**Evaluation of Tear and Aqueous Humor Level, and Genetic Variants of Connective Tissue Growth Factor as Biomarkers for Early Detection of Pseudoexfoliation Syndrome/Glaucoma**

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## **Abbreviations:**

CI: Confidence interval

CTGF: Connective tissue growth factor

DNA: Deoxyribonucleic acid

IOP: Intraocular pressure

MD: Mean deviation

OR: Odds ratio

PCR: Polymerase chain reaction

PEG: Pseudoexfoliative glaucoma

PEX: Pseudoexfoliation syndrome

PSD: Pattern standard deviation

RFLP: Restriction fragment length polymorphism

SD: Standard deviation

SNP: Single-nucleotide polymorphism

TGFβ: Transforming growth factor beta

VFS: Visual field score

## **ABSTRACT**

Pseudoexfoliation syndrome (PEX) may lead to the development of pseudoexfoliative glaucoma (PEG), a potential cause of irreversible blindness, if left untreated. This type of glaucoma often presents with much higher intraocular pressure (IOP) values than observed in primary open angle glaucoma, and patients are often unaware of their condition. Therefore, early diagnosis is of utmost importance in PEX and PEG. Unfortunately, no valid objective biomarkers are available that can be used for this purpose. The excessive synthesis and deposition of elastic microfibrillar pseudoexfoliation material is observed in the pathophysiology of PEX, therefore, growth factors may play roles in this pathology. Thus, in this study, we sought to determine the roles of phenotypes and genotypes of connective tissue growth factor (CTGF) as objective biomarkers for early diagnosis of PEX and PEG. Thus, we investigated possible associations involving tear and aqueous humor CTGF concentrations and four single nucleotide polymorphisms (SNPs) of the *CTGF* gene in PEX and PEG. The study was designed as a 2-year case-control study in the Turkish population. Study population was composed of 214 patients with PEG, 214 patients with PEX, and 214 age-matched controls for *CTGF* SNP analysis. Tear fluid study group consisted of 78 patients with PEG, 77 patients with PEX, and 78 controls. Aqueous humor analysis included 8 patients with PEG, 17 patients with PEX, and 23 controls. Tear fluid was collected using Schirmer strips, and aqueous humor samples were taken during cataract surgery. CTGF concentration was determined by ELISA, and total protein concentration was determined by Bradford assay in tear and aqueous humor samples. PCR followed by restriction fragment length polymorphism analysis was used for genotyping of rs6918698G/C and rs9399005C/T, while real-time PCR was used for rs9402373C/G and rs12526196T/C. Intraocular pressure,visual field score, mean deviation, and pattern standard deviation parameters were also evaluated. CTGF concentration in tear fluid was significantly higher in PEG patients compared with controls (*P* = 0.001), while it was lower in PEX patients. Similarly, total protein concentration in tear fluid was significantly increased in PEG patients relative to PEX patients (*P* = 0.026) and controls (*P* = 0.004). CTGF concentration in aqueous humor did not differ markedly between the groups, whereas total protein was significantly higher in the PEG group compared with the PEX group (*P* = 0.012) and controls (*P* = 0.003). Receiver operating characteristic analysis revealed that total protein in aqueous humor was a robust classifier for evaluating the presence of PEG against controls (Area under the curve = 0.897, *P =* 0.001). The genotypes of the studied SNPs were not significantly correlated with CTGF concentration in aqueous humor or tear fluid, and did not exhibit significant association with PEG or PEX. In conclusion, this was the first study to investigate tear fluid CTGF concentration in PEX and PEG, which came out not to be a good classifier for PEG or PEX. Total protein level in tear fluid and *CTGF* SNPs also did not predict PEG or PEX status successfully.

**Keywords:** aqueous humor; exfoliation syndrome; exfoliative glaucoma; CTGF; genetic polymorphism; SNP; tear

1. **Introduction**

Pseudoexfoliation syndrome (PEX) is an age-related systemic disorder of the extracellular matrix characterized by the production and progressive accumulation of abnormal fibrillary material in ocular tissues as well as in various extraocular tissues (Mitchell et al., 1999). Pseudoexfoliation material may lead to the development of pseudoexfoliative glaucoma (PEG) when it obstructs the drainage canals of the aqueous humor, causing elevation of the intraocular pressure (IOP) (Ritch and Schlotzer-Schrehardt, 2001). PEX prevalence in Turkey is 18.2% for people older than 50 years (Örgen, 1949). The incidence of glaucoma in patients with PEX is 34.3%, and PEX frequency in glaucoma patients is 46.9% in Turkey (Yalaz et al., 1992). In other words, not all PEX patients will eventually develop PEG. Unfortunately, it is currently not possible to predict which PEX patients will develop PEG. Moreover, PEX and PEG are insidious diseases, and PEG may cause vision loss if left untreated. According to the World Health Organization, glaucoma is the second leading cause of blindness after cataract (Resnikoff et al., 2002). Therefore, there is a need for objective biomarkers for early diagnosis and to predict those PEX patients prone to develop PEG.

The pathogenesis of PEX includes excessive synthesis of elastic microfibrillar components throughout the body (Streeten et al., 1986), therefore, growth factors may play roles in this process. It has been shown that transforming growth factor beta 1 (TGFβ1) is expressed at higher levels in PEX patients than controls (Koliakos et al., 2001). Moreover, connective tissue growth factor (CTGF, also known as CCN2) expression is induced by TGFβ (Chen et al., 2000), and CTGF mediates some of the downstream effects of TGFβ on proliferation, migration, and extracellular matrix production (Kothapalli and Grotendorst, 2000; Weston et al., 2003).CTGF appears to act by increasing the expression level of fibrillin, a protein shown to be present in the pseudoexfoliation material (Schlötzer-Schrehardt et al., 1997). It is expressed in a variety of tissues, including the anterior chamber of the eye, and is found in the aqueous humor (van Setten et al., 2002). In two previous studies, the CTGF concentration in aqueous humor was reported to be significantly higher in patients with PEG than in patients with PEX (Ho et al., 2005; Browne et al., 2011).

Aqueous humor is obviously a biological fluid with high potential in terms of discovery of proteins with pathological importance. However, it is not a perfect biological fluid for biomarker discovery because it can only be obtained during cataract surgery. Instead, tear fluid, which is composed of proteins, lipids, electrolytes, and small metabolites, can be obtained relatively easily and non-invasively, as opposed to aqueous humor, and can reflect the clinical status within the anterior chamber of the eye (Zhou et al., 2006). However, tear fluid CTGF levels have not been previously investigated in PEX and PEG.

The *CTGF* gene has several single nucleotide polymorphisms (SNPs), such as rs6918698 G/C, rs9399005 C/T, rs9402373 C/Gandrs12526196 T/C, which may affect mRNA transcript stability (Fonseca et al., 2007; Dessein et al., 2009; Granel et al., 2010; Suh et al., 2015). These SNPs can affect the aqueous humor and tear CTGF levels, and hence, can be associated with PEX and PEG risk. There are no previous studies concerning the association of *CTGF* SNPs with PEX and PEG, other than one report which analyzed the possible association of twelve *CTGF* SNPs with PEX and PEG in a small Korean population (Suh et al., 2015).

Therefore, in this study, we aimed to investigate CTGF and total protein concentrations in tear fluid and aqueous humor in PEX and PEG cases and to verify if there was a correlation between them. The second aim was to determine the role of *CTGF* SNPs as biomarkers for predicting susceptibility to PEX and PEG, and also to detect the effect of these SNPs on tear and aqueous humor CTGF levels.

1. **Methods**
   1. **Study population**

Patients were recruited from the Ophthalmology Unit of Gülhane Training and Research Hospital, University of Health Sciences, Ankara, Turkey, between 2015 and 2017. All subjects were Caucasian and from Central Anatolia, Turkey. The Ethics Committee of Gülhane Medical Academy approved the study, which was conducted in accordance with the principles of the Declaration of Helsinki. Each participant provided written informed consent.

Diagnosis of PEX and PEG was carried out as described previously (Ceylan et al., 2013; Can Demirdöğen et al., 2019). Briefly, cases of PEX were identified by the detection of pseudoexfoliation material during biomicroscopic examination. PEG cases were diagnosed when the anterior segment findings of PEX were accompanied by an IOP value > 21 mmHg without treatment, or of < 21 mmHg with therapy, typical optic nerve head changes, and visual field defects. Consecutive age-matched subjects with an IOP ≤ 21 mmHg, normal optic disk appearance, and no pseudoexfoliation material on the anterior lens capsule and pupil margin on dilated anterior segment examination, and no visual field defects were included as controls. All of the participants received detailed ophthalmologic examinations, including testing for best-corrected visual acuity using Snellen charts, dilated fundoscopic and biomicroscopic examinations, IOP determination by Goldmann applanation tonometry, and visual field determination using the 30-2 SITA-Standard algorithm (Humphrey Instruments, San Leandro, CA, USA).

Exclusion criteria included the presence of other eye disorders, such as age-related macular degeneration, uveitis, having kidney disease, any other systemic fibrotic disease, and any central nervous system disease that could interfere with visual field testing, and use of non-steroidal anti-inflammatory therapeutic agents, vitamin C supplementation, and/or vitamin E, or diuretic use.

The study population consisted of 214 patients with PEX, 214 patients with PEG, and 214 unrelated symptom-free controls. Peripheral whole-blood samples were collected into Na-EDTA tubes. Tear samples of 78 PEG, 77 PEX patients, and 78 controls, who had provided blood samples, were collected. Aqueous humor could only be obtained from patients who were scheduled to undergo cataract surgery: aqueous humor samples were collected from 8 PEG, 17 PEX patients, and 23 controls.

Age and sex information, and a detailed history of conventional risk factors, including diabetes, cardiovascular disorders, systemic hypertension, and smoking status, was taken from each study participant, and this is summarized in Table 1. The mean ages in the PEG, PEX, and control groups did not differ significantly and were 71.1 ± 7.7, 69.7 ± 6.7 and 70.0 ± 7.9, respectively. Men represented 59.8% (128) of PEG cases, 53.3% (114) of PEX patients, and 46.7% (100) of controls (*P* = 0.025). We did not identify any significant differences in terms of risk factors between cases and controls.

The visual clinical parameters, IOP, mean deviation (MD), pattern standard deviation (PSD), and visual field score (VFS) values are also presented in Table 1. The visual field was staged using the Advanced Glaucoma Intervention Study scoring system (The Advanced Glaucoma Intervention Study Investigators, 1994), which takes into account the number and depth of neighboring depressed test locations on the total deviation plot in the nasal area, upper, and lower hemifields, dividing the visual field into 4 concentric-like regions, with the outermost being the most sensitive to a depression. Each participant’s visual field was given a score between 0 and 20, where 0 indicates that no defective points were identified, and 20 indicates that at least 2 depressed locations involving the nasal area, and 9 depressed locations in each hemifield, were measured. The health status of a visual field is categorized based on this score: (i) a score of 0 is a normal field, categorized as stage 1; (ii) scores 1–5 are fields with mild damage, categorized as stage 2; (iii) scores 6–11 are fields with moderate damage, categorized as stage 3; (iv) scores 12–17 are fields with severe damage, categorized as stage 4; (v) scores 18–20 are end-stage fields, categorized as stage 5. All fields of the study participants were able to be classified unambiguously.

* 1. **Aqueous Humor Assays**

Aqueous humor samples were collected during cataract surgery with a syringe into a 1.5 ml tube and stored at -80°C until analysis. These samples were diluted in varying proportions depending on their initial volume before total protein and CTGF concentration determination. Total protein concentration was determined using the Bradford method (Thermo Fisher Scientific, Rockford, Illinois, USA, 23200) and CTGF concentration was measured by ELISA (Finetest, Wuhan, China, EH0702) following the instructions of the manufacturers.

* 1. **Tear Fluid Assays**

Basal tear fluid samples (without any stimulation) were collected using Schirmer tear test strips. In a dimly lit room, a Schirmer strip was placed in the inferior conjunctival fornix of one of the eyes of the subject without topical anesthesia. The strips were placed in 5 ml sterile polypropylene tubes and stored at -86°C until analysis.

Several previously reported methods (Shoji et al., 2003; Farias et al., 2013; Denisin et al., 2012) were tested, and finally a combination of these procedures was optimized to extract proteins from the strips. In this procedure, strips were cut into 0.5 cm lengths and all the pieces were put in a 2 ml tube and 400 µl extraction buffer (PBS buffer with 500 mM NaCl and 0.5% Tween20) was added. After the tubes were incubated at 25°C for 3 hours with gentle shaking, they were centrifuged at 16000 × g for 5 min. Supernatant was delivered to another tube to rest at -20°C overnight and remaining strips were discarded. The next day, tear extracts were thawed at room temperature (25°C) and centrifuged at 14000 × g for 5 min. Supernatant was transferred to another tube and used without dilution for measurement of total protein by the Bradford method (Thermo Fisher Scientific, 23200) and CTGF concentration by ELISA (Finetest, EH0702).

The CTGF concentration in tear samples of 7 controls, 3 PEX, and 2 PEG patients could not be included in statistical analysis because they were above the upper limit of the ELISA kit used, and no tear sample was left to dilute and measure again.

* 1. ***CTGF* Genotyping**

Genomic DNA was extracted from peripheral blood leukocytes using a salting-out method (Lahiri and Schnabel, 1993). CTGF rs6918698G/C and rs9399005C/T SNPs were genotyped using a PCR - restriction fragment length polymorphism method. The primer pairs used for rs6918698G/C were forward: 5’ GAGAACAAAGACGCGTGTGA 3’ and reverse: 5’AGCCCCTACCTACCCAACAC 3', and for rs9399005C/T were forward: 5’ TTGTGATGTGAAGGGTTGGA 3’ and reverse: 5’ GCAGGCATACACACCACATT 3’. For amplification of both SNP regions, the PCR reagent mixture contained 200 ng genomic DNA, 200 μM dNTPs, 20 pmol of each primer, 2.5 mM MgCl2, and 1.25 U of Taq polymerase in a total volume of 50 μl. To genotype the rs96918698G/C polymorphism, 194 bp PCR products were digested with 5 U BseRI at 37°C for 45 min, resulting in 67 and 127 bp fragments for the G allele, and a non-digested 194 bp fragment for the C allele. To genotype rs9399005C/T, 191 bp PCR products were digested with 5 U MscI at 37°C for 45 min, which resulted in 36 and 155 bp fragments for T alleles and a non-digested 191 bp fragment for C alleles. Digestion products were run in 2.5% agarose gels with ethidium bromide and visualized under UV illumination.

TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) were used to genotype rs12526196T/C (C\_1764938\_10) and rs9402373C/G (C\_30110249\_10). The assays were conducted using 1 × TaqMan genotyping PCR master mix, 1 × TaqMan genotyping assay, and 0.8 µg/ml of DNA on a StepOnePlus Real-Time PCR System, and allelic discrimination analysis was performed using StepOne v2.3 software (Applied Biosystems).

* 1. **Statistical Analysis**

SPSS version 15.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Findings were considered statistically significant when *P* values were < 0.05. The continuous variables and the interval variable, namely VFS, were given as both median and Q1 (25%) – Q3 (75%) quartiles, and means, standard deviation (SD), and their distribution was tested using Kolmogorov-Smirnov test. Mean and SD values were used for bar graphs. ANOVA tests were used for comparison of parameters which were normally distributed, and Kruskal-Wallis H tests were used for parameters which were not normally distributed. The Games-Howell or Bonferroni post-hoc test was applied for ANOVA, and Conover’s formula (Conover, 1980) was used as a post-hoc test for Kruskal-Wallis tests. Receiver operating characteristics (ROC) curves were constructed, and the area under the ROC curve (AUC) was determined for the CTGF and total protein parameters. AUC values were considered excellent when between 0.9-1, good when between 0.8-0.9, fair when between 0.7-0.8, poor when between 0.6-0.7, and failed when 0.5-0.6. The optimal cutoff point was derived from ROC analysis using the highest sensitivity and specificity combination. Categorical variables were expressed as percentages or ratios, and these were compared using the chi-squared test. The consistency of genotype frequencies with Hardy-Weinberg equilibrium was also checked using the chi-squaredtest. The odds ratio (OR) values, 95% confidence intervals (CIs) and P values for allele frequencies were calculated using Pearson χ2 test. Correction for age and sex was applied for genotype comparison, using SNPstats web tool (<https://www.snpstats.net/start.htm>). Pearson's correlation method was used to investigate the correlation between study parameters. Binary logistic regression analysis with the backward selection method was performed using the Hosmer-Lemeshow calibration to determine association of the study parameters with PEX and PEG. The “Genetic Association Study (GAS) Power Calculator” (<http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/>) was used for sample size calculation, and a power value > 50% was considered adequate. With the lowest allele frequency being 0.157, disease prevalence 10%, and significance level 0.05, the observed lowest power was 72%, with 214 subjects in each group. The sample size for the tear fluid and aqueous humor groups was calculated using GPower 3.1.9.4 (Faul et al., 2007) with the following input parameters: effect size d: 0.8, α error probability: 0.05. The test results came out to be satisfactory (over 99 % for tear fluid groups, and over 60% for aqueous humor groups).

1. **Results**

Aqueous humor CTGF concentrations did not differ significantly between the study groups and were found to be median: 4.35, quartiles (Q1-Q3): 3.01-7.30 ng/ml, (mean: 5.40, SD: 2.87 ng/ml)in PEG, median: 2.89, quartiles 2.26 - 4.49 ng/ml (mean: 3.62, SD: 2.16 ng/ml)in PEX, and median: 2.84, quartiles: 2.44 - 5.76 ng/ml (mean: 4.10, SD: 2.90 ng/ml) in controls (*P =* 0.190) (Figure 1A). AUC values did not reveal a significant output.

Total protein concentrations of the aqueous humor samples were significantly increased in patients with PEG (median: 352.50, quartiles: 238.50 - 554.85µg/ml, mean: 388.65, SD: 161.52µg/ml) compared with patients with PEX (median: 171.50, quartiles: 137.24 - 427.30 µg/ml, mean: 270.17, SD: 199.12 µg/ml, *P =* 0.012) and controls (median: 175.40, quartiles: 144.30 - 26.30 µg/ml, mean: 196.85, SD: 91.63 µg/ml, *P =* 0.003) (Figure 1B). On the other hand, aqueous humor total protein concentration did not differ significantly between PEX and control groups (*P* = 0.562). CTGF concentrations in aqueous humor did not correlate with total protein concentration in any of the study groups (data not shown). ROC analysis resulted in an AUC value of 0.897 (*P =* 0.001), and the cut-off value was determined to be 227.65 µg/ml for aqueous humor total protein, with 87.5% sensitivity and 78.3% specificity (Figure 2). Thus, total protein in aqueous humor was a good classifier for evaluating the presence of PEG against controls. In contrast, ROC analysis of this parameter for predicting PEX against controls, and for discriminating PEG and PEX, both revealed non-significant results.

Tear fluid CTGF concentrations were significantly higher in patients with PEG (median: 5.98, quartiles: 3.57-11.61 ng/ml, mean: 12.15, SD: 15.69 ng/ml) when compared with controls (median: 3.61, quartiles: 1.56-8.90 ng/ml, mean: 11.91, SD: 26.59 ng/ml, *P* = 0.001). In addition tear fluid CTGF concentration of PEX patients (median: 5.44, quartiles: 2.87-10.97 ng/ml, mean: 10.08, SD: 12.11 ng/ml) was also significantly different from that of controls (*P* = 0.025, Figure 3A). Despite, this parameter did not change significantly between PEG and PEX cases (*P* = 0.299). ROC analysis revealed an AUC value of 0.655 (*P* = 0.001) for tear CTGF concentrations for predicting the presence of PEG, and the cut-off value was determined as 4.69 ng/ml, with 65% sensitivity, and 62% specificity. However, we did not determine the cut-off values of tear CTGF concentrations for predicting PEX, nor for discriminating PEG and PEX patients, because the AUC was less than 0.5 for both.

Total protein concentrations in the tear fluid of patients with PEG (median: 16.29, quartiles: 12.91 - 23.31 mg/ml, mean: 18.91, SD: 8.91 mg/ml) were significantly higher when compared with those of the PEX group (median: 14.27, quartiles: 10.36 - 19.29 mg/ml, mean: 16.47, SD: 8.94 mg/ml, *P =* 0.026) and controls (median: 13.33, quartiles: 9.70 - 19.81 mg/ml, mean: 16.34, SD: 10.29 mg/ml,  *P =* 0.004) (Figure 3B). The difference between PEX and control groups was not statistically significant (*P* = 0.499). AUC was found to be 0.629 (*P =* 0.005) for tear total protein for predicting PEG against controls, and the best cut-off level was 15.35 mg/ml, with 63% sensitivity and 60% specificity. In addition, AUC for tear total protein for discriminating PEG and PEX was 0.607 (*P =* 0.022), and the cut-off value was 15.12 mg/ml, with 63% sensitivity, and 57 % specificity. On the other hand, this parameter failed to be a classifier for distinguishing PEX from controls, due to the AUC value of 0.535, which was not statistically significant.

Correlation analysis revealed that tear fluid total protein and CTGF concentrations were significantly correlated with each other in the overall study population (r = 0.354, *P =* 0.000), in PEG patients (r = 0.401, *P =* 0.000), and in controls (r = 0.455, *P =* 0.000), but not in the PEX group.

A cross-correlation analysis between tear and aqueous humor showed that tear fluid and aqueous humor CTGF levels and total protein concentration were not correlated with each other (data not shown).

Theallele and genotype frequencies for the four *CTGF* SNPsin patient and control groups are presented in Table 2. The observed genotype frequencies were consistent with Hardy-Weinberg equilibrium. Comparison of allele and genotype frequencies between patient and control groups revealed no significant relationship. On the other hand, combined genotype analysis for the four SNPs (rs6918698, rs9399005, rs12526196, rs9402373) revealed that the GG-CC-TT-CC (respectively) genotype was significantly more common in controls (34 controls, 15.9 %) than in PEX patients (15 PEX patients, 7 %) (OR = 0.399, *P =* 0.004). Likewise, the GC-CC-TC-CC genotype was more frequent in PEX patients (24 PEX patients, 11.2 %) compared to PEG patients (8 PEG patients, 3.7 %) (OR = 0.307, *P =* 0.003); and the GC-CT-TT-CG genotype was more common in PEX patients (35 PEX patients, 16.4 %) compared to PEG patients (16 PEG patients, 7.5 %) (OR = 0.413, *P =* 0.005). The four analyzed *CTGF* SNPs were found to be significantly correlated with each other both in the genotype group and in the tear fluid sub-group (data not shown).

We analyzed the effects of *CTGF* SNPs on aqueous humor CTGF and total protein concentrations (Figure 4) and observed that in the control group, CTGF concentrations in aqueous humor of individuals with the rs12526196 TT genotype were significantly higher than in subjects with the CC genotype (*P =* 0.018) (Figure 4A). In addition, the aqueous humor total protein levels of PEX patients with the rs12526196 TCgenotype were significantly higher than those of PEX patients with the TT genotype (*P =* 0.026) (Figure 4B). Similarly, the effects of *CTGF* SNPs on tear CTGF levels and total protein concentrations in PEG and PEX patients and control subjects are shown in Figure 5. This analysis did not reveal a coherent output. Likewise, correlation analysis also failed to show any significant relationship (data not shown).

We also analyzed correlations involving the visual clinical parameters (IOP, VFS, MD, and PSD) with the *CTGF* SNPs, CTGF levels, and total protein concentrations in tear and aqueous humor, which resulted in no significant outcome (data not shown).

Logistic regression analysis to evaluate the effects of several factors (age, gender, diabetes, hypertension, smoking, and *CTGF* SNPs) as covariates on PEX or PEG status revealed that male gender is a significant predictor of PEG (OR = 1.726, 95% CI= 1.174 - 2.537, *P =* 0.006). Likewise, a similar regression analysis in the tear fluid study group pointed out male sex to be significantly associated with PEX (OR = 2.049, 95% CI= 1.017 - 4.130, *P =* 0.045) and PEG (OR = 2.231, 95% CI= 1.153-4.316, *P* = 0.017). The calibration of the models was satisfactory, according to the Hosmer-Lemeshow goodness-of-fit test. In addition, we sought to identify the effect of gender on the visual parameters, and this analysis revealed that IOP did not differ; however, VFS and MD values were significantly different between men and women (Table 3).

1. **Discussion**

Glaucoma is the most frequent cause of permanent and irreversible blindness globally. However, those affected often do not realize their condition until it is advanced and leads to loss of vision; therefore, glaucoma is considered to be an insidious disease. It is well-established that PEX is a risk factor for glaucoma, but not all PEX patients develop this disorder. Furthermore, an objective parameter that can be used for early diagnosis of PEX patients with glaucoma risk is not available. Such a biomarker would contribute to efforts aimed at preventing the loss of vision caused by PEG, with the help of medical therapies to decrease the IOP. Furthermore, currently, due to an incomplete picture of the pathophysiology of PEX development, there is no complete cure for PEX or PEG.

Deposition of fibrillary pseudoexfoliation material throughout the eye is a hallmark of PEX, and growth factors may be related to this pathology by inducing cells to synthesize and secrete increased amounts of extracellular material. CTGF is a major fibrogenic protein known for its diverse roles in development, adhesion, mitosis, and extracellular matrix synthesis (Oemar and Lüscher, 1997; Brigstock, 1999; Shimo et al., 1999; Dhar and Ray, 2010; Jacobson and Cunningham, 2012). Overproduction of elastic microfibrils, which cross-link to form the characteristic pseudoexfoliation material, has an important role in the pathogenesis of PEX. Moreover, *in vitro* exposure of trabecular meshwork cells to CTGF results in 50% upregulation of fibrillin-1, an extracellular matrix protein previously observed to be a component of the pseudoexfoliation material (Schlötzer-Schrehardt et al., 1997; Browne et al., 2011). Furthermore, CTGF is a potent inducer of other extracellular matrix proteins, including collagens (Leask and Abraham, 2006).

Based on these findings, we hypothesized that CTGF might play a role in the pathophysiology of PEX and PEG, and thus, could be used as a biomarker for early diagnosis of these disorders. Therefore, in this study, we investigated the possible association of tear fluid and aqueous humor levels of CTGF, together with *CTGF* SNPs and PEX and PEG.

CTGF in the aqueous humor was first detected by van Setten and colleagues, and the concentration was reported to be 1.24 ± 0.26 ng/ml (van Setten et al., 2002). Later, aqueous humor CTGF was quantified in PEX and PEG, and was observed to be markedly increased in PEG (6.03 ± 1.09 ng/ml) compared with PEX (2.73 ± 0.45 ng/ml, *P* < 0.01). Furthermore, aqueous humor CTGF levels in PEX patients with or without glaucoma (4.38 ± 0.65 ng/ml) were higher than those in controls (2.35 ± 0.46 ng/ml, *P* < 0.05) (Ho et al., 2005). Similarly, CTGF levels in aqueous humor of PEG patients were highest (5.15 ± 0.79 ng/ml) compared with PEX patients (2.76 ± 0.64 ng/ml), and controls (2.60 ± 0.29 ng/ml) (Browne et al., 2011).

In the present study, aqueous humor CTGF concentrations were similar to those found in previous studies, but there was no statistically significant difference between study groups. Slight differences between our findings and previous studies’ results might be due to the differences in genetic and epigenetic background, and lifestyle habits of the study populations. In addition, we observed that total protein concentrations in aqueous humor were highest in PEG patients, followed by PEX cases, and lowest in controls, which was very similar to the observations of two previous studies (Zenkel et al., 2006; Padhy et al., 2014). ROC analysis revealed that aqueous humor total protein level was a good classifier for evaluating the presence of PEG against controls, with 87.5% sensitivity and 78.3% specificity. However, we determined that aqueous humor levels of CTGF and total protein were not correlated. Therefore, we ascribe disruption to the function of the blood-aqueous barrier in these patients for the observed increases in total protein, as also reported by Zenkel and colleagues (Zenkel et al., 2006). Nevertheless, an aqueous humor sample cannot be collected from every patient, thus, this parameter is not suitable for early diagnosis of PEG. Therefore, we turned our focus to tear fluid, the composition of which is reflective of ocular health.

The first comprehensive proteomics study of proteins in tears was carried out by de Souza et al. (De Souza et al., 2006), who reported the presence of 491 different proteins in tears. Then, in a more recent study, 1543 different proteins were detected in the tears of a healthy person (Zhou et al., 2012). When compared, 239 of these proteins were found to be common between these studies, and there are approximately 1800 different proteins in tears (Zhou et al., 2012). In contrast, there are 1929 different proteins in human plasma (Farrah et al., 2011).

The proteomic composition of tear fluid has been attracting much attention in clinical medicine in recent years because different distributions of tear proteins may provide insight into the pathophysiology of ocular diseases. In addition, some proteins in tears have the potential to be used as biomarkers in the diagnosis of ocular and systemic diseases, including thyroid eye disease (Baker et al., 2006), diabetic retinopathy (Csősz et al., 2012), diabetes (Stolwijk et al., 1994), multiple sclerosis (Salvisberg et al., 2014), and cancer (Lebrecht et al., 2009).

Moreover, tear collection is a non-surgical minimally invasive procedure, and often considered non-invasive (Csősz et al., 2012; Stolwijk et al., 1994; Salvisberg et al., 2014; Lebrecht et al., 2009; Posa et al., 2013). Interestingly, in a study questioning patient preferences, patients preferred to give tear fluid instead of a blood sample (Quah et al., 2014).

The most commonly employed methods for collecting a tear sample use a microcapillary tube (De Souza et al., 2006; Csősz et al., 2012) or a Schirmer strip (Denisin et al., 2012; Stuchell et al., 1984). The use of a Schirmer strip has some advantages for both the ophthalmologist and the patient (Posa et al., 2013). Moreover, strip-like paper-based biosensors is a rapidly growing research field (Chen et al., 2012), which, hopefully, will lead to rapid and early detection of eye disorders, as well as systemic diseases, in the long-term.

Therefore, we employed Schirmer strips to collect non-stimulated (basal) tear samples. This method can be used in different medical disciplines to investigate tear fluid proteins. We observed that tear fluid CTGF concentration in PEG patients was significantly higher than that of controls. ROC analysis results, however, indicated that tear fluid CTGF level is a poor classifier for PEG.

There is only one study in the literature that reported the presence of CTGF protein in tears (van Setten et al., 2003) which was collected with glass capillaries from healthy eyes. CTGF was detected in 7 of the 70 specimens, with a maximum level of 17 ng/ml in basal tears (van Setten et al., 2003). However, no studies have been conducted previously on CTGF protein levels in tears in PEX or PEG: such precedence can be confirmed by a Pubmed search with the keywords “Connective tissue growth factor OR CTGF” AND “tear OR tear fluid” AND “pseudoexfoliation OR exfoliation”.

Moreover, in this study, total protein concentration in tears was significantly higher in PEG patients than PEX and control groups, whereas no prominent difference was observed between PEX group and controls. Furthermore, total protein and CTGF concentrations were significantly correlated with each other in the overall study population, in PEG patients, and in controls, but not in the PEX group. This indicates that remarkable changes in the tear proteome of PEX patients take place during PEG development. As a result, total protein in tears was a poor classifier of PEG in ROC analysis. It seems that in our study, tear protein levels do not reflect the situation in the anterior chamber of the eye, because CTGF and total protein levels were found not to be correlated between tear fluid and aqueous humor.

The *CTGF* gene has several SNPs that may affect transcript stability, including rs6918698G/C, rs9399005C/T, rs9402373C/Gandrs12526196T/C. rs6918698G/C is found at position -945 in the gene promoter, where the presence of a C allele is critical for transcriptional suppression of the *CTGF* gene, which in turn would reduce CTGF production (Fonseca et al., 2007). rs9399005C/T is found in the 3’ untranslated region, where the T allele binds nuclear factors with greater affinity than the C allele, and *in silico* analysis has shown that this polymorphism may affect the secondary structure of *CTGF* mRNA, and thus the stability and half-life of the transcript (Granel et al., 2010). Both rs9402373C/G and rs12526196T/C are intronic variants that can potentially influence transcription or transcript stability by affecting the binding efficiency of nuclear factors (Dessein et al., 2009).

Moreover, these SNPs were previously analyzed in diseases with similar pathology to PEX and PEG. For example, rs6918698 was found to be associated with systemic sclerosis (Fonseca et al., 2007; Kawaguchi et al., 2008); however, this relationship has not been confirmed in other studies (Rueda et al., 2009; Louthrenoo et al., 2011). rs9399005 was also associated with systemic sclerosis (Granel et al., 2010) and hepatic fibrosis (Dessein et al., 2009). rs9402373 and rs12526196 had the strongest association with hepatic fibrosis (Dessein et al., 2009).

Therefore, we studied these four *CTGF* SNPs in PEX and PEG cases and found that alleles and genotypes were not significantly associated with these conditions. There is only one previous study (Suh et al., 2015) on the association of *CTGF* SNPs with PEX and PEG, which analyzed twelve SNPs in a small Korean population. Only rs9399005 was studied in common with the present research and, similar to our results, this SNP was not associated with PEX or PEG. It was reported that rs2151532 was significantly associated with disease in total patients (PEX and PEG), and especially with PEG (Suh et al., 2015).

In addition, we compared the minor allele frequencies obtained for the control group in this study with those obtained in other populations (Table 4), because this is the first time these SNPs are analyzed in our population. The frequency of the *CTGF* rs6918698C allele in the Turkish population was observed to be 0.449, which was similar to that found in French (0.478) (Granel et al., 2010) and Japanese populations (0.490) (Miyoshi et al., 2014), but lower when compared to that found in another study carried out in Japanese population (0.552) (Kawaguchi et al., 2008).This frequency was much lower in Thai population (0.348) (Louthrenoo et al., 2011).rs9399005 Tallele frequencywas found to be 0.287 in the Turkish population, which was similar to that found in Swedish (0.276) (Ahmad et al., 2015), French (0.275) (Granel et al., 2010), and Irish populations (0.297-0.317) (Burke et al., 2013), but much lower than found in the Korean population (0.474) (Suh et al., 2015). The minor allele frequency (C) for rs12526196was determined to be 0.227 in the Turkish population. In contrast, this frequency was much lower in Swedish (0.080) (Ahmad et al., 2015), Irish (0.087-0.059) (Burke et al., 2013), and French (0.086) (Granel et al., 2010) populations. The frequency of the rs9402373 G allele in the Turkish population was 0.182 and was very close to that found in the Irish population (0.183-0.208) (Burke et al., 2013).

In the present study, logistic regression analysis revealed that male gender is a significant risk factor for PEG. Similarly, the probability of developing PEG from PEX is greater in males in previous studies (Madden and Crowley, 1982). PEX is more prevalent in male Yugoslavs, Australian Aborigines, Peruvian Indians and Asian Indians (Forsius, 1988).In contrast, a gender relationship was not found in other studies ( Moreno-Montanis et al., 1989), although the prevalence of PEX has been found to be higher in women in some other studies (Aasved, 1969; Kozart and Yanoff, 1982).

In a previous study, high IOP was detected in 10 of 25 men (40%), and in 17 of 75 (22.7%) women (Kozart and Yanoff, 1982). It was reported that PEG occurred earlier and with higher IOP in males, and visual field defects were more common in men (Moreno-Montanis et al., 1990). In our study, we did not detect higher IOP values in males; hence, our finding of male gender as a risk factor for PEG was not related to higher IOP values. Besides, VFS and MD values were markedly different in males, potentially contributing to a higher risk for development of PEG.

Limitations of this study include the number of subjects, especially in the aqueous humor analysis. This was mainly due to technical difficulties in obtaining enough sample during cataract surgery. However, the statistical power tests confirmed the adequacy of the sample size.

1. **Conclusion**

In conclusion, in this study, the role of CTGF in PEX and PEG risk was investigated at both the phenotypic and genotypic levels, in an effort to be able to find objective biomarkers for early diagnosis of PEG and PEX. This is the first time that tear fluid CTGF levels in PEX and PEG patients have been investigated. The main findings were that 1) CTGF and total protein contents in tear fluid were significantly higher in PEG cases relative to controls; 2) tear fluid CTGF and total protein levels, as well as *CTGF* SNPs were not good predictors for PEG or PEX.

A valid biomarker for early diagnosis of PEX patients at risk of glaucoma is of paramount importance, and the need for such a biomarker persists.

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**FIGURE CAPTIONS**

Figure 1. Connective tissue growth factor (CTGF) (A) and total protein concentrations (B) in aqueous humor of patients with pseudoexfoliative glaucoma (PEG), patients with pseudoexfoliation syndrome (PEX), and the controls. Mean and SD values are presented.

Figure 2. Receiver operating characteristics curve for total protein concentration in aqueous humor for discriminating patients with pseudoexfoliative glaucoma (PEG) from the controls. Area under the curve = 0.897 (*P =* 0.001).

Figure 3. Connective tissue growth factor (CTGF) (A) and total protein concentrations (B) in tear fluid of patients with pseudoexfoliative glaucoma (PEG), patients with pseudoexfoliation syndrome (PEX), and the controls. Mean and SD values are presented.

Figure 4. Connective tissue growth factor (CTGF) (A) and total protein (B) concentration in aqueous humor of patients with pseudoexfoliative glaucoma (PEG), patients with pseudoexfoliation syndrome (PEX), and the controls stratified by *CTGF* rs6918698G/C, rs9399005C/T, rs12526196T/C and rs9402373C/G genotypes. Mean and SD values are presented.

Figure 5. Connective tissue growth factor (CTGF) (A) and total protein (B) concentration in tear fluid of patients with pseudoexfoliative glaucoma (PEG), patients with pseudoexfoliation syndrome (PEX), and the controls stratified by *CTGF* rs6918698G/C, rs9399005C/T, rs12526196T/C and rs9402373C/G genotypes. Mean and SD values are presented.