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Role of *IL6* -174 G/C, *IL10* 1082G/A and *IL10* -592C/A in the pathogenesis of keratoconus and development of recurrent erosion in Ukrainian patients with lattice corneal dystrophy

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Background: *Keratoconus (KC, or corneal ectasia) is a multifactorial disease with a genetic component and an average annual incidence rate of 2.0/100,000 persons. Lattice corneal stromal dystrophy (LCD), a monogenic disorder with varying phenotypic manifestations, is the most common hereditary corneal dystrophy associated with mutations in the TGFBI gene in Ukraine, with as much as 40.2% of cases attributed to this disease.*
Purpose: *To elucidate the role of polymorphic variants in the IL6 promoter (-174 G/C) and IL10 (-1082G/A and -592C/A) as factors of a genetic predisposition to KC and recurrent corneal erosion in Ukrainian patients with LCD.*
Material and Methods: *All patients underwent a routine eye examination including visual acuity assessment, biomicroscopy, fluorescein testing, tonometry and ophthalmoscopy. In addition, patients with KC underwent keratotopography, pachymetry, remote biometry and gonioscopy. Genotyping was done for IL6 -174 G/C, IL10 -1082G/A and IL10 -592C/A by polymerase chain reaction followed by restriction fragment length polymorphism. Fexact test was used for statistical analyses.*
Results: *The frequency of homozygotes (AA) for IL10 rs1800896 was increased, whereas the frequency of homozygotes (CC) for IL6 174G/C was decreased in patients with KC compared to controls (0.25 vs 0.19 and 0.18 vs 0.22, respectively), although the differences were not statistically significant. The frequency of IL6 C allele carriers was significantly higher among patients with LCD and recurrent corneal erosion than controls (0.78 vs 0.66, respectively; $p < 0.05$). There was a statistically significant difference in the proportion of carriers of the IL10 -592A allele between patients with recurrent corneal erosion and population sample (0.483 vs 0.327, respectively, $p < 0.05$).*
Conclusion: *IL6 174G/C, IL10 -592C/A and IL10 -1082G/C and the genes determining the pathological processes in the cornea produce a cumulative effect towards modifying the clinical phenotype in keratoconus and lattice corneal dystrophy.*

Introduction

The disorders associated with pathological changes in the cornea are a challenge for the ophthalmologist, because they substantially affect visual acuity and may lead to visual disability. Keratoconus (or corneal ectasia) is one of the most common corneal disorders with an average annual incidence rate of 2.0 per 100,000 population [1], and prevalence ranging from 50 to 230 cases per 100,00 population, with a wide variation in prevalence across ethnic populations. Keratoconus is recognized as a multifactorial disease with a genetic component, and a number of candidate genes have been linked to the disease.

Lattice corneal stromal dystrophy (LCD), a monogenic disorder with varying phenotypic manifestations, is another important issue and the most common hereditary corneal dystrophy in Ukraine, with as much as about 40.2% of hereditary corneal dystrophy cases attributed to this disease.

Keratoconus (KC) is an idiopathic, progressive, non-inflammatory corneal dystrophy that is commonly chronic. It is a condition in which the cornea assumes a conical shape because of thinning and protrusion. This results in longitudinal increases in corneal curvature and changes in corneal optical characteristics, leading to incorrect central and paracentral astigmatism with significantly decreased vision.

There is accumulating evidence supporting a genetic basis of keratoconus, while an environmental factor may be essential to act as a trigger in genetically predisposed individuals. The bilaterality of the disease strongly supports a genetic basis. In addition, the disease is frequently associated with hereditary disorders or syndromes (Down's syndrome, Marfan's syndrome, Leber's blindness, optic nerve atrophy, retinal pigment degeneration, cataract, Crouzon's syndrome, blue sclera, incomplete osteogenesis, type IV Ehlers-Danlos' syndrome, mitral valve prolapse, etc) [2-5].

In addition, a genetic predisposition to keratoconus is well documented with increased incidence in some familial groups [6, 7] and numerous reports of concordance between monozygotic twins [8-11]. It is noteworthy that positive family history has been documented in 6% to 23% KC subjects [12, 13]. First degree family members are at higher risk of keratoconus than normal population [14]. A number of candidate genes have been linked to KC.

There is a number of heritable and frequently associated with each other quantitative traits among biometric parameters of the human eye. One of them is central corneal thickness (CCT), with its heritability being as high as 0.95 [15]. Extreme CCT measurements have been associated with rare hereditary connective tissue disorders such as brittle cornea syndrome (BCS) and several types of osteogenesis imperfect [16,17], whereas moderate CCT measurements have been associated with more common eye disorders in elderly individuals. CCT is believed to be an essential trait in keratoconus [18,19].

Previous genome-wide association studies (GWAS) have identified eleven loci associated with CCT in European and Asian populations [20,21,22,23].

Association of KC with certain gene polymorphisms, such as those of hepatocyte growth factor (HGF), has been demonstrated [24]. Although KC is defined as a non-inflammatory corneal dystrophy, recent studies have shown that cytokines, proteolytic enzymes and free radicals play a role in the development of the disease [25]. Elevated tear IL6, TNF-α and MMP-9 levels have been demonstrated in patients with KC. In addition, eye rubbing contributes to increased tear IL6, TNF-α and MMP-9 levels, and is a risk factor for KC [26-31].

Interleukin-10 (IL10) is an anti-inflammatory cytokine, and the IL10 gene is highly expressed in injured corneal epithelium. Mouse models have demonstrated that *IL10* -1082 G/A (rs1800896) substantially effects the level and functional activity of the encoded protein.

The recognition site for the transcription factor C/EBP disappears in the presence of the A allele in *IL10* rs1800896. Findings of experimental studies have confirmed that such a substitution will be associated with reduced expression of the gene and production of the relevant protein [32, 33]. Given that anti-inflammatory cytokines play a crucial role in eliminating inflammatory cells and protecting against corneal ulcers and neovascularization, we have chosen *IL10* rs1800896 as a candidate gene for a genetic predisposition to keratoconus.

There have been numerous studies on the effect of cytokines on corneal epithelial repair after mechanical or chemical injury [34,35,36]. The corneal epithelial wound healing response is brought about by a complex cascade of events involving cytokine-mediated interactions between the epithelial cells, keratocytes of the corneal stroma, corneal nerves, lacrimal glands, and cells of the immune system [34]. Thus, pro-inflammatory cytokines *IL1, IL6* and *IL8* regulate proliferation and migration of corneal cells and degradation of collagen residue, and protect the site of injury from bacterial infection [34,37].

Two allelic variants in the *IL6* promoter, -597G/A and -174 G/C, have been considered as risk factors for these disorders. These promoter polymorphisms regulating *IL6* gene expression are associated with production of the relevant protein and circulating levels of C-reactive protein [38].

Based on the results of the analysis of a regulatory sequence associated with *IL6* gene, we chose a single-nucleotide guanine-to-cytosine substitution at position −174 of the *IL6* gene promoter as a subject for further analysis. Based on the results of analysis of the predicted effect of this polymorphism on the structure of transcription factor recognition sites, we found that the single-nucleotide substitution is located in the recognition site of NRD and may result in disappearance of the cis-regulatory site in the promoter region. In an in vitro study [39], the -174C construct showed lower expression than the -174G construct. In addition, it has been demonstrated that the presence of the above single-nucleotide substitution also effects the capacity of the estrogen receptor-estrogen complex to regulate the activity of *IL6* gene promoter [40].

We have previously found that cytokine genes are expressed in the corneal epithelium, and their polymorphisms are associated with the risk of and/or protection against recurrent corneal erosion in patients with LCD [41]. Given the importance of *IL6* -174 G/C and *IL10* -1082G/A and -592C/A polymorphisms for visual function, we have chosen these genetic polymorphisms as candidate SNPs for keratoconus and candidate genes modifying LCD phenotypes.

Therefore, **the purpose** of this study was to elucidate the role of polymorphic variants in the *IL6* promoter (-174 G/C) and *IL10* (-1082G/A and -592C/A) as factors of a genetic predisposition to keratoconus and recurrent corneal erosion in Ukrainian patients with LCD.

Material and Methods

Analysis of clinical data and sampling

A DNA bank of patients with keratoconus and those with LCD type I or type I/III has been established. The material for the study consisted of blood samples obtained from (a) patients with the above diseases at the Filatov Institute, and (b) blood donors from various Ukrainian regions who were not related to each other and represented the general population of Ukraine.

All participants provided informed consent before entering the study. All DNA samples were coded and tested in an anonymous manner.

Patients with keratoconus who were treated at the Filatov Institute were included in the study. Results of the control population sample were provided by the Department of Human Genetics, Institute of Molecular Biology and Genetics of the NAS of Ukraine. Table 1.1 presents characteristics of patient groups.

The results of the genetic study in 106 patients with keratoconus (212 eyes; age, 10 years to 67 years; mean age, 31.5±11.82 years) were subjected to analysis. Patients were mostly males (65%). Sixty-nine patients with LCD (46 patients with TGFBI Arg124Cys LCD type I and 23 patients with Hys626Arg LCD type I/IIIA) underwent molecular genetic studies. Of these, 56 had recurrent corneal erosion (RCE) and 13 had no history of corneal erosion.

Clinical examination

All patients underwent a routine eye examination including uncorrected (UCVA) and best-corrected visual acuity (BCVA) assessment, biomicroscopy, fluorescein testing, tonometry and ophthalmoscopy. In addition, patients with keratoconus underwent keratotopography, pachymetry, remote biometry and gonioscopy.

Twenty-four eyes had stage I keratoconus (astigmatism ≤ 5.0 D; keratometry, ≤ 48.0 D; visual acuity, 0.5 to 1.0), 52 eyes had stage II (astigmatism, 5.0 to 8.0 D; keratometry, ≤ 53.0 D; pachymetry, ≥ 400 μm; visual acuity, 0.1 to 0.4), 44 eyes had stage III (astigmatism, 8.0 to 10.0 D; keratometry, > 53.0 D; pachymetry, 300 to 400 μm; visual acuity, 0.12), and 83 eyes had stage IV (keratometry, > 55.0 D; pachymetry, <200 μm; visual acuity, 0.01 to 0.02) (Amsler-Krumeich classification).

Genetic examination

Genomic DNA samples were isolated from peripheral blood lymphocytes using proteinase K digestion of the cell lysate and subsequent phenol extraction. Spectral analysis and 0.6% agarose gel electrophoresis were used to determine sample quality. The concentration and spectral characteristics of the DNA sample were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE USA).

PCR was performed automatically on the 2720 thermal cycler (Applied Biosystems, Waltham, MA) or iCycler (Bio-Rad, Hercules, CA).

PCR amplification with subsequent hydrolysis of restriction enzyme, NlaIII, and visualization with 2% agarose gel electrophoresis were used for the analysis of the DNA sequence containing the substitution -174 G-->C in the *IL6* promoter.

The -1082G/A polymorphism in the promoter region of the *IL10* gene represents a single-nucleotide G-to-A substitution leading to the disappearance of the recognition site for restriction enzyme EcoNI. Therefore, in vitro PCR amplification with subsequent restriction digestion of PCR products with restriction enzyme, EcoNI, were used for detection of the SNP. After digestion of PCR products with restriction enzyme, EcoNI, they were separated and analyzed by 2% agarose gel electrophoresis.

Molecular genetic studies of the *IL10* -592 C/A polymorphism were conducted on the basis of PCR and subsequent restriction fragment length polymorphism (RFLP) analysis. The -592 C/A polymorphism represents a single-nucleotide C-to-A substitution in the promoter region of the *IL10* gene. As a result of this substitution, the restriction site appears which leads to differential digestion of various allelic variants in the *IL10* gene with restriction enzyme, RsaI, making them identifiable. After digestion of PCR amplification products with restriction enzyme, RsaI, they were separated and analyzed by 2% agarose gel electrophoresis.

Statistical analyses were conducted using OpenEpi (Andrew G. Dean and Kevin M. Sullivan, Atlanta, GA) software. Chi-square test and Fexact test were used.

Results and Discussion

Clinical manifestations of keratoconus

KC is a progressive and commonly bilateral corneal dystrophy that leads to corneal thinning and deformation and uncorrectable decreased visual acuity. Disease onset commonly occurs in young individuals of working age, and the rate of progression varies between individuals. In patients of this study, signs of disease were first noted at a median age of 19.6 + SD7.01 years (range 6 to 40 years).

It is important to differentiate between subclinical KC and clinical KC. Subclinical keratoconus is characterized by the appearance of indirect signs (symptoms) such as complaints of astenopia, formation of myopic (sometimes mixed) astigmatism in pubertal age or beyond it, decreased visual acuity that improves under diaphragming conditions, decreased best-corrected visual acuity, and instability of ophthalmometric measurements on the eyes. Enhanced appearance of corneal nerves and sparse stroma in the paracentral cornea are a biomicroscopic sign of early keratoconus [2].

Clinical disease is characterized by a narrow optic section in the ectactic zone and Vogt's striae, fine vertical stromal lines that are produced by Descemet's membrane compression. These lines disappear when external pressure is applied to the globe. Other signs appear as the disease progresses, including decreased visual acuity, astigmatism that is difficult or impossible to correct, myopic refractive error combined with astigmatism which is usually incorrect, increased corneal refractive power, small radius of corneal curvature, and corneal thinning. As the cone

further grows, the apex becomes scarred and opaque, with thinning involving more and more peripheral cornea [2, 3]. Of the patients enrolled in the current study, most (60%; 127 eyes) had stage III or IV keratoconus. Acute hydrops were seen in 6 eyes (2.8%) (Fig. 1).

Clinical manifestations of lattice corneal dystrophy

The complete clinical picture of lattice dystrophy type 1 generally develops in the third to fourth decade of life. The typical diagnostic triad of LCD comprises (1) transparent double-contoured radially branching lattice structures in the anterior and intermediate stroma; (2) punctate grayish opacities in the superficial stroma; and (3) diffuse, central subepithelial opacity [45, 46]. Radially oriented lattice lines with dichotomous branching near their central terminations are pathognomonic for LCD. Recurrent corneal erosions may be associated with LCD, develop as early as childhood, and are the most common major complaint in patients with LCD attending an eye department. Patients, however, vary in the number of recurrent episodes, intensity of recurrence and severity of the concurrent inflammatory response (Fig. 2 a, b). Epithelial injury is a mechanism triggering the inflammatory response in LCD. The mean age of our patients with LCD type 1 was 41.7 ±SD11.2 years.

The complete clinical picture of LCD type I/IIIA generally develops in the fourth to fifth decade of life, but the components of the typical diagnostic triad are somewhat less apparent than in LCD type I. LCD type I/IIIA is characterized by thinner lattice structures compared to LCD type I, and asymmetric manifestations with no or rare recurrence of corneal erosion [46]. The mean age of our patients with LCD type I/IIIA was 55.3±SD9.6 years, which was older that the mean age of patients with LCD type I due to later manifestation of this LCD type.

Results of studies of IL6 and IL10 polymorphisms in patients with keratoconus and LCD

IL10 rs1800896 was analyzed in 106 patients and 100 healthy controls. Table 2 presents the distribution of genotypes and alleles for *IL10* rs1800896.

Patients with keratoconus exhibited higher frequency of the homozygous (AA) genotype of rs1800896 than controls (25% vs 19%, respectively). Although the difference was not significant, but there was a tendency for association between the homozygous (AA) genotype of *IL10* rs1800896 and keratoconus.

Frequency of the homozygous (CC) genotype of *IL6* -174 G/C was higher in control subjects (22%) compared to patients with keratoconus (18%). Although the difference was not significant, but there was a tendency for association between the homozygous (CC) genotype of *IL6* -174 G/C and keratoconus.

A recent meta-analysis of genome-wide association studies identified 11 keratoconus-associated loci [42]. The findings we obtained may be considered as potential evidence of an additive cumulative effect of our investigated SNPs of *IL6* and *IL10*, and the candidate genes that have been identified by the meta-analysis.

To study such a cumulative modifying effect of the *IL6* and *IL10* SNPs on the clinical phenotype in patients with another corneal disorder, particularly, lattice corneal dystrophy, we investigated polymorphisms of these genes in patients with this diagnosis.

Hereditary stromal corneal dystrophies (particularly, lattice stromal corneal dystrophy) are a group of autosomal-dominant disorders caused by mutations in the transforming growth factor, beta-induced (TGFBI) gene [43,44]. We conducted molecular genetic studies of these variants in 56 patients with recurrent corneal erosions and 13 patients with no history of corneal erosion. Previously, we have identified the TGFBI gene mutations characteristic for lattice dystrophy in these patients [45].

To elucidate the possible role of *IL6* -174G/C and *IL10* -592C/A (the latter SNP is non-equilibrium linked with *IL10* -1082G/C that we have already investigated) as phenotype modifiers in patients with LCD, we conducted molecular genetic studies of these variants in patients with recurrent corneal erosions.

Table 4 presents the distribution of genotypes among groups.

Among the group of patients with corneal erosions, we found 9 individuals (0.18) who were homozygous for the –174C allele that had been shown to be associated with a decreased production of *IL6*. The heterozygous -174GC genotype was a major genotype (0.600) both in the group and the population sample under study. A deviation from Hardy–Weinberg equilibrium, evaluated by comparing observed and expected genotype frequencies with an exact goodness of fit test, was observed in the patients with recurrent corneal erosion associated with LCD for this polymorphic variant ($\chi 2=4.08$; $p=0.04$). Distribution of allele frequencies for the polymorphic variant was characterized based on the distribution of genotype frequencies among study patients and two control groups. The frequency of the carriers of the *IL6* -174C allele was 0.780 in the corneal erosion group compared to 0.659 in the control population sample ($p < 0.05$) (Fig. 4) and 0.500 in the no-erosion control group; the difference for the latter group, however, did not reach statistical significance ($p > 0.05$), likely due to small group size.

The distribution of genotypes for the polymorphic allele of *IL10* -592 C/A among the study group and control group was determined based on the results of molecular genetic studies. The homozygous wild-type (-592 CC) genotype was the most common (0.518) for patients with recurrent corneal erosion and population sample, whereas the heterozygous (-592 CA) genotype was the most common (0.462) for patients without corneal erosions. No deviation from Hardy–Weinberg equilibrium, evaluated by comparing observed and expected genotype frequencies with an exact goodness of fit test, was observed in the patients with recurrent corneal erosion associated with LCD for this polymorphic variant ($\chi 2=0.49$; $p=0.380$).

There was a statistically significant difference ($p < 0.05$) in the proportion of carriers of the *IL10* -592A allele

between patients with recurrent corneal erosion (0.483) and population sample (0.327) (Fig. 5).

This may be explained given that recurrent corneal erosions may differ in the degree of concomitant inflammation [34]. Impaired adhesion of basal epithelial cells to the Bowman layer due to accumulation of amyloid deposits (abnormal protein fibers) in between these structures, and secondary degenerative changes in the anterior stroma provide the morphological basis for recurrent corneal erosions in LCD [46]. Epithelial injury is a mechanism triggering the inflammatory response in LCD.

Corneal regeneration is a complex body response, with its success and timing depending on the correct combination of cytokines and growth factors expressed at specific times [34,47,35]. Pro-inflammatory cytokines co-regulate proliferation of corneal cells and degradation of necrotized cells and denaturated collagen [36,48]. Anti-inflammatory cytokines provide elimination of inflammatory cells, thus preventing corneal ulceration, melting and neovascularization [34,47].

The genes of the two major cytokines of acute inflammation (*IL1β* and *IL6*), chemokine *IL8* and anti-inflammatory cytokine *IL10* are expressed in the injured corneal epithelium [36,49]. The proteins encoded by these genes have been found in the tear fluid of patients with injured corneal tissue and shown to play a key role in corneal regeneration [37].

It may be assumed that, in patients with lattice corneal dystrophy, the degree of inflammation that rises in recurrent erosions is under control of modifying genes.

Conclusion

We analyzed, for the first time, the distribution of genotypes and alleles for *IL6* -174G/C and *IL10* -592C/A in patients with keratoconus and those with recurrent erosions (including those with and without lattice corneal dystrophy). We found that *IL6* -174C and *IL10* -592A alleles are potential inflammation modifiers and potential genetic risk factors for recurrent corneal erosions in patients with lattice corneal stromal dystrophy.

The frequency of homozygotes (AA) for *IL10* rs1800896 was increased, whereas the frequency of homozygotes (CC) for *IL6* 174G/C was decreased in patients with keratoconus compared to controls (0.25 vs 0.19 and 0.18 vs 0.22, respectively), although the differences were not statistically significant. Given the multifactorial pathogenesis of keratoconus, further research with substantially increased sample sizes is warranted to achieve statistically significant differences and verify these findings.

Therefore, we obtained one more confirmation of an additive cumulative effect of *IL6* 174G/C, *IL10* -592C/A and *IL10* -1082G/C and the genes determining the pathological processes in the cornea.

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The authors certify that they have no conflicts of interest in the subject matter or materials discussed in this manuscript.

Table 1. Characteristics of patient and control groups

Group of patients or controls	Number of patients
Patients with keratoconus	106
Unrelated blood donors (controls)	100
Lattice corneal dystrophy patients with recurrent corneal erosion	56
Lattice corneal dystrophy patients with no history of corneal erosion	13

Table 2. Distribution of genotypes and alleles for *IL10* -1082G/C single nucleotide polymorphism among Ukrainian healthy controls and patients with keratoconus

Genotype	Control group		Patients with keratoconus	
	Absolute number	Frequency	Absolute number	Frequency
GG	23	0.230	29	0.274
GA	58	0.580	51	0.481
AA	19	0.190	26	0.245
n	100		106	
G allele	104	0.520	109	0.514
A allele	96	0.480	103	0.491

Table 3. Distribution of genotypes and alleles for *IL6* -174G/C single nucleotide polymorphism among Ukrainian healthy controls and patients with keratoconus

Genotype	Control group		Patients with keratoconus	
	Absolute number	Frequency	Absolute number	Frequency
GG	30	0.341	15	0.294
GC	39	0.443	27	0.529
CC	19	0.216	9	0.177
n	88		51	
G allele	99	0.562	57	0.559
C allele	77	0.438	45	0.441

Table 4. Distribution of genotypes and alleles for *IL6* -174G/C and *IL10* -592C/A single nucleotide polymorphisms in lattice corneal dystrophy patients with recurrent corneal erosion and with no corneal erosion

Genotype / allele	Patients with recurrent corneal erosion	Patients with no corneal erosion	Population group
	Number (frequency)		
IL6 -174G/C			
Total	50	12	88
GG	11 (0.220)	6 (0.500)	30 (0.341)
GC	30 (0.600)	4 (0.333)	39 (0.443)
CC	9 (0.180)	2 (0.167)	19 (0.216)
G allele	52 (0.520)	16 (0.667)	99 (0.562)
C allele	48 (0.480)	8 (0.333)	77 (0.438)
IL10 -592C/A			
Total	56	13	101
CC	29 (0.518)	5 (0.385)	68 (0.673)
CA	24 (0.429)	6 (0.462)	30 (0.297)
AA	3 (0.054)	2 (0.153)	3 (0.030)
C allele	82 (0.732)	16 (0.615)	164 (0.820)
A allele	30 (0.268)	10 (0.385)	36 (0.180)

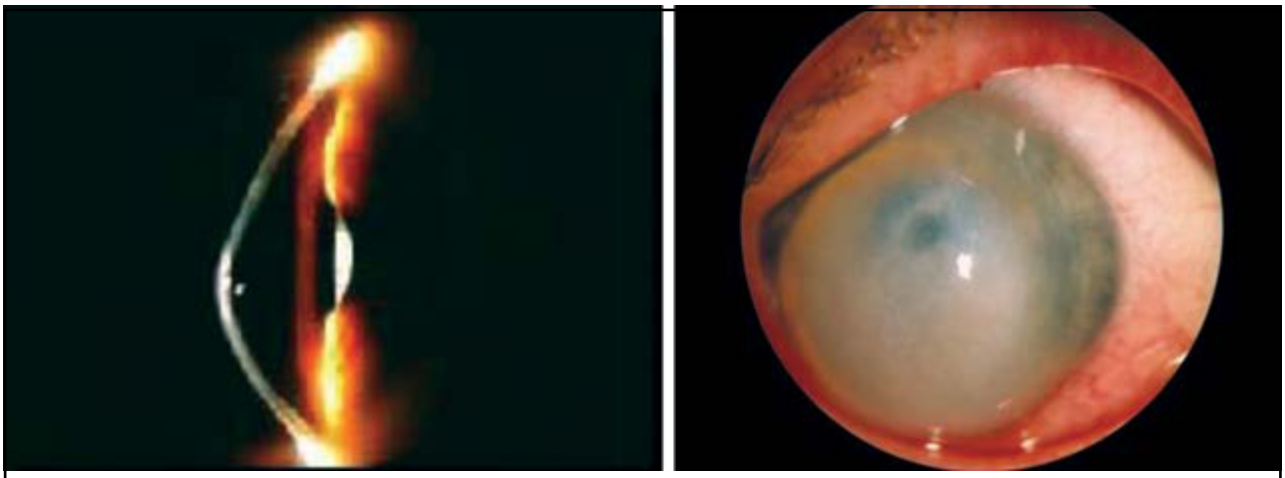

Fig. 1. (A) Stage IV keratoconus (B) Keratoconus with acute hydrops

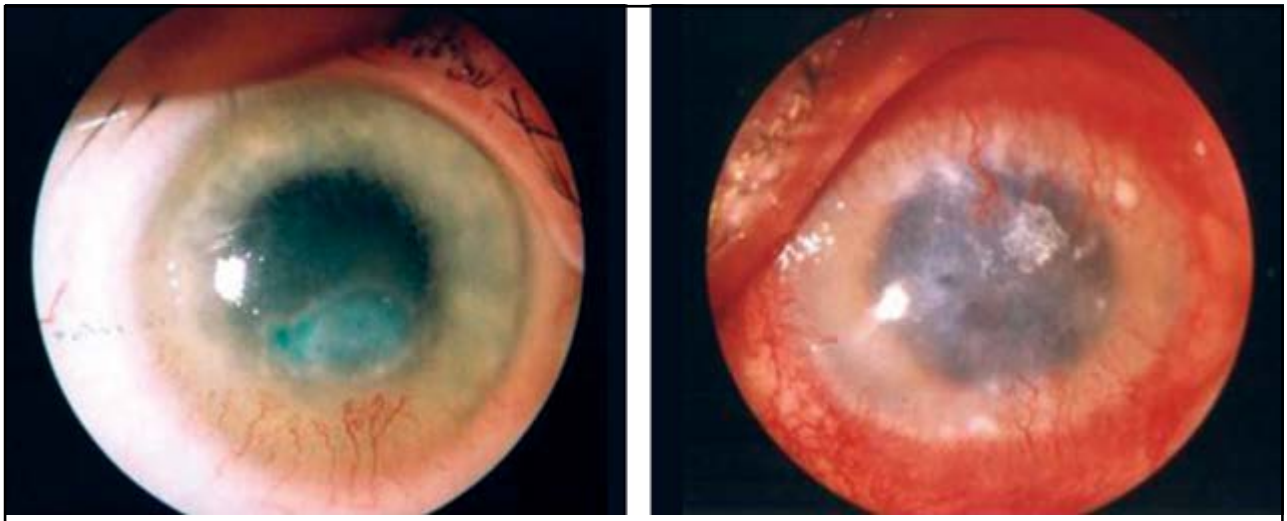

Fig. 2. Eyes of 49-year-old (A) and 56-year-old (B) patients with TGFBI Arg124Cys lattice corneal dystrophy type I. (A) Moderate stromal inflammation is present. (B) Severe stromal inflammation is present.

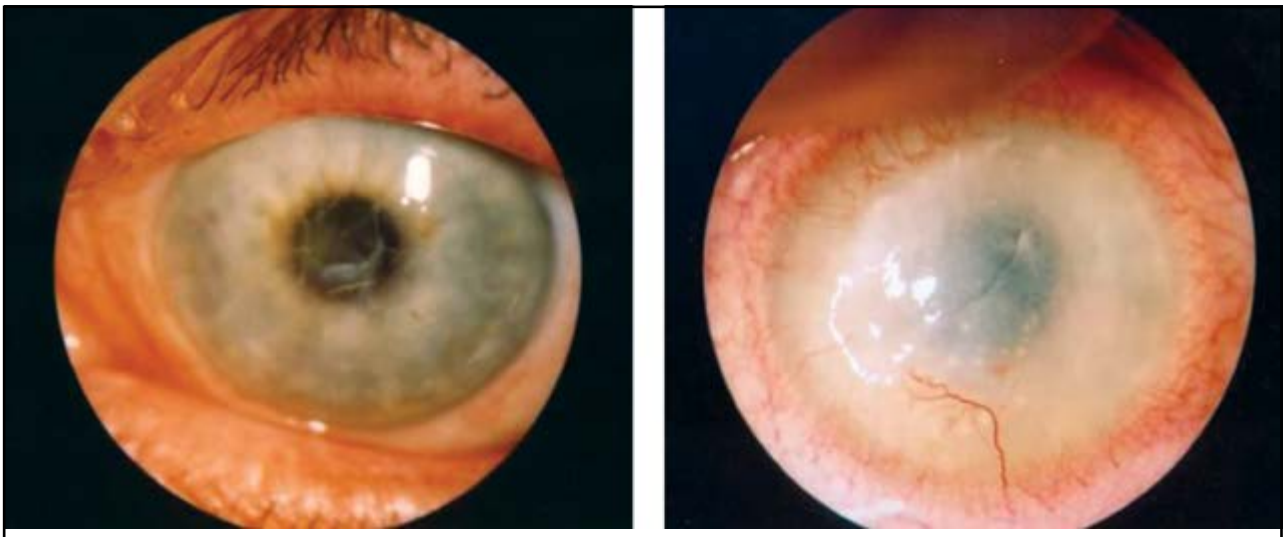

Fig. 3. Eyes of 58-year-old (A) and 48-year-old patients with TGFBI Hys626Arg lattice corneal dystrophy type I/III. (A) Moderate stromal inflammation is present. (B) Severe stromal inflammation is present.

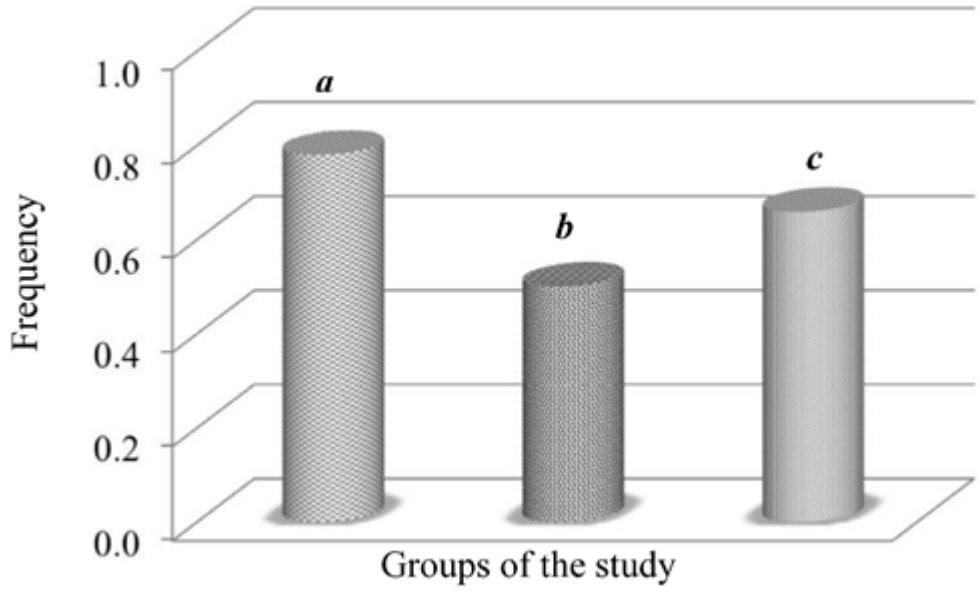

Fig. 4. Frequency of carriers of the *IL6* -174C allele among lattice corneal dystrophy patients with (a) and without (b) corneal erosion, and the population group (c)

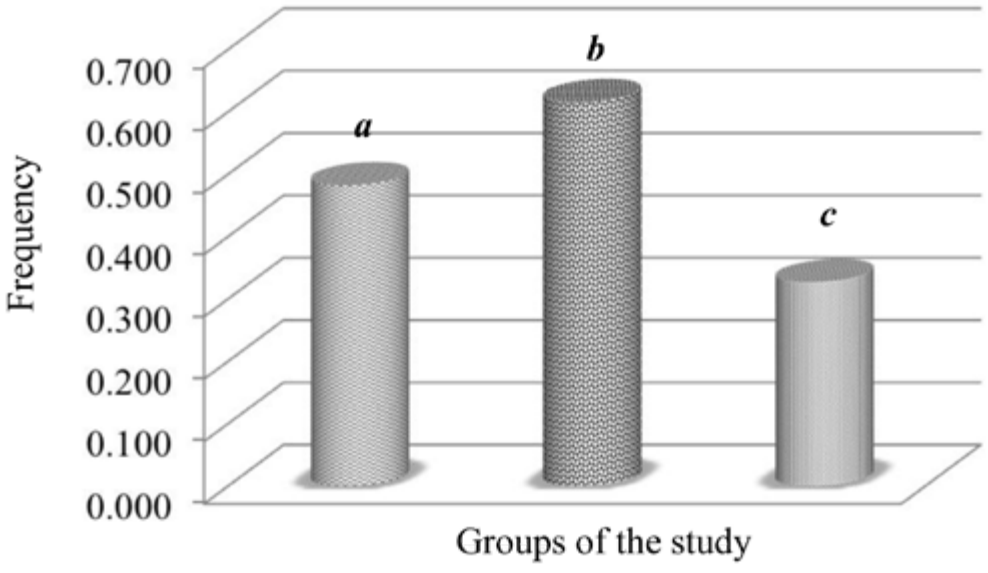

Fig. 5. Frequency of carriers of the *IL10* -592A allele among lattice corneal dystrophy patients with (a) and without (b) corneal erosion, and the population group (c)