# Primary Open-Angle Glaucoma: Association with Cholesterol 24S-Hydroxylase (CYP46A1) Gene Polymorphism and Plasma 24-Hydroxycholesterol Levels

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**PURPOSE.** Genetics has made significant contributions to the study of glaucoma over the past few decades. Cholesterol-24S-hydroxylase (CYP46A1) is a cholesterol-metabolizing enzyme that is especially expressed in retinal ganglion cells. CYP46A1 and its metabolic product, 24S-hydroxycholesterol, have been linked to neurodegeneration. A single-nucleotide polymorphism (SNP) in the *CYP46A1* gene, designated as rs754203 and associated with Alzheimer disease, was evaluated as a genetic risk factor for primary open-angle glaucoma (POAG), as well as plasma 24S-hydroxycholesterol levels.

**METHODS.** The frequency of the *CYP46\*C* and *CYP46\*T* alleles was analyzed in 150 patients with POAG and 118 control subjects. Plasma 24S-hydroxycholesterol levels were quantified. Sex, age, alleles, and genotype frequencies between patients with POAG and control subjects were compared by using the $\chi^2$ and Student's *t*-tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression to assess the relative association between disease and age, sex, and genotypes.

**RESULTS.** The frequency of the TT genotype was significantly higher in patients with POAG than in control subjects (61.3% versus 48.3%, respectively, OR = 1.26; 95% CI = 1.006–1.574, $P < 0.05$). Plasma 24S-hydroxycholesterol levels did not differ between control subjects and patients with POAG. The ratio of estimated brain weight to liver volume as an estimate of the capacity of the human body to synthesize and metabolize 24S-hydroxycholesterol was found to correlate to plasma 24S-hydroxycholesterol in control subjects and patients with POAG.

**CONCLUSIONS.** The rs754203 SNP in CYP46A1 was associated with a risk for POAG. This polymorphism was not associated with changes in plasma 24S-hydroxycholesterol, highlighting
that despite its retinal origin, 24S-hydroxycholesterol cannot be used as a biomarker for POAG. (*Invest Ophthalmol Vis Sci.* 2009;50:5712–5717) DOI:10.1167/iovs.09-3655

Glaucoma is the second leading cause of blindness worldwide, affecting more than 50 million people.[1] Glaucoma is a progressive optic neuropathy characterized by optic nerve head changes and visual field loss.[2] Improving case identification and providing a better understanding of the causes of glaucoma has brought about great progress in defining risk factors such as high intraocular pressure, age, familial history, ethnicity, and myopia, which have been associated frequently with primary open-angle glaucoma (POAG).[3] Genetics has substantially contributed to the study of glaucoma over the past few decades. Myocilin has been identified and validated as the first gene linked to more than 10% of juvenile-onset POAG cases and 4% of adult-onset POAG cases.[4,5] Other genes such as optineurin, WDR36, and CYP1B1 have been found more frequently in certain clinical presentations of glaucoma. Nevertheless, the link between the genes and the development of the disease is not always clearly demonstrated.[6] Other associations such as diabetes or systemic hypertension are still being debated. The links between dyslipidemia and particularly cholesterol abnormalities have already been described.[7] Recent studies have reported the efficacy of statins and nonstatin cholesterol-lowering drugs in the treatment of POAG.[8–10] Although the mechanism underlying this association remains unclear, these data suggest that reducing cholesterol biosynthesis may be a target for POAG treatment. Increasing cholesterol efflux and/or enhancing cholesterol catabolism may also be critical.

We[11] and others[12] recently reported that cholesterol 24S-hydroxylase (CYP46A1) is exclusively expressed in the neuronal part of the retina, most particularly in retinal ganglion cells. This finding consistently supports the possible role played by CYP46A1 in cholesterol homeostasis in ocular structures. Cholesterol-24S-hydroxylase was initially identified as a cholesterol-metabolizing enzyme in the brain. Therein, it catalyzes the formation of 24S-hydroxycholesterol, a polar metabolite of cholesterol that can cross the blood–brain barrier.[13–17] In addition to their role in disease-free conditions, cholesterol-24S-hydroxylase (CYP46A1) and 24S-hydroxycholesterol have been linked to neurologic disorders of the central nervous system[18–20] and potentially to neuronal death.[19] Single-nucleotide polymorphisms (SNPs) have been identified in the *CYP46A1* gene. In some[21–32] but not all[28,33–39] studies, this type of polymorphism has been associated with increased risk for late-onset Alzheimer disease. Surprisingly, whether this intronic polymorphism affects the P450 mRNA and protein levels has not yet been studied. Therefore, today the mechanistic relation between the polymorphism and the apparent effect on the risk of Alzheimer disease remains unknown.

The purpose of this study was to explore the association of rs754203 SNP in intron 2 of the CYP46A1 gene with the risk of POAG in a population from a single center in France and to

evaluate plasma levels of the cholesterol metabolite formed by CYP46A1 activity, 24S-hydroxycholesterol.

## METHODS

### Subjects

The protocol adhered to the tenets of the Declaration of Helsinki. This cross-sectional study was approved by the Ethics Committee of Burgundy (France). Informed consent was obtained from all subjects before participation.

Blood samples were collected from 150 patients with glaucoma and 118 control subjects after an overnight fast at the Department of Ophthalmology, Dijon University Hospital, Dijon, France. None of the subjects was related to any other. All patients included in the study were examined by a glaucoma specialist and underwent a thorough ocular examination. The patients had been observed in our clinic for several years. They did not have a history of neurologic disease or dyslipidemia. Those with angle-closure glaucomas and secondary glaucomas were excluded. POAG was defined by an open angle on gonioscopy, characteristic glaucomatous optic nerve head abnormalities, and corresponding reliable visual field defects recorded with SITA standard white-on-white perimetry (Humphrey Field Analyzer HFA-II; Carl Zeiss Meditec, Paris, France). Among the 150 patients, 18 had normal-tension glaucoma and four had exfoliative glaucoma. The glaucoma stages were defined according to the classification of Hoddap et al.[40] The control subjects were age matched and recruited among patients operated on for cataract or lid surgery and willing to participate in the study. They had an IOP below 21 mm Hg, no evidence of glaucoma on optic disc examination, and a normal frequency-doubling technique screening test result. The brain weight and liver volume were calculated using size and weight data and established formulas from the literature.[41–43]

### CYP46A1 Genotyping

Buffy coats were placed on DNA-preserving paper (FTA; Whatman, Kent, UK) and stored at room temperature in the dark according to the manufacturer's instructions. A 1.25-mm sample disc was taken for each PCR reaction. A 285-bp PCR product was amplified by using specific primers for rs754203 SNP (forward primer: 5′-TGAAAACGAGTTTC-CCGTCC-3′; reverse primer: 5′-GTGTGACCAGGTAACAGTCA-3′) in a 12.5-$\mu$L volume reaction with 1.25 U of DNA polymerase, 1.5 mM of MgCl$_2$, 1.25 $\mu$L of 10× NH$_4$ buffer, 200 $\mu$M dNTPs (BioTaq; Bioline GmbH, Luckenwalde, Germany).

After initial denaturation at 95°C for 8 minutes, the reaction mixture was subjected to 50 cycles of 1-minute denaturation at 95°C, 1-minute annealing at 53°C, and a 2-minute extension at 72°C, followed by a final extension at 72°C for 5 minutes. The PCR products were digested by *Msp*I (Promega, Charbonnières, France). The fragment amplification and digestion results were revealed by 1.8% agarose gel electrophoresis and ethidium bromide staining. The CYP46A1*T allele corresponded to the uncut 285-bp fragment, whereas the CYP46A1*C allele was characterized by two fragments of 209 and 76 bp.

### Quantification of Plasma 24S-hydroxycholesterol Levels

Plasma 24S-hydroxycholesterol levels were assayed by isotope dilution mass spectrometry using racemic [23,23,24-$^2$H$_3$]-24-hydroxycholesterol, as previously described.[14,17,18,44] Briefly, deuterated 24-hydroxycholesterol (200 ng, [23,23,24-$^2$H$_3$]-24-hydroxycholesterol) was added to 0.5 mL of plasma. After alkaline hydrolysis with 1 N KOH in 90% ethanol for 2 hours, the solution was neutralized with 65 $\mu$L of phosphoric acid, and the sterols were extracted with 9 mL of chloroform in the presence of 3 mL of 0.9% sodium chloride. The organic phase was removed, and the solvent was evaporated to dryness. The sterols were dissolved in 1 mL of toluene. A 100 $\mu$L aliquot was

removed for quantification of the cholesterol concentration. For this purpose, 30 $\mu$g of 5$\alpha$-cholestane was added, the solvents were evaporated to dryness, and the sterols were derivatized to trimethylsilyl ethers by heating at 60°C after the addition of 200 $\mu$L of pyridine and 200 $\mu$L of BSTFA (Supelco, Bellafonte, PA). The solvents were evaporated under nitrogen gas, and the samples were resuspended in hexane and analyzed on a gas chromatography-electron ionization mass spectrometer (HP6890 series II; Agilent, Palo Alto, CA) plus a chromatograph combined with a mass selective detector (HP; Agilent) operated in selected ion monitoring mode. A 1-$\mu$L aliquot was injected by automated injection in a splitless mode at an injection temperature of 300°C on a fused silica capillary column (30 m × 0.25 mm ID; 0.25 $\mu$m film thickness; DB-5MS; J&W Scientific, Agilent Technologies, Massy, France). The initial oven temperature was kept at 50°C for 1 minute; then it was increased at a rate of 20°C · min$^{-1}$ to 250°C and thereafter at 5°C · min$^{-1}$ to a final temperature of 300°C. The temperature of the transfer line was kept at 300°C. Electron impact ionization was used at 70 eV ionization energy. Trimethylsilyl-cholesterol and 5$\alpha$-cholestane were measured at *m/z* 368 and 372 atomic mass units (amu), respectively. Absolute amounts of cholesterol were determined by interpolation from a standard curve generated in each experiment.

The remainder of the saponified sterols was purified on silica columns (Supelco) for quantification of 24S-hydroxycholesterol. Cholesterol was eluted with 8 mL of 0.5% isopropanol in hexane. Purified oxysterols were subsequently eluted with 5 mL of 30% isopropanol in hexane and derivatized to trimethylsilyl ether as just described. The samples were analyzed by gas chromatography-mass spectrometry as described. Both 24S-hydroxycholesterol and deuterated 24-hydroxycholesterol were measured at *m/z* 413 and 416 amu, respectively. Absolute amounts of 24S-hydroxycholesterol were determined by interpolation from a standard curve generated in each experiment. A typical mass spectrometry-gas chromatogram obtained from a plasma sample is displayed in Figure 1, as well as a typical standard curve in the inset in Figure 1.

### Statistical Analysis

The Student's *t*-test and the $\chi^2$ test were performed to compare sex, age, alleles, and genotype frequencies between POAG cases and controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression analysis to assess the relative association between disease and age, sex, and genotypes. The percentage of population-attributable risk of carrying the TT genotype was calculated with the formula $f(R - 1)/R$, where $f$ is the fraction of cases with the TT-genotype and $R$ is the measure of OR. The SAS Software (SAS Institute, Cary, NC) was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

## RESULTS

The clinical characteristics of control subjects and patients with POAG are presented in Table 1. Both groups were similar except for the female-to-male ratio in moderate POAG compared with control subjects and other POAG subgroups (Table 1). The genotypic OR and 95% CI for POAG were estimated assuming a dominant genetic model (major TT homozygote versus the others). The frequency of the TT-genotype and T-allele in the CYP46A1 intron 2 rs754203 polymorphism was significantly higher in the population of patients with POAG than in the control group: 61.3% versus 48.3%, respectively, for the TT genotype ($P < 0.05$). This frequency was even greater in patients with normal-tension glaucoma since 13 of the 18 carried the TT genotype. The rs754203 SNP in intron 2 of CYP46A1 was associated with a significant risk for POAG (OR = 1.26; 95% CI = 1.006–1.574, $P < 0.05$, Table 1).

Patients with POAG had no significant differences in plasma 24S-hydroxycholesterol levels and the 24S-hydroxycholesterol-to-cholesterol ratio compared with control subjects ($P > 0.05$,
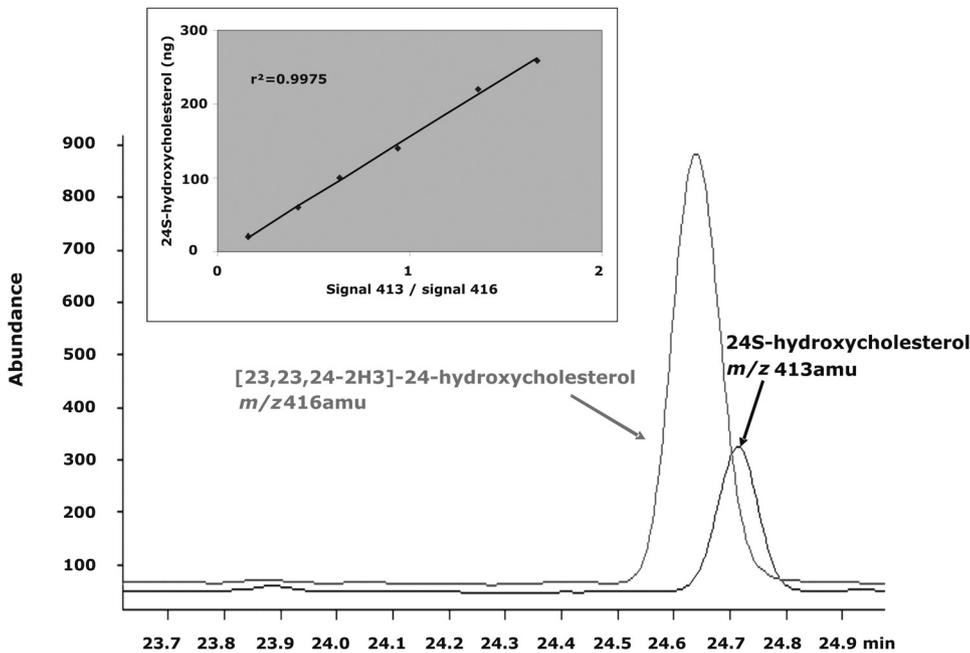
Table 1). Similarly, the plasma 24S-hydroxycholesterol levels and the 24S-hydroxycholesterol-to-cholesterol ratio were not different between CC, CT, and TT carriers when considering patients with POAG and control subjects independently ($P > 0.05$, Table 2) or together ($P > 0.05$, data not shown). Plasma absolute and cholesterol-related 24S-hydroxycholesterol levels did not correlate to visual field parameters such as MD (mean defect) or PSD (pattern standard deviation) (data not shown). The subjects' ages, corresponding to the age when blood was sampled, were used as the cofactor. Figure 2 shows the plasma cholesterol-related levels of 24S-hydroxycholesterol in control subjects (Fig. 2A) and patients with POAG (Fig. 2B) from the age of 44 years onward. The ratio between brain weight and liver volume for the different ages, based on data from the literature,[41–43] is also indicated in the figure. The levels of 24S-hydroxycholesterol closely followed the brain weight-to-liver volume ratio during the different decades of life. The only exception was observed in patients with POAG older than 80 years who had lower plasma 24S-hydroxycholesterol levels than expected from their brain weight-to-liver volume ratio.

## DISCUSSION

Glaucoma encompasses a group of diseases that appear to be genetically heterogeneous. Different glaucoma genes have been identified, but these genes account for only a small proportion of glaucomas.[45] Myocilin mutations have been linked to POAG and associated with 10% of juvenile-onset and 4% of adult-onset cases of POAG.[4,5] Nevertheless, contradictory data have been obtained on other genes such as apolipoprotein E.[46–56] Most glaucoma cases appear to be multifactorial and are probably affected by multiple interacting loci. Several genetic susceptibility factors have been suggested to contribute to glaucoma. These factors fit into two broad groups, those affecting intraocular pressure and those that are important in modulating retinal ganglion cell viability.[45] Recent data suggest that cholesterol and cholesterol metabolism may be involved in the pathogenesis of glaucoma.[8,9,57] Cholesterol-24S-hydroxylase (CYP46A1) is a cholesterol-metabolizing enzyme involved in the removal of cholesterol from neuronal structures.[13,14,17] Among various SNPs in the CYP46A1

**TABLE 1.** Clinical Characteristics, Plasma 24S-Hydroxycholesterol Levels and SNP in Intron 2 of the *CYP46A1* Gene in the Study Subjects

|  | Control Subjects | Patients with POAG | | | |
|---|---|---|---|---|---|
|  |  | All | Early* | Moderate* | Advanced* |
| *n* | 118 | 150 | 31 | 49 | 70 |
| Female-male ratio | 1.3 | 1.1 | 1.1 | 1.6 | 0.9 |
| Age, y (mean $\pm$ SEM) | 70.9 $\pm$ 1.1 | 69.5 $\pm$ 1 | 66.1 $\pm$ 0.9 | 68.4 $\pm$ 0.9 | 71.5 $\pm$ 1 |
| Plasma 24S-hydroxycholesterol |  |  |  |  |  |
| ng $\cdot$ mL of plasma$^{-1}$ (mean $\pm$ SEM) | 97.4 $\pm$ 3.7 | 94 $\pm$ 2.6 | 102.8 $\pm$ 3.2 | 92.1 $\pm$ 2.5 | 91.5 $\pm$ 2.5 |
| ng $\cdot$ mg of cholesterol$^{-1}$ (mean $\pm$ SEM) | 123.1 $\pm$ 5.1 | 126 $\pm$ 5.6 | 120.6 $\pm$ 4.9 | 124.6 $\pm$ 5.7 | 129 $\pm$ 5.8 |
| rs754203 SNP in CYP46A1 gene (percentage of total samples in each category) |  |  |  |  |  |
| CC | 2.5 | 3.3 | 3.2 | 4.1 | 2.9 |
| CT | 49.2 | 35.3 | 29 | 42.9 | 32.9 |
| TT | 48.3 | 61.3 | 67.7 | 53.1 | 64.3 |
| Allele frequency |  |  |  |  |  |
| CYP46A1*C | 0.27 | 0.19 | 0.18 | 0.26 | 0.19 |
| CYP46A1*T | 0.73 | 0.81 | 0.82 | 0.74 | 0.81 |

* Stages of glaucoma were defined according to the classification of Hoddap et al.[40]

TABLE 2. 24S-hydroxycholesterol Levels as a Function of rs754203 SNP in Intron 2 of the *CYP46A1* Gene in Control Subjects and Patients with POAG

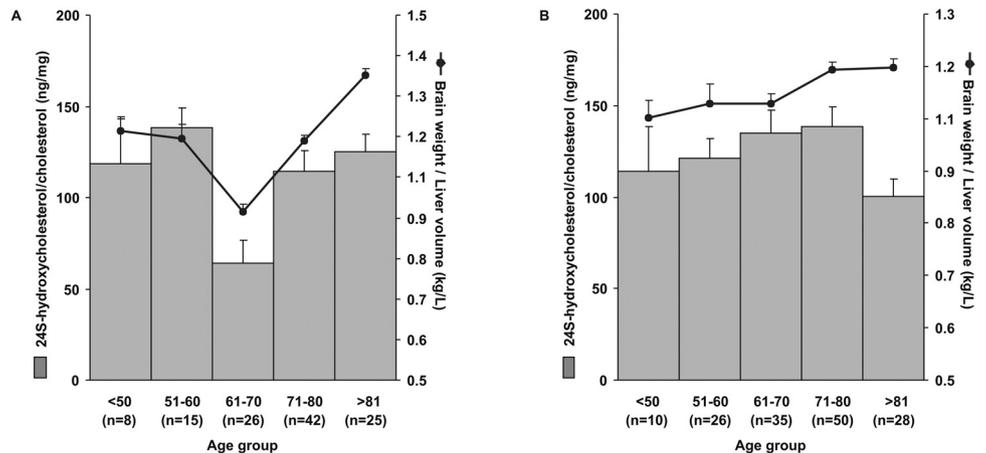| | Plasma 24S-hydroxycholesterol | | |
| | ng · mL plasma$^{-1}$ | ng · mg cholesterol$^{-1}$ | Age (y) |
|---|---|---|---|
| Control subjects (*n* = 118) | 97.4 ± 3.7 | 123.1 ± 5.1 | 70.9 ± 1.1 |
| TT (*n* = 57) | 104 ± 6.6 | 123 ± 7.5 | 71.2 ± 1.6 |
| CC+CT (*n* = 61) | 91 ± 3.5 | 123.2 ± 6.7 | 71 ± 1.4 |
| All POAG patients | 94.2 ± 2.6 | 124.8 ± 5.5 | 69.5 ± 0.9 |
| TT (*n* = 92) | 98.3 ± 3.4 | 123.7 ± 6.9 | 68 ± 1.2 |
| CC+CT (*n* = 58) | 87.9 ± 3.1 | 126.5 ± 9.7 | 68.2 ± 1.6 |
| Classified in early glaucoma* | 103.4 ± 1.3 | 122.8 ± 11.1 | 66.3 ± 0.4 |
| TT (*n* = 21) | 106.8 ± 9.8 | 126.35 ± 14.6 | 65.2 ± 2.3 |
| CC+CT (*n* = 10) | 94.9 ± 7.2 | 109.15 ± 15.6 | 67.8 ± 3.7 |
| Moderate glaucoma* | 92.1 ± 2.1 | 124.6 ± 10 | 68.3 ± 1.6 |
| TT (*n* = 26) | 96.8 ± 6.9 | 127.7 ± 14.1 | 70.3 ± 2.1 |
| CC+CT (*n* = 23) | 86.9 ± 5.1 | 121.2 ± 14.5 | 66 ± 2.3 |
| Advanced glaucoma* | 91.4 ± 4.6 | 128.9 ± 8.5 | 71.5 ± 1.2 |
| TT (*n* = 45) | 94.4 ± 5 | 122.4 ± 9.9 | 72.2 ± 1.8 |
| CC+CT (*n* = 25) | 86.5 ± 4.9 | 139.9 ± 15.7 | 70.2 ± 2.7 |

Results are expressed as the mean ± SEM.
* Stages of glaucoma were defined according to the classification of Hoddap et al.[40]

gene,[21-39,58] the TT genotype in intron 2 of CYP46A1, designated as rs754203 SNP, has been found to be a risk factor for Alzheimer disease.[25,26,30,32] Several observations have suggested a possible relationship between Alzheimer disease and glaucoma,[47,59-63] with possible pathogenic similarities. Among the common features of these two diseases are overproduction of Alzheimer-A$\beta$ peptide, which has been reported in the aqueous humor of patients with glaucoma,[64] and deposition of which has been associated with retinal degeneration.[65] Brown et al.[66] reported that 24S-hydroxycholesterol inhibits A$\beta$ and amyloid precursor protein secretion in cortical neurons via LXR activation. Increased 24S-hydroxycholesterol production would therefore be expected to decrease neuron degeneration.

Most 24S-hydroxycholesterol in human circulation originates from the brain[14,17] and is metabolized in the liver.[14,67] Hence, the ratio of brain weight-to-liver volume has been found to be a powerful indicator of the age-related variations of plasma 24S-hydroxycholesterol levels in humans.[18] In our population of patients with POAG and control subjects, we also found a correlation between plasma 24S-hydroxycholesterol and subject age (Fig. 2), suggesting that even though the neural retina also expresses CYP46A1 and is enriched in 24S-hydroxycholesterol,[11] it accounts for only a minor part of the plasma 24S-hydroxycholesterol levels. Furthermore, our data showed that 24S-hydroxycholesterol was not a relevant glaucoma biomarker.

The biological importance of 24S-hydroxycholesterol in the eye has not been clearly elucidated until now. We[11] and others[12] have found that the neural retina exclusively expresses CYP46A1 and is enriched in 24S-hydroxycholesterol.[11] Of note, Ikeda et al.[68] reported the existence of an enzyme in the nonpigmented ciliary epithelium capable of converting 24S-hydroxycholesterol into 7$\alpha$,24S-dihydroxycholesterol. 24S-hydroxycholesterol may also bind oxysterol-binding protein, which has already been reported to be expressed in the retina,[69] and thereby participate in cellular signaling. As in the brain,[13,14,17] production of 24S-hydroxycholesterol may be considered a metabolic pathway for neurons to eliminate cholesterol. Indeed, by triggering the addition of a hydroxyl group, CYP46A1 renders the cholesterol molecule more hydrophilic and facilitates its flux through biological membranes. Based on data from animal models and human observations, other mechanisms involving lipoproteins are also crucial for cholesterol metabolism in the retina.[70-74]

In view of the present controversy with respect to the possible link between the intronic polymorphism in the CYP46A1 gene and Alzheimer disease,[21-31,33-39] it is important that the results of the present study be confirmed in other populations of patients with glaucoma. Indeed, glaucoma can be considered an optic nerve neuropathy characterized by neurodegenerative processes.[2] But among the various subtypes of glaucoma, normal-tension glaucoma may share the closest



FIGURE 2. Plasma cholesterol-related 24S-hydroxycholesterol levels in normal subjects (A) and patients with POAG (B) as a function of age. Results are expressed as the mean ± SEM. Estimated ratio between brain weight and liver volume is indicated.

features with Alzheimer disease. Therefore, further investigations of CYP46 polymorphisms in those patients are warranted to verify the higher frequency of the TT genotype. Since we did not find a correlation between the levels of 24S-hydroxycholesterol in the circulation and the polymorphism, we do not have a mechanistic explanation for the apparent link between glaucoma and the polymorphism. If the present results can be confirmed, however, the polymorphism can be added as an additional risk factor for the disease.

## Acknowledgments

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