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

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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 ε2 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 ultrasoundography 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, Apolipoprotein 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 β-VLDL and homozygosity for the ε2 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 ε2 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 ss1 SNP (rs5128) with 85% of the chromosomes with APOA5*2 containing the minor allele of ss1. It is therefore of interest to determine the frequency of the APOC3 ss1 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 Probands 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 ss1 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±3.5) and those without (28.0±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 without plaque lipid values were obtained in the absence of lipid lowering therapy. Patients with plaque had higher total cholesterol, 400±174mg/dl compared to patients without, 291±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.

| Pat | AGE (years) | SEX | IMT (mm) | IMT-bulb (mm) | Lp(a) (mg/dl) | Smoker | D (s/ex/n) | HT | KHK | AVK | CVI | Chol (mg/dl) | Trig (mg/dl) | HDL (mg/dl) |
|-----|-------------|-----|----------|--------------|--------------|---------|-----------|----|-----|-----|-----|-------------|-------------|------------|
| A) Plaque present | | | | | | | | | | | | | | |
| Num | 101 | 37 | M | 0.75 | 0.90 | 105 | N | - | - | - | - | 481 | 588 | 69 |
| | 76 | 41 | M | 0.93 | 1.83 | 3 | 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 |
| | 45 | 45 | M | 0.83 | 1.48 | 3 | Y | - | - | - | - | 439 | 476 | 36 |
| | 28 | 47 | M | 0.85 | 1.50 | 3 | Y | - | - | - | - | 316 | 634 | 53 |
| | 86 | 50 | F | 0.80 | 0.95 | 2 | Y | - | - | - | - | 484 | 636 | 71 |
| | 63 | 44 | M | 0.78 | 1.10 | <2 | EX | - | - | - | - | 240 | 361 | 37 |
| | 77 | 45 | F | 0.95 | 1.75 | 11 | N | - | + | - | - | 651 | 433 | 44 |
| | 18 | 45 | M | 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 | 568 | 41 |
| | 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 | EX | - | - | - | - | 137 | 69 | 57 |
| | 38 | 48 | M | 0.83 | 1.23 | 11 | N | - | - | - | - | 292 | 428 | 39 |
| | 93 | 52 | F | 1.08 | 1.90 | 4 | Y | - | - | - | - | 651 | 433 | 44 |
| | 87 | 52 | M | 0.83 | 1.03 | <2 | EX | - | - | - | - | 331 | 548 | 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 |
| | 37 | 57 | F | 0.90 | 1.43 | 11 | Y | - | - | - | - | 435 | 388 | 46 |
| | 52 | 59 | M | 1.08 | 1.63 | <2 | EX | - | - | - | - | 137 | 69 | 57 |
| | 38 | 48 | M | 0.83 | 1.23 | 11 | N | - | - | - | - | 292 | 428 | 39 |
| | 93 | 52 | F | 1.08 | 1.90 | 4 | Y | - | - | - | - | 651 | 433 | 44 |
| | 87 | 52 | M | 0.83 | 1.03 | <2 | EX | - | - | - | - | 331 | 548 | 41 |
| | Mean | 53.5 | 0.88 | 1.40 | | | | | | | | | | |
| | n | 25 | 17/8 | | | | | | | | | | |

| B) Plaque absent | | | | | | | | | | | | | |
| Num | 46 | 28 | M | 0.80 | 1.58 | 10 | Y | - | - | - | - | 246 | 143 | 41 |
| | 100 | 30 | M | 0.73 | 1.05 | 19 | Y | - | - | - | - | 654 | 151 | 44 |
| | 70 | 31 | M | 0.77 | 0.80 | 4 | Y | - | - | - | - | 201 | 261 | 57 |
| | 72 | 32 | M | 0.67 | 0.85 | 3 | Y | - | - | - | - | 446 | 729 | 51 |
| | 33 | 34 | M | 0.70 | 0.88 | 7 | Y | - | - | - | - | 201 | 261 | 57 |
| | 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 | 494 | 40 |
| | 97 | 38 | M | 0.60 | 0.83 | 2 | EX | - | - | - | - | 408 | 402 | 69 |
| | 21 | 39 | M | 0.70 | 0.98 | 3 | Y | - | - | - | - | 341 | 778 | 33 |
| | 62 | 39 | F | 0.70 | 1.05 | 22 | N | - | - | - | - | 336 | 411 | 50 |
| | 16 | 40 | M | 0.80 | 1.13 | <2 | N | - | - | - | - | 416 | 405 | 49 |
| | 89 | 41 | M | 0.83 | 1.28 | 9 | N | - | - | - | - | 393 | 555 | 44 |
| | 3 | 42 | M | 0.92 | 2.15 | 10 | EX | + | - | - | - | 331 | 548 | 41 |
| | 91 | 56 | F | 0.90 | 1.55 | 7 | N | + | - | - | - | 367 | 507 | 47 |
| | Mean | 53.5 | 0.88 | 1.40 | | | | | | | | | | |
| | n | 25 | 17/8 | | | | | | | | | | |

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 | Pat | APOC3 | APOA5 | APOA5 |
|-------------------|-----|--------|--------|--------|
| Num               |     | sstI   | 1131T>C| S19W   |
|                   |     | rs5128 | rs662799| rs3135506|
| 101               |     | GC     | TC     | TC     |
| 76                |     | GC     | TC     | TC     |
| 90                |     | GC     | TC     | SW     |
| 36                |     | GC     | TC     | TC     |
| 35                |     | GC     | TC     | SW     |
| 28                |     | GC     | TC     | SW     |
| 86                |     | GC     | TC     | TC     |
| 63                |     | GC     | TC     | SW     |
| 4                |     | GC     | TC     | TC     |
| 87                |     | GC     | TC     | TC     |
| 20                |     | GC     | TC     | SW     |
| 45                |     | GC     | TC     | TC     |
| 71                |     | GC     | TC     | SW     |
| 27                |     | GC     | TC     | SW     |
| 48                |     | GC     | TC     | SW     |
| 37                |     | GC     | TC     | SW     |
| 52                |     | GC     | TC     | SW     |
| 38                |     | GC     | TC     | SW     |
| 93                |     | GC     | TC     | SW     |
| 6                |     | GC     | TC     | SW     |
| 16                |     | GC     | TC     | SW     |
| 89                |     | GC     | TC     | SW     |
| 3                |     | GC     | TC     | SW     |
| 91                |     | GC     | TC     | SW     |
| n 10 12 5          |     | GC     | TC     | SW     |
| B) Plaque absent  | Pat | APOC3 | APOA5 | APOA5 |
| Num               |     | sstI   | 1131T>C| S19W   |
|                   |     | rs5128 | rs662799| rs3135506|
| 46                |     | GC     | TC     | WW     |
| 50                |     | GC     | TC     | SW     |
| 84                |     | GC     | TC     | SW     |
| 5                |     | GC     | TC     | SW     |
| 102               |     | GC     | TC     | SW     |
| 5                |     | GC     | TC     | SW     |
| 14                |     | GC     | TC     | SW     |
| 50                |     | GC     | TC     | SW     |
| 8                |     | GC     | TC     | SW     |
| 95                |     | GC     | TC     | SW     |
| 61                |     | GC     | TC     | SW     |
| 12                |     | GC     | TC     | SW     |
| 64                |     | GC     | TC     | SW     |
| 47                |     | GC     | TC     | SW     |
| 85                |     | GC     | TC     | SW     |
| 94                |     | GC     | TC     | SW     |
| 11                |     | GC     | TC     | SW     |
| 1                |     | GC     | TC     | SW     |
| 53                |     | GC     | TC     | SW     |
| 25                |     | GC     | TC     | SW     |
| 98                |     | GC     | TC     | SW     |
| 34                |     | GC     | TC     | SW     |
| 59                |     | GC     | TC     | SW     |
| 9                |     | GC     | TC     | SW     |
| 5                |     | GC     | TC     | SW     |
| n 5 7 7/1          |     | GC     | TC     | SW     |

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 sst1 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 the 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 an 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 promoter 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 sst1 polymorphism and two promoter 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 promoter 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 sst1 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 sst1 SNP was significantly high 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/APOA5STC/APOA5SSS 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 small but homogeneous patient groups 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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