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ORIGINAL ARTICLE

Apolipoprotein D is associated with long-term outcome in patients with schizophrenia

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Accumulating evidence implicates deficiencies in apolipoprotein D (ApoD) function and arachidonic acid signaling in schizophrenic disorders. We addressed two hypotheses in relation to ApoD: first, polymorphisms in the *ApoD* gene confer susceptibility to or are markers of disease, and, second, genetic variation in the ApoD is associated with long-term clinical outcome to antipsychotic treatment. We genotyped two single-nucleotide polymorphisms in the *ApoD* gene in 343 chronic patients with schizophrenia spectrum disorders (ICD-10) and 346 control subjects of Danish origin. We did not find *ApoD* alleles, genotypes or haplotypes to be associated with disease. However, we did find that long-term clinical outcome was associated with the *ApoD* polymorphism rs7659 ($P = 0.041$) following adjustment for lifetime clinical global impression, age at first admission and gender.
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Introduction

A substantial body of evidence has implicated dysfunction of the fatty acid and phospholipid pathways in psychiatric diseases.[1,2] Numerous studies have examined lipid-carrying and -recruiting proteins expressed in the brain as possible risk factors of schizophrenia. Recently, several studies have shown brain content of these proteins to be altered in schizophrenia.[3] The causal relation between brain lipids and the development of schizophrenia is unknown, but membrane proteins – and thus the membrane-associated receptors affected in this disease – are generally regulated by the lipid composition of the surrounding lipid bilayer.[4,5]

Apolipoprotein D (ApoD) is such a lipid carrier protein, initially found as a component of high-density lipoproteins in human plasma, in complex with apolipoprotein A-II, and subsequently shown to be highly expressed in the brain.[6,7] Although the function of ApoD is not fully understood, it is known to bind and transport cholesterol,[8] steroid hormones[9,10] and arachidonic acid (AA).[3,11–13] ApoD is structurally similar to the plasma-retinol-binding protein and other members of the alpha-2 microglobulin family.[14,15] Sequence analyses places ApoD as a member of the ancient lipocalin superfamily with no marked similarity to other and more extensively studied apolipoproteins, such as ApoE associated with Alzheimer's disease.[16]

Like other lipocalins, ApoD is associated with pathological conditions, including developmental and degenerative processes in the brain. For example,

elevated plasma levels of ApoD have been found in the brain and cerebrospinal fluid in patients suffering from Alzheimer's disease.[17,18] Similarly, ApoD has been implicated in psychiatric illnesses, where elevated levels of ApoD have been detected in plasma from drug-naive schizophrenic patients.[19] Furthermore, analyses of post-mortem brains documented increased expression levels of *ApoD* in patients with bipolar disorder and schizophrenia.[20] Interestingly, the regional patterns of increased *ApoD* expression differed between the two patient categories (bipolar and schizophrenia), suggesting that ApoD may be a disease marker.[21,22]

The notion that ApoD might be implicated in schizophrenia has gained support from expression analyses in rodents demonstrating that antipsychotic drugs regulate *ApoD* expression in plasma and the brain.[23] Interestingly, the atypical antipsychotic clozapine increases *ApoD* levels in several brain regions both during acute and chronic administration of the drug, whereas the typical antipsychotic drug haloperidol appears to reduce *ApoD* expression.[24] Hence, animal studies suggest that regulation of ApoD might be an important factor relating to the distinct therapeutic effects of antipsychotic medication.[19] If so, genetically influenced treatment outcome in psychotic patients (reviewed in Mancama *et al*.[21]) might, in part, be defined by gene variants affecting ApoD expression or activity.

The gene encoding ApoD is located on chromosome 3q26.2, a region tightly linked to schizophrenia in genome-scan meta-analysis,[25] and is thus consistent with the involvement of ApoD in the etiology of schizophrenia. Further genetic support of an involvement of ApoD in developmental and degenerative brain diseases is provided by the finding that a potential regulatory polymorphism in the *ApoD* gene is associated with Alzheimer's disease in an Afro-American sample, although the associated polymorphisms are not present in Caucasians.[26]

In order to clarify the role of ApoD in schizophrenia, we assessed two hypotheses: first, polymorphisms in the *ApoD* gene confer susceptibility to schizophrenia, and, second, such polymorphisms are associated with long-term clinical outcome of antipsychotic treatment.

Materials and methods

Subjects and characteristics

This study included 343 schizophrenic in- and outpatients, who had previously been recruited to the Danish Psychiatric Biobank from psychiatric departments at the five hospitals in the Copenhagen area. All patients had a history of psychotic illness (average = 13.1 ($\pm$8.9) years of treatment since first contact to psychiatric care), and had been clinically diagnosed with schizophrenia (F20, F22 or F25) according to the ICD-10 criteria. The reliability of the clinical diagnoses was confirmed in 100 of these patients by an experienced consultant psychiatrist (KDJ) using the semistructured interview of the OPCRIT instrument[27] (positive predictive value >98%).[28]

Also included in the study were 346 unrelated, anonymous blood donors serving as healthy control subjects. Patients (137 females and 206 males) and controls (139 females and 207 males) were not different with respect to sex ratio ($P = 0.90$), whereas the mean age of patients (42.2 $\pm$ 12.1 years) was slightly lower than in the control sample (49.7 $\pm$ 12.8 years). Both samples were ethnically highly homogeneous. Most patients (87%) were ethnically Danish (i.e. patient and both parents were born in Denmark), whereas in a minor fraction of cases (13%), one parent was born outside Denmark in another northwestern European country. The healthy control subjects were also Danish and recruited from the same geographical area of Greater Copenhagen as the patients.

Key anamnestic, epidemiological and clinical variables were obtained through a review of case records and hospital discard registers by an experienced consultant psychiatrist (AGW). Estimates were obtained for age at first admission (AFA; $n = 308$), clinical global impression (lifetime CGI[29]; rated as 1–4 or 5–7; $n = 227$), substance abuse (clinically relevant, consistent with an ICD-10 diagnosis of F1X.2; $n = 308$) and suicidal behavior (presence/absence of documented suicidal attempts; $n = 228$). Long-term outcome was estimated based on medical history of 308 patients. Owing to the extensive time period that elapsed since the first hospital admission, we defined operational criteria of long-term clinical outcome as follows: *Good outcome patients* were defined as subjects in long-term pharmacotherapy with traditional or atypical antipsychotics and with a satisfactory clinical outcome. *Poor outcome patients* were defined as individuals (previously treated with standard antipsychotics without satisfactory clinical outcome) in long-term clozapine therapy. The definition of these criteria for long-term outcome status was based on the treatment guidelines from Danish[10] and American[30] psychiatric associations, which state that clozapine is a late treatment option allowed only in hard-to-treat or treatment-resistant schizophrenic patients. Thus, the responsible clinician has established that the patient has not responded clinically satisfactorily to several distinct classes of antipsychotics in adequate doses, before shifting the patient onto clozapine. Patients who were treated with clozapine owing to side effects from standard antipsychotics and patients in whom clozapine treatment had been discontinued owing to side effects were excluded from the study.

The two long-term outcome groups did not differ with respect to recruiting hospital ($P = 0.071$), gender ($P = 0.64$), age ($P = 0.16$), substance abuse ($P = 0.53$) or suicidal behavior ($P = 0.055$). AFA was significantly lower in the poor outcome group than in individuals with good clinical outcome ($P = 0.007$), as was the CGI score ($P < 0.0001$). These findings confirm the well-established epidemiological knowledge that early onset of illness and a poor CGI score are predictors of poor long-term clinical outcome and thus validate the operational criteria that we have used in this study.[31]

Ethics
The study was approved by the Danish Scientific–Ethical Committees and the Danish Data Protection Agency. All patients had given written informed consent before inclusion into the project.

Genotyping
The single-nucleotide polymorphisms (SNPs) markers were selected from the NCBI SNP database applying a minor frequency cutoff of 0.2. Two SNPs spanning 4.5 kb of the 5′-end of the *ApoD* gene fulfilled these criteria in white Caucasians. The relative short distance between SNPs was chosen to evaluate linkage disequilibrium (LD) for the region, which is not available in public databases. The first SNP, *rs7659*, was positioned in the 3′-untranslated region of the *ApoD* gene, and was found to be situated in a putative binding site for the human splice factor SR SC35 (SFRS2).[32] The second SNP, *rs1464505*, was located in the 3rd intron and selected in order to span the 3′ portion of the *ApoD* gene. Other SNPs, as mentioned earlier, have previously been examined in relation to Alzheimer's disease and speculated to be associated with schizophrenia,[26] but these polymorphisms have not been detected in Caucasians . An additional SNP located in exon's of the *ApoD* gene had frequencies below 0.1 and was therefore not assessed in this study.

Total genomic DNA was extracted from venous blood. A TaqMan® system from Applied Biosystems (Foster City, CA, USA) was used to genotype the two SNPs situated in the 3′-untranslated region (rs14644505) and intron 3 (rs7659). The former of these two SNPs was genotyped by Assay-on-Demand® reagents, whereas the analysis of the other was carried out by Assay-by-Design®, using the following primers: forward: gtgtacatttctattactgagggcttct; reverse: ggtgg gtaggaaggagctctt; and the probe FAM/VIC: ctttttggatt[a/t] attattg.

Statistical analysis
Chi-square (χ^2) analysis was used to assess whether the distribution of genotypes in the control sample corresponded to those expected under conditions of Hardy–Weinberg proportions (HWP) and to compare distributions of genotypes, alleles and haplotypes between study groups. Yates corrections were used whenever appropriate. An EM algorithm was used to estimate the haplotype frequencies, and the extent of LD was determined by calculation of D' and r^2.[33] Long-term outcome patient groups were compared by ANOVA analysis and stepwise logistic regression using SYSTAT® (Systat Software Inc., Point Richmound, CA, USA) including *P*-value of 0.15. To test the goodness of fit of our disease model, we used the program provided by Wittke-Thompson *et al*.[34]

Results

The genotype counts and corresponding allele frequencies of the *ApoD* polymorphisms, *rs7659* and *rs1464505*, are given in Table 1. The allele frequencies among control and schizophrenic subjects of both markers did not deviate significantly from those previously reported in Caucasians and other populations.[35] Both polymorphisms were found to be in HWP in the control sample. *rs7659* deviated marginally from HWP in the schizophrenic sample ($P=0.049$).

The HWP in the patient group could potentially produce a spurious genotype–disease association owing to genotyping errors, and chance or failure to meet HWP prerequisites. However, a goodness-of-fit test[34] was used to challenge this hypothesis and show that the observed departure from HWP was compatible with an underlying genetic model of disease transmission ($\chi^2=1.14$, df$=1$, $P=0.29$). Furthermore, neither the *rs7659* genotype nor allele frequencies were significantly different between control and schizophrenic subjects (Table 1).

A maximum-likelihood procedure was used to estimate the frequencies of haplotype phases of the polymorphic sites in the control and patient samples. LD was found between the two loci ($\chi^2=0.895$, df$=3$, $P=0.83$; $D'=0.66$ and $r^2=0.63$), and no significant association was detected between haplotypes and disease status ($P=0.95$; Table 2). These findings do not provide evidence to support our first hypothesis that ApoD is associated with schizophrenia as such.

In order to challenge the second hypothesis, that is, polymorphisms are associated with long-term clinical outcome of antipsychotic treatment, 308 patients were stratified according to their long-term clinical outcome (see also Materials and methods for details). Both polymorphisms were in HWP in both long-term outcome groups. The initial analysis of the genotype, allele and haplotype frequencies did not show any statistically significant differences across controls and the two outcome categories of schizophrenic patients (Tables 1 and 2). However, several epidemiological and clinical variables are known to affect long-term outcome and could obscure a putative genetic effect. We therefore performed a forward logistic regression analysis using the demographic and clinical variables described in Materials and methods. CGI, AFA and gender remained in the final model, with CGI being most strongly associated with long-term outcome as shown in Table 3. Interestingly, the rs7659 locus was also included in the model in a manner indicating a recessive effect (OR$\sim$0.4 for both AA and AG genotypes with respect to the GG genotype; Table 3). Further analysis, in which these two genotypes were combined, supported this notion (AA/AG vs GG: OR$\sim$0.4, $P=0.041$; Table 3).

Discussion

This study was designed to assess the potential importance of ApoD in schizophrenia spectrum disorders, challenging the hypotheses that alterations in ApoD activity may be related to disease status or long-term outcome.

We failed to provide evidence for the first hypothesis, as the SNP genotype, allele frequencies and the haplotype did not translate into an association with the disease or long-

Table 1 Disease and outcome status vs *ApoD* genotypes and alleles[a]

	Controls (n = *346*)	*Cases* (n = *343*)	*Good outcome*[b] (n = *204*)	*Poor outcome*[c] (n = *104*)	P-*value*[d] (χ^2-*value*)
Genotypes					
rs7659 AA	159 (0.43)	161 (0.47)	94 (0.46)	49 (0.47)	
rs7659 AG	149 (0.43)	136 (0.40)	88 (0.43)	38 (0.37)	
rs7659 GG	38 (0.11)	46 (0.13)	22 (0.11)	17 (0.16)	
χ^2 test (df = 2)	—	—			0.51 (1.35)
χ^2 test (df = 4)[e]	—		—	—	0.54 (3.12)
χ^2 *test* (df = 2)			—	—	0.30 (2.43)
Alleles					
rs7659 (G)	0.33	0.33	0.32	0.35	
χ^2 test (df = 1)	—	—			0.78 (0.08)
χ^2 test (df = 2)[e]	—		—	—	0.83 (0.37)
χ^2 test (df = 1)			—	—	0.57 (0.32)
Genotypes					
rs1464505 AA	21 (0.06)	23 (0.07)	16 (0.08)	5 (0.05)	
rs1464505 AT	154 (0.45)	140 (0.41)	79 (0.39)	47 (0.45)	
rs1464505 TT	171 (0.49)	180 (0.52)	109 (0.53)	52 (0.50)	
χ^2 test (df = 2)	—	—			0.61 (0.98)
χ^2 test (df = 4)[e]	—		—	—	0.61 (2.17)
χ^2 test (df = 2)			—	—	0.41 (1.79)
Alleles					
rs1464505 (A)	0.28	0.27	0.27	0.27	
χ^2 test (df = 1)	—	—			0.48 (0.50)
χ^2 test (df = 2)[e]	—		—	—	0.75 (0.57)
χ^2 test (df = 1)			—	—	0.96 (0.003)

[a]Genotypes are presented as raw counts with frequencies given in parentheses, while only the minor allele frequencies are listed.
[b]*Good long-term outcome*: subjects responding clinically satisfactorily to either traditional or atypical antipsychotic drugs.
[c]*Poor long-term outcome*: subjects treated with standard antipsychotics without adequate clinical response, but improved clinically satisfactorily treated with clozapine or with clozapine and one or more additional antipsychotic drugs.
[d]*P*-values are uncorrected for multiple testing.
[e]Further analyses between controls and each of the two patient groups did not indicate differences between groups.

Table 2 Disease and outcome status vs two-loci haplotype[a] (rs7659–rs1464505)

	Controls (n = *346*)	*Cases* (n = *343*)	*Good outcome*[b] (n = *204*)	*Poor outcome*[c] (n = *104*)	P-*value*[d] (χ^2 *test*)
A–A	0.242	0.241	0.256	0.222	
A–T	0.426	0.434	0.421	0.432	
G–A	0.029	0.042	0.016	0.052	
G–T	0.303	0.283	0.307	0.294	
χ^2 test (df = 3)	—	—			0.95 (0.32)
χ^2 test (df = 6)	—		—	—	0.75 (3.45)
χ^2 test (df = 3)			—	—	0.35 (3.28)

[a]Haplotype frequencies are estimated by EM algorithm.
[b]*Good long-term outcome*: subjects responding clinically satisfactorily to either traditional or atypical antipsychotic drugs.
[c]*Poor long-term outcome*: subjects treated with standard antipsychotics without adequate clinical response, but improved clinically satisfactorily treated with clozapine or with clozapine and one or more additional antipsychotic drugs.
[d]*P*-values are uncorrected for multiple testing.

term outcome. This might raise concern for the validity of the operational criteria applied in this study to estimate long-term outcome. However, both the clinical CGI rating and the demographic estimate of age at onset (AFA) are strongly correlated to long-term outcome, confirming the usefulness of this assessment.

Table 3 Multivariate logistic regression analysis of risk factors for long-term outcome[a]

Parameter	*Odds ratio[b] (95% CI[c])*	P-*value*[d]
Gender (male)[e]	1.7 (0.87–3.13)	0.13
CGI (5-7)[f]	7.4 (3.81–14.5)	0.000
AFA (cont.)[g]	0.96 (0.93–1.0)	0.025
rs7659 (three levels)[h]		0.11
−rs7659 (AA)	0.37 (0.14–0.98)	−0.045
−rs7659 (AG)	0.40 (0.15–1.1)	−0.068
rs7659 (two levels)[i]	0.39 (0.16–0.96)	0.041

[a]Overall significance of multivariate model: $\chi^2 = 54.774$; df = 5; $P < 0.0001$ (in the stepwise forward logistic regression analysis *P* inclusion value was set to 0.15)
[b]Odds ratio >1 indicates risk of having poor long-term outcome.
[c]Upper and lower 95% confidence interval are given in parentheses.
[d]*P*-values are uncorrected for multiple testing.
[e]Odds ratio refers to being male.
[f]Odds ratio refers to having a CGI score of 5, 6 or 7.
[g]AFA is used as a continuous variable, with higher AFA giving a marginal protection against poor long-term outcome.
[h]Genotypes are used as three categories using the GG genotype as reference. The overall *P*-value of this variable ($P = 0.11$) is given as well as individual *P*-values and odds ratios of the AA and AG genotypes.
[i]In a subsequent logistic analysis, the AA and AG genotypes were combined. The odds ratio and *P*-value of the combined AA/AG genotypes are given with respect to the reference GG genotype.

This also raised the possibility that differences in CGI or AFA might be used to fine-tune the long-term outcome estimate and uncover a putative association with the genotype. In fact, once these variables are included in a multivariate analysis, the GG genotype at the rs7659 locus does indeed confer risk of poor long-term outcome. In light of effect sizes of well-established genetic risk factors in complex disorders, the OR (~2.5) of the *ApoD* GG genotype represents quite a considerable risk of poor long-term outcome.

We cannot exclude that the signal detected in this study may originate from a nearby functional polymorphism in LD with this *ApoD* genotype. However, the *rs7659* polymorphism is positioned in a putative binding site for the human splicing factor SR SC35 (T Hansen, unpublished observation) and may therefore itself affect processing of the *ApoD* mRNA and give rise to genotype-specific ApoD activities. This argues that ApoD is involved in the pathology of schizophrenia either as a factor conferring increased risk of illness with poor long-term outcome or as a modulating factor affecting the responsiveness of otherwise identical disease entities.

In summary, our analyses did not indicate that *ApoD* genotypes were differentially distributed between healthy controls and each of the two disease groups, implying that ApoD does not define an etiologically distinct disease entity of refractory schizophrenia, but rather affects the regulatory potential of antipsychotics in the patient. Thus, it would be highly interesting to compare long-term clinical outcome and ApoD response to antipsychotic treatment in chronic patients. If acute changes in ApoD level are indeed indicative of therapeutic response, one may speculate that monitoring of ApoD may be used to guide pharmacotherapy even in recent onset patients.

Duality of interest

None declared.

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