A Novel *ABCR* Nonsense Mutation Responsible for Late-Onset Fundus Flavimaculatus

Eric H. Souied,[1,2] *Dominique Ducroq,*[1] *Jean-Michel Rozet,*[1] *Sylvie Gerber,*[1] *Isabelle Perrault,*[1] *Margaret Sterkers,*[2] *Nathanael Benhamou,*[2] *Arnold Munnich,*[1] *Gabriel Coscas,*[2] *Gisèle Soubrane,*[2] *and Josseline Kaplan*[1]

Purpose. To report the ophthalmologic features of a novel truncating mutation in the *ABCR* gene in a patient affected with late-onset fundus flavimaculatus (FFM).

Methods. A complete ophthalmologic examination was performed in a 70-year-old patient, including best-corrected visual acuity measurement, slit lamp and fundus examination, fundus photographs, frequent fluorescein and indocyanine green angiographies, visual field testing, color vision analysis, electroretinogram, and electro-oculogram. The 50 exons of the *ABCR* gene were analyzed using direct sequencing.

Results. Fluorescein and indocyanine green angiographies confirmed the diagnosis of FFM. A heterozygous base change was found, resulting in the substitution of an arginine to a stop at codon 152 of the *ABCR* gene.

Conclusions. A heterozygous nonsense *ABCR* gene mutation was found in a patient affected with FFM. No other mutation has been identified in the entire coding sequence and the promoter region, suggesting that a heterozygous severe *ABCR* mutant may be responsible for a mild and delayed FFM phenotype, different from that of age-related macular degeneration. (*Invest Ophthalmol Vis Sci.* 1999;40:2740–2744)

Stargardt disease (STGD) is an autosomal recessive macular dystrophy of childhood, characterized by juvenile onset, a rapidly progressive course, and poor visual outcome.[1] The STGD gene has been mapped to 1p22.1, with evidence of genetic homogeneity of the disease, and has been physically mapped to a specific YAC contig.[2,3] In contrast, fundus flavimaculatus (FFM), an STGD-like phenotype, described by Franceschetti and François, is characterized by late-onset and a more slowly progressive course.[4] The disease is usually termed STGD when visual acuity loss begins in the first 2 decades, whereas the term FFM is favored when the disease begins at the end of the second decade or within the third decade and has a slowly progressive course.[5,6] The FFM gene has been mapped to the STGD locus, supporting the idea that the two conditions are allelic disorders.[3,7]

The ABCR protein belongs to a superfamily of membrane proteins involved in energy-dependent transport of a wide variety of substrates across membranes.[8] Mutations in the *ABCR* gene encoding a rod cell–specific ATP-binding cassette (ABC) transporter have been associated recently with at least four inherited retinal dystrophies: STGD, FFM, cone–rod dystrophy, and retinitis pigmentosa (RP19).[8–13] Mutation screening of the *ABCR* gene in large series of patients affected with STGD, FFM, or retinitis pigmentosa allowed genotype–phenotype correlations.[8–13] These studies suggest that homozygosity for truncating *ABCR* gene mutations results in retinitis pigmentosa phenotypes, whereas compound heterozygosity for frameshift and missense mutations or two missense mutations at this locus results in STGD disease. Conversely, FFM phenotype has always been reported as the result of two missense mutations. We report the ophthalmologic consequence of a novel mutation truncating *ABCR* in a patient affected with late-onset FFM.

Methods

Patient

A 70-year-old man attended our department for moderate loss of central vision in the left eye (LE; 20/30). He did not mention any prior visual symptom. A complete ophthalmologic examination was performed including best-corrected visual acuity measurement, slit lamp and fundus examination, fundus photography, fluorescein angiography (FA), indocyanine green (ICG) angiography, visual field testing, color vision analysis, electroretinogram (ERG) and electro-oculogram. This patient was subsequently examined at ages 71, 72, 73, 74, 75, and 76 years. Fundus photography and FA were performed using a Topcon camera (model 50IA; Tokyo, Japan). ICG angiography was performed using a scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany). Visual field analysis (static perimetry), color vision analysis (28-hue Farnsworth–Munsell), ERG, and electro-oculogram were performed according to standardized protocols.

The patient had no siblings and was born to nonconsanguineous parents. His two healthy sons underwent best-corrected visual acuity measurement, slit lamp and fundus examination, and FA.

Mutation Analysis

The *ABCR* gene was analyzed using both single-strand conformation polymorphism (SSCP) and sequencing of each exon. Informed consent was obtained, as required by French bioethics legislation, in agreement with the Declaration of Helsinki for research involving human subjects. The entire coding region and intronic sequences flanking the 50 exons were analyzed using the previously described primers.[14] The DNA (100 ng) from peripheral blood leukocytes was amplified using the primers and 0.1 μl [α-^{33}P]dCTP (10 mCi/ml) in a 25-μl amplification mixture containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 units *Taq* polymerase. The PCR conditions were set for each exon. Amplified DNA (6 μl) was mixed with an

From the [1]Institut National de la Santé et de la Recherche Médicale U-393, Hôpital des Enfants-Malades, Paris, and the [2]Clinique Ophthamologique Universitaire de Créteil, France.

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Corresponding author: Gisèle Soubrane, Clinique Ophthamologique Universitaire de Créteil, 40 Avenue de Verdun, 94010 Créteil, France.

E-mail: soubranegisele@europost.com

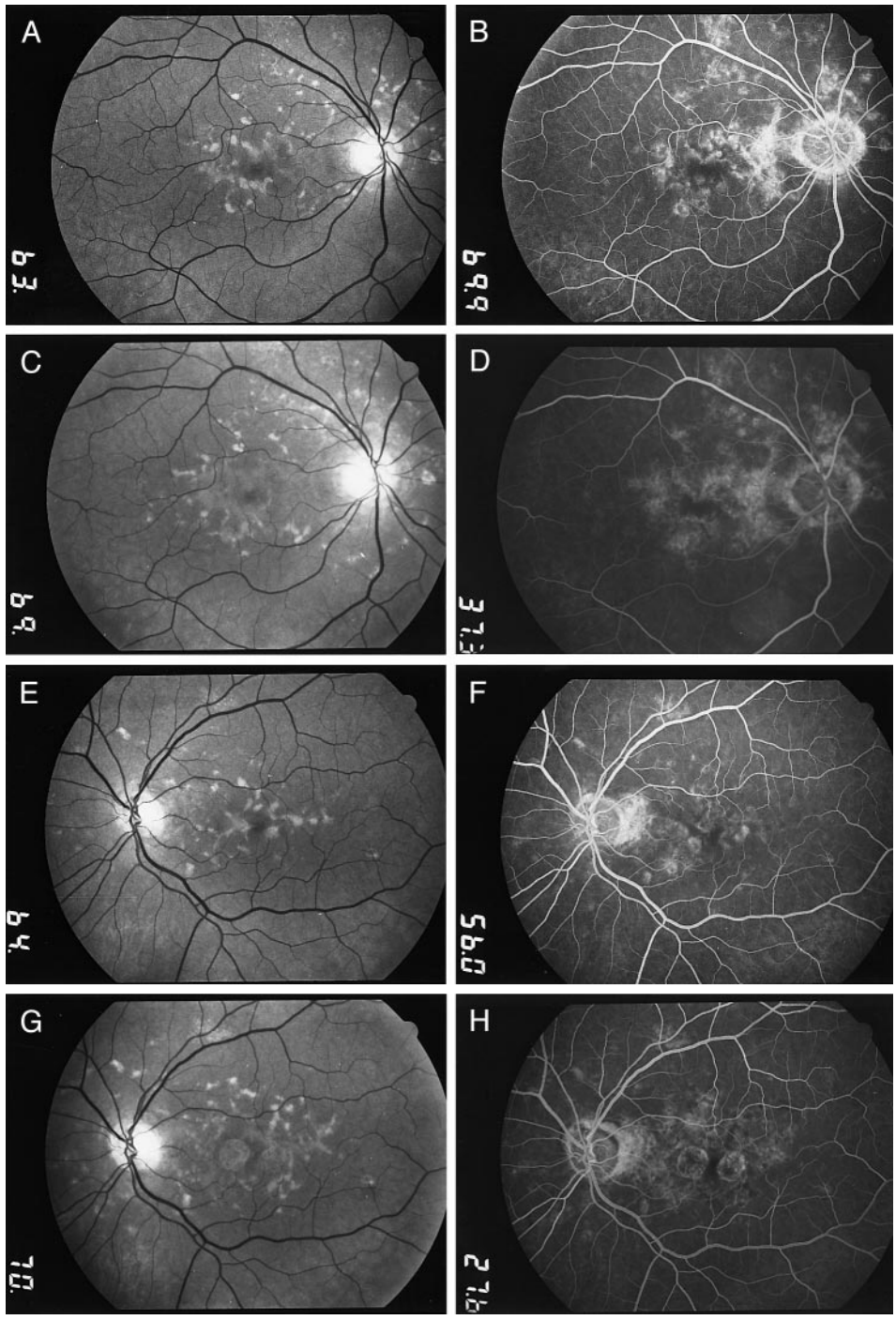

FIGURE 1. Natural course of the proband over a 6-year period. The red-free frame of the right eye visualized perimacular flecks, at age 70 (**A**). On the fluorescein angiogram (69 seconds), the large flecks were hypofluorescent (**B**). The red-free frame 6 years later, at age 76, shows that the previous well-defined flecks had become fuzzy (**C**). Similarly, on the fluorescein angiogram (37 seconds) the area of interest was hyperfluorescent (**D**). Note the dark choroid obvious in the fundus. The left eye also showed perimacular flecks on the initial red-free photograph (**E**). The fluorescein frame (56 seconds) disclosed the hypofluorescence related to the blockage of the flecks (**F**). Six years later, a round area of retinal pigment epithelial atrophy had replaced the previously confluent nasal flecks on the red-free frame (**G**). In addition, FA (103 seconds) demonstrated the occurrence of a second area of atrophy temporal to the fovea.

equal volume of formamide loading dye (95% formamide, 20 mM EDTA, 0.005% bromophenol blue, and 0.05% xylene cyanol). The samples (5 μl) were denatured for 10 minutes at 95°C, quickly cooled, loaded onto a polyacrylamide gel, and electrophoresed at 4 W for 18 hours at room temperature in 0.6× TBE running buffer. Gels were transferred onto Whatman paper (Maidstone, UK), dried, and autoradiographed (X-OMAT film; Eastman Kodak, Rochester, NY) for 24 hours. Exons that displayed abnormal profiles were compared with control samples from 90 unrelated and healthy French subjects.

Purified fragments were directly sequenced using the specific primers (3.2 picomoles) and a sequencing kit (PRISM Ready Reaction; Perkin Elmer–Cetus, Überlingen, Germany) on an automatic fluorometric DNA sequencer (Applied Biosystems, Foster City, CA). Direct sequencing was repeated twice, to rule out errors of the *Taq* polymerase.

RESULTS

Ophthalmologic Examination

Best-corrected visual acuity slowly decreased from 20/20 right eye (RE) and 20/30 LE at age 70, to 20/30 RE and 20/40 LE at age 76. No cataract was detected at slit lamp examination. Fundus exam-

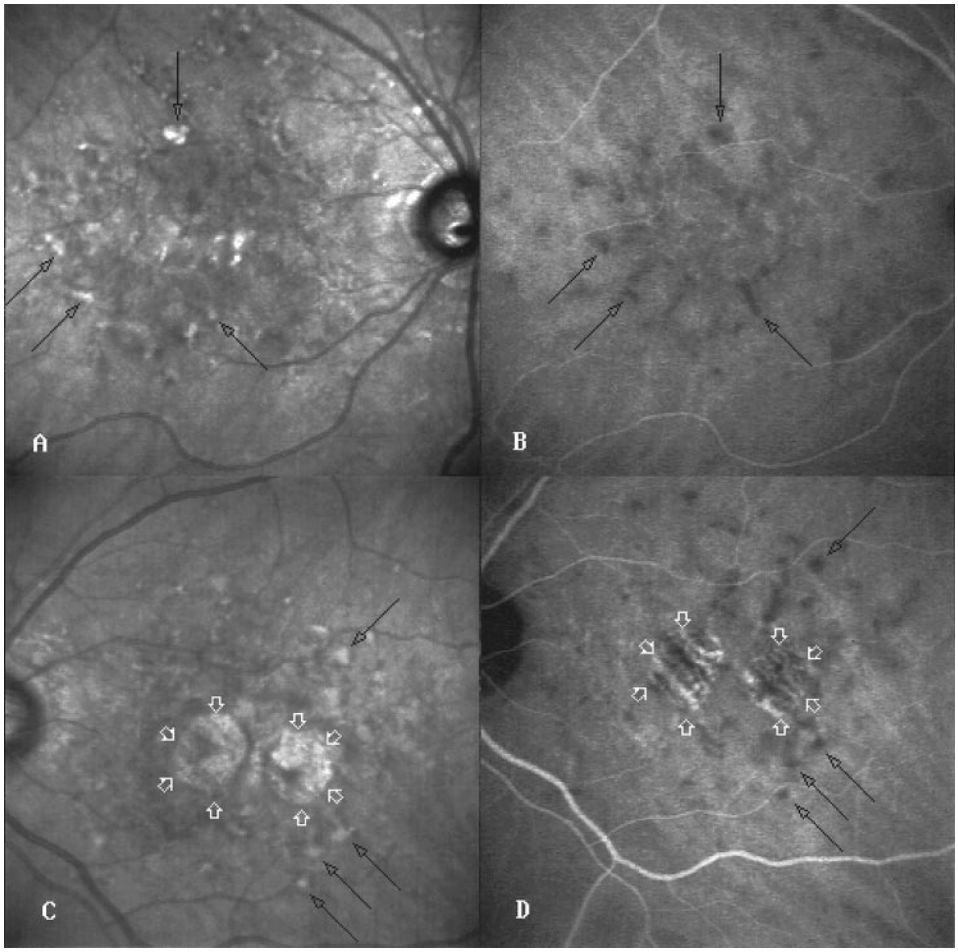

FIGURE 2. ICG angiogram of the patient at age 76. The infrared frame of the right eye (**A**) showed typical FFM flecks (*arrows*). The hypofluorescence, 5 minutes after ICG injection, showed precisely the extent of the flecks (**B**). The left eye showed on the infrared frame (**C**) the two well-delimited areas of retinal pigment epithelial atrophy (*open arrows*). Some peripheral flecks (*arrows*) were still obvious. The two macular atrophic areas (*open arrows*), suggesting AMD in the 76-year-old patient, were surrounded with hypofluorescent spots typical of FFM on ICG angiograms at 5 minutes (**D**).

ination revealed the presence of bilateral FFM yellowish flecks in the posterior pole. FA confirmed the diagnosis of FFM (Fig. 1). The "young" flecks, yellowish, sharply outlined and hypofluorescent on FA, turned to "old" flecks, gray, less defined and hyperfluorescent on FA. Two patches of perimacular retinal pigment epithelial atrophy were noted in the left eye at age 76. Dark choroid slowly increased at FA examinations. Retinal flecks, whatever their age, were constantly hypofluorescent on ICG angiography, as previously described[15] (Fig. 2). Findings in visual field analysis, color vision analysis, ERG, and electro-oculogram, recorded at age 70, were all perfectly normal. FA performed in the two healthy sons (aged 44 and 38 years) showed normal eyes.

ABCR Gene Mutation

One abnormal pattern of migration was found in exon 5 of the *ABCR* gene by SSCP analysis. DNA sequence analysis showed that this bandshift resulted from a heterozygous C-to-T transition, at nucleotide 450, resulting in the substitution of an arginine to a stop codon at position 152 of ABCR (Fig. 3). Subsequently, the patient was carefully examined for a mutation on the second allele by direct sequencing of the entire promoter sequence and the 49 remaining exons. The *ABCR* gene promoter sequence[16] was amplified as four partially overlapping segments (Table 1). No polymorphism, variant, or deleterious base change was found in this careful analysis. Of the two children, only the youngest (aged 38) harbored this truncating mutation. This sequence change was not observed in the 90 healthy control subjects (180 chromosomes).

DISCUSSION

We report a first case of an *ABCR* nonsense mutation in a patient affected with late-onset and mild FFM, subtly different from AMD. Previous screening of the *ABCR* gene in large series of STGD and FFM patients allowed the identification of numerous mutations.[8–13,17,18] Phenotype–genotype correlations in a

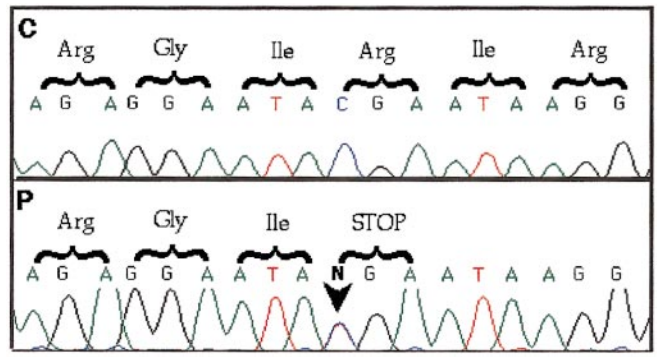

FIGURE 3. Identification of the heterozygous mutant genotype of the *ABCR* gene. *Top*: the normal sequence of exon 5 of the *ABCR* gene. *Bottom*: the automatic sequence analysis of the patient. *Arrowhead* indicates the heterozygous base substitution shown by the superposition of a normal C in blue and an abnormal T in red. The abnormal amino acid sequence resulting from the base substitution Arg→Stop is represented.

TABLE 1. *ABCR* Gene Oligonucleotides Used for Analysis of the Promoter Gene

Position (ATG = +90)		Primers Pairs (sense/antisense)	Size of PCR Product
Start	End		
−792	−760	CAAAAAGTTCCACTGCGAAGTGC	238 bp
−574	−554	GAAAAGGCACTGGAACACTC	
−606	−586	AAGTCAGTCACGGCTCTGTC	298 bp
−328	−308	CATTTGTCCTCGGCAAGAGC	
−376	−356	GATTGGACTTAAAGGGCCAG	263 bp
−134	−113	CTCCCTTTTATGGAGAGAAAG	
−160	−140	CCAGCCACTTGTGTCTTTAG	282 bp
+102	+122	GCAAAAGCTGTATCTGTCTC	

The PCR primers for exon amplification of the *ABCR* promoter gene are shown in 5′-to-3′ orientation. ATG = +90, initiator codon[16]; PCR, polymerase chain reaction.

large series of patients showed that compound heterozygosity for frameshift and missense mutations or two missense mutations results in STGD, whereas the FFM phenotype is always associated with two missense mutations.[8–13,17,18]

Surprisingly, in this sporadic late-onset case of FFM, a heterozygous truncating mutation was identified in the *ABCR* gene. STGD and FFM are usually considered to be autosomal recessive conditions, and it cannot be ruled out that we failed to detect a mutation in the other allele. Indeed, previous studies using SSCP screening identified only 60% of the disease-causing mutations.[8,12] In the present study, we subsequently carefully screened the 50 exons and the promoter region of the *ABCR* gene using direct sequencing, but no other mutation was found on the second allele.

However, according to the previous phenotype–genotype correlations, a compound heterozygosity for frameshift and missense mutations would be expected to induce a severe form of the disease. Conversely, our patient was affected with very late onset of FFM and a slowly progressive course. Age at first symptoms was 70 years, which was very late, even for FFM. It is notable that the retinal dystrophy turned into retinal pigment epithelium atrophy in the left eye and that the functional symptoms of this patient were very similar to an atrophic form of age-related macular degeneration (AMD). This patient's disorder could have been misdiagnosed as AMD without the results of FA and ICG angiography. Nevertheless, at age 70, only oval, elongated, and pisciform flecks were observed (Fig. 1). In addition, the dark choroid observed in this patient confirmed the diagnosis of FFM (Fig. 1). Finally, ICG angiography revealed the typical features of the FFM flecks (Fig. 2).

In contrast, Allikmets et al.[19] identified 13 mutant alleles of the *ABCR* gene in 16% (26/167) of patients affected with AMD, suggesting that heterozygous missense *ABCR* mutations could lead to late-onset macular degeneration. The relevance of *ABCR* missense mutations in atrophic forms of AMD has been questioned by some investigators.[20–22] Nevertheless, based on our data, we can speculate that heterozygous *ABCR* missense mutation may be responsible for AMD, whereas heterozygous mutations truncating *ABCR* may lead to a different but close phenotype, late-onset FFM. Furthermore, previous descriptions of FFM inherited as autosomal dominant traits strongly support this hypothesis.[23,24] In our patient, we could not determine the mode of inheritance of the disease. Indeed, his maternal grandmother, who died at age 92, had loss of central vision (no ophthalmologic data available), whereas his parents, who died at ages 74 and 72, did not mention any visual impairment. Moreover, findings in fundus examination and FA of his two sons were normal, without pigment epithelium atrophy, retinal flecks, or dark choroid. However, analysis of the *ABCR* gene revealed that the youngest, aged 38, had inherited the deleterious mutation. Considering the age of onset of the proband, we cannot exclude that macular dystrophy would appear later in life.

In conclusion, we have found a heterozygous truncating *ABCR* gene mutation associated with a very mild and late-onset form of FFM. Although the question of whether the *ABCR* gene is involved in AMD has become controversial, we demonstrated in this study that the *ABCR* gene was involved in this "age-related FFM." No other mutation on the second allele has been detected in the entire coding sequence, splice junctions, or the promoter region, suggesting that a truncating mutation could lead to a FFM phenotype late in life. This hypothesis remains open to debate. Further *ABCR* gene analyses of late-onset FFM cases are awaited.

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Enhanced Secretory Group II PLA_2 Activity in the Tears of Chronic Blepharitis Patients

Cheol Hwa Song,[1] *Jae Sin Choi,*[2] *Dae Kyong Kim,*[2] *and Jae Chan Kim*[1]

Purpose. Phospholipase A_2 (PLA_2) hydrolyzes phospholipids, one of the important constituents of human meibomian gland secretions. This study was performed to investigate PLA_2 type and activity in the tears of chronic blepharitis patients compared to those of normal persons.

Methods. Tear samples of 36 patients and 10 normal persons were collected in non-heparinized microcapillary tubes. PLA_2 activity in the tears was measured by Dole's method, and the results of the blepharitis patients were compared to those of the normal persons. The characterization of PLA_2 was performed by the head group preference test and the dithiothreitol (DTT) sensitivity test. The classification of PLA_2 type was done using Western blot analysis with anti-human secretory PLA_2 antibody.

Results. No statistically significant differences were found among the six categories of chronic blepharitis. However, the mean PLA_2 activity in the tears of the chronic blepharitis patients was about two times higher than that of the normal controls with statistical significance ($P < 0.05$). The PLA_2 substrate specificity test revealed group II PLA_2 activity. Furthermore, the group II PLA_2 was identified as a 14 kDa band in Western blot analysis using an antibody raised against human secretory group II PLA_2.

Conclusions. Secretory group II PLA_2 activity was significantly enhanced in the tears of the chronic blepharitis patients compared with that of the normal controls. It is suggested that this increased enzymatic activity may decrease the tear film stability through increased hydrolysis of phospholipids. (*Invest Ophthalmol Vis Sci.* 1999;40: 2744–2748)

Chronic blepharitis, a commonly encountered condition, is characterized by variable pictures of eyelid inflammation and is difficult to manage.

There are several classifications of chronic blepharitis. Dougherty and McCulley[1] have expanded the two fundamental categories of marginal lid inflammation into six categories, while at the same time placing a strong emphasis on the role of the meibomian glands in the inflammatory picture.

The tear film lipid layer, derived primarily from secretions of meibomian glands, is important in tear film stability.[2] The composition of human meibomian gland secretions have been reported as hydrocarbons (7%), sterol esters (27%), wax esters (32%), triglycerides (4%), polar lipids (15%), free sterols (2%), and free fatty acids (2%).[3] Significant differences have been discovered in meibomian lipid composition between chronic blepharitis patients and normal individuals. The constituents of polar lipids as well as sterol esters and wax esters are important

From the [1]Department of Ophthalmology, Chung–Ang University Hospital and [2]Department of Environmental and Health Chemistry, College of Pharmacy, Chung–Ang University, Seoul, Korea.

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Corresponding author: Jae Chan Kim, Chung–Ang University Hospital, Hangang–Ro 3ka 65-207, Yongsan 140-757, Seoul, Korea.