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Apolipoprotein E and Complement C3 Polymorphism and Their Role in the Response to Gemfibrozil and Low Fat Low Cholesterol Therapy

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Dedicated to Prof. Dr. E. Kaiser on the occasion of his 70th birthday

Summary: Three different allelic variants of apolipoprotein E determine, in concert with other gene products, the levels of plasma lipoproteins. Recently, cleavage products of the complement C3 molecule have also been implicated in determining plasma triacylglycerol concentrations.

This study presents data of an ongoing study to dissect the role of the apolipoprotein E gene locus in the response to low fat/low cholesterol diet combined with gemfibrozil treatment. In addition, for the first time, the significance of C3 allelic variants to such hypolipidaemic therapy response was analysed. To this end data from 81 obese hyperlipoproteinaemic patients (*Fredrickson* type II/A and B and type IV and V) confirmed the usefulness of the combined gemfibrozil/diet treatment and unveiled apolipoprotein E allele group specific therapy responses. The mean changes of lipid properties due to combined treatment was 15% for total cholesterol, 48% for triacylglycerols and 28% for atherogenic index. Division into hyperlipidaemia types according to *Fredrickson* and subgrouping into E2, E3 and E4 groups (apolipoprotein E2/2 and 2/3, apolipoprotein E3/3 and apolipoprotein E4/2 and 4/3 phenotype groups respectively) exposed pronounced differences from these mean changes, suggesting substantial influence of apolipoprotein E variants on this therapy. We observed triacylglycerol reductions of from 17% in type IIA-apolipoprotein E3 group patients up to 78% in the type IV and V-apolipoprotein E2 group. Thus it might be concluded the apolipoprotein E genotyping aides therapy success prediction. Although, low sample numbers in some subgroups obscures significance in this pilot study, significant therapy success emerges for the E3 and E4 group in type IV and V hyperlipidaemia and type IIB-apolipoprotein E3 homozygous patients can be predicted to respond better than apolipoprotein E2 carriers.

Finally, we present evidence that positive changes of lipid properties are also determined by the "fast" complement C3 allel (C3-F). Patients with complement factor C3-FS pattern respond better to treatment than patients with C3-SS configuration. In summary these data endorse the genotyping of apolipoprotein E alleles to predict maximal success of "fibrate" treatment. In addition they argue strongly for further assessment of the involvement of complement C3 allelic variations in lipid homeostasis.

Introduction

Apolipoprotein E, a M_r = 34 000 protein, is one of the key molecules involved in lipid metabolism. Apolipoprotein E controls the catabolism of chylomicrons, VLDL (very low density lipoprotein) and the remnants thereof (IDL), thereby controlling plasma triacylglycerol levels by its capacity to bind to both the apolipoprotein

E LDL receptor related protein/α_2-macroglobulin-receptor and the LDL receptor (1, 2). The amino-terminal domain of apolipoprotein E, around arginine 158, contains the region that binds to the cysteine rich repeats on the LDL and LDL receptor related protein/α_2-macroglobulin-receptor (2) which bears homologous repeats. The human apolipoprotein E gene spans 3.7×10^3 bases including four exons (3, 4) and is located on chromosome 19 (4) in a gene family that is also close to the locus of the complement factor C3 gene (5). Apolipoprotein E polymorphic with three common isoforms, ε2 (112cys, 158cys), ε3 (112cys, 158arg, the most common) and ε4 (112arg, 158arg), which are determined by separate alleles at the apolipoprotein E locus. There are thus six widespread apolipoprotein E phenotypes in the population: homozygous E2/2, E3/3, E4/4 and heterozygous E3/2, E4/2 and E4/3 (6). The apolipoprotein E4 with two arginines in the binding region has higher affinity and apolipoprotein E2 with two cysteins has a lower affinity for LDL receptor than apolipoprotein E3.

As the lipoprotein levels in blood are governed by their affinity for receptors, low density lipoprotein (LDL) cholesterol concentrations vary with apolipoprotein E. Lipoprotein levels are highest in people bearing the allele ε4 (but apolipoprotein E serum levels are lower (7)), intermediate in those with ε3 and lowest in those with ε2 (8, 9). This occurs because in persons carrying the allele ε4, the high affinity of apolipoprotein E4 carrying particles leads to an oversupply of liver cells with cholesterol. As a consequence, LDL receptors are made inactive which leads to less efficient removal of IDL and enhanced conversion to LDL. Consequently accumulation and prolonged circulation of LDL in the bloodstream is observed (10). The genotype homozygous for allele ε2 is associated with delayed chylomicron clearance but raised apolipoprotein E levels (7) after high-fat load. The ε2 allele is also associated with delayed clearance of the IDL/VLDL remnant fraction and LDL receptor levels are not down regulated as no oversupply of cells with IDL exists. In the treatment with anti-hyperlipidaemic drugs a more precise prediction of efficiency is desirable and might be possible by grouping patients according to apolipoprotein E genotypes (11−14). For the frequently used anti-hyperlipidaemic drugs (hydroxymethylglutaryl-CoA reductase inhibitors, type simva-, lova-, pravastatin and chlorophenoxy methylpropionate derivatives like clo-, feno-, etofibrate) predictable effects on type II/B and IV forms of hyperlipidaemia are highly desirable but not readily available (15, 16). It would be useful to predict the individual reaction to various classes of lipid lowering drugs thereby facilitating their selection. Recent findings suggest that the human complement factors C3 and C4 (C3, C4) are markers of a genetic predisposition to myocardial infarction. C3 is located in the vicinity of the apolipoprotein E gene locus on chromosome 19 and C4 in the vicinity of the 21-hydroxylase gene locus on chromosome 6. C3 levels measured in sera from subjects without previous ischaemic events are independently associated with the risk of myocardial infarction (17). A certain C4 allotype (C4B*Q0) had higher prevalence in men with myocardial infarction (18). C3 was reported to be involved in the regulation of the adipsin/acylation stimulating protein system which seems to play a pivotal role in regulation of triacylglycerol removal from plasma (19). It was found that acylation stimulating protein is identical with the cleavage product of complement factor C3, named C3a-desArg, and stimulates triacylglycerol synthesis in human adipocytes. We therefore tested whether alleles of C3 or C4 are involved in plasma lipid regulation and thus serve as risk factors for myocardial infarction by analysing the lipid properties changes linked to the occurrence of the C4B*Q0 allele and the "fast" and "slow" alleles of complement C3 in our study group.

Methods

Patients

The study group consisted of 81 unrelated obese (body weight 30% above ideal) outpatients, 27 females and 54 males with hyperlipidaemia (*Fredrickson* phenotype II/A = 13, II/B = 33, IV = 15, V = 20), who were attending the lipid clinic of St. Emeric Hospital, Budapest. The principal criteria for entry was a cholesterol concentration above 7.8 mmol/l and/or triacylglycerol above 5.0 mmol/l. In all cases, analyses were performed before and after interventions. The patients had been requested to consume a cholesterol-lowering diet (less than 30% of daily energy as fat and daily cholesterol intake < 200 mg, (19)) and were treated with gemfibrozil (Gevilon®, Gödecke Inc.) 900 mg at bedtime. They were advised to adhere to this diet during the experimental period (6 months). Dietary advice was provided by a co-ordinated group of registered dietitians throughout the study. None of the study group had heart failure, renal, liver, or thyroid disease, or diabetes mellitus. Height and weight were obtained with a balance beam scale, and body mass index (BMI), a measure of body fat, was obtained by dividing body weight in kilograms by height in meters squared. All patients volunteered for the study, which had been approved by the Ethical Committee of the hospital.

Measurement of lipids, lipoproteins, and apolipoproteins

Subjects were evaluated in the morning after a 12-hour fast for the following measures: the concentrations of serum total cholesterol and triacylglycerol were assayed enzymatically with a Hitachi 717 automated analyzer according to the Manual of laboratory Operations of the Lipid Research Clinics Program (20, 21).

High density lipoprotein (HDL) was isolated in the supernatant after precipitation of apolipoprotein B-containing lipoproteins with heparin and manganese chloride (22). Immunonephelometric assay (Orion Inc./Turox Inc.) was used to measure the apolipoproteins AI, AII and B. Apolipoprotein E isoforms were characterised by means of polymerase chain reaction (PCR) and restricion fragment length polymorphism (RFLP) (23). Internal quality control was

evaluated throughout the study with the use of on the one hand *Taq*-DNA polymerase and template DNA-free samples, and on the other, intra- and inter-observer crossover investigation. C3 and C4 (the third and fourth complement of the human complement system) phenotypes were determined by high voltage electrophoresis using the method of *Teisberg* (24, 18).

Monitoring for adverse events

At each visit, the pulse rate, sitting blood pressure and body weight were recorded, and patients were questioned about adverse events. Routine biochemical and haematological analyses (including liver and muscle functions) were performed by the central laboratory at the run-in and at the end-period.

Statistical analyses

Statistical analyses were carried out with the BMDP statistical software package (University of California, Los Angeles, CA, 1985). Analysis of variance (ANOVA) was used for comparison of means. For pairwise comparison of means *Wilcoxon* rank sum test was applied, multiple stepwise regression analysis was done to differentiate the influences affecting serum lipid levels ($p < 0.05$ was considered statistically significant).

Results

Clinical characteristics and lipid- and apolipoprotein properties of the study subjects before treatment (Baseline) and after therapy (Intervention) are depicted in table 1. The pre-treatment levels of total plasma cholesterol, non-HDL cholesterol and apolipoprotein A-I were significantly higher in females (not shown). The mean changes of lipid properties in men and women due to combined treatment was 15% for total cholesterol, 48% for triacylglycerol and 28% for atherogenic index. Division into hyperlipidaemia types according to *Fredrickson* and subgrouping into E2, E3 and E4 groups (apolipoprotein E2/2 and 2/3, apolipoprotein E3/3 and apolipoprotein E4/2 and 4/3 phenotype groups) exposed pronounced differences from this mean changes, suggesting substantial influence of apolipoprotein E variants on therapy efficacy. In table 2, triacylglycerol reductions of from 17% in type IIA-apolipoprotein E3 group patients up to 78% in the type IV and V-apolipo-

Tab. 1 Clinical characteristics, lipid and apolipoprotein properties of the study subjects.

Variables (Units)	Men (n = 54)		Women (n = 27)		p values*
	Limits		Limits		
	mean ± SD	(max, min)	mean ± SD	(max, min)	
Independent variables					
Age (a)	49.0 ± 8.2	(66.9, 34.0)	51.2 ± 11.4	(72.0, 26.0)	0.22
BMI (kg/m² height)					
A. Baseline	29.4 ± 4.9	(45.4, 27.2)	26.4 ± 4.3	(44.3, 20.1)	0.008
B. Intervention	28.2 ± 5.5				
Dependent variables					
Total-cholesterol (mmol/l)					
A. Baseline	7.5 ± 2.5	(19.3, 4.5)	8.4 ± 2.8	(20.3, 5.2)	0.49
B. Intervention	6.7 ± 1.7	(15.0, 4.3)	6.8 ± 1.2	(8.9, 4.5)	
Triacylglycerol (mmol/l)					
A. Baseline	6.4 ± 5.7	(31.9, 1.2)	7.7 ± 8.6	(31.3, 1.3)	0.59
B. Intervention	4.1 ± 4.4	(25.3, 1.0)	3.2 ± 3.7	(19.9, 1.0)	0.06
HDL cholesterol (mmol/l)					
A. Baseline	0.8 ± 0.3	(1.9, 0.4)	0.9 ± 0.2	(1.5, 0.5)	0.14
B. Intervention	1.1 ± 0.4	(2.6, 0.4)	1.1 ± 0.3	(2.2, 0.6)	0.22
Non-HDL cholesterol (mmol/l)					
A. Baseline	6.6 ± 2.6	(18.7, 3.7)	7.4 ± 2.0	(14.6, 4.9)	0.02
B. Intervention	5.6 ± 1.7	(13.6, 3.6)	5.6 ± 1.4	(8.2, 3.1)	0.84
Atherogenic Index					
A. Baseline	9.8 ± 5.1	(39.0, 3.1)	9.7 ± 3.6	(18.7, 4.9)	0.83
B. Intervention	7.3 ± 2.6	(13.0, 3.1)	6.8 ± 3.4	(18.7, 2.4)	0.26
Apolipoproteins (g/l)					
-A-I	1.5 ± 0.5	(3.0, 0.6)	1.7 ± 0.4	(2.5, 1.0)	0.02
-A-II	0.3 ± 0.2	(1.1, 0.1)	0.3 ± 0.1	(0.4, 0.2)	0.11
-B	1.1 ± 0.5	(2.3, 0.4	1.0 ± 0.5	(1.9, 0.4)	0.40
-B/A-I	0.8 ± 0.4	(1.9, 0.3)	0.7 ± 0.4	(1.7, 0.3)	0.22

Non-HDL cholesterol (VLDL cholesterol + LDL cholesterol), BMI (Body Mass Index),
* p values (*Mann-Whitney* u test), $p < 0.05$ significant.

protein E2 group can be observed. Similar trends were obvious with cholesterol and atherogenic index. They are currently under investigation in the extended sample pool and a complete table will provide a useful therapy decision tool when *Fredrickson* Type of hyperlipidaemia and apolipoprotein E genotype of a patient is known. The apolipoproteins showed no statistically significant distribution differences among apolipoprotein E genotypes. Individual lipid and clinical data analysis for each apolipoprotein E group and *Fredrickson* type of this pilot study cohort are not shown but are available upon request.

In figure 1 the significant changes of properties occurring in *Fredrickson* types along the different apolipoprotein E genotype permutations are detailed. The numbers of patients (n) for each group are given. Values of changes ((A-B)/A%, A: Baseline values, B: Post-intervention values) that reached statistical significance (*Wilcoxon* rank sum test, $p < 0.05$ significant) are lettered S. in type IV and V the triacylglycerol values were significantly lowered, while in IIB, with the same number of patients, no significant changes were seen in the E2 group. Thus it seems conceivable that the apolipoprotein E allelic variation will govern the therapy response in the respective *Fredrickson* type hyperlipidaemias. The apolipoprotein E alleles pooled frequencies were according to the *Hardy-Weinberg* equilibrium, $\varepsilon_2 = 0.20$, $\varepsilon_3 = 0.67$ and $\varepsilon_4 = 0.13$ thus showing independence. In summary figure 1 gives an overview of the relevant properties cholesterol, triacylglycerols and atherogenic index across the allele groups and *Fredrickson* types. Responsiveness to therapy within identical apolipoprotein E genotypes and significant beneficial therapeutic trends could be seen in most properties with decreasing order of allele influence: $\varepsilon_3 > \varepsilon_4 > \varepsilon_2$. These positive changes could be seen in spite of decreasing BMI values during therapy.

Tab. 2 Changes in triacylglycerol (mmol/l) during therapy between different *Fredrickson* phenotypes within apolipoprotein E genotype groups.

Variables	E2/E2, E2/E3 E2-Group (n = 16)	E3/E3 E3-Group (n = 45)	E4/E3, E4/E2 E4-Group (n = 20)
II/A	25.0	16.66	20.0
II/B	20.0	45.23	52.38
IV + V	77.86	60.58	76.66

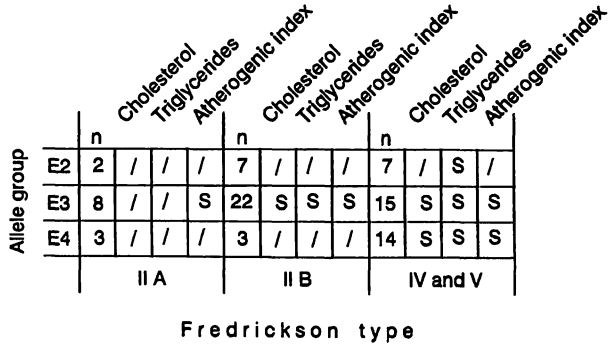

Fig. 1 Pattern of significance of lipid properties changes along apolipoprotein E allele groups and *Fredrickson* type hyperlipidaemias (n = number of patients in group). Baseline and post-intervention values have been analysed by *Wilcoxon* rank sum test for significance of difference. Cells were lettered with S when significant changes were reached. Numbers of observations are given to indicate where low sample size may obscure significance.

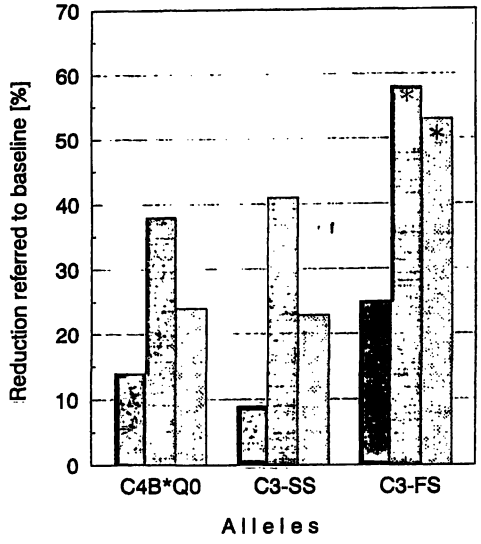

Fig. 2 Reduction of lipid properties observed in the different complement genotype groups. Bars marked with star show significant changes. (left bar of triade = cholesterol, middle = triacylglycerols, right = atherogenic index).

The frequencies of the two most interesting C3 and C4 alleles (18), the first lying in the vicinity of the apolipoprotein E gene locus on chromosome 19 and the latter in the vicinity of the steroid 21-hydroxylase gene locus on chromosome 6 ware also determined and related to lipid properties. In the study group the frequencies of C3-S and C3-F were 0.84 and 0.16, respectively, identical to those found in the healthy population. In figure 2, cholesterol, triacylglycerol and atherogenic index changes are given in the various allele groups. Significant decrease in atherogenic index could be seen only in heterozygotes carrying C3-FS. The mean atherogenic index during dietary measures for homozygous (C3-SS) individuals decreased from 9.40 to 7.01 and for heterozygotes (C3-FS) from 11.08 to 5.33 ($p < 0.01$). Changes seen in carriers of the C4B*Q0 were not significant.

Discussion

Our results have shown that in obese hyperlipidaemic Hungarian Caucasians the influence of apolipoprotein E genotypes on baseline and post intervention lipid prop-

erties differed from that found in previous studies carried out in various populations with different ethnic and socio-economic backgrounds (25–28). In our survey the relative frequency of the ε_2 allele was as much as 3 times higher than in previous studies dealing with normolipidaemic subjects from different ethnic groups (0.20 vs. 0.06) (29), whereas no difference in the relative frequency of the ε_4 allele between two study groups was seen (0.13 vs. 0.13). The ε_4 allele carriers were prone to higher initial lipid properties and BMI values and they also showed a pronounced positive therapy response. By using ANOVA we could not detect significant differences in therapy response between various apolipoprotein E genotypes in *Fredrickson* type II/A and IV + V. In type IIA this might be explained by the relatively small number in the subgroup and was predicted to reach significant levels at total sample sizes above 300 by cluster analysis technique. In accordance with previous findings we could also detect the coincidence of ε_2 allele and higher cholesterol levels mainly in type IV and V hyperlipidaemia (compare l. c. (29)).

Allelic variation in apolipoprotein E has the greatest impact on plasma levels of cholesterol, triacylglycerol, and apolipoprotein B, C-II, and E (11, 12, 31–33). It has been suggested that ε_4 could tip a compromised lipolytic system in obese (relative insulin insufficiency, decreased lipoprotein lipase activity, prolonged postprandial lipaemia) into frank phenotypic expression of dyslipidaemia. The triacylglycerol substrate bound to apolipoprotein E has a greater capacity to activate lipoprotein lipase and the extent of this capacity may be related to the amount of apolipoprotein E bound to triacylglycerol (34–36, 38). The triacylglycerol-rich particles in individuals with ε_4 are relatively poor in apolipoprotein E, as are the VLDL from hyperlipoproteinaemia type IV subjects (37). This metabolic alteration could unveil insufficient lipoprotein lipase activation and/or the ineffective clearance of lipid particles due to apolipoprotein E. Such mechanisms might be operative in the type IV + V-E2 group leading to unconvincing response to therapy.

As a first consequence typing of ε alleles in obese *Fredrickson* type II/B has been statistically established to successful predict treatment when ε_3 is detected. For these patients, 32 out of 81 in our group, a convincing correlation of the therapy success with the ε_3 genotype (22 of 32) justifies putting such patients on combined treatment.

Plasma C3 levels are associated with the risk of myocardial infarction. The local activity of the complement system may affect the response of the plasma lipid regulatory systems. The two common phenotypes of the apolipoprotein E neighboring C3 gene (C3-SS and C3-FS) on chromosome 19 have different functional activity (30). On the other hand, a cleavage product of human complement C3 generated during complement activation – or perhaps due to plasma lipid feedback mechanisms – regulates plasma triacylglycerol removal. Thus, our finding that the functionally more active C3-FS has a better influence on diet may have not only a predictive role, but also suggests that the fast complement C3 allele product is more advantageous in adipsin/acylation stimulating protein mediated plasma triacylglycerol regulation. Our findings also motivate the elucidation of this allele's participation in the adipsin/acylation stimulating protein system.

In conclusion our results support the contention that apolipoprotein E polymorphism modulates the therapy response to combined dietary and gemfibrozil therapy. Thus genotyping would, in the long run, be a useful tool in selecting an effective antihyperlipidaemic therapy.

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