

ARTICLE

# Polymorphisms in *APOA1* and *LPL* genes are statistically independently associated with fasting TG in men with CAD

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The objective of this paper was to identify the single nucleotide polymorphisms (SNPs) that show unshared effects on plasma triglyceride (TG) levels and to investigate whether these SNPs show statistically independent effects on plasma TG levels. In total, 59 polymorphisms in 20 genes involved in lipid metabolism were investigated. Polymorphisms were selected for a multivariate ANOVA model if they showed an univariate association with TG (after adjustment for HDL-C and LDL-C) in more than 50% of bootstrap samples that were made from the original data. The multivariate model included 512 men with coronary artery disease from the REGRESS study who were completely genotyped for eight polymorphisms selected in the univariate procedure (ie, *APOA1* G(-75)A, *ABCA1* C(-477)T, *ABCA1* G1051A, *APOC3* T3206G, *APOE* Arg158Cys, *LIPC* C(-514)T, *LPL* Asn291Ser and *LPL* Ser447Stop). The gene variants *APOA1* G(-75)A ( $P=0.04$ ) and *LPL* Asn291Ser ( $P=0.03$ ) were significantly associated with plasma TG levels in this multivariate analysis. The eight polymorphisms explained 8.9% of the variation in plasma TG levels. In conclusion, this study showed statistically independent effects of gene variants in the *APOA1* and *LPL* genes on fasting plasma levels of TG. Nevertheless, only a small part of variation in TG levels could be explained by the polymorphisms.

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## Introduction

High plasma triglyceride (TG) levels are a risk factor for coronary artery disease.<sup>1</sup> The regulation of TG levels, and fat metabolism in general is a complex process in which many enzymes, receptors and proteins play a role. For a more elaborate description of the TG level regulation, we refer to Gurr<sup>2</sup> and Mead *et al.*<sup>3</sup> In short, the major carrier of

triglycerides in (fasting) blood is very-low-density lipoprotein (VLDL), which is mainly secreted by the liver.<sup>2</sup> TG from VLDL is hydrolysed by lipoprotein lipase (LPL), which is associated with capillaries mostly in muscle and adipose tissue.<sup>3</sup> The cells of these tissues take up fatty acids and VLDL is degraded to intermediate density lipoprotein (IDL) and by further hydrolysis to low-density lipoprotein (LDL).<sup>2,3</sup> VLDL, like all lipoproteins, is coated with phospholipids and apolipoproteins.<sup>2</sup> These apolipoproteins stabilize lipids in an aqueous environment and recognize and interact with specific receptors on cell surfaces.<sup>2</sup> There are a number of enzymes (eg LPL, CETP, PLTP, LCAT), transport proteins (eg ABCA1) and receptors

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(eg SR-B1, LDL-receptor) that play an important role in the transport and metabolism of TG and cholesterol.<sup>2,4-6</sup>

Fasting TG levels vary considerably between individuals. This variation can be explained by a combination of environmental and genetic factors. Twin studies have estimated the heritability of TG to be more than 50% (reviewed by Snieder *et al*<sup>7</sup>). Several studies have investigated the effect of different polymorphisms on plasma TG levels eg.<sup>8-10</sup> However, these studies were usually restricted to one gene and one or a few polymorphisms within a gene. Whereas, given the complexity of TG regulation, many genes probably play a role. Some of these genes probably show (pleiotropic) effects on TG as well as on HDL-C or LDL-C (which we call shared genes), while others do not (which we call unshared genes). In this study we are interested in the effects of unshared genes. Arya *et al*<sup>11</sup> show that in univariate linkage analysis, the power to detect the unshared genes can be increased by incorporating other correlated traits as covariates. In this view, covariates are used to intensify a genetic effect that may be hidden by the relationship between the phenotype and the covariate.<sup>12</sup> The same is true for association studies where the power of detecting association between an unshared gene (polymorphism) and a phenotype can be increased by reducing residual variation through including correlated traits as covariates.<sup>13</sup> The present study was conducted to identify the single nucleotide polymorphisms (SNPs) that show unshared effects on plasma TG levels and to investigate whether these SNPs show statistically independent effects (ie, effects not caused by linkage disequilibrium (LD)) on plasma TG levels.

## Methods

### Study population

The REGRESS study was a double-blind, placebo-controlled, multicentre trial to assess the effects of pravastatin on progression and regression of angiographically documented coronary atherosclerosis. The study consisted of 884 unrelated Dutch male patients with serum cholesterol between 4 and 8 mmol/l and TG levels lower than 4.0 mmol/l. Details of the study design have been reported previously.<sup>14</sup>

### Selection of genes/polymorphisms

Many polymorphisms in different candidate genes for cardiovascular disease have been determined in the REGRESS study. We identified 20 (candidate) genes that are reported to be involved in lipid metabolism. The 59 polymorphisms that were available in these 20 genes and for which at least 600 patients had been genotyped, were analysed in the present study (see Table 1).

**Table 1** The 59 polymorphisms examined in this study

Gene	Polymorphism	Frequency of least common allele	% bootstrap samples where $P < 0.05$	
APOA1	G(-75)A	0.105	86.2	
	C83T	0.029	12.5	
APOC3	C(-641)A	0.421	18.7	
	C(-482)T	0.246	10.7	
	T(-455)C	0.339	6.7	
	C1100T	0.260	29.1	
	C3175G (SstI)	0.087	12.3	
APOA4	T3206G	0.336	61.5	
	Thr347Ser	0.169	10.1	
APOA5	Gln360His	0.080	37.6	
	T(-1131)C	0.070	42.3	
APOB	Ser19Trp	0.072	36.5	
	Thr71Ile	0.288	16.0	
APOE	Arg3500Gln	0.001	0.0	
	Cys112Arg	0.167	9.9	
ABCA1	Arg158Cys	0.072	85.6	
	C(-477)T	0.448	54.7	
	C17G	0.323	41.5	
	G1051A	0.255	89.1	
	G2706A	0.029	32.6	
	A2715C	0.003	16.4	
	G2868A	0.080	3.0	
	G3911C	0.026	11.6	
	G5155A	0.259	30.9	
	CETP	G(-2708)A	0.310	14.1
		G(-972)A	0.490	7.6
		C(-630)A	0.079	6.6
		C(-629)A	0.437	9.6
Ile405Val		0.281	17.3	
Asp442Gly		0.001	0.0	
CCC784A		0.390	8.0	
ECONI		0.532	6.8	
MSPI		0.182	6.4	
TAQIB		0.399	9.9	
CYP7A1	A278C	0.374	10.7	
LCAT	Ser208Thr	0.030	16.1	
LDLR	C1959T	0.449	11.4	
	NCOI	0.295	6.6	
LIPC	TAQI	0.090	11.4	
	T(-710)C	0.213	49.3	
LPA	C(-514)T	0.213	66.5	
	C93T	0.118	6.0	
LPL	G121A	0.124	29.1	
	T(-93)G	0.018	41.0	
	Asp9Asn	0.024	18.0	
	Asn291Ser	0.029	60.2	
	Ser447Stop	0.095	63.6	
	HIND3	0.289	19.9	
	PVUI	0.491	36.2	
MTP	G(-493)T	0.267	5.6	
PON1	Leu55Met	0.367	25.1	
	Gln192Arg	0.290	41.3	
PON2	Ser311Cys	0.234	12.3	
	PPARA	G2176A	0.078	4.7
PPARG	Leu162Val	0.057	30.7	
	Pro12Ala	0.113	13.5	
	Gly2Ser	0.086	5.2	
SCARB1	C1050T	0.485	34.2	
	Intron 5 (C/T)	0.086	21.3	

### Biochemical and DNA analysis

All lipid laboratory tests were carried out at the Lipid Reference Laboratory. Serum cholesterol, high-density lipoprotein cholesterol (HDL-C), and triglycerides were measured in fasting blood samples by standard techniques and LDL was calculated according to the Friedewald formula.<sup>14</sup>

Within REGRESS, patients have been genotyped for several polymorphisms in genes associated with lipid metabolism. The genotypes were obtained as described earlier.<sup>9,15–32</sup>

### Statistical analysis

A  $\chi^2$  test with one degree of freedom was performed to evaluate whether the genotype counts of the selected polymorphisms were different from those expected under Hardy–Weinberg equilibrium. Baseline TG values were logarithmically transformed to represent a normal distribution. All analyses were performed with the transformed values, but the geometric means and their 95% confidence intervals (CI) are presented. All statistical analyses have been performed using the SAS System (version 8.2 for Windows). In general, genotyping of many polymorphisms leads to few completely genotyped individuals. In this study, the 59 available polymorphisms could not be analysed simultaneously in a multivariate model due to missing values (ie, too few completely genotyped patients were available). Therefore, a polymorphism selection process preceded the multivariate model. This was done in the following way, based on an idea of Hoh *et al.*<sup>33</sup> A thousand bootstrap samples were made from the original data set. In each set, each polymorphism was tested for its effect on TG while the model was adjusted for HDL-C baseline and low-density lipoprotein cholesterol (LDL-C) baseline values (ie, to increase the power to detect unshared effects). A polymorphism was selected for further analyses if a statistically significant ( $P < 0.05$ ) association was found in more than 50% of the bootstrap samples. The bootstrap method was used to minimize the effect of very influential patients and possible departures from ANOVA model. Thus, a more robust selection of polymorphisms was made. The selected polymorphisms were analysed together in a multivariate model, which was again adjusted for HDL-C and LDL-C. This was done, both in the patients from the original REGRESS study that were completely genotyped for the selected polymorphisms, as well as in the thousand bootstrap samples, similar to the univariate procedure. To evaluate whether possible loss of significance in the multivariate analyses was not just an effect of loss of patients compared to the univariate analyses, we also calculated the univariate  $P$ -value in the patients that were completely genotyped for the selected polymorphisms.

For the selected polymorphisms, all possible two-way gene–gene interactions were assessed by backward selec-

tion. The main effect of the selected polymorphisms as well as HDL-C and LDL-C were forced into the model.

To examine whether the patients available for multivariate analysis differed from the others for baseline characteristics, comparisons between selected (ie, those that are completely genotyped for the polymorphisms in the multivariate model) and not selected individuals were made with  $t$ -tests for age, body mass index (BMI) and baseline lipid values (total cholesterol, HDL-C, LDL-C and TG) and with  $\chi^2$  tests (or Fisher's exact where appropriate) for history of smoking, smoking presently, use of several drugs (ie, nitrates, beta blockers, CA inhibitors and ACE inhibitors) and genotype counts.

### Results

A total of 59 polymorphisms have been examined in this study (Table 1). Eight polymorphisms were significantly associated with TG after adjustment for HDL-C and LDL-C in more than 50% of the bootstrap samples. These were *APOA1* G(–75)A, *ABCA1* C(–477)T, *ABCA1* G1051A, *APOC3* T3206G, *APOE* Arg158Cys, *LIPC* C(–514)T, *LPL* Asn291Ser and *LPL* Ser447Stop. The eight polymorphisms were then analysed together in a multivariate analysis, which included a total of 512 men, who were completely genotyped for the eight polymorphisms. Their baseline characteristics are presented in Table 2. These 512 men were not statistically significantly different for age, BMI, total cholesterol, HDL-C, LDL-C and TG baseline levels, history of smoking, present smoking or intake of nitrates, beta blockers, CA inhibitors and ACE inhibitors, from the 372 men that were not available for multivariate analysis. Genotype counts of the eight polymorphisms selected for multivariate analyses differed between these two groups for *APOA1* G(–75)A ( $P < 0.001$ ), but were comparable for the other polymorphisms. Except *ABCA1* C(–477)T, *ABCA1* G1051A and *LPL* Asn291Ser, the observed genotype counts were not statistically significantly different from those expected under Hardy–Weinberg equilibrium.

In the multivariate analysis *APOA1* G(–75)A ( $P = 0.04$ ) and *LPL* Asn291Ser ( $P = 0.03$ ) were statistically significantly associated with TG (Table 3). These were also statistically

**Table 2** Baseline characteristics of the patients in the multivariate analyses ( $n = 512$ )

Characteristic	Selected population ( $n = 512$ )
Age (years) (mean $\pm$ SD)	55.8 $\pm$ 8.0
BMI (kg/m <sup>2</sup> ) (mean $\pm$ SD)	26.0 $\pm$ 2.7
Total cholesterol (mmol/l) (mean $\pm$ SD)	6.0 $\pm$ 0.9
HDL (mmol/l) (mean $\pm$ SD)	0.9 $\pm$ 0.2
LDL (mmol/l) (mean $\pm$ SD)	4.3 $\pm$ 0.8
Triglycerides (mmol/l) (median (range))	1.7 (3.6)

**Table 3** Genotype counts and TG levels as well as results from univariate and multivariate analyses for eight selected polymorphisms

	Homozygous wildtype		Heterozygous		Homozygous mutant		Univariate P-value <sup>b</sup>	Multivariate P-value <sup>b</sup>	Multivariate % bootstrap samples where P < 0.05
	Mean (95% CI) <sup>a</sup>	N	Mean (95% CI) <sup>a</sup>	N	Mean (95% CI) <sup>a</sup>	N			
<i>APOA1</i> G(-75)A	1.63 (1.56–1.70)	394	1.63 (1.51–1.77)	107	2.49 (2.25–2.77)	11	0.02	0.04	64.3
<i>ABCA1</i> C(-477)T	1.58 (1.47–1.71)	142	1.67 (1.59–1.76)	281	1.66 (1.52–1.82)	89	0.22	0.15	34.9
<i>ABCA1</i> G1051A	1.66 (1.58–1.75)	277	1.65 (1.56–1.76)	218	1.34 (1.12–1.60)	17	0.21	0.13	40.8
<i>APOC3</i> T3206G	1.55 (1.46–1.64)	227	1.72 (1.62–1.82)	222	1.77 (1.59–1.97)	63	0.03	0.10	45.5
<i>APOE</i> Arg158Cys	1.63 (1.56–1.70)	446	1.78 (1.60–1.97)	64	1.60 (0.78–3.26)	2	0.13	0.13	48.1
<i>LIPC</i> C(-514)T	1.61 (1.53–1.69)	320	1.71 (1.60–1.82)	168	1.70 (1.45–1.98)	24	0.18	0.13	44.0
<i>LPL</i> Asn291Ser	1.63 (1.56–1.69)	482	1.97 (1.76–2.22)	28	2.76 (1.69–4.52)	2	0.03	0.03	64.8
<i>LPL</i> Ser447Stop	1.67 (1.60–1.74)	421	1.52 (1.38–1.67)	85	2.14 (1.45–3.14)	6	0.02	0.14	36.9

<sup>a</sup>TG levels are presented as geometric mean and 95% confidence interval (CI).<sup>b</sup>In total, 512 complete cases.

significantly associated in more than 50% of the multivariate analyses in the bootstrap samples and in the univariate analysis in the 512 complete cases (Table 3). *APOC3* T3206G and *LPL* Ser447Stop were statistically significantly associated with TG in the univariate analysis in the 512 complete cases ( $P=0.03$  and  $0.02$  respectively), but were not in the multivariate analyses. *ABCA1* C(-477)T, *ABCA1* G1051A, *APOE* Arg158Cys and *LIPC* C(-514)T were not statistically significantly associated with TG in the univariate analysis in the 512 complete cases or in the multivariate analyses (Table 3). Mean TG values (95% CI) and number of cases per genotype for the eight polymorphisms are also presented in Table 3. In the multivariate analysis, after correction for HDL-C and LDL-C, the polymorphisms explained 8.9% of the remaining variation of baseline TG values.

A statistically significant gene-gene interaction between *ABCA1* C(-477)T and *APOC3* T3206G ( $P=0.03$ ) was found. The other gene-gene interactions did not reach statistical significance. When this interaction was included in the multivariate model, the main effects of the polymorphisms and interaction together explained 11.1% of the remaining variation of baseline TG values.

Using the two statistically significantly associated polymorphisms (ie, *APOA1* G(-75)A and *LPL* Asn291Ser), a distinction is possible between two genetic subgroups with clinically relevant differences in TG values. The 471 patients, who were homozygous wildtype or heterozygous for *APOA1* G(-75)A and homozygous wildtype for *LPL* Asn291Ser, had mean TG level of 1.61 mmol/l. In contrast, the 41 patients with at least one 'deleterious' genotype (ie, homozygous mutant for *APOA1* G(-75)A or heterozygous or homozygous mutant for *LPL* Asn291Ser) had average TG levels of 2.14 mmol/l.

## Discussion

This study investigated the effect of multiple SNPs on plasma TG levels. The *APOA1* G(-75)A and *LPL* Asn291Ser polymorphisms were statistically independently related to plasma TG values. *APOC3* T3206G (in LD with *APOA1* G(-75)A) and *LPL* Ser447Stop did not show independent effects. No firm conclusion can be drawn about *ABCA1* C(-477)T, *ABCA1* G1051T, *APOE* Arg158Cys and *LIPC* C(-514)T, because their lack of significance in the multivariate model might be caused by loss of power. The bootstrapping method we used to identify polymorphisms with an unshared effect on TG does not account for polymorphisms that only have significant effects when taking into account the effect of other polymorphisms nor does it identify gene-gene interactions without marginal effects. These kinds of effects are likely in a biological system such as lipid metabolism, but the problem of

missing data, which is very common in genetic association studies, prevented us from investigating these aspects.

To investigate whether the difference in genotype counts between selected and not selected cases for *APOA1* G(-75)A influenced the results, we performed multiple imputation of the missing genotypes of polymorphisms in all 686 patients of whom the *APOA1* G(-75)A polymorphism was available, and subsequently we performed the same multivariate regression analysis on all 686 patients. We found again that *APOA1* G(-75)A was statistically significantly associated with TG in this multivariate analysis and that parameter estimates were very similar to the parameter estimates from analysis with the set of 512 patients (data not shown). Therefore, we conclude that the effect of *APOA1* G(-75)A was estimated correctly.

Three of the eight polymorphisms (ie, *ABCA1* C(-477)T, *ABCA1* G1051A and *LPL* Asn291Ser) selected for the multivariate analyses showed deviations from Hardy–Weinberg equilibrium. In general, deviations from Hardy–Weinberg equilibrium may point to genotyping errors, subpopulation structure or they might occur when the study population is not a random selection of the general population. Since we observed an excess of heterozygotes instead of homozygotes, the deviations from Hardy–Weinberg equilibrium are unlikely caused by genotyping errors. Furthermore, if genotyping errors were present, this would probably decrease power to detect association, since genotyping errors introduce more noise. We checked whether subpopulation structure was present with the method of Pritchard *et al*,<sup>34</sup> but this did not seem to be the case. The subjects in this study all have established coronary artery disease and, therefore, the study population is not a random selection of the general population. This might have caused deviations from Hardy–Weinberg equilibrium. Even so, these deviations from Hardy–Weinberg equilibrium are less relevant to our study since our statistical analyses nowhere assume Hardy–Weinberg equilibrium as we investigate genotype effects and not allele effects.

Homozygous carriers of the A allele in *APOA1* G(-75)A showed higher levels of TG. This was also found by Xu *et al*,<sup>35</sup> but other studies found no association between *APOA1* G(-75)A and TG levels.<sup>36,37</sup> *APOA1* G(-75)A is located in the promoter region of the *APOA1* gene and could therefore influence the expression of the *APOA1* gene. Whether this polymorphism is functional or in LD with a functional polymorphism is uncertain. A possible biological explanation of how this gene variation elevates plasma TG levels is that the binding capacity of the protein to lipases is impaired, resulting in decreased lipolysis and elevated plasma TG levels. On the other hand, Yamamoto *et al*<sup>38</sup> showed that apolipoprotein C-III, apolipoprotein E and apolipoprotein A-I work as a potential inhibitor for the LPL-mediated lipolysis of TG-rich emulsions. This implies that elevated lipoprotein levels in blood lead to elevated

TG levels. For *APOA1* G(-75)A, results concerning the effects of this SNP on apolipoprotein A-I levels are inconsistent.<sup>24,36,39–41</sup> In the present study, no data were available on plasma levels of apolipoproteins. Therefore, the association between the *APOA1* G(-75)A polymorphism and plasma levels of apolipoproteins could not be studied.

In this study, carriers of the *LPL* Asn291Ser mutant allele showed higher levels of TG. Several studies found a similar association,<sup>8,42–44</sup> while others did not find an association between *LPL* Asn291Ser and TG levels.<sup>45,46</sup> Carriers of *LPL* Asn291Ser are found to have modest decreases of LPL activity and higher TG levels.<sup>47</sup> In the present study, data on LPL activity were available. Carriers of the least common allele in the *LPL* Asn291Ser polymorphism showed lower LPL activity, but this was not statistically significant ( $P=0.17$ ).

It is often found in association studies that effects of polymorphisms differ between men and women (eg by Dallongeville *et al*<sup>48</sup>) and between different ethnic groups. Therefore, generalization of the results to healthy individuals, women or ethnic groups other than Caucasian is difficult since this is a study with Caucasian male patients with established coronary artery disease. The sample of men with CAD was chosen, because high TG is a known risk factor for CAD and therefore, rare genotypes will probably have an increased prevalence in such a population. The fact that men were included in the study based on their baseline total cholesterol value poses another problem with generalization to the broad population. The patients all had screening total cholesterol levels between 4.0 and 8.0 mmol/l and therefore these men are not fully representative of the general population. The maximum TG value in the REGRESS population is 4.0 mmol/l. However, using mean and SD for TG from the Framingham Offspring Study<sup>10</sup> and the STANILAS cohort,<sup>49</sup> it is estimated that only 2% of the population had fasting TG levels above 4 mmol/l.

In this study, an explained variance of approximately 9% was found. This percentage is small compared to a heritability of more than 50% that is estimated by twin studies. To identify genes that have previously been associated with TG, we performed a search in Pubmed (MESH headings: polymorphism or mutation and triglycerides) and OMIN (keyword: triglyceride). This search identified 30 genes of which eight were not directly involved in lipid metabolism (eg, *ACE*, *interleukin-6*). Of the remaining 22 genes, only five had not been analysed in the present study (ie, *APOH*, *APOA2*, *FABP2*, *FATP*, *VLDLR*). Besides these 17 genes, we also analysed three additional genes involved in lipid metabolism (ie, *LCAT*, *PON1* and *PON2*). Therefore, the most important candidate genes were included in the present study. Nevertheless, there could be a number of reasons why only a minor part of variation in TG levels could be explained in our study.

Probably environmental factors are very important in determining variation in TG levels and the heritability of more than 50% may be an overestimation. Possibly, (complex) gene-gene interactions or presently unknown genes influence plasma TG levels. The REGRESS study was conducted to evaluate the effect of pravastatin, a lipid-lowering drug. In the present study, baseline TG values were used for analyses, so the results are not biased by the treatment effect.

In conclusion, significant evidence for statistically independent effects of gene variants in the *APOA1* and *LPL* gene on fasting plasma levels of TG has been provided by this study. However, only a small part of variation in TG levels could be explained by the gene variants. Therefore, it can be concluded that a large part of the variation in TG is caused by environmental factors and unknown gene variants.

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