**Abstract.** Background/Aim: Fuchs’ endothelial corneal dystrophy (FECD) is a hereditary, progressive, bilateral, and irreversible disorder of the corneal endothelium. The purpose of this study was to develop a novel, accurate and high-throughput real-time polymerase chain reaction (PCR) method and melting-curve analysis in order to genotype the rs613872 polymorphism in the transcription factor 4 (TCF4) gene and to implement it on a well-ascertained sample of 22 Greek FECD patients and 58 healthy individuals, age- and sex-matched. Patients and Methods: DNA was extracted from blood samples, which were screened with the DNA sequencing method in order to detect the g.31753T>G/p.L450W (rs8035192) and g.31767C>A/p.Q455K (rs8035191) mutations in a COL8A2 genomic region. Results: TCF4 risk G allele frequency increased to 48% in FECD patients compared to 17% in healthy-subjects [OR=4.82 (95% CI=1.98-11.73)]. No p.L450W and p.Q455K COL8A2 gene mutations were detected. Conclusion: We confirmed that rs613872 in the TCF4 gene is strongly and statistically associated with late-onset FECD in a Greek population.

Fuchs’ endothelial corneal dystrophy (FECD) is a hereditary, progressive, bilateral, and irreversible disorder of the corneal endothelium (1, 2). FECD is characterized by thickening of the Descemet’s membrane and microscopic collagenous protuberances known as guttae (3). Progressive and accelerated loss of corneal endothelial cells and a concomitant increase in the extracellular matrix deposition at the level of Descemet’s membrane take place, resulting in morphological changes in the size (polymegethism) and shape (pleomorphism) of the remaining endothelial cells (4, 5). As a consequence, the corneal endothelium is no longer able to support corneal deturgescence, leading to corneal edema, which causes the development of folds in Descemet’s membrane, while increased endothelial pigmentation may also be present (4).

Patients initially complain of blurred vision (often worse in the morning), usually during the fourth and the fifth decade of their life. As the disease progresses, epithelial edema develops, leading to mycrocystic edema and epithelial bullae, which may rupture causing pain. Untreated cases of FECD often result in blindness (4). The only definitive treatment is corneal transplantation, either in the form of penetrating keratoplasty or Descemet’s stripping endothelial keratoplasty (6, 7).

FECD is also the most common form of corneal dystrophies, being more frequent in women compared to men (2.5-3:1) (3, 8-12). The disease prevalence varies around the world and it is reported to affect 4% of the population over 40 years of age in the United States (13), whereas it is uncommon in Japan, Saudi Arabia, and in the Chinese of Singapore (14, 15).

The FECD grading scale, which was described and utilized by Krachmer, consists of 5 stages according to the number and distribution of guttae (13). More specifically, a severity score of 1 reflects minimal and asymptomatic disease and is defined as more than 12 central and scattered guttae; a cluster of central confluent guttae are scored as 2, and higher scores represent increasing diameters of distribution of confluent guttae (13).
Based on the age of disease onset, FECD is divided into two categories, early- and late-onset. Early-onset FECD is rare, usually begins in the first decade of life, and becomes clinically detectable during the second and the third decade (4). Late-onset FECD is either sporadic or familial and is generally apparent in the fifth decade of life, progressing over the next two to three decades (8). It is a primarily autosomal-dominant condition (16-18), with incomplete penetrance (13, 19, 20).

Although the exact etiology of FECD is still unclear, several studies suggested that FECD is a genetically heterogeneous disease associated with multiple genetic variations, which could be identified as possible contributors to its pathogenesis. Early-onset FECD has been associated with mutations in the collagen VIII α2 gene (COL8A2) (9, 21). Late-onset FECD is more genetically heterogeneous and several causal DNA mutations have been identified by the candidate-gene approach in families, or by genome-wide association study (GWAS) in patients, in genes such as transcription factor 4 (TCF4), zinc finger E-box-binding homeobox 1 (ZEB1), ATP/GTP Binding Protein Like 1 (AGBL1), solute carrier family 4 sodium borate transporter member 11 (SLC4A11), and lipoxigenase homology domain-containing 1 gene (LOXHD1) (21-24).

TCF4 gene is located on chromosome 18q (25), encodes a transcription factor protein E2-2, which is expressed in the cornea during development, and is involved in regulating cellular growth and differentiation (26). TCF4 gene mutations have been associated with several diseases, such as schizophrenia, primary sclerosing cholangitis and Pitt-Hopkins syndrome (2, 24). TCF4 polymorphisms have also attracted attention for their association with FECD (24, 27). More specifically, it has been suggested by Baratz et al. that a significant association exists between FECD and four single nucleotide polymorphisms (SNPs) (rs17595731, rs613872, rs9954153, and rs2286812) of the TCF4 gene (2, 24). However, this suggestion was rejected by a study performed in India, underlying that differences in ethnic groups may affect this association (28).

The corneal endothelium secretes type VIII collagen (COL8), which is a significant component of normal Descemet’s membrane. COL8 has two isoforms, α1 (COL8A1) and α2 (COL8A2), which interact to form a hexagonal lattice structure (29). The COL8A2 gene is located on chromosome 1p at position 34.3. As we previously mentioned, it is well established that mutations in the COL8A2 gene are linked with the development of early-onset FECD (30). The Gln455Lys (p.Q455K) missense mutation of COL8A2 gene, which encodes the α2 chain of type VIII collagen, involves replacement of highly conserved glutamine with negatively charged lysine, which may alter the tertiary structure of the protein. The Leu450Trp (p.L450W) mutation involves the substitution of leucine with tryptophan (4). These mutations result in abnormal intracellular accumulation of mutant COL8A peptides, affecting triple helical stability (30, 31), while they have been associated with posterior polymorphous dystrophy (32).

Based on the aforementioned observations, we investigated the possible associations between the rs613872 polymorphism of TCF4, and p.L450W and p.Q455K mutations or other polymorphisms in a COL8A2 genomic area, with late-onset FECD in a well-defined Greek population. To the best of our knowledge, our study is the first in the literature to conduct such genotyping in Greek patients with FECD.

Materials and Methods

Patients. The study was conducted on a cohort of 58 healthy controls (mean age 72±8 years, 63.6% female) and 22 Greek patients with late-onset FECD (Krachmer scale ≥2) (mean age 74±10 years, 56.9% female), who were recruited during a 1-year period (2016-2017). All participants were selected after ophthalmological evaluation and clinical data collection. Ophthalmological evaluation included visual acuity and slit-lamp anterior segment examination. Grading of disease severity was determined using a modified version of the Krachmer scale classification system, which classifies severity on a scale of 0 to 5; Grade 0: no central cornea guttae, grade 1: scattered central cornea guttae, grade 2: 1 or 2 mm of central cornea guttae, grade 3: 2 to 5 mm of grouped cornea guttae, grade 4: 5 mm of grouped central cornea guttae, grade 5: cornea guttae with corneal edema. Individuals were classified as being affected when the grade was 2 or more. The peripheral blood samples were obtained after approvals from the “G. Gennimatas” General Hospital Scientific and Bioethics committees and after obtaining signed informed consent from each participant.

Genomic DNA isolation. Blood samples were collected in EDTA tubes at “G. Gennimatas” General Hospital and stored at −20°C until DNA extraction. Genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The absolute measurement of DNA concentration was calculated by the Quant-IT dsDNA BR Assay Kit in a Qubit fluorimeter (ThermoFisher Invitrogen, Waltham, MA, USA) which employs a dye specific for DNA. The DNA was stored at −20°C until further use.

TCF4 (rs613872) novel real-time polymerase chain reaction (PCR) assay. In order to develop a novel, rapid and accurate, high-throughput TCF4 methodology for the rs613872 polymorphism, we selected the real-time PCR LightCycler instrument (Roche Applied Science, Penzberg, Germany) and the format of dual hybridization probes (e.g. anchor and sensor probes labeled with fluorescein and LC640 fluorescent dyes) that provides additional specificity (33). Only when both anchor and sensor probes locate their specific targets within the PCR product does fluorescence resonance energy transfer emission occur for the LC640 probe, which can then be continuously monitored in the F2 detection channel of the instrument. The sequences of the primers and hybridization probes for the proposed real-time PCR assay were designed in silico and

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and 72˚C for 1 min, followed by a final extension at 72˚C for 5 min. 40 PCR cycles were performed as follows: 95˚C for 30 sec, 60˚C for 30 sec, and extension at 72˚C for 20 sec. The temperature activation of the polymerase enzyme, the three-step cycling protocol included 45 cycles of denaturation at 95˚C for 10 min for hot-start activation of the polymerase enzyme, the three-step cycling protocol included 45 cycles of denaturation at 95˚C for 10 min, annealing at 59˚C for 20 sec, and extension at 72˚C for 20 sec. The temperature ramp rate was 20˚C/sec.

Allele discrimination was achieved with melting-curve analysis of the PCR product after the cycling ended. The melting-curve analysis started by raising the temperature to 95˚C for 1 min, maintaining it at 43˚C for 60 sec and then proceeded with a slow heating step up to 80˚C at a ramp rate of 0.4˚C/sec. The first derivative plot of the collected fluorescent measurements (−d(F2)/dT) was then used for the easy identification of wild-type and mutant alleles by their different melting temperature (Tm). The efficiency (E) of the real-time PCR assay was calculated with the equation E=10−1/slope as previously described (34).

**COL8A2 assay.** A COL8A2 genomic region was screened using the DNA sequencing method in order to detect the g.31753T>G/p.L450W (rs8035192) and g.31767C>A/p.Q455K (rs8035191) COL8A2 mutations that are associated with early FECD, but also for other mutations or polymorphisms in the same area (sequences of the primer pair used are shown in Table I). The conventional PCR protocol was performed in 20 μl volumes containing genomic DNA (100 ng/μl), 8 pmoles COL8A2-F primer, 8 pmoles COL8A2-R primer, 10 μl enzyme MyTaq™ Red Mix Bioline 2X, 2 μl dimethyl sulfoxide, and water to supplement up to 20 μl. After an initial denaturation step at 95˚C for 5 min, 40 PCR cycles were performed as follows: 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min, followed by a final extension at 72˚C for 5 min.

**DNA sequencing.** After purification of PCR amplicons (High Pure PCR Cleanup Micro kit; Roche Applied Science), cycle sequencing reaction was performed with the Big Dye 1.1 reagent (Applied Biosystems, Waltham, MA, USA) in both directions with the use of either the forward or the reverse primer. 10 μl of the purified cycle sequencing reactions (by NucleoSeq columns; Macherey-Nagel, Germany) were heated at 95˚C for 2 min and cooled immediately at 4˚C for 2 min with 10 μl formamide and then run in capillaries of an ABI Prism 310 Genetic Analyzer. For the analysis of DNA sequencing electropherograms, Chromas 2.01 software was used (Technelysium Pty Ltd, Australia) and results were compared with the expected gene sequences with NCBI BLAST.

**Statistical analysis.** Statistical analysis was performed using the SPSS 23.0 software package for Windows (IBM Armonk, NY, USA). The criterion used for statistical significance was p<0.05. Normality of distribution of age was assessed with the Kolmogorov-Smirnov test and the Shapiro-Wilk test. Median age between patients and controls was compared with the Mann-Whitney test and sex proportions with the Chi-squared test. Logistic regression analysis for FECD prediction was also performed. Conformance with Hardy-Weinberg equilibrium (HWE), allelic count, genotypic frequencies and odds ratio (OR) calculation with 95% confidence intervals (CI) were examined with the SNPstats software (35).

**Results**

**TCF4 rs613872 real-time method validation.** Our novel methodology was easy to perform and rapid (within 40 min after DNA isolation). The proper size (307 bp) and purity of the expected PCR product of the TCF4 assay was confirmed after inverting capillaries and 2% agarose electrophoresis of the collected amplicons. Characteristic melting curves for the TCF4 rs613872 SNP assay are shown in Figure 1. The peaks for the two alleles were clearly separated since the Tm was 56.1˚C for the T allele (SD=0.99, CV=1.7% n=10) and 56.4˚C for the G allele (SD=0.80, CV=1.25% n=8) and, therefore, ΔTm was 7.39˚C (SD=0.18, CV=2.44% n=6). The areas under the curve of the two allele melting peaks in the heterozygote samples were nearly equal, with a G/T ratio of 0.982 (SD=0.078, CV=7.9% n=10). The method was accurate when compared to the gold standard method in
genetic analysis: DNA sequencing (100% concordance, Figure 2 for the forward primer). Reproducibility as judged from between-run precision and repeatability from within-run precision were excellent (CVCq of the standards: <1.2%, n=8 and <1.9%, n=3 respectively). The method was linear: in a representative standard curve, the slope and intercept were −3.623 and 42.17 respectively, while the PCR reaction efficiency was calculated to be 1.86 (Figure 3).

TCF4 results. TCF4 genotyping results were obtained from 22 patients with late–onset FECD and 58 healthy controls. Age (Mann-Whitney-test, p>0.05) and sex (Chi-squared test, p>0.05) did not differ significantly between the two groups. From the allelic count, the frequency of the risk G TCF4 allele of the rs613872 SNP increased significantly from 17% in controls to 48% in patient samples. All genotypic frequencies were in HWE equilibrium for both controls and patients (p>0.05). In patients, 7 (31.8%) wild-type TT were detected, 9 (40.9%) GT heterozygotes, and 6 (27.3%) homozygotes GG. Among the 58 healthy controls, 38 (65.5%) were wild-type TT and 20 (34.5%) were GT heterozygotes, while no homogeneous mutant GG was observed. According to SNPStats, both the dominant and the log-additive model were statistically significant models of inheritance; with the latter being the selected one since it possesses the least Akaike information criterion (AIC). This model estimates an OR=4.82 (95%CI=1.98-11.73) for FECD (Table II). When examining the effect of the presence of risk G allele along with age in binary logistic regression for FECD prediction, it was found that only the presence of G allele remained a potent and independent predictive risk factor with p=0.003 and OR=5.98 (95%CI=1.82-19.60). The risk G allele constituted 56% of total TCF4 alleles in FECD men and only 46% of total TCF4 alleles in women but this sex-specific trend did not reach statistical significance.

COL8A2 results. No individuals with COL8A2 g.31753T>G/p.L450W (rs8035192) or g.31767C>A p.Q455K (rs8035191) were detected in our late-onset FECD population, as expected. However, two sequence changes were detected in
a 78-year-old patient with FECD: a silent one (NM_005202.3:c1526C>A → p.P508P, reported previously as rs560539803 with minor allelic frequency MAF 0.0004) and one novel missense (NM_005202.3:c1491G>A → p.A497T) (Figure 4). This amino acid change from alanine to threonine was examined in online prediction programs (PMut, Mutationt@sting, Provean) to determine whether it might cause damaging changes in the structure and function of the COL8A2 protein; however, it was considered benign or tolerated.

Discussion

As we previously mentioned, the exact etiology of late-onset FECD still remains obscure. The theory of a genetic influence on FECD development is well established, while the role of several gene mutations has been evaluated by multiple studies. We aimed to determine whether gene polymorphisms of TCF4 (rs613872) and COL8A2 [g.31753T>G/p.L450W (rs8035192) and g.31767C>A/p.Q455K (rs8035191) mutations] are associated with late-onset FECD in a well-ascertained Greek population.

Initially, we developed and validated an accurate (100% concordance with DNA sequencing), linear, reproducible and fast methodology (<1 h after DNA extraction of 32 samples) for TCF4 genotyping in the LightCycler platform. Considering the effect size of the polymorphism and the frequency of the disease, it is our belief that soon this assay could be transferred to the higher throughput LC480 or LC1536 instruments. There, a significantly larger number of samples could be accommodated (up to 1,536), and this assay can find its place in clinical (patient population screening) as well as research applications. We
demonstrated that TCF4 rs613872 GT and GG genotypes are positively and strongly associated with late-onset FECD in a Greek cohort of patients. Our findings suggest that a selective link exists between the presence of a G allele at rs613872 in TCF4 gene and late-onset FECD, since the carriers of a G allele are almost five times more likely to develop the disease compared to individuals with the wild-type TT genotype. Our findings support recent studies reporting that rs613872 polymorphism in the TCF4 gene is strongly associated with FECD (24, 36) and suggesting that TCF4 polymorphisms contribute to FECD pathogenesis. However, this association is not present in every ethnic group, since this association was rejected in a cohort of patients in India (28), while this polymorphism was not detected in Chinese patients with FECD (37). To the best of our knowledge, this is the first time that this polymorphism has been studied in relation to FECD in a Greek population.

Figure 3. Representative standard curve for the rs613872 real-time PCR TCF4 assay in the LightCycler. Different concentrations of a quantitated DNA standard were used ranging from $28 \times 10^1$ to $28 \times 10^5$ pg DNA per reaction and Cqs were recorded and the calibration plot was calculated with linear regression from the LightCycler software.

Figure 4. DNA sequencing analysis of a 78-year-old patient with FECD: wild-type GG and AA for COL8A2 gene rs80358192 and 80358191 SNPs but with a silent (NM_005202.3:c1526C>A→p.P508P) and a novel missense COL8A2 mutation (NM_005202.3:c1491G>A→p.A497T, using the forward primer COL8A2-F).
The TCF4 gene encodes the E2-2 protein, a member of the ubiquitously expressed class I basic helix–loop–helix transcription factors, which are implicated in cellular growth and differentiation (37). E2-2 protein is expressed in developing corneal endothelium and is involved in endothelium growth, proliferation, and differentiation (38). Mutations in TCF4 gene result in dysfunction of E2-2 protein, and as a consequence the number of endothelial cells is decreased, a situation which is observed in FECD (24). Furthermore, E2-2 protein up-regulates the expression of zinc finger E-box binding homeobox 1 (ZEB1) protein. ZEB1 protein binds to E-box promoters and is implicated in epithelial-to-mesenchymal transition (EMT), through the repression of E-cadherin (39). Through these mechanisms, ZEB1 protein has been identified as the pathogenic protein of FECD (2). Taking the aforementioned proofs into consideration, it has been suggested that mutations in the TCF4 gene, with the rs613872 polymorphism being one of the most prominent, significantly elevate the risk for the development of late-onset FECD. In our study, risk TCF4 allele was detected more frequently in men with FECD compared to women, however, this sex-specific trend did not reach statistical significance, most probably due to the limited sample number of our cohort. In the largest GWAS study to date on FECD, another TCF4 polymorphism (rs784277) conferred a significantly higher risk for FECD development on men (OR=7.56, 95%CI=5.96-9.57) compared to women (OR=5.06, 95%CI=4.29-5.96) (40).

Another TCF4 polymorphism frequently assessed in various FECD populations is a trinucleotide repeat –termed as CTG18.1– which is deleterious when present in above 40 repetitions, probably due to either RNA toxicity or toxic dipeptide formation, an analogous situation to C9orf72 repeats for frontotemporal dementia (41). This polymorphism, in general, confers greater risk (OR=32.3, 95%CI=13.4-77.6) compared to the aforementioned rs613872 TCF4 SNP. The two polymorphisms are in significant linkage disequilibrium ($r^2=0.47$), but still far from absolute; therefore, testing for both is warranted in patients with FECD in order to detect all cases that might be attributed to the TCF4 gene (40, 42, 43).

We did not detect COL8A2 g.31753T>G/p.L450W (rs8035192) nor g.31767C>A/p.Q455K (rs8035191) polymorphisms in the study population of patients with late-onset FECD. Although these two mutations are well-established risk factors for the development of early-onset FECD (4), no role for them has been recognized in the development of the much more frequent late-onset FECD (4).

Both these mutations affect the triple helical stability of COL8 through the abnormal accumulation of mutant COL8 peptides (30, 31). Chronic intracellular accumulation of COL8 is likely to be injurious to the fragile corneal endothelium and the unfolded protein stress response and increased endothelial cell apoptosis have been noted in patients with FECD (44-47). Although the pathological mechanisms of both early- and late-onset FECD are similar, including progressive loss of endothelial cells associated with characteristic excrescences and thickening of Descemet’s membrane, COL8A2 g.31753T>G/p.L450W (rs8035192) and g.31767C>A/p.Q455K (rs8035191) polymorphisms have been associated only with early-onset FECD.

Transparency of the human cornea is necessary for vision. FECD is usually a bilateral, heritable degeneration of the corneal endothelium, and a leading indication for corneal transplantation in developed countries. While the early-onset, and rarer, form of FECD has been linked to COL8A2 mutations, the more common, late-onset form of FECD has genetic mutations linked to only a minority of cases. It is therefore important to identify the genetic background of patients suffering from FECD in order to better understand the importance of clinical findings, their onset and their progression.

In conclusion, in our study of a Greek population, the rs613872 SNP in TCF4 gene was confirmed to be strongly and statistically associated with late-onset FECD. It was also confirmed that the early-onset causative p.L450W and

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Table II. Genotypic frequencies, inheritance models, and calculated ORs for the rs613872 SNP in the TCF4 gene from the SNPsStats software.

| Model      | Genotype | Controls | Patients with FECD | OR (95%CI) | p-Value | AIC  |
|------------|----------|----------|-------------------|------------|---------|------|
| Codominant | T/T      | 38 (65.5%) | 7 (31.8%)           | 1.00       | NA      | 80.8 |
|            | G/T      | 20 (34.5%) | 9 (40.9%)           | 2.44 (0.70-7.54) |         |      |
|            | G/G      | 0 (0%)    | 6 (27.3%)           | NA (0.00-NA) |         |      |
| Dominant   | T/T      | 38 (65.5%) | 7 (31.8%)           | 1.00       | 0.0065  | 90.7 |
|            | G/T-G/G  | 20 (34.5%) | 15 (68.2%)          | 4.07 (1.43-11.61) |       |      |
| Recessive  | T/T-G/T  | 58 (100%)  | 16 (72.7%)          | 1.00       | NA      | 81.3 |
|            | G/G      | 0 (0%)     | 6 (27.3%)           | NA (0.00-NA) |         |      |
| Overdominant | T/T-G/G  | 38 (65.5%) | 13 (59.1%)          | 1.00       | 0.6     | 97.8 |
|            | G/T      | 20 (34.5%) | 9 (40.9%)           | 1.32 (0.48-3.60) |       |      |
| Log-additive | N/A     | N/A       | N/A                | 4.82 (1.98-11.73) | 1e-04  | 83.5 |

T: Wild-type allele; G: risk-mutant allele; AIC: Akaike information criterion; FECD: Fuchs Endothelial Corneal Dystrophy; CI: confidence interval; OR: odds ratio; SNP: single-nucleotide polymorphism; N/A: not applicable. Bold values indicate statistically significant correlations.
pQ455K COL8A2 gene mutations are not associated with late-onset FECD. A potential limitation of this study is its relatively small sample size.

Certainly, in the future, with the advent of next-generation sequencing methods, we could expand our genetic testing to other implicated genes (ZEB1, AGBL1, SLC4A11, and LOXL1DI) that can be assessed simultaneously in a large number of DNA samples suitably-barcoded. In the most recent and largest of all FECD GWAS studies involving 2,075 patients and 3,342 controls of Caucasian descent, besides the confirmation of the aforementioned gene associations, another three gene associations were detected in KN motif- and ankyrin repeat domain-containing protein 4 (KANK4), laminin gamma 1 (LAMC1) and Na+/K+ transporting ATPase, beta-1 polypeptide (ATP1B1) when a p-value significance threshold was set at $10^{-7}$ (40). These latest findings could also be included in future studies in order to detect the full spectrum of genes attributing to late-onset FECD.

Conflicts of Interest

No potential conflict of interest in regard to this study was reported by the Authors.

Authors’ Contributions

MMM wrote and reviewed the manuscript. AD did the experimental (lab) work, performed the statistical analysis and wrote the manuscript. NG performed the statistical analysis and wrote the manuscript. KD, EB, KC, and GK collected data and reviewed the manuscript. CK selected the genes to be analyzed, designed the experimental work, wrote and reviewed the manuscript.

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