

## CEREBROVASCULAR ATHEROSCLEROSIS IN TYPE III HYPERLIPIDEMIA IS MODULATED BY VARIATION IN THE APOLIPOPROTEIN A5 GENE

D. Evans<sup>1</sup>, A. Bode<sup>1</sup>, G. von der Lippe<sup>1</sup>, F. U. Beil<sup>1</sup>, W. A. Mann<sup>2</sup>

<sup>1</sup>Endokrinologie und Stoffwechsel, Medizinische Klinik III, Zentrum für Innere Medizin, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany

<sup>2</sup>Endokrinologikum, Frankfurt, Germany

### Abstract

**Objective:** Type III Hyperlipoproteinemia is a rare lipid disorder with a frequency of 1-5 in 5000. It is characterized by the accumulation of triglyceride rich lipoproteins and patients are at increased risk of developing atherosclerosis. Type III HLP is strongly associated with the homozygous presence of the  $\epsilon 2$  allele of the *APOE* gene.

However only about 10% of subjects with *APOE2/2* genotype develop hyperlipidemia and it is therefore assumed that further genetic and environmental factors are necessary for the expression of disease. It has recently been shown that variation in the *APOA5* gene is one of these co-factors. The aim of this study is to investigate the development of cerebrovascular atherosclerosis in patients with Type III hyperlipoproteinemia (Type III HLP) and the role of variation in the *APOA5* gene as a risk factor.

**Methods:** 60 patients with type III hyperlipidemia and *ApoE2/2* genotype were included in the study after informed consent. The presence of cerebrovascular atherosclerosis was investigated using B-mode ultrasonography of the carotid artery. Serum lipid levels were measured by standard procedures. The *APOE* genotype and the 1131T>C and S19W SNPs in the *APOA5* gene and the *APOC3* sstI SNP were determined by restriction isotyping. Allele frequencies were determined by gene counting and compared using Fisher's exact test. Continuous variables were compared using the Mann Whitney test. A p value of 0.05 or below was considered statistically significant. Analysis was performed using Statistica 7 software.

**Results:** The incidence of the *APOA5* SNPs, -1131T>C and S19W and the *APOC3* sstI SNP were determined as a potential risk modifier. After correction for conventional risk factors, the C allele of the -1131T>C SNP in the *APOA5* gene was associated with an increased risk for the development of carotid plaque in patients with Type III HLP with an odds ratio of 3.69. Evaluation of the genotype distribution was compatible with an independent effect of *APOA5*.

**Conclusions:** The development of atherosclerosis in patients with Type III HLP is modulated by variation in the *APOA5* gene.

**Key words:** Type III Hyperlipoproteinemia, Apolipo-

protein A5, Apolipoprotein C3, atherosclerosis, gene variation

### INTRODUCTION

Type III HLP is a rare disorder of lipid metabolism with a frequency of approximately 1-5 in 5000 and is characterized by the accumulation of triglyceride rich lipoprotein remnant particles in the form of  $\beta$ -VLDL and homozygosity for the  $\epsilon 2$  allele of the *APOE* gene [reviewed in 1]. Patients present with elevated and approximately equal levels of plasma cholesterol and triglycerides and are at increased risk of developing atherosclerosis. Almost all patients with Type III HLP are homozygous for the  $\epsilon 2$  allele of the *APOE* gene although only about 10% of subjects with *APOE2/2* genotype develop the condition [1] and it is assumed that further genetic and/or environmental factors are necessary for the expression of disease. It has been shown that variation in the *APOA5* gene is one of these co-factors [2, 3]. Apolipoprotein A5 (apoA5) contributes to triglyceride metabolism in both humans and animal models. In humans, apoA5 mutations show an association with elevated plasma triglyceride levels in most studies confirming the role of apoA5 in lipid metabolism. Family studies of apoA5 variants indicated a variable mode of inheritance and a low penetrance of hyperlipidemia. Thus co-existence of other hyperlipidemic factors such as diabetes mellitus, environmental factors or additional disturbances in lipid metabolism are required for expression of hyperlipidemia. The recessive mode of inheritance and the low penetrance may explain why the results from human studies are not as clear as predicted from animal studies [4]. This is especially true for clinical consequences of hyperlipidemia: whereas some studies showed an association of apoA5 with atherosclerosis, others did not [5-11]. One explanation of the variable findings is the complex association of *APOA5* variants with other apolipoprotein genes: the *ApoA5* gene is part of the *APOA/APOC3/APOA4/APOA5* gene cluster on chromosome 11q23. A number of studies, reviewed by Lai et al [12], have investigated the haplotype structure of this region. Whereas the *APOA5\*3* haplotype, defined by the S19W SNP, is independent of the *APOC3* SNPs, the *APOA5\*2* haplotype defined by the -1131T>C SNP is strongly associated with the

APOC3 sstI SNP (rs5128) with 85% of the chromosomes with APOA5\*2 containing the minor allele of sstI. It is therefore of interest to determine the frequency of the APOC3 sstI SNPs in order to evaluate the effect of variation in the APOA5 gene. Further insight into the role of apo A5 can be obtained by analysing well defined patient entities in order to restrict other variables. As it is unclear if the overrepresentation of ApoA5 variants in patients with Type III HLP is associated with increased atherosclerosis, we investigated this question in a patient collective with Type III HLP.

The aims of our study were therefore firstly to determine the incidence of atherosclerosis as determined by B-mode ultrasonography of the carotid artery and secondly to investigate the factors which lead to the development of carotid plaque in this high risk population with a special focus on *APOA5* variants.

## METHODS

### PARTICIPANTS

Patients with mixed hyperlipidemia and *APOE* 2/2 genotype who have attended the lipid clinic, Universitätsklinikum Hamburg-Eppendorf, since 1997 were eligible for the study. Details of this patient group have been described previously [2, 13]. 60 patients with type III HLP were included into the study. All Probanda gave informed consent and the study was approved by the Ethik-Kommission of the Ärztekammer Hamburg.

### CAROTID ATHEROSCLEROSIS

To eliminate inter-operator variability, carotid b-mode ultrasonography was performed by a single trained sonographer unaware of the study design using a Vivid III expert from GE, using a 7,5 MHz transducer. Patients were measured bilaterally at three levels, following the protocol used in the Framingham study [14]. Intima-media thickness (IMT) is presented as the mean in mm of the measurements obtained for the common carotid artery (CCA) and the internal carotid artery (ICA). The common carotid arteries, both bifurcations and the internal carotid arteries were evaluated for the presence of plaque at the time of the ultrasound measurement.

### BIOCHEMICAL MEASUREMENTS

Plasma cholesterol (TC) and triglycerides (TG) were determined using the GPO-PAP and CHOD-PAP kits respectively from Boehringer Mannheim. HDL was determined following precipitation of apo B containing lipoproteins with phosphotungstate (Boehringer Mannheim). Lp(a) was determined using the Beckman Array 360 (Beckman Instruments).

### GENOTYPING

The *APOE* genotype, the 1131T>C and S19W SNPs in the *APOA5* gene, and the sstI SNP in the *APOC3* gene were determined as described [2, 15,16].

## STATISTICAL METHODS

Allele frequencies were determined by gene counting and compared using Fisher's exact test. Continuous variables were compared using the Mann Whitney test. A p value of 0.05 or below was considered statistically significant. Analysis was performed using Statistica 7 software.

## RESULTS

The clinical characteristics of the individual patients are presented in Table 1. Plaque was detected in the carotid bulb in 25 (42%) of patients. The mean IMT of patients with plaque was greater (ACC 0.88 mm, bulb 1.40 mm) than those without (ACC 0.75 mm bulb 0.99 mm),  $p = 0.0001$  for the difference in ACC and 0.00002 for the difference in the carotid bulb.

Comparing the incidence of the traditional risk factors for the development of atherosclerosis in Type III HLP patients with and without plaque there was no significant difference in the proportion of smokers/ex-smokers to never smokers, (15/25 and 24/35 respectively) or women (8/25 and 10/35). There were no significant differences in the levels of Lp(a) or in the occurrence of type 2 diabetes, (2/25 and 6/35), and hypertension, (8/25 and 7/35). Although, as is usual with Type III HLP, mean BMI was high there was no significant difference between patients with plaque ( $27.9 \pm 3.5$ ) and those without ( $28.0 \pm 4.3$ ). By definition all patients had elevated plasma levels of total cholesterol and triglycerides. There were no significant differences in total cholesterol, triglycerides and HDL cholesterol between patient groups. However, comparison is difficult since for clinical reasons lipid lowering therapy could not be discontinued for a proportion of the patients. However for 16 patients with and 24 without plaque lipid values were obtained in the absence of lipid lowering therapy. Patients with plaque had higher total cholesterol,  $400 \pm 174$ mg/dl compared to patients without,  $291 \pm 124$ ,  $p = 0.047$ . There was no significant difference in triglyceride levels. No further subgroup analysis of lipid levels was performed due to small patient group size. Patients with plaque were significantly older, mean age 54 years compared to 46 years for those without,  $p = 0.002$ .

The frequency of the -1131T>C SNP in *APOA5* was compared in patients with plaque to that in those without (Table 2 a,b). The C allele was significantly more frequent in patients with plaque, 0.24 (12/25), than in those without, 0.10 (7/35),  $p = 0.035$ . The odds ratio for carriers of the C allele to develop plaque was 3.69. In multiple regression analysis with presence or absence of plaque as dependent variable and age, BMI, smoking, hypertension, DM2, sex, Lp(a) and *APOA5*-1131T>C genotype as independent variables only age ( $p = 0.002$ ) and *APOA5*-1131T>C genotype ( $p = 0.041$ ) were significant factors. Inclusion of total cholesterol and triglycerides in the analysis but exclusion of patients from whom we had no lipid values in the absence of lipid lowering therapy ( $n = 40$ ) reduced the significance of age,  $p = 0.032$  and increased the significance of *APOA5* genotype,  $p = 0.023$ . No other factors were statistically significant.

Table 1. Clinical characteristics of patients with bulbar plaque present or absent.

<b>A) Plaque present</b>														
Pat Num	AGE years	SEX M/F	IMT ACC mm	IMT -bulb mm	Lp(a) mg/dl	Smoker s/ex/n	D M 2	HT	KHK	AVK	CVI	Chol	Trig	HDL
101	37	M	0.75	0.90	105	N	-	-	-	-	-	481	588	69
76	41	M	0.93	1.83	6	Y	-	+	-	+	-	265	674	54
90	44	M	0.75	0.88	54	Y	-	-	+	-	-	426	418	41
36	44	M	0.67	1.45	2	Y	-	-	-	-	-	418	983	53
35	45	M	0.70	1.50	<2	EX	-	+	+	+	-	268	558	46
44	45	M	0.83	1.48	3	Y	-	-	-	-	-	439	476	36
28	47	M	0.85		15	Y	-	-	-	-	-	316	634	53
86	50	F	0.80	0.95	2	Y	-	-	-	-	-	484	636	71
63	50	M	0.78	1.10	<2	N	-	-	-	-	-	240	364	37
4	52	F	0.95	1.75	11	N	-	+	-	-	-	651	433	44
87	52	M	0.83	1.03	<2	N	-	+	-	-	-	182	418	41
20	53	F	1.08	1.90	4	Y	-	-	-	-	-	503	360	47
45	53	M	0.95	1.00	8	Y	-	-	-	+	-	273	315	37
71	55	M	1.10	1.60	3	EX	-	+	-	-	+	441	716	68
27	55	M	0.95	1.45	<2	N	+	+	+	-	-	317	560	40
48	55	F	1.25	1.83	<2	N	-	-	-	-	-	428	460	42
37	57	F	0.90	1.43	11	Y	-	-	-	-	-	435	388	46
52	59	M	1.08	1.63	<2	Y	-	-	-	-	+	137	69	57
38	48	M	0.83	1.23	15	Y	-	-	-	-	-	292	428	39
93	61	F			32	Y	-	+	-	-	-	341	777	33
6	62	F	0.70	1.05	22	N	-	-	-	-	-	336	411	50
16	62	M	0.80	1.13	<2	N	-	-	-	-	-	416	405	49
89	65	M	0.83	1.28	9		-	-	+	-	-	393	555	44
3	69	M	0.92	2.15	10	EX	-	+	-	-	-	331	548	41
91	76	F	0.90	1.55	7	N	+							
<b>Mean n</b>	<b>53.5 25</b>	<b>17/8</b>	<b>0.88</b>	<b>1.40</b>	<b>13</b>		<b>12/3/9 2</b>	<b>8</b>	<b>4</b>	<b>3</b>	<b>2</b>	<b>367</b>	<b>507</b>	<b>47</b>
<b>B) Plaque absent</b>														
Pat Num	AGE years	SEX m/f	IMT ACC mm	IMT- Bulbus mm	Lp(a)	Smoker s/ex/n	DM2	HT	KHK	AVK	CVI	Chol	Trig	HDL
46	28	M	0.80	1.58	10	Y	-	-	-	-	-	246	143	41
100	30	M	0.73	1.05	19	Y	-	-	-	-	-	694	1118	44
40	31	M	0.77	0.80	4	Y	-	-	-	-	-	201	261	29
72	32	M	0.67	0.85	3		-	-	-	-	-	446	729	51
33	34	M	0.70	0.88	7	Y	-	-	-	-	-			
62	36	M	0.60	0.67	10	N	-	-	-	-	-	202	177	65
60	37	M	0.67	1.05	3	Y	-	-	-	-	-	225	293	37
65	38	F	0.72	0.93	<2	EX	-	+	-	-	-	377	949	40
97	38	M	0.60	0.83	2	EX	-	-	-	-	-	408	402	69
21	39	M	0.70	0.98	3	Y	-	-	-	-	-	143	266	32
73	39	F	0.75	0.88	10	EX	-	-	-	-	-	579	519	55
102	40	M	0.78	0.93	19	N	-	-	-	-	-	196	165	52
5	41	F	0.65	0.65	27	N	+	-	-	-	-	598	939	57
14	41	F	0.73	0.98	9	EX	-	-	-	-	-	255	120	63
50	42	M	0.68	1.05	15	EX	-	+	+	-	-	251	407	29
8	42	M	0.65	0.65	<2	Y	-	-	-	-	-	154	258	41
95	42	F	0.90	1.55	11	EX	-	+	+	-	-	286	473	35
61	43	M	0.63	0.92	4	EX	-	-	-	-	-	360	678	57
12	43	M	0.80	0.98	7	Y	-	-	-	-	-	149	92	56
64	44	M	0.80	0.73	17	Y	+	+	-	-	-	444	932	49
47	47	M	0.78	0.95	4	N	-	-	-	-	-	483	641	35
85	47	M	0.73	0.78	<2	EX	-	-	-	-	-	380	727	50
94	48	M	1.05	1.03	<2	N	-	+	-	-	-	285	313	54
11	50	M	0.77	0.93	14	Y	-	-	-	-	-	343	513	40
1	50	F	0.60	0.85	8	Y	-	-	-	-	-	532	311	57
53	51	M	0.92	1.00	49	EX	-	-	+	-	-	252	198	41
25	51	M	0.70	0.98	<2		-	+	-	-	-	414	682	38
98	56	M		0.70	<2	Y	+	-	-	-	-	399	762	48
34	56	F	0.77	1.40	8	EX	+	-	+	-	-	665	1640	78
59	58	M	0.85	1.30	<2	N	-	-	-	-	-	204	282	62
31	60	F	0.80	1.05	52	N	-	-	-	-	-	397	283	70
23	61	M	0.73	1.03	56	EX	+	+	-	-	-	442	709	50
74	63	F	0.80	1.23	15	EX	-	-	-	-	-	211	323	42
70	70	F	0.83	1.43	13	N	-	-	-	-	-	473	609	65
66	74	M	0.85	1.08	5	N	+	-	+	-	+	276	752	56
<b>Mean n</b>	<b>46 35</b>	<b>25/10</b>	<b>0.75</b>	<b>0.99</b>	<b>12</b>		<b>12/12/10 6</b>	<b>7</b>	<b>5</b>	<b>0</b>	<b>1</b>	<b>354</b>	<b>558</b>	<b>50</b>

Patients are listed in order of increasing age. + = presence of DM2 or hypertension ( HT); IMT in mm; Lp(a) in mg/dl

Table 2. APOA5 and APOC3 polymorphism frequencies in patients with bulbar plaque present or absent.

A) Plaque present				C: Summary of APOA5 and APOC3 polymorphism frequencies				
Pat Num	APOC3 sstI rs5128	APOA5 1131T>C rs662799	APOA5 S19W rs3135506	APOC3 sstI	APOA5 -1131T>C	APOA5 S19W	Plaque Present	Plaque Absent
101				GG	TT	SS	9	18
76				GG	TC	SS	2	4
90	GC	TC		GG	TT	SW	4	5
36		TC		GG	TC	SW	0	2
35				GG	TT	WW	0	1
44	GC	TC						
28				GC	TT	SS	0	4
86			SW	GC	TC	SS	9	1
63	GC	TC		GC	TC	SW	1	0
4	GC	TC						
87	GC	TC						
20	GC	TC						
45	GC	TC						
71		TC						
27								
48			SW					
37	GC	TC						
52								
38			SW					
93	GC	TC						
6								
16								
89								
3			SW					
91	GC	TC	SW					
n	10	12	5					
B) Plaque absent								
Pat Num	APOC3 sstI rs5128	APOA5 1131T>C rs662799	APOA5 S19W rs3135506					
46								
100								
40								
72			WW					
33		TC						
62	GC							
60								
65								
97	GC							
21	GC	TC						
73								
102								
5			SW					
14		TC						
50			SW					
8		TC						
95								
61		TC						
12								
64								
47	GC							
85		TC	SW					
94								
11								
1								
53								
25								
98								
34	GC							
59								
31								
23			SW					
74		TC	SW					
70			SW					
66			SW					
n	5	7	7/1					

There was no significant difference in the frequency of the W allele of the S19W SNP between patients with plaque 0.10 and those without 0.14. The frequency of the minor allele of the APOC3 sstI SNP was significantly higher in Type III HLP patients with plaque, 0.2, compared to those without, 0.07 ( $p = 0.03$ , Fisher's exact, two-tailed, Table 2c). Comparing the distribution of the genotypes APOC3GG/APOA5TT/APOA5SS and APOC3GC/APOA5TC/APOA5SS amongst patients with or without plaque, we found an equal distribution in patients with plaque, 9 with each genotype whereas in patients without plaque there was only one double heterozygote compared to 18 with the common haplotype ( $p = 0.003$ , Fisher's exact, two-tailed). All four patients with genotype APOC3GC/APOA5TT/APOA5SS did not have plaque.

## DISCUSSION

The principal finding of this investigation is that the -1131T>C SNP in the APOA5 gene is a risk factor for the development of carotid plaque in patients with Type III HLP. Although this SNP has been consistently associated with elevated triglycerides and lower HDL in a number of studies [13, 17-20], its association with the development of atherosclerosis has been inconsistent with positive associations being reported in some, but not all studies [5-12]. The inconsistent results reported may be explained by a relatively modest functional influence of such a common genetic variant and by the recessive mode of disease expression as well as inheritance. These characteristics of apoA5 will result in considerable genetic and phenotypic variability in the population under study. With this background a major advantage of our study is that Type III HLP patients represent a well-defined, homogeneous group. This patient collective has a number of conventional risk factors in common providing a uniform background in which to investigate the role of variants in candidate genes. By investigating patients with the same APOE genotype one source of genetic variability is eliminated. This applies not only to the reduction of variability for dyslipidemia but also for the clinical consequence of atherosclerosis since the APOE genotype has been associated in a number of studies with carotid phenotype [reviewed in 21].

Although APOE 2/2 genotype is required for the development of Type III HLP only approximately

10% of *APOE* 2/2 subjects suffer from the condition implying that additional genetic and/or environmental factors are necessary for its expression [1]. We suggest that variation of apoA5 may be one of these factors not only for expression of hyperlipidemia [2] but also for the clinical endpoint of atherosclerosis. How could apoA5 modulate disease expression in type III HLP? It has been shown that apoA5 upregulates lipolysis by binding to heparin sulphate proteoglycans (HSPG, [22]). The interaction of apoE with HSPG plays an important role for the expression of type III HLP in addition to its reduced binding to lipoprotein receptors [23]. We postulate that intact apoA5 may be able to at least in part compensate the reduced HSPG interaction of ApoE2, whereas apoA5 variants with either reduced synthesis or structural defects may not, thus resulting in the necessary second defect for expression of type III HLP. Proof of this principle has been shown for lipoprotein lipase (LPL) variants, where defects in LPL were detected in patients with type III HLP as second dyslipidemic factor in addition to the presence of apoE 2 [24]. Recent in vitro structure – function analysis of apoA5 variants is compatible with the postulated coordinated interaction of apoE, LPL and apoA5 [25]. These variants, which do not induce hyperlipidemia in isolated form or may not be identifiable as risk factor in a unselected population, may be unmasked in combination with variants in other target genes as in the presented study. In case of the -1131T/C exchange, which is located in the promotor region of ApoA5 reduced synthesis of apoA5 may result in decreased protein levels and thus reduced coordinated protein-protein or protein-HSPG interactions. Alternatively synthesis rates may be influenced by the A>G exchange at position – 3, which is linked to the -1131T/C exchange as part of the APOA5\*2 haplotype. Results from in vitro expression studies do not show a functional effect of the single variant, however a cooperative effect of these variants on protein synthesis cannot be ruled out [26]. In addition the APOA5\*2 haplotype is in significant linkage disequilibrium with the sstI polymorphism and two promotor variants in the APOC3 gene, the 482 T and the -455 C allele [27]. These variants are located in the insulin responsive element of the promotor region of the APOC3 gene and are associated with plasma triglyceride levels [28]. To evaluate the separate effects of variation in the *APOA5* and *APOC3* gene it is therefore important to determine the frequency of the APOC3 sstI SNP in our Type III HLP patients in addition to the analysis of the 1131T/C exchange. The frequency of the minor allele of the APOC3 sstI SNP was significantly higher in Type III HLP patients with plaque, 0.2, compared to those without, 0.07 ( $p = 0.03$ , Fisher's exact, two-tailed), which is not unexpected and reflects the linkage of the alleles as part of the APOA5\*2 haplotype. In order to dissect the effects of the GC allele in APOC3 and the TC allele in APOA5 the distribution of the genotypes APOC3GG/APOA5TT/APOA5SS and APOC3GC/APOA5TC/APOA5SS amongst patients with or without plaque was analysed. Both variant alleles were significantly more frequent in patients with plaque as compared to patients without plaque. However the presence the GC allele was not associated with

plaque, when combined with a TT allele, whereas a significant association of the GC allele with plaque was observed in the presence of a TC allele. These data imply that it is the 1131T/C exchange in the *APOA5* gene which is associated with plaque in patients with Type III HLP.

The major weakness of our study is the small number of patients available for analysis. This is due to the rarity of Type III HLP, (in our clinic 72/2545 patients attending over a ten year period), with a frequency of Type III HLP of 1-5 in 5000. The 60 patients included in this study therefore represent an estimated population of 60,000-300,000. Confirmation of our findings in other populations is necessary. The analysis of the frequency of candidate SNPs for atherosclerosis in a small but homogeneous patient group adds important information to studies in large more heterogeneous groups when investigating the effect of common polymorphisms on complex traits such as atherosclerosis.

## CONCLUSION

In conclusion we present evidence that -1131T>C SNP in the *APOA5* gene influences the development of coronary plaque in a group of patients at high risk, namely those with Type III HLP indicating a role of apoA5 not only in dyslipidemia but also in the pathophysiology of atherosclerosis.

*Competing interests:* The authors declare that they have no competing interests.

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*Address for correspondence:*

W. A. Mann  
Endokrinologikum Frankfurt  
Stresemannallee 3  
60596 Frankfurt  
Germany  
Tel.: (49) 69-6959792420  
Fax: (49) 69-6959792466  
E-mail: alexander.mann@endokrinologikum.com