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## The expression of type III hyperlipoproteinemia: involvement of lipolysis genes

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## ABSTRACT

Type III hyperlipoproteinemia (HLP) is mainly found in homozygous apolipoprotein (APO) E2 (R158C) carriers. Genetic factors contributing to the expression of type III HLP were investigated in 113 hyper- and 52 normolipidemic E2/E2 subjects, by testing for polymorphisms in *APOC3*, *APOA5*, *HL* and *LPL* genes. In addition 188 normolipidemic Dutch controls (NDCP) and 141 hypertriglyceridemic (HTG) patients were genotyped as well.

No associations were found for four *HL* gene polymorphisms and two *LPL* gene polymorphisms and type III HLP. The frequency of the rare allele of *APOC3* 3238 G>C and *APOA5* -1131 T>C (in linkage disequilibrium) was significantly higher in type III HLP patients compared with normolipidemic E2/E2 subjects, 15.6% vs. 6.9% and 15.1% vs. 5.8% respectively ( $P < 0.05$ ). Furthermore, the frequencies of *APOA5* c.56 G>C polymorphism and *LPL* c.27 G>A mutation were higher in type III HLP patients, though not significant. Some 58% of the type III HLP patients carried either the *APOA5* -1131 T>C, c.56 G>C and/or *LPL* c.27 G>A mutation as compared to 27% of the normolipidemic APOE2/2 subjects (odds ratio 3.7, 95% confidence = 1.8 – 7.5,  $P < 0.0001$ ). The HTG patients showed similar allele frequencies of the *APOA5*, *APOC3* and *LPL* polymorphisms, whereas the NDCP showed similar allele frequencies as the normolipidemic APOE2/2. Patients with the *APOC3* 3238G>C / *APOA5* -1131 T>C polymorphism showed a more severe hyperlipidemia than patients without this polymorphism.

Polymorphisms in lipolysis genes associate with the expression and severity of type III HLP in APOE2/2.

## INTRODUCTION

Patients with type III hyperlipoproteinemia (HLP) are characterized by elevated levels of total cholesterol and triglycerides due to high plasma levels of chylomicron and very low density lipoproteins (VLDL) remnants enriched in cholesterol esters and APOE.<sup>1,2</sup>

APOE, a major constituent of chylomicron and VLDL remnants, serves as a ligand for the receptor-mediated uptake of these particles by the liver.<sup>3</sup> In type III HLP, APOE mutations lead to an impaired clearance of remnant lipoproteins by hepatic lipoprotein receptors. There are three common genetic variants of APOE: APOE2 (Arg158→Cys), APOE3 (Cys112; Arg158) and APOE4 (Cys112→Arg). These isoforms are encoded by three co-dominant alleles that are located at one single gene locus on chromosome 19. In comparison to the other two isoforms, APOE2 has less than 1% binding capacity for the hepatic low density lipoprotein receptor (LDLR).<sup>4</sup> Most type III HLP patients (> 90%) are homozygous carriers of APOE2 (Arg158→Cys).<sup>4</sup> In Caucasian populations, APOE2 homozygosity occurs with a frequency of about 1%, whereas the frequency of type III HLP is about 1 to 7 per 5000.<sup>5,6</sup> A minority of the APOE2 homozygous subjects will develop type III HLP, indicating that type III HLP is a multifactorial disorder requiring additional genetic and environmental factors for its clinical manifestation.<sup>4,7,8</sup> It has been suggested that contributors to the expression of type III HLP include factors causing (1) an overproduction of lipoproteins, (2) an impaired lipolysis of lipoproteins or (3) an impaired hepatic uptake of remnants.<sup>4,9</sup> Insulin resistance is associated with high triglyceride levels caused by an increased VLDL production.<sup>10,11</sup> Earlier we found an association of high insulin levels with the expression of type III HLP.<sup>5,12</sup>

Several groups studied genetics factors that may contribute to the expression of type III HLP.<sup>13-16</sup> However, in the present study we have studied a larger type III HLP cohort. In addition, we used a

normolipidemic APOE2 homozygote cohort as control group. Mutations in genes involved in lipolytic conversion, such as *LPL* (lipoprotein lipase), *HDL* (hepatic lipase) and *APOC3* have been associated with hyperlipidemia (for reviews see references).<sup>17-20</sup> In addition, Zhang *et al.*<sup>13</sup> observed an increased allele frequency for the *LPL* N291S mutation in type III HLP patients when compared with the general population. SNPs in the *APOA5* gene (11q23) were found to be strongly associated with plasma triglyceride levels.<sup>21,22</sup>

Data from family studies on type III HLP indicate that one or more genes are possible additional genetic factors predisposing to type III HLP.<sup>4</sup> However, from these studies it was not evident which additional genes were involved or the study population was very small. In the present study, a significant population of both normolipidemic and hyperlipidemic E2/2 subjects was collected to determine additional genetic risk factors contributing to the expression of type III HLP in APOE2 homozygotes. For comparison, these genetic risk factors were also typed in hypertriglyceridemic (HTG) patients<sup>23,24</sup> - they partly match their elevated VLDL/TG phenotype with type III HLP patients – and a normolipidemic Dutch control panel (NDCP).

## METHODS

### SUBJECTS

The study population consisted of 167 unrelated homozygous carriers of APOE2 (Arg158 → Cys). Type III HLP patients were defined as having total cholesterol and triglyceride levels  $\geq$  90<sup>th</sup> percentile, VLDL cholesterol/triglyceride ratio  $>$  0.3 (mg.dL<sup>-1</sup>/ mg/dL) and/or VLDL cholesterol/VLDL triglyceride ratio of  $>$  0.8 (mmol/L/mmol/L) whereas normolipidemic E2/2 subjects had total cholesterol and triglyceride levels  $<$  90<sup>th</sup> percentile according to the age- and sex-related percentile levels of the Prospective Cardiovascular Münster Study (PROCAM).<sup>25</sup> Ultracentrifuge data were not available from 24 HLP type III patients. Two HLP APOE2 homozygotes were excluded from the analysis because their plasma triglyceride levels were normal.

In total, 102 type III HLP patients and 9 normolipidemic E2/2 subjects were recruited from the outpatient Lipid Clinics of the University Medical Centers of Leiden, Nijmegen, Amsterdam and Rotterdam. Ten unrelated carriers (two type III HLP patients and eight normolipidemic E2/2 subjects) were detected during a population-based study among 2018 randomly selected 35-year-old men.<sup>26</sup> Forty-four E2/2 subjects (9 type III HLP patients and 35 normolipidemic E2/2 subjects) were collected from the Rotterdam Study, a prospective cohort study of 6870 healthy participants aged 55 years and older, investigating determinants of chronic diseases.<sup>27</sup>

In this study we included 113 type III HLP patients and 52 normolipidemic E2/2 subjects. Clinical data and blood samples from type III HLP patients were collected before lipid-lowering medication. One normolipidemic E2/2 subject and one type III HLP patient with a history of pancreatitis were not included in the study.

The selection of the 141 hypertriglyceridemic (HTG) patients was described earlier.<sup>23</sup> The 188 normolipidemic Dutch controls (NDCP) were selected on APOE genotype, and total plasma cholesterol, triglycerides and HDL levels, between the 25<sup>th</sup> and 75<sup>th</sup> percentile. Informed consent was given by each participant and the study was approved by the Ethics Committee of our hospital.

Vascular disease was defined as the presence of coronary artery disease (angina pectoris, 70% stenosis on coronary arteriography, myocardial infarction, coronary by-pass or percutaneous

transluminal coronary angioplasty) and/or cerebrovascular disease (stroke or transient ischaemic attack) and/or peripheral vascular disease. Hypertension was defined as systolic blood pressure of  $\geq 160$  mm Hg, or diastolic blood pressure of  $\geq 90$  mm Hg, or the use of medication for hypertension.<sup>28</sup> Less than 20% of the Type III HLP patients were treated with antihypertensive medication: 11% with a  $\beta$ -blocker and/or diuretic and 8% with other antihypertensive drugs. About 6% of the normolipidemic subjects were treated with antihypertensive medication. Four patients received anti-diabetic medication.

Hyperinsulinemia was defined as fasting insulin concentrations  $\geq 100$  pmol/L. The diagnostic criterion for diabetes mellitus was fasting blood glucose  $\geq 7$  mmol/L. Smoking was defined as the consumption of at least ten cigarettes per day. The non-smokers also included ex-smokers, who stopped smoking for at least one year. Alcohol consumers were defined as subjects with alcohol consumption of two or more grams per day.

#### APOE PHENOTYPING AND GENOTYPING

APOE phenotypes were determined by isoelectric focusing of delipidated serum samples and after cysteamine treatment followed by immunoblotting with a polyclonal anti-APOE antiserum as described.<sup>29</sup> The results were confirmed by APOE genotyping.<sup>30</sup>

#### LIPID AND LIPOPROTEIN ANALYSIS

With the exception of the sampling of 38 E2/2 subjects originating from the Rotterdam Study, all blood samples were collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 min at room temperature. Total serum cholesterol and triglyceride levels were measured enzymatically, using commercially available kits. Serum HDL-C concentration was measured after precipitation of VLDL and LDL with phosphotungstic acid and  $MgCl_2$ .<sup>31</sup> Three milliliters of serum was ultracentrifuged for 15 hours at 232000 g at 15°C in a TL-100 tabletop ultracentrifuge. The ultracentrifugate was divided in a density ( $d$ )  $< 1.006$  and  $1.006 < d < 1.25$  g/mL fraction, designated as the VLDL and IDL+LDL-HDL fraction, respectively.

#### SERUM INSULIN AND GLUCOSE MEASUREMENTS

Insulin and glucose concentrations were only measured in fasting blood samples. The insulin concentration was determined by a radioimmunoassay (Ins-Ria-100, Medgenix). The antibody of this assay cross-reacts with proinsulin (40%) but not with C-peptide ( $< 0.001\%$ ).<sup>32</sup> Serum glucose was determined by the automated hexokinase method of Hitachi 747, Boehringer Mannheim-Hitachi.

#### DNA ANALYSES

Genomic DNA was isolated from leucocytes according to Miller *et al.*<sup>33</sup> The following mutations or polymorphisms were indentified with PCR followed by restriction enzyme analysis as previously described: The 3238 C>G (Sst-1; rs5128)<sup>34</sup> polymorphism in 3'-UTR of exon 4 of the APOC3 gene, LPL c.27 G>A (D9N; rs1801177), LPL c.1342 C>G (S447X; rs328)<sup>35</sup>, LPL c.874 A>G (N291S; rs268)<sup>13</sup>, HL c.219 G>A (V73M; rs6078), HL -480 C>T (rs8192701)<sup>36</sup>, HL c.1005 A>G (L334F; rs3829462)<sup>37</sup> and HL c.609 C>G

(T202T; rs6084)<sup>38</sup> in respectively the *LPL* and *HL* gene. Analysis of both the *APOA5* polymorphisms -1131 T>C (SNP3; rs662799) in the *APOA5* promoter region and the c.56 G>C (S19W, rs3135506) in exon 3 of the *APOA5* gene, were analyzed as described earlier.<sup>23</sup>

#### STATISTICAL ANALYSES

Differences between groups were tested with the  $\chi^2$  test for dichotomous and categorical variables and the unpaired Student's *t*-test for continuous variables. As total cholesterol, total triglyceride and plasma insulin levels showed non-Gaussian distributions, these parameters were logarithmically transformed before analysis. Untransformed levels are shown in the tables.

For each polymorphism or mutation, the Hardy-Weinberg equilibrium was calculated using the gene-counting method and differences were assessed by  $\chi^2$  test. The  $\chi^2$  test or Fisher's exact test were applied to compare genotype and allele frequencies between type III HLP patients, normolipidemic E2/2 subjects, NDCP and HTG patients. A logistic regression model was used to examine the association between the presence of a mutation and the occurrence of type III HLP. The strength of the association was estimated as the odds ratio (OR) with 95% confidence intervals (95% CI). *P*-values lower than 0.05 were considered significant. Selection of all SNPs in this study was based on literature data, describing association of these SNPs with lipid phenotype. Therefore adjustment for multiple testing was not applied (replication). Statistical analyses were performed with SPSS statistical software (version 14.01; SPSS, Chicago, IL). Linkage disequilibrium (LD) estimations in the *APOC3/A5* region were estimated using Haploview 3.32.<sup>39</sup>

## RESULTS

#### BASELINE CHARACTERISTICS OF THE STUDY POPULATIONS

The mean age of type III HLP patients was lower and their BMI was higher compared with normolipidemic E2/2 subjects (Table 1). The prevalence of vascular disease was increased in type III HLP patients, whereas no differences in the occurrence of hypertension, diabetes mellitus and the number of smokers and alcohol consumers were found between the groups. Furthermore, type III HLP patients had significantly higher total triglyceride and insulin levels compared with normolipidemic E2/2 subjects, whereas HDL-C was decreased. Characteristics of the normolipidemic panel (NDCP) and hypertriglyceridemic (HTG) patients are summarised in Table 1.

#### GENOTYPE AND ALLELE FREQUENCIES

Table 2 shows the genotype distributions and allele frequencies of polymorphisms in the *APOC3*, *APOA5*, and the *LPL* genes in the type III HLP patients, normolipidemic E2/2 subjects, NDCP and the HTG patients. The genotype or allele frequencies of all *HL* polymorphisms and a subset of the analyzed *LPL* polymorphisms did not differ significantly between the study populations and are not shown in Table 2. These include: *HL* -480 C>T, *HL* c. 219 G>A, *HL* c.1005 A>G, *HL* c.609 C>G, *LPL* c.874 A>G and *LPL* c.1342 C>G. All polymorphisms, in all groups, were in Hardy-Weinberg equilibrium.

Distribution of the genotypes of the *APOC3* 3238 G>C, *APOA5* -1131 T>C and *LPL* c.27 G>A polymorphisms differed significantly between the normolipidemic E2/2 subjects and the type III HLP

Table 1 Clinical and biochemical characteristics of type III HLP patients, normolipidemic E2/2 subjects, normal Dutch controls and hypertriglyceridemic patients

	Type III HLP	Normo		NDCP		HTG		N
		N	N	N	N	N	N	
Male	76 (67%)	113	32 (62%)	52	90 (48%)	188	119 (84)	141
Female	37 (33%)	113	20 (39%)	52	97 (52%)	188	22 (16%)	141
Age mean (years)	50.4 **	113	64.0	52	41	187	53	141
Age range (years)	27-80	113	27-80	52	6-78	187	26-78	141
Body mass index (kg/m <sup>2</sup> )	26.9 ± 3.8*	113	25.3 ± 4.0	49	-	-	-	-
Vascular disease	46 (41%)*	113	7 (15%)	39	-	-	-	-
Hypertension	31 (27%)	113	14 (26%)	42	-	-	-	-
Diabetes mellitus	11 (10%)	113	5 (10%)	40	-	-	-	-
Smokers	45 (40%)	112	19 (39%)	43	-	-	-	-
Alcohol consumers	78 (75%)	105	29 (78%)	30	-	-	-	-
Total cholesterol (mmol/L)	11.0 ± 3.5 <sup>a</sup> ,**	113	5.7 ± 1.0	52	5.1 ± 0.72 <sup>a</sup>	188	8.8 ± 3.89	141
Total triglycerides (mmol/L)	6.7 ± 4.7 <sup>a</sup> ,**	113	2.0 ± 0.7	47	1.1 ± 0.35 <sup>a</sup>	188	14.3 ± 13.80 <sup>a</sup>	137
HDL-C (mmol/L)	1.0 ± 0.3 **	113	1.4 ± 0.3	48	1.3 ± 0.23 <sup>a</sup>	188	0.7 ± 0.20	137
Glucose (mmol/L)	5.4 ± 1.2	100	5.2 ± 0.8	14	-	-	-	-
Insulin (pmol/L)	157.2 ± 138.0**	74	62.5 ± 41.4	14	-	-	-	-

Type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects; NDCP: normal Dutch control panel; HTG: hypertriglyceridemic patients. N, total number of subjects with available data about the respective parameter. Body mass index and plasma parameters are presented as mean ± SD.

<sup>a</sup> Selection criterion.

\*  $P < 0.05$ , significantly different from normolipidemic E2/2 subjects.

\*\*  $P < 0.001$ , significantly different from normolipidemic E2/2 subjects.

patients (Table 2). Distribution of the genotypes of the *APOC3* 3238 G>C, *APOA5* -1131 T>C and *LPL* c. 27 G>A, but not the *APOA5* c.56 G>C polymorphisms, differed significantly between NDCP and type III HLP patients. We observed a significant difference in genotype frequency between HTG patients and type III HLP patients for the *APOA5* -1131 T>C polymorphisms but we did not observe such difference for the *APOC3* 3238 G>C, *APOA5* c.56 G>C and *LPL* c.27 G>A polymorphisms.

The frequency of the minor allele of the *APOC3* 3238 G>C polymorphism and the (in LD) *APOA5* -1131 T>C polymorphism were increased in type III HLP patients and the HTG patients (15.6% and 15.1% in the type III HLP, 21.7% and 23.5% in the HTG patients, vs. a mean NDCP / normolipidemic *APOE2* homozygotes of ~7.2 and ~6.1%, respectively). The *APOA5* c.56 G>C polymorphism showed a significantly increased frequency of the minor allele in type III HLP patients (11.8%) and in HTG patients (18.7%) when compared with the mean minor allele frequency of the NDCP / normolipidemic *APOE2* homozygotes (~6.2%). A significant enrichment of the minor allele of the *LPL* c.27 G>A mutation was observed among type III HLP patients and HTG patients (respectively, 6.2% and 4.8%), compared to the other study populations (NDCP and normolipidemic *APOE2* homozygotes respectively, 2.6%, and 1.0%). LD estimation within the tightly linked cluster of the *APOC3/A5* region, confirmed earlier findings of others that the *APOA5* -1131 T>C and *APOC3* 3238 G>C polymorphisms are in strong LD ( $D' = 0.8$ ), whereas the *APOA5* c.56 G>C and -1131 T>C polymorphisms are not.

### ASSOCIATION OF APOC3 3238 G>C, APOA5 -1131 T>C, APOA5 C.56 G>C AND LPL C.27 G>A WITH TYPE III HLP

Association between polymorphisms and the expression of type III HLP was tested by logistic regression analysis using a dominant model (i.e. 1/1 vs. 1/2 + 2/2). Table 3 shows the association of the APOC3 3238 G>C polymorphism (OR = 2.7, 95% CI 1.1 – 6.7, P = 0.03), the APOA5 -1131 T>C polymorphisms (OR = 3.1 95% CI 1.2 – 8.0, P = 0.02), the APOA5 c.56 G>C polymorphism (OR = 2.4, 95% CI 0.9 – 6.7, P =

Table 2 Genotype and allele frequencies

Genotype / Allele frequency <sup>a</sup>	APOE2 homozygotes			
	Type III HLP, N (%)	NORMO, N (%)	NDPC, N (%)	HTG, N (%)
<b>APOC3 3238 G&gt;C</b>				
GG	78 (69.6)	44 (86.3) *	123 (83.9) **	71 (61.7)
GC	33 (29.5)	7 (13.7)	23 (15.4)	38 (33.0)
CC	1 (0.9)	0 (0.0)	1 (0.7)	6 (5.2)
1	189 (84.4)	95 (93.1) *	273 (92.6) *	180 (78.3)
2	35 (15.6)	7 (6.9)	25 (7.4)	50 (21.7)
95 % CI	1.11 – 1.20	1.02 – 1.12	1.05 – 1.12	1.16 – 1.27
<b>APOA5 -1131 T&gt;C</b>				
TT	75 (70.8)	45 (88.2) *	127 (88.2) **	67 (58.3) *
TC	30 (28.3)	6 (11.8)	16 (11.1)	42 (36.5)
CC	1 (0.9)	0 (0.0)	1 (0.7)	6 (5.2)
1	180 (84.9)	96 (94.1) *	270 (93.7) **	176 (76.5) *
2	32 (15.1)	6 (5.9)	18 (6.3)	54 (23.5)
95 % CI	1.10 – 1.20	1.01 – 1.10	1.03 – 1.09	1.18 – 1.29
<b>APOA5 c.56 G&gt;C</b>				
GG	84 (79.2)	46 (90.2)	114 (85.1)	85 (67.5)
GC	19 (17.9)	5 (9.8)	20 (14.9)	35 (27.8)
CC	3 (2.8)	0 (0.0)	0 (0.0)	6 (4.8)
1	187 (88.2)	97 (95.1)	248 (92.5)	205 (81.3)
2	25 (11.8)	5 (4.9)	20 (7.5)	47 (18.7)
95 % CI	1.01 – 1.16	1.01 – 1.10	1.04 – 1.1	1.14 – 1.24
<b>LPL C.27 G&gt;A</b>				
GG	100 (88.5)	51 (98.1) *	144 (94.7) *	103 (90.4)
GA	12 (10.6)	1 (1.9)	8 (5.3)	11 (9.6)
AA	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)
1	212 (93.8)	103 (99.0) *	296 (97.4) *	217 (95.2)
2	14 (6.2)	1 (1.0)	8 (2.6)	11 (4.8)
95 % CI	1.03 – 1.09	0.99 – 1.03	1.01 – 1.04	1.02 – 1.08

95% CI: 95% confidence interval; type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects; NDPC: normal Dutch control panel; HTG: hypertriglyceridemic patients.

<sup>a</sup> 1 represent common allele frequency, 2 rare allele frequency.

\* Significant different genotype / allele frequency (P < 0.05) with type III HLP.

\*\* Significant different genotype / allele frequency (P < 0.01) with type III HLP.

Differences between genotype or allele frequencies are based on linear-by-linear association.



o.12) and the *LPL* c.27 G>A mutation (OR = 6.6, 95% CI 0.8 – 52.1, *P* = 0.07) with the occurrence of type III HLP.

The *APOA5* -1131 T>C and *APOC3* 3238 G>C polymorphisms (in LD) showed a similar significantly increased risk on the occurrence of type III HLP. A significant association between the *APOA5* c.56 G>C polymorphism and the *LPL* c.27 G>A mutation and expression of type III HLP was not seen. However, the association (Table 3, cumulative) of carrying a rare allele of either *APOA5* c.56 G>C, -1131 T>C, *APOC3* 3238 G>C polymorphisms or *LPL* c.27 G>A mutation (OR = 3.7, 95% CI 1.8 – 7.5, *P* < 0.0001) and the expression of type III HLP was significantly increased.

To investigate the effect of the polymorphisms on plasma lipid levels, type III HLP patients were divided in carriers and non-carriers of the polymorphisms. Type III HLP patients with the *APOC3* 3238 G>C polymorphism and (in LD) the *APOA5* -1131 T>C polymorphism showed increased levels of total cholesterol compared with their counterparts without the polymorphism (Figure 1). Total triglyceride levels were significantly increased in carriers of the *APOA5* -1131 T>C polymorphism compared with non-carriers, whereas the increased levels of total triglyceride levels in the *APOC3* 3238 G>C carriers did not reach statistical significance (Figure 2). To further determine the association of the different cholesterol-rich lipoprotein fractions with genetic variation, we also investigated the effect of the polymorphisms, *APOC3* 3238 G>C, *APOA5* -1131 T>C and c.56 G>C, and *LPL* c.27 G>A on plasma HDL and VLDL cholesterol (Figure 3). We observed no effect of the polymorphisms on plasma HDL cholesterol (data not shown) whereas carriers of rare variants of both *APOC3* 3238 G>C and *APOA5* -1131 T>C, showed a significant association with plasma VLDL cholesterol levels. Evaluation of the association of the *APOA5* c.56 G>C, *APOA5* -1131 T>C, *APOC3* 3238 G>C and the *LPL* c.27 G>A variants with plasma lipid levels in the NDCP did not reveal any significant associations. The effect of the polymorphisms on plasma lipid (TG) levels in the HTG patients has been described previously.<sup>23,24</sup>

Table 3 Association of genotypes with the expression of type III HLP

	Type III HLP, N (%)	Normo, N (%)	OR (95% CI)	P-value
<i>APOC3</i> 3238 G>C				
1/1	78 (69.6)	44 (86.3)	2.7 (1.1– 6.7)	0.03
1/2 + 2/2	34 (30.4)	7 (13.7)		
<i>APOA5</i> -1131 T>C				
1/1	75 (70.8)	45 (88.2)	3.1 (1.2–8.0)	0.02
1/2 + 2/2	31 (29.2)	6 (11.8)		
<i>APOA5</i> c.56 G>C				
1/1	84 (79.2)	46 (90.2)	2.4 (0.9–6.7)	0.12
1/2 + 2/2	22 (20.8)	5 (9.8)		
<i>LPL</i> c.27 G>A				
1/1	100 (88.5)	51 (98.1)	6.6 (0.8– 52.1)	0.07
1/2 + 2/2	13 (11.5)	1 (1.9)		
Cumulative †				
non-carrier	48 (42.5)	38 (73.1)	3.7 (1.8 – 7.5)	< 0.0001
Carrier	65(57.5)	14 (26.9)		

Type III HLP: type III hyperlipidemic patients; normo: normolipidemic E2/2 subjects. OR, odds ratio; 95% CI: 95% confidence interval. 1/1: homozygous for the common allele; 1/2: heterozygous for the common and rare allele; 2/2: homozygous for the rare allele. † Cumulative: non-carrier represents non-carrier of the rare allele of either the *APOA5* c.56 G>C, *SNP3*, *APOC3* -1131 T>C or *LPL* c.27 G>A and carrier represents carrier of the rare allele of either the *APOA5* c.56 G>C, *SNP3*, *APOC3* -1131 T>C or *LPL* c.27 G>A.

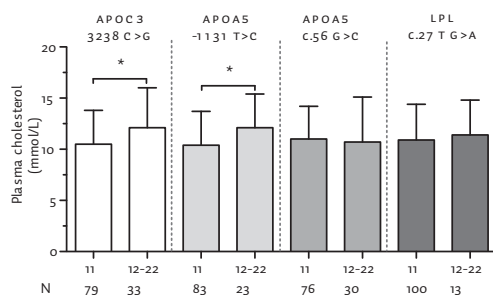


Fig.1. Effect of the *APOC3* 3238 G>C, the *APOA5* -1131 T>C and *APOA5* c.56 G>C, and the *LPL* c.27 G>A polymorphisms on plasma cholesterol levels in type III HLP patients. N represents number of type III HLP patients.

\*P < 0.05.

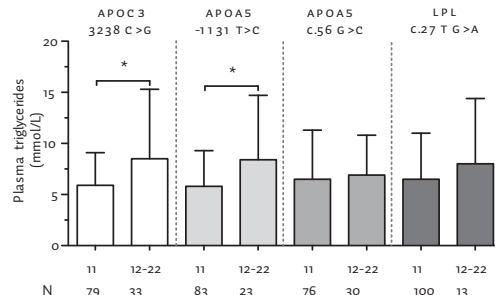


Fig.2. Effect of the *APOC3* 3238 G>C, the *APOA5* -1131 T>C and *APOA5* c.56 G>C, and the *LPL* c.27 G>A polymorphisms on plasma triglyceride levels in type III HLP patients. N represents number of type III HLP patients.

\*P < 0.05.

## DISCUSSION

The clinical manifestation of type III HLP, in addition to defective hepatic clearance of remnant lipoproteins in E2/2 subjects, may be caused by impaired lipolysis.<sup>4,9,40</sup> Several groups reported about LPL and proteins involved in LPL activity, as apoCIII and apoAV in type III patients.<sup>13-16</sup> In accordance with this hypothesis we replicated in the largest type III cohort reported thus far, associations for polymorphisms in proteins involved in the lipolytic conversion of lipoproteins: the 3238 G>C polymorphism in the *APOC3* gene (in LD with *APO5* -1131 T>C polymorphism) and the c.27 G>A mutation in the *LPL* gene. Our results show that the *APOC3* 3238 G>C/*APOA5* -1131 T>C polymorphism exacerbates the hyperlipidemic phenotype of type III HLP patients, while the *LPL* c.27 G>A mutation has no additional effect on plasma lipid levels.

In an earlier study, we found an interaction between hyperinsulinemia and the *APOC3* 3238 G>C polymorphism, associated with severe hyperlipidemia in *APOE2* homozygotes.<sup>12</sup> By increasing the number of E2/2 subjects, data from the present study demonstrate that the *APOC3* 3238 G>C polymorphism *per se* is also an important contributor to type III HLP expression. The *APOC3* 3238 G>C polymorphism is strongly associated with elevated plasma triglyceride levels.<sup>41</sup> In accordance, our type III HLP patients carrying the *APOC3* 3238 G>C polymorphism showed a more severe hyperlipidemia, i.e. higher VLDL-cholesterol levels and a tendency of higher triglyceride levels than non-carriers. The molecular mechanism underlying the association with HTG is still unclear. The *APOC3* 3238 G>C polymorphism is located in the 3'-UTR of the *APOC3* gene and could be a causal variant→ by acting on mRNA stability which results in increasing apoCIII plasma levels. However, it is also possible that the *APOC3* 3238C>G polymorphism itself is not responsible for the triglyceride-raising effect. Other mutations within the *APOA1-C3-A4* gene cluster, located near the *APOC3* 3238 G>C polymorphic site, could be candidates for being the causative mutations leading to HTG. Two polymorphisms located

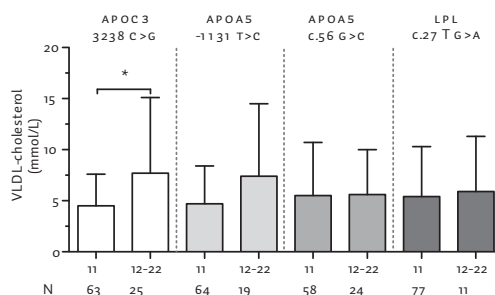


Fig.3. Effect of the *APOC3* 3238 G>C, the *APOA5* -1131 T>C and *APOA5* c.56 G>C, and the *LPL* c.27 G>A polymorphisms on VLDL-cholesterol levels in type III HLP patients. N represents number of type III HLP patients.

\*P < 0.05.

at positions –482 and –455 in the promoter region of the *APOC3* gene showed substantial linkage disequilibrium with the 3238 G>C polymorphism, as reported by others.<sup>41-43</sup> However, we found in a much smaller subgroup of our type III HLP patients, no evidence of an association between these polymorphisms and increased triglyceride levels<sup>12</sup>, whereas Miller *et al.* suggested an association with these two *APOC3* polymorphisms and the metabolic syndrome.<sup>40</sup> Functional studies to elucidate the exact role on apoCIII protein levels of each of these three polymorphisms should be performed.

The *LPL* c.27 G>A mutation leads to an increased bridging of LDL and monocyte adhesion and is also associated with elevated levels of plasma triglycerides and reduced plasma HDL-cholesterol levels.<sup>44</sup> In our type III HLP patients, a tendency towards increased plasma cholesterol and triglyceride levels was observed in *LPL* c.27 G>A carriers, but this did not reach statistical significance, possibly due to the size of our groups. In Caucasians, the –93T G transition in the *LPL* gene promoter is in linkage disequilibrium with the *LPL* c.27 G>A mutation.<sup>18,45</sup> However, several studies have reported that the triglyceride-raising effect was solely attributable to the presence of the *LPL* c.27 G>A mutation.<sup>45</sup>

Data from genetic association studies on type III HLP are limited due to the low prevalence of *APOE2* homozygosity in the population. To circumvent this problem, most studies have compared the allele frequency of mutations in type III HLP patients to that of the general population. Zhang *et al.*<sup>13</sup> found an increased allele frequency for the *LPL* N291S mutation in type III HLP patients compared with the Dutch population. However, this approach does not include the possibility that the mutation is also more prevalent in normolipidemic *APOE2* homozygotes. Conversely, Evans *et al.*<sup>16</sup> showed that the *LPL* mutations D9N, in particular N291S and S447X (c.27 G>A, c.874 A>G and c.1342 C>G resp.) were not associated with type III HLP.

Comparison of the Dutch population frequency for the *HL* –480 C>T mutation (19%) with that of our type III HLP patients (31%) revealed that the mutation was significantly more prevalent in patients ( $P < 0.001$ ).<sup>36</sup> However, the fact that we did not find such a difference between patients and normolipidemic E2/2 subjects indicates that the *LPL* c.874 A>G and the *HL* –480 C>T mutations are not major contributors to the expression of type III HLP (data not shown). Moreover, also no association with type III HLP was found for *HL* c. 219 G>A, *HL* c.1005 A>G, *HL* c.609 C>G and *LPL* c.1342 C>G.

To further study the patient and population prevalences of the *APOA5*, *APOC3* polymorphisms and the *LPL* c.27 G>A mutation, two additional populations were screened: the NDCP and HTG patients. The prevalences of the *APOC3* 3238 G>C/*APOA5* –1131 T>C polymorphism, *APOA5* c.56 G>C and the *LPL* c.27 G>A mutation in the NDCP cohort are similar to those observed in the normolipidemic *APOE2* homozygote individuals. This indicates that the normolipidemic *APOE2* homozygotes were not a selected sub-population. In contrast, the prevalences of the *APOC3*, *APOA4* and *LPL* mutations in the HTG patients are similar to those of the type III patients, indicating that these polymorphisms are universal risk factors for hypertriglyceridemia.

Earlier studies have reported frequencies for the rare variant of the c.56 G>C polymorphism in type III patients.<sup>14,15,46</sup> In our type III patients, the rare variant of *APOA5* c.56 G>C polymorphism is far less frequent (11.8%) than reported thus far. In addition, 4.9% of the normolipidemic *APOE2* homozygotes still was carrier of the rare allele of the *APOA5* c.56 G>C polymorphism. Moreover, we found no association between plasma triglyceride and cholesterol levels and the c.56 G>C polymorphism in hyper- and normolipidemic FDS. The fact that this association is not present and the frequency of the c.56 C-allele is somewhat increased in the type III HLP population suggests that the *APOA5* c.56 G>C polymorphism is a weak contributor to the clinical manifestation of type III HLP. The mechanism how this variant acts remains to be solved. It has been suggested that the *APOA5* c.56 G>C polymorphism

alters the signal peptide which results in a decreased secretion of apoAV protein. However, such correlation between APOA5 rare variant carriers and plasma apoAV, was not found in a cohort of HTG patients.<sup>23</sup>

As reported by others, our data show that the APOC3 3238 G>C and APOA5 -1131 T>C polymorphisms show a high linkage disequilibrium.<sup>41,47</sup> Fifteen percent of type III HLP patients are carriers of the APOA5 -1131 T>C polymorphism and 15.6% are carriers of the APOC3 3238 G>C polymorphism. In the present study we did not aim to elucidate a causal role of each polymorphism.

In total 58% of type III HLP patients vs. 27% of normolipidemic APOE2 homozygotes are carrier of the APOC3 3238 G>C/APOA5 -1131 T>C polymorphism, APOA5 c.56 G>C and/or the LPL c.27 G>A mutation, indicating that these mutations could partly explain the expression of type III HLP. Additional genetic and environmental risk factors remain to be identified. Earlier, we found that hyperinsulinemic APOE2 homozygotes have an increased risk for type III HLP.<sup>6</sup> It is likely that the suppression of VLDL-production by insulin in these hyperinsulinemic patients is reduced, resulting in chronically elevated VLDL-production.<sup>10</sup> Another possible mechanism is through an interaction between insulin and genes involved in the lipolysis of lipoproteins. Several studies support the hypothesis that insulin is involved in the regulation of the APOC3 gene. The presence of the APOC3 -455 and -482 polymorphisms may abolish the insulin responsiveness of the APOC3 promoter.<sup>48,49</sup> Since the APOC3 3238 G>C polymorphism in E2/2 subjects is almost exclusively found in combination with the presence of the -455 and -482 promoter variants, it seems possible that in E2/2 subjects, who are carriers of the 3238 G>C polymorphism, the loss of insulin regulation results in overexpression of the APOC3 gene and, as a consequence, in overt hyperlipidemia.<sup>12,34</sup> In addition, insulin is a major regulator of LPL activity. The combination of high insulin levels and decreased LPL activity, as found in carriers of LPL mutations, may further exacerbate the expression of HTG.<sup>50</sup>

In conclusion, our data indicate that the APOC3 3238 G>C, APOA5 -1131 T>C and to a lesser extent the LPL c.27 G>A mutation associate with the elevated plasma TG phenotype of type III HLP patients, whereas APOA5 c.56 G>C is perhaps a weak modifier.

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