Corneal Abnormalities Are Novel Clinical Feature in Wolfram Syndrome

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• PURPOSE: To evaluate corneal morphology among patients with Wolfram syndrome (WFS).

• DESIGN: Comparative observational longitudinal case series of WFS patients with a laboratory approach in the *WFS1* gene knockout (Wfs1KO) mouse model.

• METHODS: A group of 12 patients with biallelic mutations in the *WFS1* gene recruited from the whole country and a control group composed of 30 individuals with type 1 diabetes (T1D) were evaluated in a national reference center for monogenic diabetes. All subjects (n = 42) underwent a complete ophthalmic examination, computer videokeratography, and corneal thickness and endothelial measurements. Additionally, WFS patients (n = 9) underwent longitudinal videokeratography and Pentacam evaluation. Corneal characteristics were assessed and compared between both groups. Human and mouse corneas were subjected to immunohistochemistry to detect wolframin expression and microscopic evaluation to study corneal morphology ex vivo.

• RESULTS: Clinical and topographic abnormalities similar to keratoconus were observed in 14 eyes (58.3%) of 8 WFS patients (66.7%). Flat keratometry, inferior-superior dioptric asymmetry, skewed radial axis, logarithm of keratoconus percentage index, index of surface variance, index of vertical asymmetry, keratoconus index, central keratoconus index, index of height asymmetry, and index of height decentration differed between WFS and T1D patients. Immunohistochemistry demonstrated wolframin expression in human and mouse corneas. Compared with Wfs1WT mice, Wfs1KO mice also presented corneal abnormalities.

• CONCLUSIONS: Patients with WFS present a high prevalence of changes in corneal morphology compatible with the diagnosis of early stages of keratoconus. Observations in a mouse model suggest that a mutation in the *WFS1* gene may be responsible for corneal abnormalities similar to keratoconus. (Am J Ophthalmol 2020;217: 140–151. © 2020 Elsevier Inc. All rights reserved.)

WOLFRAM SYNDROME (WFS, OMIM: #222300) IS AN ultrarare genetic inherited disease also known as DIDMOAD syndrome, with an estimated prevalence in European populations of approximately 1 per 800,000 individuals.[1] Insulin-dependent diabetes mellitus and optic atrophy together with diabetes insipidus and neurosensorial deafness are the most frequent clinical features. At the molecular level, WFS result from biallelic defects of the *WFS1* gene, which lead to increases in endoplasmic reticulum (ER) stress and mitochondrial dysfunction.[2] Apart from optic atrophy (OA), other visual system abnormalities, such as maculopathy, retinopathy, and cataracts, have also been observed in WFS patients.[3,4] Interestingly, during routine annual examination of our national cohort of patients with WFS, corneal abnormalities were observed in a single patient.

Keratoconus is a multifactorial, chronic, noninflammatory disorder. Although the etiology of keratoconus is still unknown, there is increasing evidence that supports a genetic link.[5] Corneal abnormalities, including keratoconus, are reported in many genetic syndromes, including Down syndrome,[6] Turner syndrome,[7] Noonan syndrome,[8] and Bardet-Biedel syndrome.[9]

Thus, since corneal topography has yet to be studied among patients with WFS, we performed a comprehensive evaluation of corneal features and found that corneal anomalies were frequently linked with WFS. Moreover, we demonstrated that wolframin is expressed in human and mouse corneas and that *WFS1* gene knockout (Wfs1KO) and wild-type (Wfs1WT) mice differed in corneal thickness (CT) and epithelial features.

METHODS

COMPARATIVE OBSERVATIONAL LONGITUDINAL CASE SEries of WFS patients was approved by the Research Ethics Board of the Medical University of Lodz (RNN/140/13/KE). Consent to include clinical information in scientific

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studies was obtained from all the participating patients, following the tenets of the Helsinki declaration.

All experiments in this study involving animals were performed in accordance with the European Parliament Directive 2010/63/EU and under permit (no. 86, May 4, 2016) from the Estonian National Board of Animal Experiments.

• **STUDY GROUP:** Patients with WFS of Polish origin were clinically and genetically identified and recruited based on the Nation-wide genetic screening of monogenic diabetes–TEAM program (January 2005 and December 2015)[10,11] and the EURO-WABB initiative.[12]

The present study included 12 patients with biallelic mutations in the *WFS1* gene and clinical symptoms of WFS. Because all patients with WFS suffered from diabetes treated with insulin, we included a control group composed of 30 individuals with type 1 diabetes (T1D). Detailed characteristics of the study groups are shown in Table 1. All patients were referred for prospective and complete ophthalmologic examination in a detailed study protocol by a team from the Department of Ophthalmology.

Diabetes was typically recognized according to World Health Organization (WHO) criteria. At the onset of T1D, autoantibodies and decreased C-peptide levels were detected in all patients, and diabetic ketoacidosis was present in 34.5% of patients. Glycated hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography using the Bio-Rad VARIANT Hemoglobin A1c Program (Bio-Rad Laboratories, Inc, Hercules, California, USA) with its values represented as percentages. All WFS patients had DM recognized according to WHO criteria and OA recognized using ophthalmologic examination, visual evoked potentials, and/or magnetic resonance imaging and confirmed by optical coherence tomography.[13] The causative mutations in the *WFS1* gene detected in all WFS patients were recognized as described previously.[14]

• **OPHTHALMOLOGIC EVALUATION:** All subjects (WFS patients and T1D patients) underwent a complete ophthalmic examination that included spherical equivalent manifest refraction and manifest cylinder measurement with autorefractometry (RC-800; Tomey, Phoenix, Arizona, USA), logMAR best-corrected distance visual acuity measurement with an ETDRS chart (LCD Frey CP-400; Frey Sp.J., Piaseczno, Poland), slit-lamp biomicroscopy (SL-D2; Topcon Inc, Paramus, New Jersey, USA) with anterior segment and fundus examination, tonometry (Goldmann applanation tonometer), and retinoscopy (Keeler, Windsor, UK).

• **CORNEA EXAMINATION:** To detect keratoconus, all patients from both groups (n = 42) underwent slit-lamp biomicroscopy to determine whether stromal corneal thinning, Vogt striae, Fleischer ring, Descemet breaks, prominent nerve fibers, apical scars, and subepithelial fibrosis were present. None of the WFS or DM patients had previous ocular surgery, corneal opacities, or a history of contact lens wear of any type.

• **TOPOGRAPHIC, KERATOMETRIC, AND PACHYMETRIC EXAMINATION:** Topographic, keratometric, and pachymetric analyses were performed for all subjects on each eye in WFS and T1D patients using Placido disk videokeratography (Keratograf 4; Oculus Inc, Wetzlar, Germany) and a noncontact specular microscope (EM-3000; Tomey, Nagoya, Japan).

Nine patients with WFS underwent Pentacam HR Scheimpflug tomography (Oculus Optikgerate GmbH, Wetzlar, Germany; software version 6.07r29). The analyzed parameters are described in the Supplemental Appendix (Supplemental Material available at AJO.com).

• **KERATOCONUS AND SUBCLINICAL KERATOCONUS CLASSIFICATION SCHEME:** For the diagnosis of keratoconus (KC) and subclinical keratoconus (SKC) based on clinical, topographic, keratometric, and pachymetric evaluation,[15,16] each eye of all participants was grouped into 1 of 3 groups: KC, SKC, and normal, according to the classification scheme described in the Supplemental Appendix.

• **CORNEAL ENDOTHELIAL MEASUREMENTS:** Specular microscopy (using the noncontact specular microscope, EM-3000; Tomey Corporation, Nagoya, Japan) was performed on all patients. The image with the highest quality in terms of contrast and illumination was automatically selected by the instrument and subsequently verified manually by the examiner. We used automated cell detection and counting implemented in the built-in manufacturer's software. Data collected from specular microscopy included cell size (minimum, maximum, average, standard deviation [SD] and coefficient of variation [CV] in cell area), endothelial cell density (ECD) and CT.

• **HISTOPATHOLOGIC EXAMINATION OF THE CORNEA:** *Human cornea.* A normal human cornea, serologically not suitable for transplantation, obtained from the Eye Bank of Warsaw, Poland, was used as a control. This cornea was obtained from a male cadaver 52 years of age within 10 hours of death.

Exclusion criteria for the donor of the examined cornea were the following: previous ocular surgery, uveitis, glaucoma, chronic use of topical medications, connective tissue disease, rheumatologic disorders, thyroid eye disease, diabetes mellitus, congenital malformations, psychiatric diseases, and a history of medications that might affect hormone levels (eg, systemic steroids).

The cornea was stored at 4 C in corneal storage medium Eusol-C (Alchimia S.r.l, Ponte S. Nicolò, Italy) and was used for immunohistochemical examinations within 48 hours.

TABLE 1. Clinical and Ophthalmologic Features of the Patients With Wolfram Syndrome and of the Reference Group of Patients With Type 1 Diabetes

Patient ID	Sex	Age at Examination (Years)	Mutation - Amino Acid Change	Optic Atrophy (Age at Diagnosis)	Diabetes Mellitus (Age at Diagnosis)	Diabetes Insipidus	Hearing Impairment	Renal Tract Disorders	Neurologic/ Psychiatric Disorders	Time of Follow-up (Months)	BCVA (LogMAR), OD/OS	Corneal Abnormalities, OD/OS
WFS1	F	24.5	Homozygous V412S	9	5	Yes	No	Neurogenic bladder	-	6	HM/HM	SKC/No
WFS2	M	19.3	W648X V779G	5.8	3.8	No	No	Atonic bladder	-	33	1.7/1.7	KC/KC
WFS3	F	21.3	R558C V412S	6	8	No	No	Neurogenic bladder	Ataxia. depression	LOF	0.6/0.5	No/No
WFS4	F	19.7	Homozygous S443R	5.5	5	Yes	No	-	-	6	2/2	SKC/SKC
WFS5	F	16.8	Homozygous S443R	5	4	Yes	No	-	Dysautonomia	6	CF/1.7	SKC/SKC
WFS6	F	19.5	Homozygous W540X	9	4	No	Yes	-	-	30	0.9/0.9	No/No
WFS7	F	21.3	Homozygous W540X	10	5	No	Yes	-	-	30	HM/HM	KC/SKC
WFS8	F	18	S167E W648X	9.7	8.7	No	Yes	Nocturnal enuresis	-	31	1.4/1.1	No/No
WFS9	F	21.7	Homozygous W648X	9	5	Yes	Yes	Neurogenic bladder	-	6	0.6/0.5	No/No
WFS10	M	21.4	R558C V412S	9.5	8.5	Yes	Yes	-	Ataxia	5	0.5/0.4	KC/KC
WFS11	F	16	Homozygous W540X	9	6	No	Yes	-	-	LOF	1.3/1.3	No/SKC
WFS12	F	22.1	Homozygous W540X	6	4.5	-	-	-	-	LOF	HM/HM	KC/KC
Control group of patients with diabetes	18 F/12 M	20.4 (range, 16.6-21.3)	NA	NA	5.9 (3.4-10.0)	NA	NA	NA	NA	NA	0	None

BCVA = best-corrected visual acuity; CF, counting fingers; HM, hand movement; KC, keratoconus; LOF = lost to follow-up; NA = not applicable; SKC = subclinical keratoconus; WFS = Wolfram syndrome.

Mouse corneas. Wfs1-deficient (Wfs1KO) mice were generated as described previously.[17,18] Experiments were performed with Wfs1KO male mice with a 129S6/SvEvTac background and their wild-type (Wfs1WT) littermates. The animals were housed under standard laboratory conditions, kept on a 12-hour light-dark cycle (lights on at 7:00 AM), and given free access to food and water.

Three Wfs1WT and 3 Wfs1KO mice were sacrificed at 109 ± 2 days of age, and both eye bulbs and optic nerves were taken for histopathologic examination.

Reagents for the preparation of histopathologic specimens were purchased from Leica Biosystems (Wetzlar, Germany) and from Sigma-Aldrich (St. Louis, Missouri, USA), unless otherwise stated.

• **HISTOPATHOLOGIC EXAMINATION:** The bulbs of eyes were fixed in 10% neutral buffered formalin for at least 24 hours. Following macroscopic evaluation, the bulbs were routinely processed into paraffin-embedded tissue blocks. Six consecutive 4-μm-thick sections were taken at the largest longitudinal diameter of each bulb and subsequently stained with hematoxylin-eosin in a routine procedure. The most representative sections were selected by standard light microscopy examination (Light Microscope BX43; OLYMPUS Europa SE & Co, Hamburg, Germany). The selected sections were scanned, and a detailed morphologic analysis of corneas was conducted using an UltraFast Scanner (Philips IntelliSite Solution, Best, Netherlands) with DigiPath Professional Production Software (Xerox, Norwalk, Connecticut, USA).

In the morphologic examination, the following qualitative and quantitative parameters were selected for examination: CT, corneal epithelial thickness, corneal stromal thickness, corneal endothelial thickness, lateral CT (μm) (each parameter measured in 3 independent locations), longitudinal diameter of eye (μm) (optic nerve to cornea), lateral diameter of eye (μm) (cornea-cornea), CT/eye longitudinal diameter ratio, lateral CT/eye longitudinal diameter ratio, and number of cells per 0.1 mm of basement membrane (Figure 1).[19,20]

• **IMMUNOHISTOCHEMISTRY:** The immunohistochemical expression of Wolfram syndrome protein 1 (WFS1) and glial fibrillary acidic protein (GFAP) was evaluated following the standard protocol.[21]

Immunohistochemical staining was performed on 5-μm-thick sections collected on polylysine-covered microscopic slides (Accu-Cut SMR 200 rotary microtome; Sakura Finetek, Tokyo, Japan). The following antibodies were used: for WFS1, catalog number 11558-1-AP, Immunogen catalog number AG2114, WFS1 polyclonal rabbit antibody (Proteintech Group Inc, Rosemont, Illinois, USA); for GFAP, M0761, Clone GF2, Monoclonal Mouse Anti-Human with cross-reactivity to mice (Dako-Agilent, San

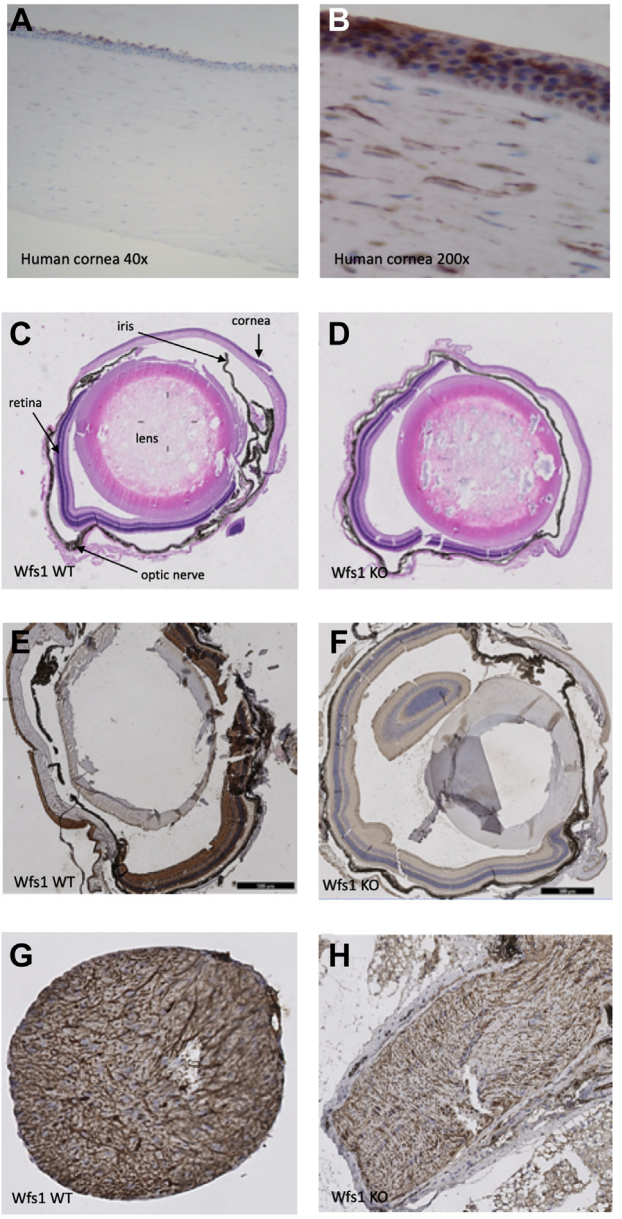

FIGURE 1. Panel of 8 digital immunohistochemical images of the human cornea (A, B) as well as Wfs1WT (C, E, G) and Wfs1KO (D, F, H) mouse eyes. A, B. WFS1 staining of the human cornea at low (A) and high (B) magnification showing intense expression of wolframin in all layers, mainly in the epithelium. C, D. Examples of hematoxylin-eosin staining of mouse Wfs1WT (C) and Wfs1KO (D) eyes in a low-power view (10×). E, F. Wfs1WT mouse (E) and Wfs1KO mouse (F) eye staining for wolframin. The Wfs1KO eyes show no or weak expression of wolframin in the cornea and retina compared to the Wfs1WT eyes. G. GFAP-positive staining of Wfs1WT mouse optic nerve. H. Degeneration of the optic nerve in Wfs1KO mice depicted by GFAP staining. GFAP, glial fibrillary acidic protein; WFS1 = Wolfram syndrome 1 gene; Wfs1KO = Wolfram syndrome 1 gene knockout mice; Wfs1WT = Wolfram syndrome 1 gene wild-type mice.

TABLE 2. Comparison of Keratometric, Topometric, and Pachymetric Indices and Endothelial Parameters in Patients With Wolfram Syndrome and Control Group of Patients With Type 1 Diabetes in Computer Videokeratography and Specular Microscopy Examinations

Parameter	WFS Patients (N = 24 Eyes) Median (Lower-Upper Quartile)	Controls (N = 60 Eyes) Median (Lower-Upper Quartile)	*P* Value
Keratometric indices			
K_1 (D)	42.4 (41.75-42.9)	43.1 (42.6-43.55)	.0216
K_2 (D)	43.3 (42.4-45.6)	43.9 (43.2-44.5)	.383
K_{apex} (D)	43.25 (42.2-43.95)	43.6 (42.9-44.3)	.2086
Astigmatism (D)	0.86 (0.615-1.355)	0.71 (0.515-0.98)	.14
Topometric indices			
I-S (D)	0.345 (0.232-0.662)	0.179 (0.063-0.429)	.009
SRAX	17.5 (9.5-42.5)	10 (3.5-17.5)	.013
Log (KISA)	2.01 (1.61-2.39)	1.27 (0.71-1.62)	.000012
ISV	27.5 (20.5-30.0)	18.5 (16-22)	.00017
IVA	0.19 (0.12-0.285)	0.001 (1.00-1.02)	.0024
KI	1.03 (1.02-1.055)	1.01 (1.00-1.01)	.000067
CKI	1.01 (1.01-1.01)	1.01 (1.00-1.01)	.0579
IHA	10.95 (6.50-20.05)	5.900 (3.350-10.450)	.00523
IHD	0.01 (0.005-0.013)	0.005 (0.003-0.007)	.0044
R_{min}	7.64 (7.395-7.765)	7.590 (7.465-7.715)	.893
Pachymetric indices			
CT (μm)	541.5 (527-568)	550 (527-574)	.610
Endothelial parameters			
ECD/mm^2	2888 (1783-3352)	2891.5 (2740.5-3081.5)	.779
AVG in μm^2 (cell size)	346 (327.5-355)	346 (324-365)	.809
SD in μm^2 (cell size)	134.5 (116.5-149)	11.5 (97-136)	.0056
CV (%)	39 (36-41)	32 (28.5-38)	.000273
Max in μm^2 (cell size)	960.5 (872.5-1121)	843 (704.5-995)	.0132
Min in μm^2 (cell size)	90 (79-104)	93 (81.5-105.5)	.764

AVG = average cell size; CKI = central keratoconus index; CT = corneal thickness; CV = coefficient of variation in cell area; D = diopters; ECD = endothelial cell density; IHA = index of height asymmetry; IHD = index of height decentration; I-S = inferior-superior dioptric asymmetry; ISV = index of surface variance; IVA = index of vertical asymmetry; K_1 = keratometry values in the flat meridian; K_2 = keratometry values in the steep meridian; K_{apex} = keratometry values at the corneal apex; KI = keratoconus index; log(KISA) = logarithm of keratoconus percentage index; Max = maximum cell area; Min = minimum cell area; R_{min} = minimum radius of curvature; SD = standard deviation in cell size; SRAX = skewed radial axis; T1D = type 1 diabetes; WFS = Wolfram syndrome.

Jose, California, USA). GFAP antigens were unmasked by incubating the sections in pH 8.0 citrate buffer (S1699; Dako-Agilent, San Jose, California, USA) in PT Link (20 minutes, 97 C, Dako-Agilent, San Jose, California, USA), and WFS1 was unmasked by incubating the sections in pH 6.0 citrate buffer (S1699; Dako-Agilent) in a water bath (30 minutes, 98 C). Visualization of staining was performed using specific detection systems—EnVision FLEX+ (Dako-Agilent, San Jose, California, USA). The reactions were carried out using Autostainer Link (Dako-Agilent, San Jose, California, USA). The EnVision+ System-HRP antibody with DAB as a chromogen (K4011; Dako-Agilent, San Jose, California, USA) was used according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin.

The staining results were evaluated qualitatively using light microscopy (Light Microscope BX43; OLYMPUS Europa SE & Co), and images of representative samples were taken using an UltraFast Scanner (Philips IntelliSite Solution) with DigiPath Professional Production Software (Xerox).

• **STATISTICAL ANALYSIS:** For statistical analyses of the computer videokeratography examination results, the parameters were summed for both eyes of all patients from each study group. Statistical analyses of Pentacam results were performed for each eye of WFS patients separately at the follow-up time point and grouped into the SKC, KC, and normal subgroups.

Continuous variables are presented as medians followed by interquartile ranges, while nominal variables are presented as numbers followed by percentages in brackets. The Shapiro-Wilk test was used to assess the normality of distribution. Continuous variables were compared using the Mann-Whitney *U* test, Wilcoxon and Kruskal-Wallis analysis of variance (ANOVA) in the case of a non-

TABLE 3. Comparison of Keratometric and Topometric Indices in Patients With Wolfram Syndrome in Videokeratography Longitudinal Analysis

Parameter	First Examination (N = 18 Eyes) Median (Lower-Upper Quartile)	Follow-up (N = 18 Eyes) Median (Lower-Upper Quartile)	*P* Value
Keretometric indices			
K_1 (D)	42.6 (41.7-43.7)	42.4 (41.8-44.1)	.21
K_2 (D)	43.3 (42.5-45.2)	43.3 (42.7-45.7)	.46
K_{apex} (D)	43.3 (41.9-44.7)	43.45 (42.4-45.2)	.72
Astigmatism (D)	0.79 (0.61-1.04)	0.81 (0.59-1.04)	.41
Topometric indices			
I-S (D)	0.284 (0.156-0.400)	0.59 (0.324-0.85)	.000134
SRAX	17.5 (11.0-43.0)	20.5 (12-48)	.003807
Log (KISA)	1.82.00 (1.53-2.34)	2.25 (1.69-2.64)	.0012
ISV	25.5 (18.0-30.0)	34 (22-38)	$<10^{-5}$
IVA	0.175 (0.120-0.270)	0.27 (0.19-0.38)	$<10^{-5}$
KI	1.03 (1.02-1.05)	1.04 (1.03-1.07)	.00001
CKI	1.01 (1.01-1.01)	1.01 (1.01-1.01)	.00817
IHA	10.15 (3.80-19.60)	18.950 (9.8-27)	$<10^{-5}$
IHD	0.006 (0.004-0.012)	0.011 (0.007-0.015)	.000044
R_{min}	7.65 (7.41-7.85)	7.615 (7.38-7.88)	.8079

CKI = central keratoconus index; D = diopters; IHA = index of height asymmetry; IHD = index of height decentration; I-S = inferior-superior dioptric asymmetry; ISV = index of surface variance; IVA = index of vertical asymmetry; K_1 = keratometry values in the flat meridian; K_2 = keratometry values in the steep meridian; K_{apex} = keratometry values at the corneal apex; KI = keratoconus index; KISA = keratoconus percentage index; R_{min} = minimum radius of curvature.

normal distribution, or *t* test in case of normal distribution. In the case of ANOVA, a *P* value < .1 subgroup analysis was also performed. The Statistica 12.5 PL package (Statsoft, Tulsa, Oklahoma, USA) was used for the analysis. *P* values < .05 were considered statistically significant. In the case of ANOVA with a *P* value < .1, subgroup post hoc analysis was also performed.

RESULTS

THE DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF the patients with WFS and T1D are demonstrated in Table 1. The WFS patient group and control T1D group were matched for sex, age, and age at diabetes diagnosis. Ophthalmologic examination results are collected in the Supplemental Appendix.

• **CORNEA ASSESSMENT:** Ophthalmologic examination with slit-lamp biomicroscopy showed clearly visible corneal nerves and prominent nerve fibers in 5 eyes (20.8%) of 3 WFS patients.

• **KERATOCONUS DETECTION:** Clinical and topographic abnormalities similar to keratoconus were observed in 14 (58.3%) eyes of 8 (66.7%) WFS patients. Bilateral keratoconus was detected in 3 (25%) WFS subjects. One (8.3%) WFS patient with unilateral keratoconus had subclinical keratoconus in the fellow eye. One eye of 1 keratoconus WFS patient had stage 2 KC according to the Amsler-Krumeich classification. The remaining keratoconus patients had stage 1 KC.

The final qualification of each eye to a specific subgroup was made after videokeratography and/or Pentacam analyses.

Analysis of the topographic pattern of the anterior corneal surface showed a keratoconus-suspect pattern only in the WFS group, such as abnormal localized steepening in 2 (8.3%) eyes of 2 (16.7%%) WFS patients, asymmetric bow-tie with skewed radial axes pattern in 3 eyes (12.5%) of 3 (25%) WFS patients, and asymmetric bow-tie with inferior steepening pattern in 9 eyes (37.5%) of 8 (66.7%) WFS patients. Detailed analysis of keratometric, topometric, pachymetric, and elevation parameters between WFS patients and control patients is presented in Table 2.

Nine WFS patients (75%) underwent several months of follow-up (median 6 months; range 5-33 months). Analysis of follow-up videokeratography results did not reveal any statistically significant differences in keratometric parameters in the WFS patient group, but we observed a significant progression of topometric indices. Detailed differences between the first and last videokeratography examination results are presented in Table 3. Figure 2 shows the progression of corneal abnormalities in a 25-month follow-up in 7 WFS patients. Paired comparisons of each single eye at study entry and at follow-up for all

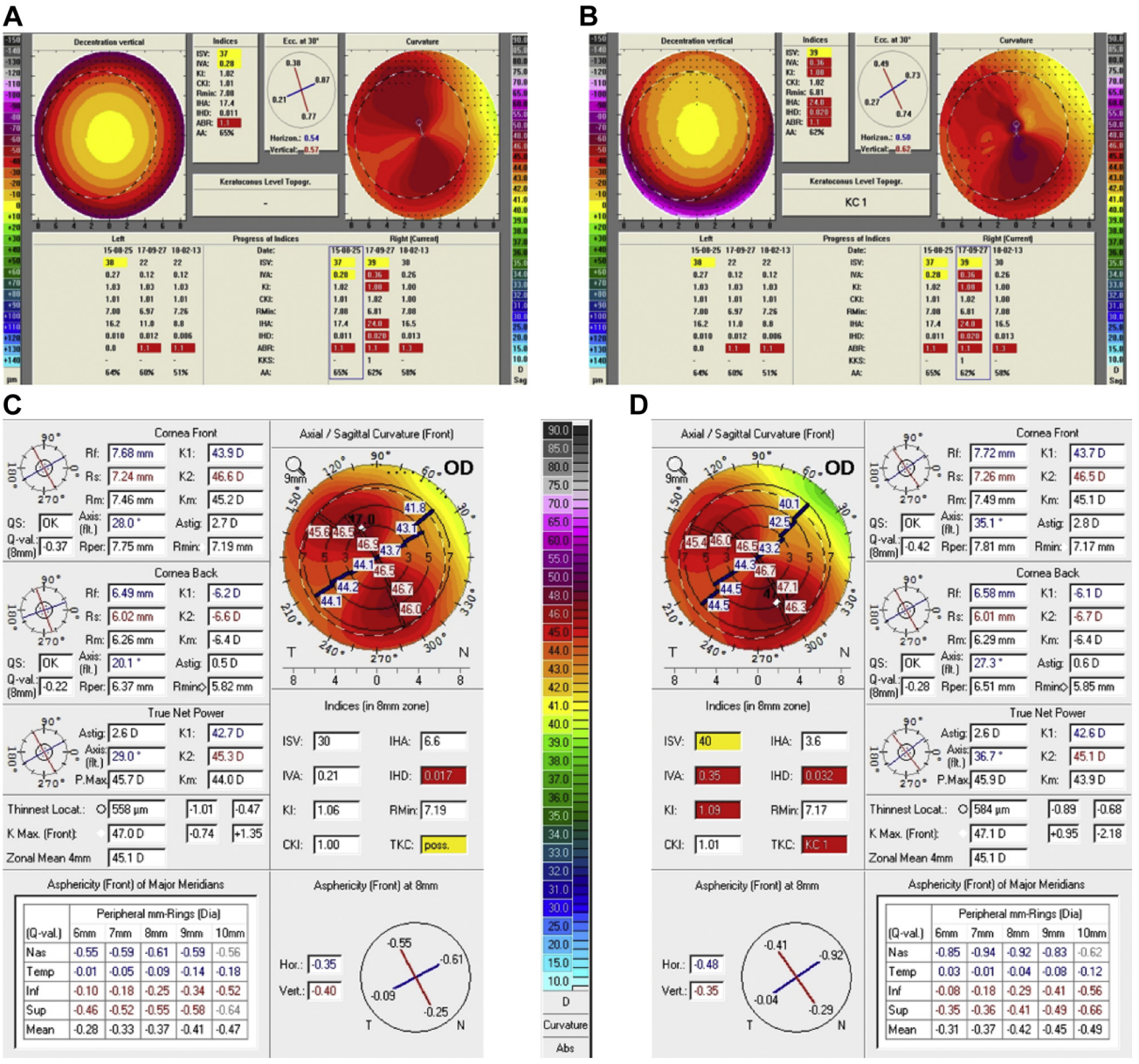

FIGURE 2. Progression of corneal abnormalities in a 25-month follow-up in 2 Wolfram syndrome patients using computer videokeratography (A, B) and Pentacam (C, D) examinations.

videokeratography parameters, which differed significantly between these 2 time points, are presented in Supplemental Figure 1 (Supplemental Material available at AJO.com). To increase a statistical power, we performed an analysis for the differences in videokeratography parameters, presented as a delta split by a median follow-up. This revealed significant differences in index of vertical asymmetry (IVA) and index of height decentration (IHD) parameters, suggesting an association between follow-up time and progression of corneal abnormalities. These results are presented in Supplemental Figure 2 (Supplemental Material available at AJO.com).

Supplemental Table 1 (Supplemental Material available at AJO.com) presents the detailed results of Pentacam parameters measured in WFS patients. Based on the criteria reported in the Methods, the corneal abnormalities diagnosed in WFS patients were categorized as subclinical keratoconus (8 eyes, 44.4% of eyes), keratoconus (5 eyes, 27% of eyes), and normal (5 eyes, 27% of eyes). Mean comparisons for subgroups were performed by ANOVA. There were significant differences among all subgroups for flat front keratometry ($K_{1\ front}$, $P = .042$), mean front simulated keratometry ($K_{mean\ front}$, $P = .077$), flat back keratometry ($K_{1\ back}$, $P = .044$), IVA ($P = .005$), and IHD ($P =$

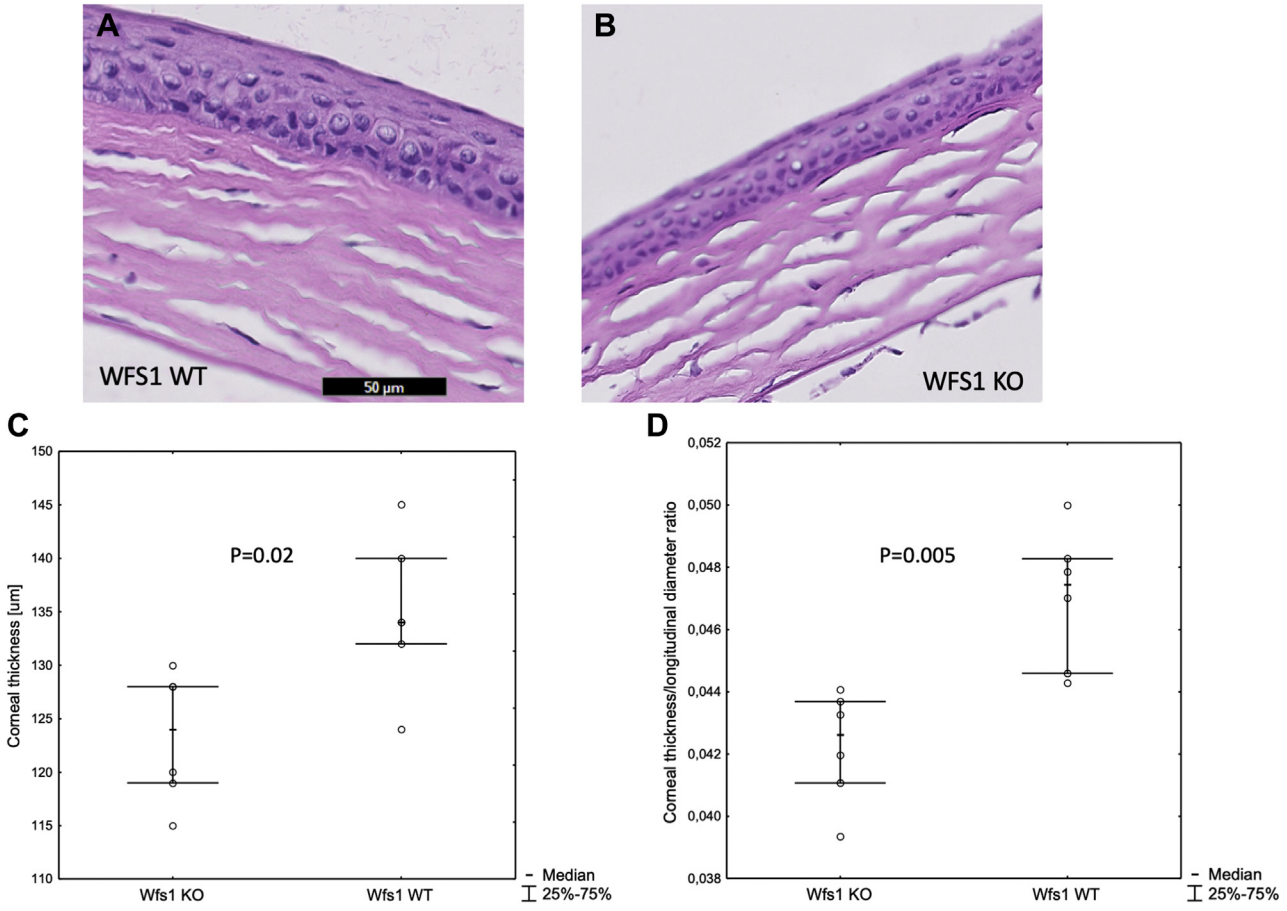

FIGURE 3. A, B. Examples of hematoxylin-eosin-stained Wolfram syndrome 1 gene wild-type (Wfs1WT; panel A) and Wolfram syndrome 1 gene knockout (Wfs1KO; panel B) mouse corneas. C, D. Comparison of corneal thickness (C) and corneal thickness/longitudinal diameter ratio (D) between Wfs1KO and Wfs1WT mice, Mann-Whitney *U* test.

.052). An analysis of the eyes qualified for the SKC and KC subgroups showed significant differences between them in $K_{1\ front}$ ($P = .016$), $K_{mean\ front}$ ($P = .048$), $K_{1\ back}$ ($P = .013$), steep back keratometry ($K_{2\ back}$, $P = .023$), and IVA ($P = .016$). There were no significant differences among the subgroups, including CT ($P = .832$), both at the apex ($P = .701$) and at the thinnest point ($P = .837$); deviation of normality (D values, all $P > .1$); pachymetric progression indices (all $P > .1$); or Ambrosio's relational thickness (ART, $P = .495$). Supplemental Figure 3 (Supplemental Material available at AJO.com) shows sample results of keratometric, elevation, and pachymetric indices in the Pentacam study of WFS patients classified into 3 different subgroups: KC, SKC, and normal.

• **SPECULAR MICROSCOPY:** Significant differences in maximum cell size ($P = .01$), SD of cell size ($P = .005$), and CV indices (0.0003) between WFS and T1D patients were found, revealing an increase in percentage of large cells in the corneal endothelium among patients with WFS. In WFS patients, eyes in the keratoconus subgroup had significantly higher CV ($P = .049$) and maximum cell size values ($P = .027$) than eyes in the normal subgroup. The results of the endothelial parameters are shown in Table 2 and Supplemental Table 1.

• **IMMUNOHISTOCHEMISTRY ANALYSIS:** The WFS1 protein, also known as wolframin, is expressed in human and mouse corneal cells, predominantly in the corneal epithelium (Figure 1). In a mouse model of WFS, we confirmed that WFS1 is not expressed in the cornea or retina of Wfs1KO mice, unlike in the cornea and retina of mice with the normal *WFS1* gene (Wfs1WT) (Figure 1E and 2F). Moreover, GFAP staining as a neuronal marker showed nerve degeneration in Wfs1KO mice, confirming that this mouse model presented similar ophthalmologic features to those observed among patients with WFS (Figure 1G and H).

• **MORPHOMETRIC ANALYSIS OF MOUSE CORNEAS:** Detailed qualitative and quantitative morphometric analysis revealed some signs of degeneration among Wfs1KO

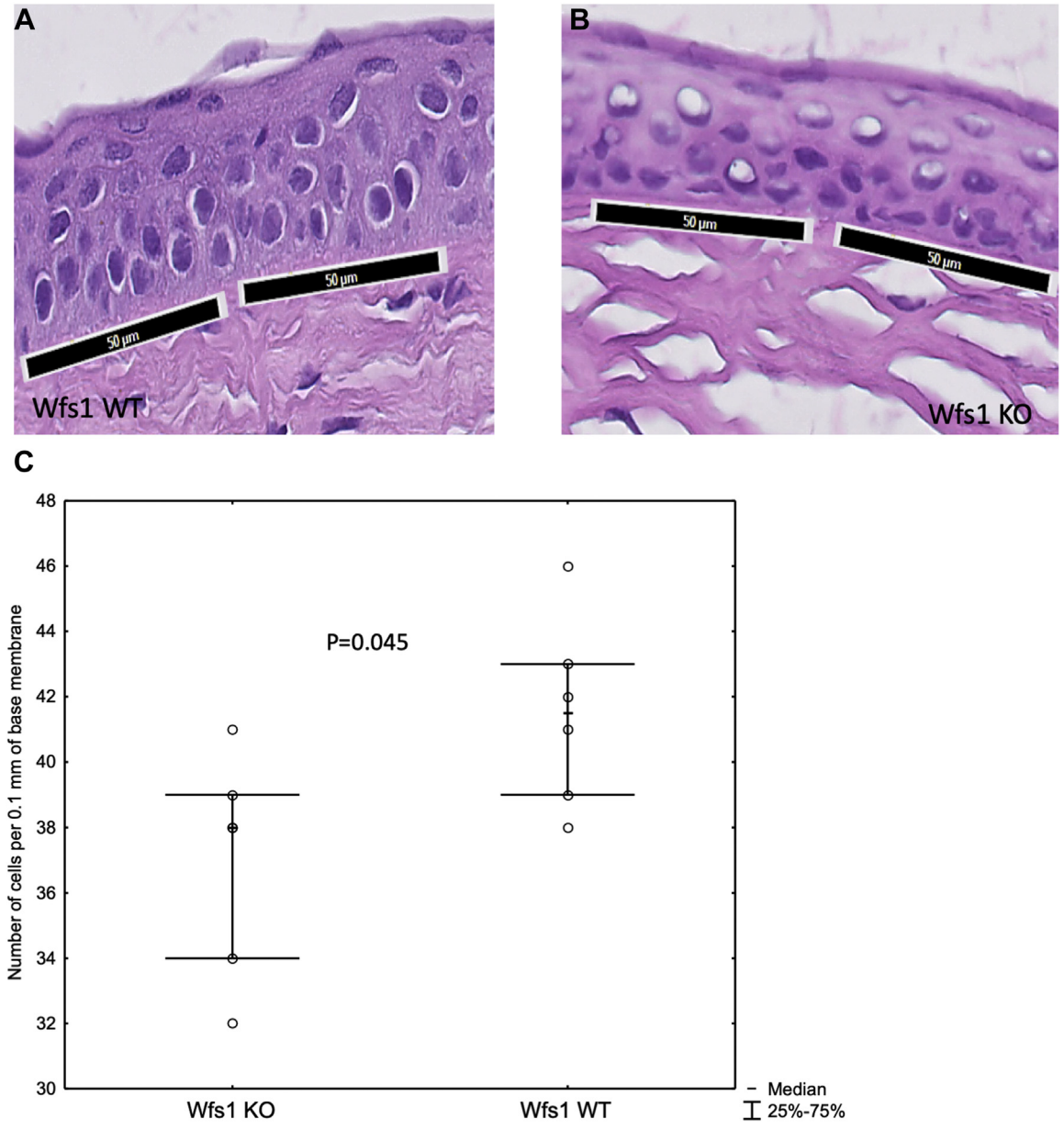

FIGURE 4. A, B. Examples of hematoxylin-eosin-stained Wolfram syndrome 1 gene wild-type (Wfs1WT; panel A) and Wolfram syndrome 1 gene knockout (Wfs1KO; panel B) mouse corneas. C. Comparison of corneal cell number per 0.1 mm of basal membrane between Wfs1KO and Wfs1WT mice, Mann-Whitney *U* test.

mouse corneas (Supplemental Table 2; Supplemental Material available at AJO.com). There were no signs of inflammation or clear shape changes in either group. The Wfs1KO corneas were significantly thinner than the Wfs1WT corneas (Figure 3C and D). This difference was mostly apparent in the stromal layer; however, a tendency for thinning was also observed in the epithelial and endothelial layers of the Wfs1KO mice (data not shown). The difference was stable when measured centrally and laterally. In the Wfs1KO group, collagen fibers appeared loosely arranged, and there were interlamellar clefts, and signs of neovascularization and edema, whereas these features were not observed in the Wfs1WT group (Figure 3A and B). The number of cells within the corneal epithelium per 0.1 mm of basal membrane was significantly lower in Wfs1KO mice than in Wfs1WT mice (Figure 4 and Supplemental Table 2).

DISCUSSION

WE DOCUMENTED FOR THE FIRST TIME THE PRESENCE OF corneal abnormalities similar to keratoconus in patients

with WFS. The observed high percentage of patients with KC prompted us to broaden the research to determine whether keratoconical changes in the corneal topography are linked with WFS. Our comparative analysis with the control group of T1D patients showed significant differences in corneal topography between the study groups. In the group of WFS patients, the incidence of corneal abnormalities was 66.7%, whereas in the control group we did not observe any changes similar to SKC or KC. Early keratoconus or other corneal abnormalities can be overlooked on a routine examination, which is why we are the first to describe corneal topography disorders strongly associated with WFS. We observed topographic patterns similar to early keratoconus only in the WFS group. We did not observe differences in CT between the examined groups. No differences in pachymetric measurements in the case of mild keratoconus advancement compared to that in normal corneas have been confirmed by other authors.[22]

Previous studies on ocular wolframin expression in both human and animal models focused on the retina and optic nerve, showing the presence of wolframin in human and mouse retinas, including retinal pigment epithelium, retinal ganglion cells, optic axons, and the proximal optic nerve.[23,24] In the present study, we showed, for the first time, that wolframin is abundantly expressed in a healthy human cornea, especially in epithelial and endothelial cells.

Consistently, our study performed on a mouse model with a normal *WFS1* gene revealed wolframin expression in the mouse cornea. Wfs1KO mice have been generated in several laboratories.[17,18,25] Exon 8 of the gene is disrupted in the WFS1-deficient mice used in the current study, resulting in the deletion of amino acids 360-890, and the mice show clinical features similar to those of patients with WFS.[17,18] In the cornea of our Wfs1KO mouse model, wolframin expression was weak or not present. A comparison of Wfs1-deficient vs WT mice also demonstrated substantial morphologic differences in the corneas. Some features of degeneration in Wfs1KO mice were also noted, including a low number of cells in the epithelium, loosely arranged collagen fibers, and interlamellar clefts in the stroma. These observations in WFS1-deficient mouse corneas may be regarded as reminiscent of human keratoconus. Unfortunately, we do not have topographic studies of mouse corneas to determine the degree of advancement of the corneal abnormalities, and mice were not longitudinally examined. Histopathologic features of the human keratoconus corneas have been documented in several studies. The most common features include epithelial thinning, breaks in the Bowman layer, compaction of stromal fibrosis, central stromal thinning, and folds in the Descemet membrane. Decreased collagen lamellae, abnormal organization of the collagen fiber network, loss of collagen fibrils in the stroma, and the presence of hyperdense nuclei mimicking apoptotic or preapoptotic cells in the anterior stroma and Bowman layer have also been reported.[26] Finally, the lack of WFS human corneal material hampered keratoconus progress in our study. We are unable to correlate the histopathologic results of WFS1-deficient mouse corneas with the human corneas from WFS patients. Our observations in WFS1-deficient mouse corneas are suggestive of human keratoconus. However, we are aware of the differences in the pathology of murine and human corneas and possible differences in the pathogenesis of WFS in humans and animals.

Tachibana and associates presented 2 mouse strains with corneas that demonstrated a keratoconical appearance.[27] However, some distinctions between keratoconus in these mice and in humans were observed; most notably, the corneas in the mouse were often cloudy and revealed leukocyte infiltration, implying an inflammatory background of the disease. In our study, histologic corneal changes did not include corneal haze or inflammatory infiltration, which is not concordant with the study by Tachibana and associates but seems to be more coherent with findings in human keratoconic corneas.

The mechanism by which wolframin deficiency may cause changes in corneal morphology is not clear. Increased ER stress, activating an ER stress signaling network, also called the unfolded protein response, and the autophagic lysosomal pathway seem to be among the possible mechanisms. Consistently, a role of autophagy in neurodegenerative diseases and keratoconus human corneal epithelial cells has been documented.[28]

Additionally, wolframin is associated with the function of the Na^+-K^+-ATPase β1 subunit[29] of the endothelial Na+-K+-ATPase active transport pump, which plays a key role in the regulation of fluid secretion from the stroma to the aqueous humor.[30] The most strongly expressed corneal decompensation owing to corneal endothelial dysfunction and acute pseudokeratoconus has been reported in mitochondrial disorders such as Kearns-Sayre syndrome,[31] Pearson syndrome,[32] and Leber hereditary optic neuropathy (LHON).[33] Our results of specular microscopy indicate significantly higher indices of endothelial cell polymegathism in WFS patients, further demonstrating that the disturbances in this layer of the cornea may be a starting point for the disturbances in corneal topography. Interestingly, recent studies have shown a similarity in the histopathologic pattern of OA between a WFS case and an LHON case.[34] Moreover, keratoconus is frequently present in LHON.[35] However, the described KC in LHON is more advanced than in our WFS cases. Interestingly, we did not observe any significant changes in slit-lamp biomicroscopy examination of WFS patient corneas. In 20% of WFS patients, we noticed clearly visible nerves only. The presence of pathologic changes in the keratoconus corneas is known to be correlated with disease severity.[36] However, a diagnosis of KC can also be made in the absence of corneal signs. Approximately 15% of patients with diagnosed keratoconus are estimated to have no pathologic changes of the anterior segment of the eye

upon biomicroscopic assessment.[37] Moreover, the literature reports that all histopathologic changes typical of keratoconus, such as epithelial thinning, breaks in the Bowman layer, folds in the Descemet membrane, stromal fiber compaction, superficial iron deposition, and epithelial and stromal scars, are not observed in stage 1 according to the Amsler classification of KC.[38] Our results have shown that corneal abnormalities similar to KC observed in patients with WFS were characterized by a low degree of progression.

The coexisting diabetes can be suspected to be responsible for the lack of significant progression of the keratoconus. Some studies have demonstrated the protective effect of diabetes, especially HbA1 levels, on keratoconus occurrence.[39] Among the reasons for this protective effect is the increased glycosylation of proteins and thus increased biomechanical immunity of diabetic corneas. According to the literature, keratoconus is observed 4 times less frequently in patients with diabetes.[40]

Owing to the low degree of corneal abnormalities similar to keratoconus and the lack of changes in CT in relation to the control group, we are careful in naming these changes keratoconus. We applied very stringent criteria for subclinical keratoconus, which might result in overestimation of corneal abnormalities among patients with WFS. The same criteria are used for refractive surgery screening and corneal ectatic disorder recognition.[15,16]

Finally, the small number of WFS patients recruited should be acknowledged as a limitation of the study. However, this disease is very rare, and the 12 patients included in this study are one of the largest cohorts identified in a single center based on long-term national screening. Moreover, the lack of long-term follow-up, in particular in changes in CT, excludes the possibility of observing a gradual reduction in the thickness of the cornea in patients with keratoconus. Despite the apparent progression of corneal topographic indices in WFS patients, we did not perform any therapeutic procedures related to halting keratoconus progression because we are aware that the main reason for the visual acuity decrease is atrophy of the optic nerves as a natural course of this disease.

Our findings suggest that patients with WFS present a high prevalence of changes in the corneal morphology compatible with the diagnosis of subclinical or early keratoconus, and thus, a close corneal evaluation should be carried out in these patients. It seems necessary to conduct long-term prospective studies to better understand the findings from our report.

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