriers than in *C*/*C* homozygotes, but there was no

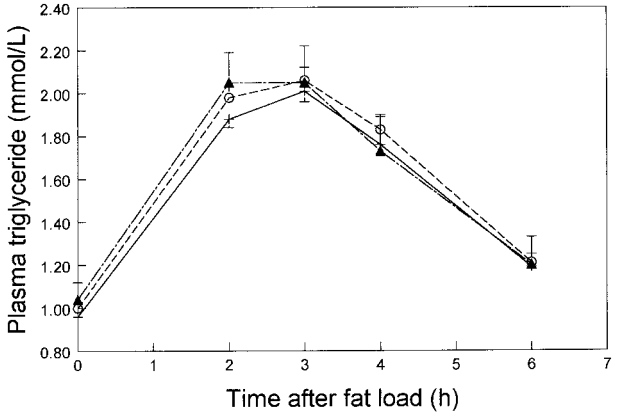

Figure 1. Postprandial changes in plasma triglyceride concentration after the fat tolerance test according to C−480T polymorphism. Mean plasma triglyceride concentrations ±SD are given. -+- indicates *CC* genotype; -○-, *CT* genotype; and -▲-, *TT* genotype. Values are adjusted for age, center, and case/control status. All associations were not significant, whether tested as 3 codominant classes or *T*+ versus *CC*. There were no significant interactions with status, body mass index, waist-hip ratio, or fasting triglyceride level.

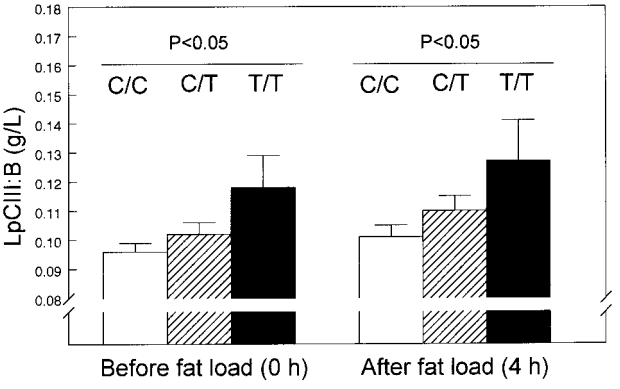

Figure 2. LpC-III:B concentrations according to C−480T polymorphism. LpCIII:B concentrations in different genotypes were measured before and 4 hours after an oral fat load. Open bars indicate *C/C* genotype (n=395); hatched bars, *C/T* genotype (n=244); and solid bars, *T/T* genotype (n=32). Values are means adjusted for age, center, and case/control status. C−480T was tested after assuming codominance. The test was performed on logarithmically transformed values. There were no significant interactions with status, body mass index, or waist-hip ratio.

difference between the *C/T* and *T/T* genotypes. There was no genotype effect on body mass index, waist-hip ratio, or plasma glucose or insulin concentrations.

Postprandial Triglyceride and LpC-III:B Response

Because HL is involved in postprandial lipid clearance, we studied the effects of the C−480T polymorphism on postprandial triglyceride concentrations after a fat tolerance test (Figure 1). There were no significant differences in the mean triglyceride concentrations between the different genotypes when tested either directly or after adjustment for fasting triglyceride. Differences in the AUC, peak height minus fasting concentration, or the time at which the triglyceride value reached its peak did not reached statistical significance among genotypes (Table 4). The association of the polymorphism with the concentration of LpC-III:B was also studied before and after the fat load. Before the fat load, the concentration of LpC-III:B was dose-dependently higher in the −480*T* allele carriers than in noncarriers. Homozygous *T* allele carriers had a 23% higher value than did individuals homozygous for the −480*C* allele ($P<0.05$; Figure 2). After the fat load this difference in LpC-III:B concentration remained, with −480*T* homozygotes having the highest levels of LpC-III:B ($P<0.05$).

TABLE 4. Plasma Triglyceride Concentration and LpC-II:B After the Fat Tolerance Test According to C−480T Genotype

	Genotype			
	CC	*CT*	*TT*	*P**
Cases:controls	207:237	137:129	17:14	
AUC†	3.82 (0.11)	3.99 (0.14)	3.71 (0.41)	NS
Peak height minus fasting value†	1.20 (0.03)	1.25 (0.04)	1.20 (0.12)	NS
Time to peak‡	2.91 (0.04)	2.92 (0.05)	2.52 (0.16)	NS
Cases:controls	184:211	129:115	18:14	
LpC-III:B, g/L				
0 Hour	0.096 (0.003)	0.102 (0.004)	0.118 (0.011)	<0.05
4 Hours	0.101 (0.004)	0.110 (0.005)	0.127 (0.014)	<0.05

*The *P* values refer to the levels of significance in the different variables after pooling the data for cases and controls.

†Test performed on square-root values.

‡Wilcoxon rank-sum test.

Discussion

We determined the frequency of the *T* allele of the HL gene promoter polymorphism, referred to as C−480T[24] or −514C/T,[30] and lipid associations in young, healthy men whose fathers had had a premature myocardial infarction (cases) and in age-matched controls (EARS II; L. Tiret et al, unpublished observations, 1998). Overall, the allele frequencies found were in the same range (≈0.2) as those previously reported in the Netherlands, Finland, and the United States.[25,30,31] The study population consisted of university students recruited in northern (the Baltic), central, and southern Europe and the United Kingdom. When all 4 regions were pooled, no significant difference in the frequency of the *T* allele between cases and controls was found ($P=0.08$). The *T* allele was associated with an increased concentration in plasma triglyceride, total cholesterol, HDL cholesterol, apoA-I, and apoB. Whether or to what extend the changes in plasma lipids are related to atherosclerosis is not clear. On the one hand, HDL cholesterol and apoA-I were increased in *T* allele carriers, suggesting that the *T* allele may be associated with a reduced atherogenic risk. On the other hand, the *T* allele was also associated with higher plasma triglyceride and cholesterol. Moreover, in carriers of the *T* allele, the concentration of LpC-III:B was significantly increased both preprandially and postprandially. ApoC-III is an important determinant of triglyceride-rich lipoprotein clearance. In vitro studies have shown that apoC-III inhibits lipoprotein lipase[35] and HL activities[36] and displaces apoE from the surface of triglyceride-rich lipoproteins. Therefore, in metabolic conditions in which lipolytic activity is decreased, lipoprotein particles that are enriched in apoC-III tend to accumulate. LpC-III:B is associated with CAD.[37–40] Thus overall, the *T* allele is

associated with a number of potentially antiatherogenic and proatherogenic lipoprotein changes in our population. In a population of normolipidemic men with CAD[25] and in white Americans,[30] the *T* allele was also associated with enhanced HDL, but not with total cholesterol and triglyceride. In our population, why effects of the *T* allele on total cholesterol and triglyceride were also found is not clear. Possibly, differences in selection criteria (age, normolipidemia) between the different studies may play a role. Although we did not determine HL activity in our population, the changes in plasma lipids are presumably related to a lower HL activity in the *T* allele carriers, as has been shown to exist in a number of other populations.[25–27] In which way the C−480T substitution or other polymorphisms lead to lowered HL expression is not known. To date, no effects of any of these substitutions on HL promoter activity in vitro have been reported. It is interesting to note that the C-to-T substitution at −480 disrupts a potential upstream stimulatory factor binding site. Upstream stimulatory factor is an insulin-inducible transcription factor involved in the regulation of hepatic lipid and carbohydrate metabolism.[41–43] However, whether this is related to or causes the lowered HL activity in the *T* allele carriers is not clear. Studies on this subject are in progress.

Appendix

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