Increased Brain β-Amyloid Load, Phosphorylated Tau, and Risk of Alzheimer Disease Associated With an Intronic CYP46 Polymorphism

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Background: CYP46, the gene encoding cholesterol 24-hydroxylase, plays a key role in the hydroxylation of cholesterol and thereby mediates its removal from brain.

Objective: To study the association of polymorphic sites on CYP46 with Alzheimer disease (AD) traits and with the risk of the development of AD.

Design: Alzheimer disease traits (β-amyloid load, β-amyloid peptides, hyperphosphorylated tau protein) were assessed in brain tissues and in the cerebrospinal fluid of patients with AD and control subjects. Genetic associations were studied in 2 independent populations.

Setting: Specialized centers for memory disorders in Switzerland, Greece, and Italy.

Participants: Fifty-five brain tissues from nondemented elderly patients for the histopathological studies; 38 patients with AD and 25 control subjects for the cerebrospinal fluid studies; 201 patients with AD and 248 control subjects for the genetic association studies.

Results: A polymorphism of CYP46 was associated with increased β-amyloid load in brain tissues as well as with increased cerebrospinal fluid levels of β-amyloid peptides and phosphorylated tau protein. Moreover, this CYP46 polymorphism was associated with higher risk of late-onset sporadic AD in 2 independent populations (odds ratio, 2.16; 95% confidence interval [CI], 1.41-3.32; P<.001). The additional presence of 1 or 2 apolipoprotein E ε4 alleles synergistically increased the risk of AD to an odds ratio of 9.6 (95% CI, 4.9-18.9; P<.001) as compared with 4.4 for apolipoprotein E ε4 alone (95% CI, 2.8-6.8; P<.001).

Conclusion: CYP46 influences brain β-amyloid load, cerebrospinal fluid levels of β-amyloid peptides and phosphorylated tau, and the genetic risk of late-onset sporadic AD.

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The CYP46 gene encodes cholesterol 24-hydroxylase, the rate-limiting enzyme for cholesterol removal from the brain.1-3 Cholesterol 24-hydroxylase mediates the hydroxylation of brain cholesterol to 24-hydroxycholesterol that can be transported readily through the blood-brain barrier.4 Because depletion of brain cholesterol levels reduces the generation of β-amyloid peptides (Aβ),5,6 and because cholesterol-lowering drugs may reduce the risk of dementia,7,8 we tested whether polymorphisms of CYP46 are associated with brain β-amyloid load in humans by measuring the sequential pattern of β-amyloid deposition in the medial temporal lobe. Brain β-amyloid deposits contain large amounts of Aβ42. Consequently, we next tested whether CYP46 genotypes affected levels of Aβ42 in cerebrospinal fluid (CSF) obtained from patients with mild to moderate Alzheimer disease (AD).

Because Aβ42 fibrils can cause the formation of neurofibrillary tangles9,10 composed of phosphorylated tau protein, and because such cholesterol-related brain diseases as Niemann-Pick type C are paralleled by hyperphosphorylation of tau,11,12 we measured CSF levels of tau phosphorylated at threonine 181 (phospho-tau 181) and stratified the sample according to the CYP46 genotypes.

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Given the reported importance of brain cholesterol metabolism on Aβ production and phosphorylation of tau, CYP46 is a candidate gene for AD. In agreement with accepted recommendations for genetic association studies,13,14 we performed a

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case-control association study with 2 independent samples collected in Zurich, Switzerland, and in Southern Europe (Table 1) to study possible associations of CYP46 polymorphisms with AD. Herein we report that a genetic variation of CYP46 influences brain β-amyloid load, CSF levels of Aβ42 and phosphorylated tau, and the genetic risk of late-onset sporadic AD.

**METHODS**

**GENETIC SCREENINGS**

Information on polymorphic sites was derived from the database of single nucleotide polymorphisms (SNPs) established by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/index.html). Five SNP candidates within the genomic region of CYP46 and 2 SNP candidates 1 centimorgan 5’ to exon 1 of CYP46 were selected for genotyp-
dentata, by bandlike Aβ deposits in the subpial portion of the molecular layer of both the entorhinal region and the temporal neocortex, and by confluent lake-like Aβ deposits in the paraventricular layer of the presubiculum region. Diffuse and core-only plaques in the corpus ammonis 4 region are features of the fourth phase.

In addition to the 35 brains from elderly individuals without AD pathological findings, amyloid staging was also done in 21 brains meeting CERAD criteria for definite AD (mean age at death, 81.4 years; range, 69–90 years; 13 women). All neuropathological evaluations were done in a blinded manner with respect to genotype.

**GENETIC ASSOCIATION STUDIES**

Genetic studies were conducted on 2 independent European populations: a hypothesis testing sample (n=183) and a hypothesis confirming sample (n=266). The clinical diagnoses of AD were made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association and were based on medical interview and results of physical examination, neuropsychological testing, brain magnetic resonance imaging or computed tomography, and blood and CSF tests. The control group (n=248) consisted of elderly individuals who were either the spouses of patients with AD or subjects recruited from the outpatient clinics of the participating institutions. Dementia and memory deficits in control subjects were excluded by neuropsychological testing, consisting of the CERAD neuropsychological test battery 21 and the Mini-Mental State Examination. 20 Only participants with a Mini-Mental State Examination score equal to or greater than 27 were included in the control group.

**CSF MEASUREMENTS**

The CSF was obtained by lumbar puncture in a subset of the participants of the genetic studies in Zurich, Switzerland. All participants gave informed written consent. Thirty-eight patients with AD (mean age, 70.1 years; 16 women) and 25 control subjects (mean age, 66.0 years; 7 women) were included. The CSF samples were aliquoted immediately on withdrawal at the bedside and stored at −85°C until biochemical analyses were performed.

Levels of Aβ42 were determined by a commercial enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Innogenetics, Gent, Belgium). Tau phosphorylated at threonine 181 was determined by an enzyme-linked immunosorbent assay with AT270 for capture as described previously. 22 The CSF concentrations of 24S-hydroxycholesterol were measured by a modified highly sensitive method using combined gas chromatography–mass spectrometry as described previously. 22 All analyses were performed blinded to diagnosis and genotype.

**STATISTICS**

Genotype and allelic frequencies between patients with AD and control subjects were compared by Pearson χ² tests. Forward and backward unconditional logistic regression analyses were done for the simultaneous assessment of the influence of age, sex, APOE, and CYP46 genotypes on the risk of AD. The estimated haplotype frequencies program was used to test for LD between SNPs. It computes the maximum-likelihood estimators for the haplotype frequencies assuming no association (H0) and allelic association (H1) and calculates the χ² statistic as the 2-fold difference between the log likelihoods. 23 Stages of neurofibrillary tangles and phases of β-amyloidosis between groups were compared with the Mann-Whitney (Wilcoxon rank sum) test. Statistical significance was assumed at P=0.05.

**RESULTS**

**BRAIN β-AMYLOID LOAD AND CSF Aβ42 CONCENTRATIONS**

Brain β-amyloid load (ie, evolutionary phases [0-4] of β-amyloid load) in nondemented elderly subjects with the CYP46*TT genotype (the CYP46 gene encodes cholesterol 24-hydroxylase) was significantly higher than in CYP46*TT-negative subjects (n=28) (mean±SEM, 1.46±0.28 vs 0.48±0.09, respectively; P=0.005) (Figure 2A). The combined presence of 1 or 2 APOE4 alleles and the CYP46*TT genotype was associated with the highest levels of brain β-amyloid load, while β-amyloid load was intermediate in the presence of either APOE4 or CYP46*TT and lowest in subjects without APOE4 and CYP46*TT alleles (Figure 2B). In patients with AD, β-amyloid load reached maximum levels (phases 3 and 4) with low variances (3.40±0.15; n=21) throughout the sample. Because of this ceiling effect, the sample was ill suited to genotype...
that this gradual difference was highly significant.

24-hydroxylase) and patients with Alzheimer disease (AD) with the apolipoprotein E
CYP46*TT

We observed the highest CSF levels of phospho-tau 181

The CSF levels of phospho-tau 181 were markedly higher

0.05

Figure 3. A, Higher cerebrospinal fluid concentration of tau phosphorylated at threonine 181 (phospho-tau 181) in both nondemented control subjects with the CYP46*TT genotype (the CYP46 gene encodes cholesterol 24-hydroxylase) and patients with Alzheimer disease (AD) with the CYP46*TT genotype. Bars represent mean ± SEM. B, Higher cerebrospinal fluid levels of phospho-tau 181 in patients with AD with both CYP46*TT and apolipoprotein E e4 (APOE4) alleles. Bars represent mean ± SEM. Minus sign indicates negative; plus sign, positive.

PHOSPHO-TAU 181 LEVELS IN THE CSF

The CSF levels of phospho-tau 181 were markedly higher in patients with AD (n = 38) than in controls (n = 26) (t test, P < .001). Carriers of the CYP46*TT genotype had higher CSF levels of phospho-tau 181 both in AD and in the control groups (F1.24 = 9.0, P = .004, multifactorial analysis of variance controlled for age, sex, and APOE4 effects). We observed the highest CSF levels of phospho-tau 181 in patients with AD with CYP46*TT (20 ± 2 pg/mL; n = 19), followed by CYP46*TT-negative patients with AD (12 ± 2 pg/mL; n = 19), followed by control subjects with CYP46*TT (6 ± 2 pg/mL; n = 10), and followed by CYP46*TT-negative control subjects (3 ± 2 pg/mL; n = 15) (Figure 3A). Forward and backward linear regression analysis controlled for age, sex, and APOE4 effects showed that this gradual difference was highly significant (r standardized = 0.62; n = 63; P < .001). The additional presence of 1 or 2 APOE4 alleles resulted in the highest CSF levels of phospho-tau 181 in patients with AD (0.028 ± 0.003 ng/mL; n = 11), intermediate levels in those carrying either the APOE4 allele (0.017 ± 0.003 ng/mL; n = 12) or CYP46*TT genotype (0.017 ± 0.003 ng/mL; n = 8), and lowest levels in those negative for both APOE4 and CYP46*TT (0.006 ± 0.004 ng/mL) (F1.24 = 6.2, P = .002; age- and sex-corrected analysis of variance) (Figure 3B).

Post hoc least significant difference tests for pairwise comparisons confirmed statistically significant differences between carriers of both APOE4 and CYP46*TT vs APOE4 carriers only (P = .04), patients carrying neither APOE4 nor CYP46*TT vs those carrying APOE4 only (P = .01), and patients carrying neither APOE4 nor CYP46*TT vs those carrying CYP46*TT only (P = .02). Thus, the CYP46*TT and APOE4 alleles were associated with high CSF levels of phospho-tau 181 both in patients with AD and in control subjects.

24S-HYDROXYCHEOLESTEROL AND CHOLESTEROL LEVELS IN THE CSF

Cholesterol-corrected CSF levels of 24S-hydroxycholesterol were significantly higher in patients with AD (0.56 ± 0.19 ng/µg; n = 24) than in controls (0.44 ± 0.42 ng/µg; n = 22) (P = .001, Mann-Whitney test). There were no differences in the CSF levels of 24S-hydroxycholesterol between CYP46*TT-positive and -negative patients with AD or control subjects. CYP46*TT-positive patients with AD had higher CSF cholesterol concentrations (0.54 ± 0.20 mg/dL [0.0140 ± 0.0052 mmol/L]; n = 15) than CYP46*TT-negative patients with AD (0.42 ± 0.12 mg/dL [0.0109 ± 0.0031 mmol/L]; n = 16). However, this difference failed to reach statistical significance (P = .07; t test).

GENETIC ASSOCIATION STUDIES

CYP46 genotype distribution in both samples was as expected under Hardy-Weinberg equilibrium conditions both in patients with AD and in control subjects (P ≥ .45 for each comparison). The frequencies of CYP46*TT were higher in patients with AD as compared with control subjects in both samples (60.7% vs 46.1%; P = .049; 58.5% vs 43.0%; P = .02; respectively) (Table 2). The distributions of APOE and CYP46 genotypes were similar in both samples (P > .2). We therefore combined the samples and confirmed significant associations of APOE and CYP46 genotypes with AD (both P < .001) (Table 3). The age- and sex-adjusted odds ratio (OR) for the risk of AD in homozygous carriers of the CYP46*TT allele was 2.16 (95% confidence interval [CI], 1.41-3.32). The OR for APOE4 allele carriers was 4.38 (95% CI, 2.83-6.77). Separate analysis of the 2 independent samples resulted in similar ORs (Table 3). The OR for the presence of both the CYP46*TT and the APOE4 genotypes was 9.63 as compared with the absence of these genotypes (95% CI, 4.89-18.96; P < .001). The OR for APOE4 carriers without the CYP46*TT genotype was 4.06 (95% CI, 2.22-7.44; P < .001). The OR for CYP46*TT in APOE4-negative subjects was 2.03 (95% CI, 1.17-3.53; P = .01). These data suggest synergistic interactions between APOE and CYP46 on the risk of AD.

In a sample of 334 participants, the frequency of the SERPINA3*A allele was 55.0% in 181 control subjects and 55.2% in 153 patients with AD (P = .95). Neither a significant interaction between SERPINA3 and CYP46
Combined Sample, No. (%)

Pearson

Hypothesis-Testing Sample
Control Subjects (n = 76) Patients With AD (n = 107)

<table>
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<th>CYP46 Genotype</th>
<th>Control Subjects</th>
<th>Patients With AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>6 (7.9)</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>CT</td>
<td>35 (46.1)</td>
<td>37 (34.6)</td>
</tr>
<tr>
<td>TT</td>
<td>35 (46.1)</td>
<td>65 (60.7)</td>
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</table>

Statistics

Pearson $\chi^2 = 4.01$  
$P = .14$

Hypothesis-Confirming Sample
Control Subjects (n = 172) Patients With AD (n = 94)

<table>
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<th>Patients With AD</th>
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<tbody>
<tr>
<td>CC</td>
<td>19 (11.0)</td>
<td>7 (7.4)</td>
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<tr>
<td>CT</td>
<td>79 (45.9)</td>
<td>32 (34.0)</td>
</tr>
<tr>
<td>TT</td>
<td>74 (43.0)</td>
<td>55 (58.5)</td>
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Statistics

Pearson $\chi^2 = 5.87$  
$P = .05$

Combined Sample (201 Patients With AD, 248 Control Subjects)

<table>
<thead>
<tr>
<th>CYP46 Genotype</th>
<th>Control Subjects</th>
<th>Patients With AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>25 (10.1)</td>
<td>12 (6.0)</td>
</tr>
<tr>
<td>CT</td>
<td>114 (46.0)</td>
<td>69 (34.3)</td>
</tr>
<tr>
<td>TT</td>
<td>109 (44.0)</td>
<td>120 (59.7)</td>
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</table>

Statistics

Pearson $\chi^2 = 11.37$  
$P = .003$

Abbreviations: AD, Alzheimer disease; CYP46, gene encoding cholesterol 24-hydroxylase.

There is growing experimental evidence that elevated cholesterol levels may increase amyloid production, possibly by modulating $\alpha$- and $\beta$-secretase activities in the endoproteolytic processing of amyloid precursor protein. In humans, statins may lower the lifetime risk of dementia. Among all human tissues, the relative concentrations of cholesterol are highest in the central nervous system, possibly because of the large surface-to-volume ratios of brain cells along with their high density. Despite these high concentrations, the mechanisms that regulate central nervous system levels of cholesterol are limited to hydroxylation to 24-hydroxycholesterol by cholesterol 24-hydroxylase. In humans, APOE4 and CYP46 are expressed predominantly in the brain, with messenger RNA mainly found in the gray matter. In situ hybridizations of mouse brains showed abundant messenger RNA in neurons of the cerebral cortex, hippocampus, dentate gyrus, and the thalamus, suggesting CYP46 expression in neurons of brain structures preferentially affected in AD. It is therefore intriguing to speculate that functional alterations of cholesterol 24-hydroxylase may modulate cholesterol concentrations in vulnerable neurons, thereby leading to altered membrane composition and associated changes in amyloid precursor protein processing and in $\alpha$-secretase production.

We observed that brain $\beta$-amyloid load in subjects with the CYP46*TT genotype was significantly higher than in CYP46*TT-negative subjects. Moreover, the genetic combination of APOE4 and CYP46*TT was associated with the highest levels of brain $\beta$-amyloid load. In addition, CYP46*TT resulted in elevated levels of CSF $\beta$-42 in patients with AD. These observations underscore a possible relationship between cholesterol and brain amyloid formation. Further in vitro experiments (e.g., neuronal transfections) should elucidate the role of CYP46 in $\beta$-secretase generation.

We also observed that CYP46*TT and APOE4 were associated with high CSF levels of phospho-tau 181 in both patients with AD and control subjects. Because threonine 181 of tau is hyperphosphorylated in neurofibrillary tangles in AD and because hyperphosphorylation may precede tangle formation, our findings possibly relate CYP46 and APOE to neurofibrillary tangle formation. Interestingly, Niemann-Pick type C disease, which is caused by disturbances of cholesterol distribution and cholesterol accumulation in neurons, is characterized by hyperphosphorylation of tau and the development of brain tauopathy. Measurements of 24S-hydroxycholesterol in CSF failed to demonstrate significantly different levels among CYP46 genotype groups. Neuronal levels of cholesterol 24-hydroxylase reportedly are decreased in AD and are paralleled by increased levels of cholesterol 24-hydroxylase in reactive astrocytes. In addition, levels of 24S-hydroxycholesterol vary across different severity
stages of AD. Therefore, measurements of 24S-hydroxycholesterol in CSF may not be sufficient to determine changes in cholesterol metabolites in neurons. We also observed slightly elevated (yet not significantly different) levels of CSF total cholesterol in demented CYP46*TT carriers. This elevation might mirror CYP46 genotype–dependent differences in brain cholesterol homeostasis in AD and should be further examined in larger samples.

Finally, we demonstrated that the CYP46*TT genotype is associated with the risk of late-onset, sporadic AD in 2 independent populations and observed a genetic synergism between APOE4 and CYP46*TT on the risk of AD. Heterozygous individuals or CYP46*C allele homozygotes were at decreased risk of AD. The SNP rs754203 is located 5′ to exon 3 of CYP46. Intrinsic SNPs have been shown to modulate genetic risk of AD, possibly through alternative splicing or altered RNA stability, or through LD with other causal loci. Sequencing of all 15 exons of CYP46 in 40 chromosomes derived from subjects homozygous for either T (n = 20) or C (n = 20) alleles failed to identify additional linked exon SNPs. CYP46 maps to chromosome 14q32.1, 6.4 cM to SNP3A3, which encodes α1-antichymotrypsin. Because SERPINA3 was previously associated with AD, we determined whether SERPINA3 and CYP46 are in LD and whether SERPINA3 is associated with AD in our sample. The frequency of the SERPINA3*A allele was similar in controls and patients with AD. In addition, neither a significant interaction between SERPINA3 and CYP46 nor significant LD between SERPINA3 and CYP46 was observed. The possibility that CYP46 is a risk gene for AD warrants replication in independent populations from independent groups.

In conclusion, our findings indicate that CYP46 polymorphisms are associated with brain β-amyloid load, CSF levels of both Aβ42 and phospho-tau 181, and the risk of late-onset sporadic AD. Our findings also suggest a synergistic interaction of CYP46 with APOE4 on the risk of AD. Therefore, these data are consistent with the possibility that CYP46 is a novel susceptibility gene for AD.

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Author contributions: Study concept and design (Drs Papassotiropoulos, Strefer, Schmid, Thal, Bruhl, Nitsch, and Hock); acquisition of data (Drs Papassotiropoulos, Strefer, Tsolaki, Schmid, Thal, Nicosia, Iakovidou, Maddalena, Lütjohann, Ghebremedhin, Hegi, Pasch, Benussi, Binetti, Braak, Nitsch, and Hock and Ms Traxler); analysis and interpretation of data (Drs Papassotiropoulos, Strefer, Schmid, Thal, Lütjohann, Nitsch, and Hock and Ms Traxler); drafting of the manuscript (Drs Papassotiropoulos, Schmid, Bruhl, and Nitsch); critical revision of the manuscript for important intellectual content (Drs Papassotiropoulos and Nitsch); obtaining funding (Drs Papassotiropoulos, Nitsch, and Hock); administrative, technical, or material support (Drs Papassotiropoulos, Strefer, Tsolaki, Schmid, Thal, Nicosia, Iakovidou, Maddalena, Lütjohann, Ghebremedhin, Hegi, Pasch, Benussi, Binetti, Braak, Nitsch, and Hock and Ms Traxler); study supervision (Drs Papassotiropoulos, Nitsch, and Hock).

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