Characteristics of the cornea and ocular surface in patients with congenital aniridia

Introduction

Congenital aniridia is a rare, bilateral, genetic disorder with variable phenotypic expression. It results, although not exclusively in aniridia, from different mutations in the PAX6 gene, which is located in the short arm of chromosome 11 and controls ocular development of the corneal epithelium, iris, lens, ciliary body and retina (Prosser and van, V, 1998). Congenital aniridia exists mainly in two forms: (1) familial and (2) sporadic. The familial pattern can be autosomal dominant (85%) or inherited in an autosomal recessive manner (2%) in Gillespie's syndrome, which is associated with mental retardation and cerebellar ataxia. It is already known that the PAX6 gene is not involved in this syndrome (Glaser et al., 1994b). In the sporadic form, it can be found in WAGR syndrome (Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation) as a result of alterations in the PAX6 and WT1 genes of chromosome 11p or alone without extraocular disorders (Miller et al., 1964; Eden et al., 2008).

Review of Related Literature

Congenital aniridia is characterized by partial or total absence of the iris, although it is frequently seen as a hypoplastic iris stump when examined through gonioscopy (Nelson et al., 1984). See Figure 1. Other associated abnormalities include dry eye syndrome, meibomian gland dysfunction, corneal disease (aniridia-related keratopathy), glaucoma, lens disorders (cataracts, lens subluxation or luxation and microphakia), pendular nystagmus and strabismus (Nelson et al., 1984; Tseng and Li, 1996; Jastaneiah and Al-Rajhi, 2005; Rivas et al., 2003; Mackman et al., 1979; Nishida et al., 1995). The posterior segment can equally be affected by pathologies such as optic nerve hypoplasia, foveal hypoplasia, and/or retinal dysfunction (Nelson et al., 1984; McCulley et al., 2005). As a consequence, it results in poor visual potential.

The degree of severity of corneal involvement has been studied and linked to dysfunction in limbal stem cells, meibomian gland damage and abnormal production of tear film components, with cytological changes in the ocular surface. (Rivas et al., 2003; Jastaneiah and Al-Rajhi, 2005; Lopez-Garcia et al., 2006a; de la Paz et al., 2008)The instability of the ocular surface determines the onset of secondary dry eye and dysfunction of the corneal epithelial barrier, both leading to conjunctival and corneal metaplastic transformation.

Penetrating keratoplasty has been proven ineffective for the long-term treatment of this disorder because it does not address the stem cell deficiency that is the primary etiologic factor. In the past decade, much has been published about the success of limbal allografts to address the problem of limbal stem cell deficiency. In a

study published by the author and colleagues (de la Paz et al. 2008) on 24 patients who underwent procedures such as penetrating keratoplasty, limbal cadaveric allograft or HLA-matched living-related limbal allograft for advanced aniridia keratopathy, we were able to demonstrate that long-term visual prognosis was the same whether or not the patient underwent surgery for the corneal problem. Limbal transplant and penetrating keratoplasty had comparable results through several years of follow-up (mean follow-up time of 14.7 years) due to failure of transplanted allografts.

It is known that several factors like deficiency in tear production, poor quality of tears, lack of corneal sensitivity and presence of ocular inflammation are risk factors for graft failure. Perhaps the microenvironment of the ocular surface in a patient with congenital aniridia is abnormal and leads to the failure of the said surgical procedures. This has led us to study the characteristics of the cornea and ocular surface in patients with congenital aniridia, in the hope that someday we can find a treatment for aniridia related keratopathy, whether it be medical or surgical.

Hypothesis

Congenital aniridia patients develop aniridia related keratopathy due to anomalies in the ocular surface such as limbal stem cell deficiency and dry eye.

Study Objectives

To describe the characteristics of the cornea and ocular surface in patients with congenital aniridia using simple office diagnostic procedures.

To describe associated clinical ophthalmological findings with the degree of aniridia related keratopathy

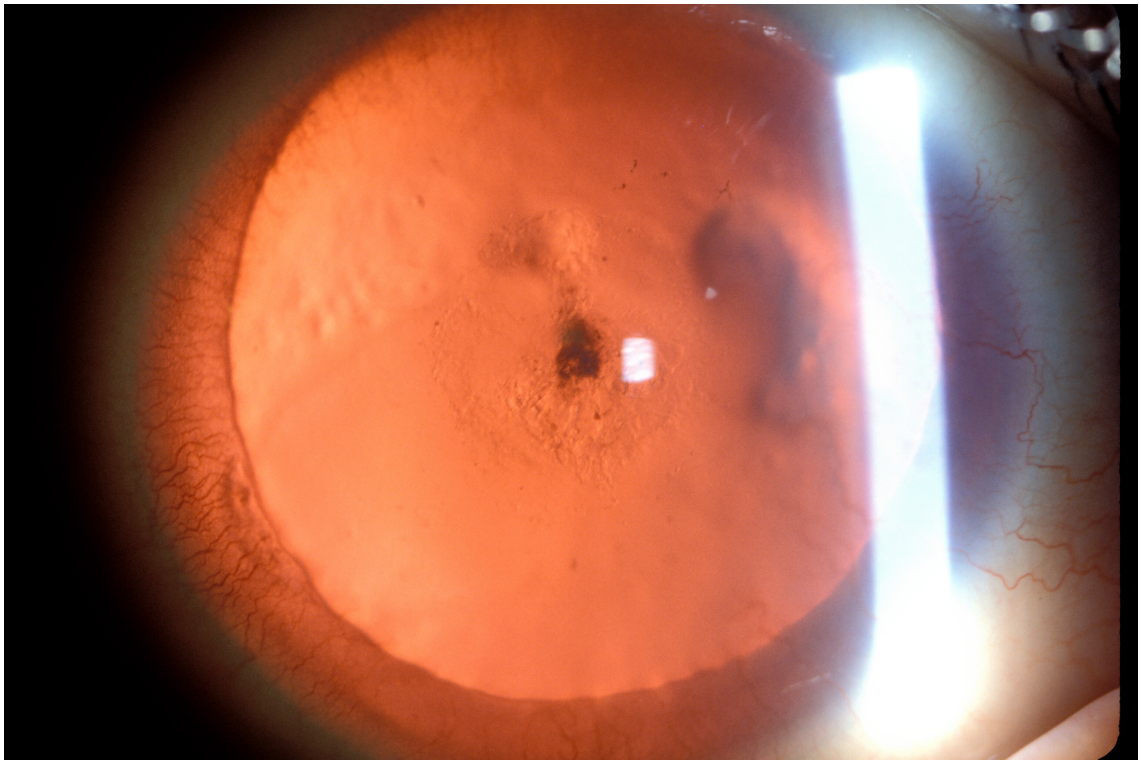


Figure 1. Congenital aniridia is the absence of iris as can be seen in this retroillumination photo showing the borders of the crystalline lens with complete absence of iris tissue in 360 degrees.

Patients and methods

Fifty eyes in 25 patients were assessed. Members of the Asociación Española de Aniridia were recruited to participate in this descriptive study. All patients/parents or guardian of minor patients expressed their consent to participate in the diagnostic study. The study included 6 male (24%) and 19 female (76%) patients, their ages ranging from 3 to 54 years and averaging 20.04 years (SD = ± 15.7). A total of 18 patients (72%) presented with a sporadic inheritance pattern, while the remaining 7 patients (28%) expressed a familial pattern. We found 7 patients (28%) with associated extraocular pathologies. For patients with a sporadic inheritance pattern, 2 (8%) had Wilms' tumour, 1 (4%) had Hashimoto's thyroiditis, and 1 (4%) had trigger thumb. For patients with a familial pattern, 1 (4%) had congenital angioma, and 1 (4%) had type 2 diabetes mellitus. A 3-year-old (4%) patient presented with cardiovascular and genitourinary abnormalities (gonadal dysgenesis), without Wilms' tumour, and demonstrated a sporadic inheritance pattern. Ten patients (40%) had a history of eye surgery.

All patients underwent complete clinical histories and ocular examinations, including measurement of visual acuity — with and without correction and using eye charts for visually impaired people, based on age and expressed as a LogMAR score (Schulze-Bonsel et al., 2006) — refraction (Figure 2) , slit-lamp biomicroscopy of the anterior segment (Figure 3), specular microscopy (Topcon non-contact specular microscope, SP-1000) (Figure 4) and corneal ultrasonic pachymetry (Pocket II Precision Pachymeter, Quantel Medical, Inc.) (Figure 5) to obtain central corneal thickness.

Due to time constraints in performing the cornea and ocular surface tests, some tests were performed in only one eye to avoid affecting the outcome of subsequent tests.

To evaluate the ocular surface, the following tests were performed: 1) Schirmer's test I (without anaesthesia) to measure basal and reflex tear secretion; 2) Schirmer's test II (with anaesthesia) to measure basal tear secretion, where a value of ≥10 mm/5 min for both tests was considered normal (Figure 6); 3) tear film break-up time (TFBUT) (Figure 7), where a value of ≥10 seconds was defined as normal; 4) corneal staining with 2% sodium fluorescein drop to assess epithelial defects (Figure 8) using the Oxford Scheme (DEWS, 2007a) (Figure 9); 5) conjunctival staining with 1% Rose Bengal paper strips (Figure 10) using the Van Bijsterveld Scheme (DEWS, 2007a; van Bijsterveld, 1969; Klaassen-Broekema and van Bijsterveld, 1992) (Figure 11); 6) tear ferning pattern test to assess the mucous layer (Rolando's classification grades I to IV (Norn, 1994; Rolando et al., 1988) (Figure 12 a and 12b); and 7) conjunctival and corneal impression cytology using Tseng's classification system (Tseng, 1985) (Figure 13).

Schirmer's test I, TFBUT and fluorescein (Oxford scheme) and Rose Bengal (van Bijsterveld scheme) staining were used to assess dry eye disease based on the severity levels defined in the Dry Eye Severity Grading Scheme of the Delphi Panel report (Behrens et al., 2006; DEWS, 2007b). This scheme consists of five levels, where 0 is normal, 1 is mild, and 4 is the most severe (Table 1). The cut-off values for Schirmer's test I and TFBUT were defined based on the Delphi Panel report. Corneal fluorescein staining has six grades (0–5; Oxford schema) and was multiplied by 0.8 for conversion into five grades (0–4; Delphi Panel report). Rose Bengal staining has 10 grades (0–9; Bijsterveld schema); they were multiplied by 0.44 to

convert them into 5 grades (0–4; Delphi Panel report). The four tests with 5 levels (0–4) were joined, and the mean of the four tests was calculated to obtain the dry eye score. We considered dry eye scores between 0 and 0.02 normal, 0.021 and 1 mild, 1.01 and 2 moderate, 2.01 and 3 severe, and 3.01 and 4 very severe.

Central corneal aesthesiometry (Cochet-Bonnet) was also performed in both eyes of 20 patients (40 eyes) (Figure 14). The test started with the aesthesiometry at the maximal length of 60 mm, reducing it gradually until a positive response (blinking) was obtained.

We performed impression cytology (IC) on 26 eyes of 16 patients using 5-x-5-mm nitrocellulose filter papers (Millipore Millicell-CM, 0.4 μm pore size). Samples were obtained from the central cornea and the superotemporal area of the bulbar conjunctiva. Histological examination was performed according to Tseng's staining protocol (Tseng, 1985), and microscopic assessment was performed by a single pathologist using Tseng's classification system (Tseng, 1985).

The main factors studied were as follows: the degree of aniridia-related keratopathy, age, history of eye surgery and quantitative and qualitative tests of the ocular surface and cornea, as discussed above.

Statistical analysis was performed using SPSS, version 13.0, software and the Kruskal-Wallis non-parametric test for correlation analyses. A p value <0.05 was considered statistically significant.

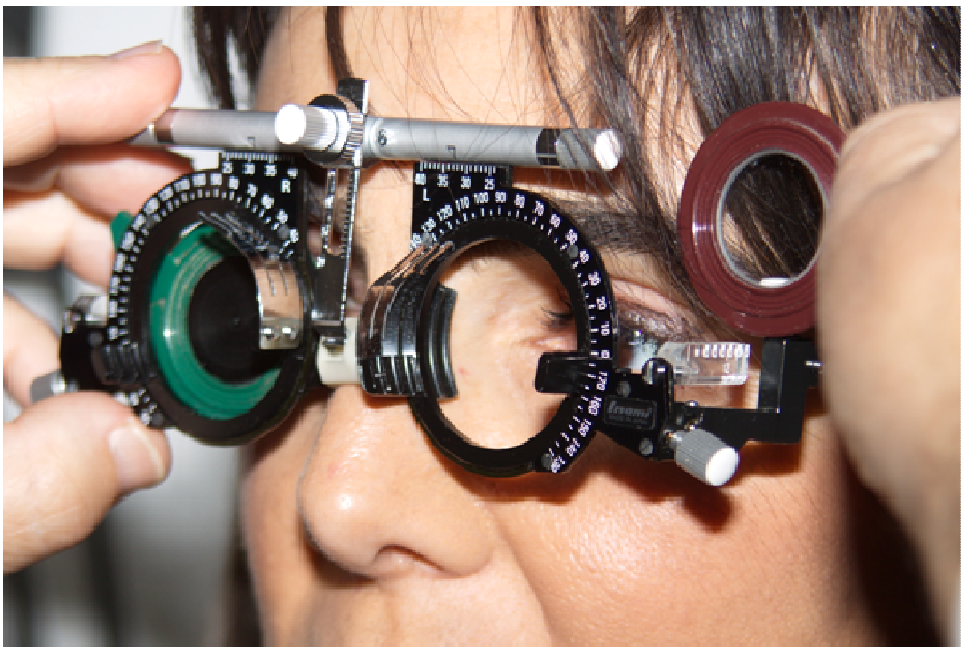


Figure 2. Visual acuity is measured with and without correction. Refraction is performed to find out the optical defects of the eye and to evaluate the best corrected visual acuity.

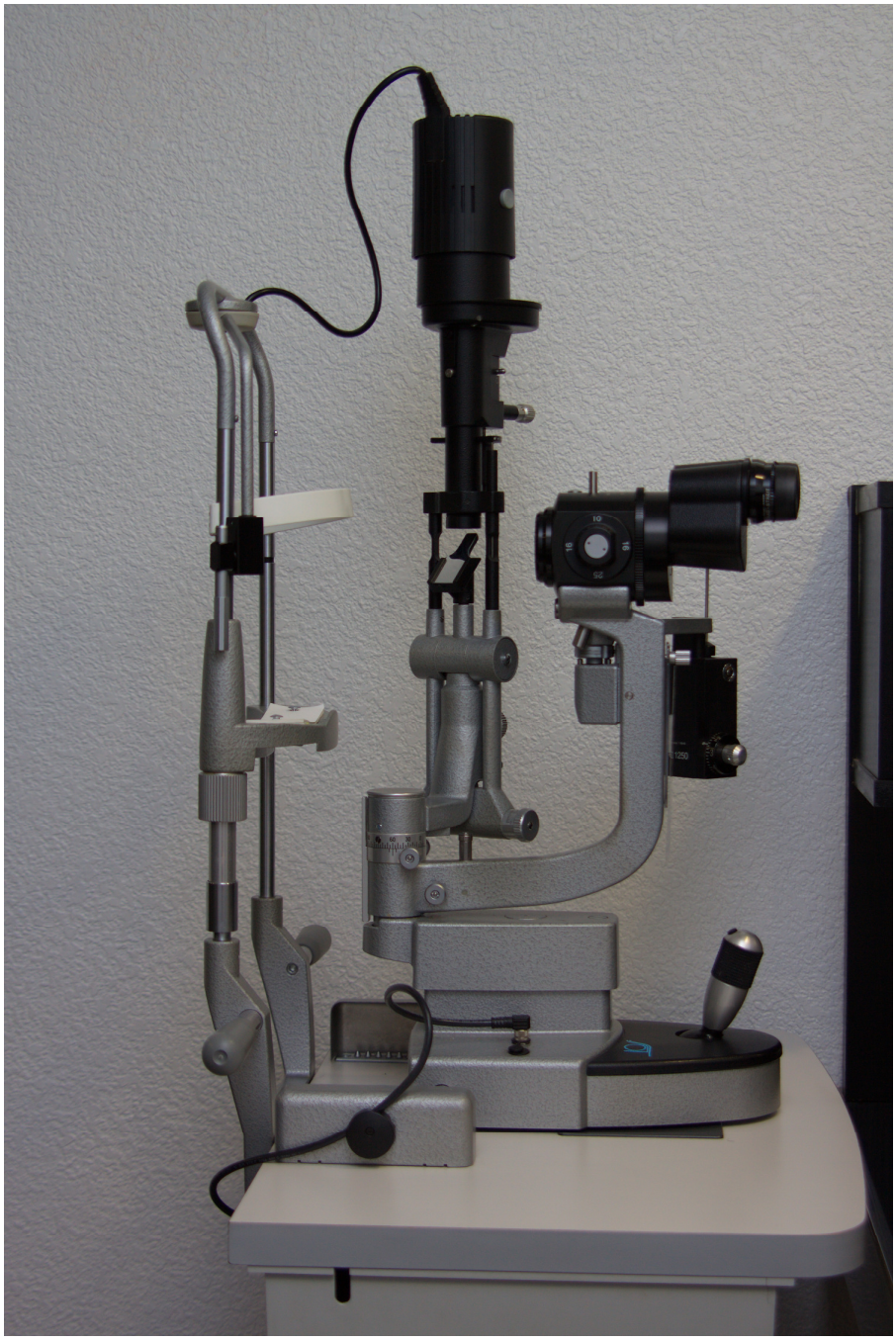


Figure 3. Slit lamp examination of the anterior segment allows us to assess the general status of the ocular surface which includes the upper and lower lid margins, the conjunctiva enveloping the globe in the anterior part and tear film and the cornea.

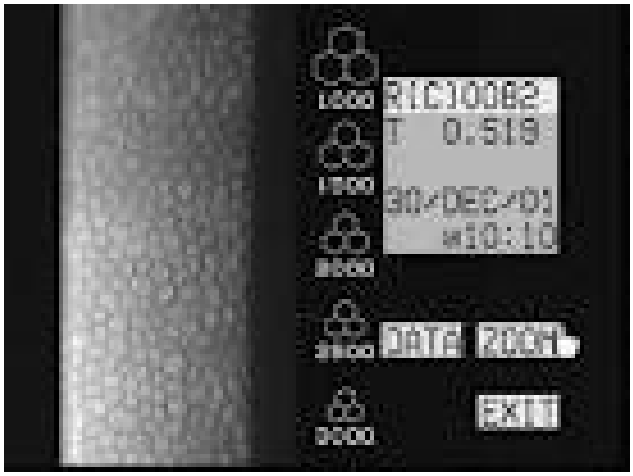


Figure 4. Corneal specular microscopy is a tool to image light reflected from an interface such as the endothelium-aqueous interface. In this study we analysed the endothelial cell count which is measured in cells/millimeter^2. Normal endothelial cell count is approximately 4,000 -5,000 cells/mm^2 at birth and declines to about 2,000-3,000 cells/mm^2 in late adulthood.

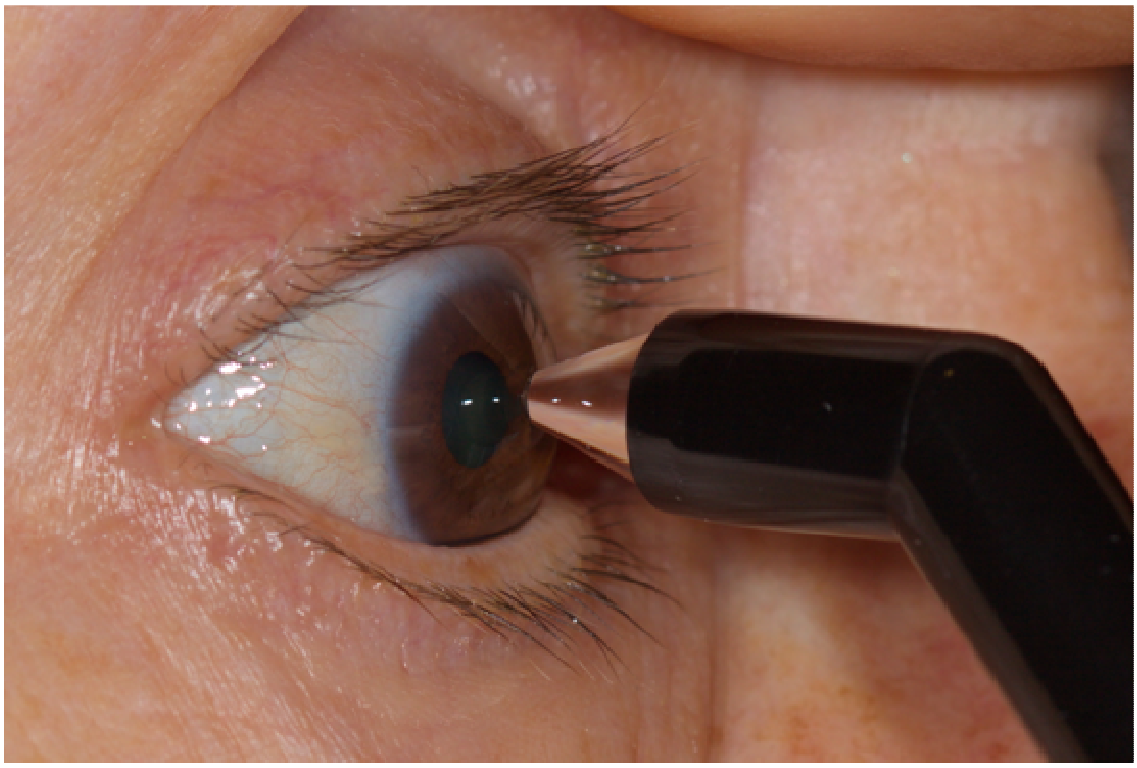


Figure 5. The measurement of corneal thickness is termed pachymetry. Ultrasound pachymetry as shown in the figure measures the corneal thickness from the tear film to the posterior face of the endothelium. The average normal corneal thickness is about 540 microns in the center.

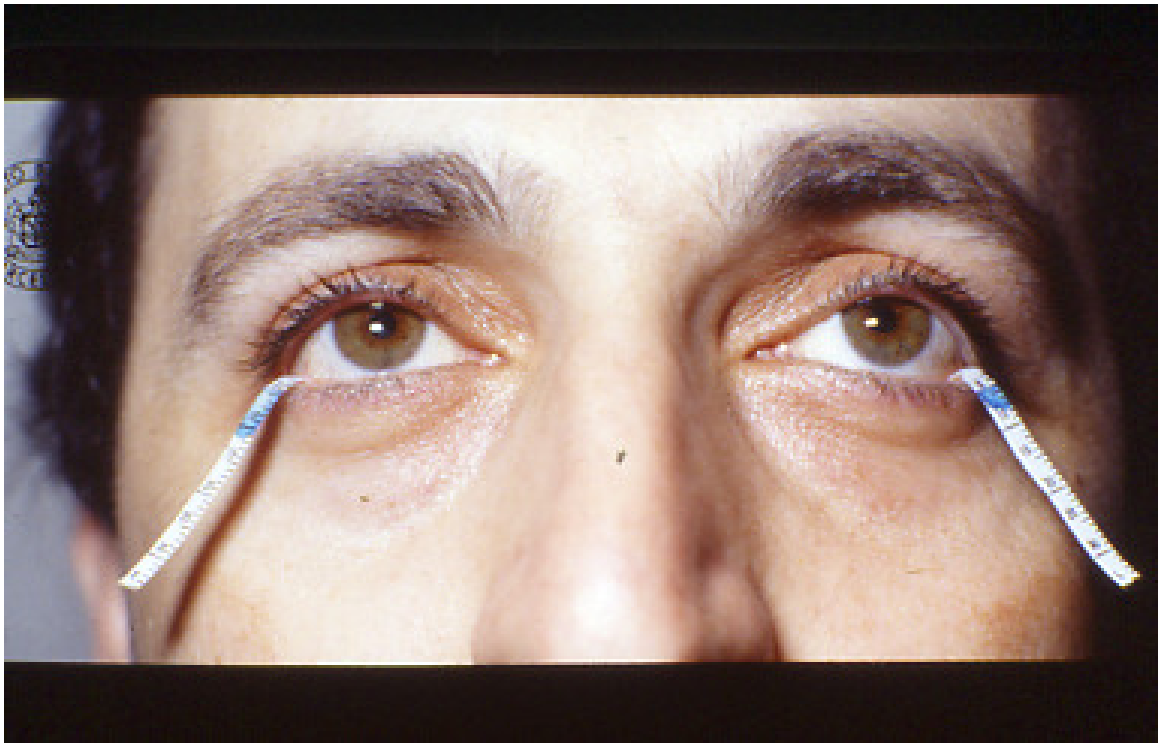


Figure 6. Schirmer´s Test evaluates the amount of aqueous tear production using ---- filter paper strips placed at the inferior temporal fornix. Schirmer´s test Type I (without anesthetic) measures the reflex tear secretion while Schirmer´s test Type II (with anesthetic) measures the basal tear secretion. The amount of tears produced after 5 minutes is seen at the marks in millimetres and is read as millimetres per minute. Normal value is > 10 millimeters in 5 minutes. Values lower are considered abnormal and signifies low aqueous tear production.

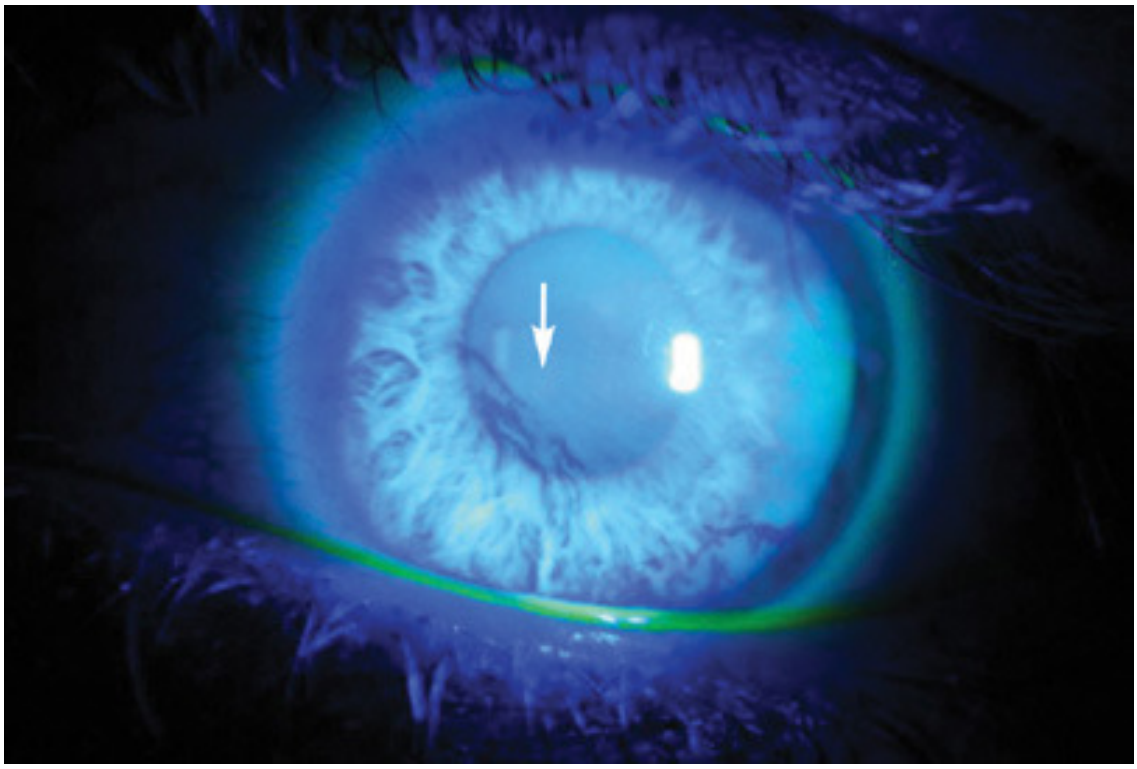


Figure 7. Tear Film Break-up time or TBUT evaluates the lipid layer of the tear film. Fluorescein strips are applied into the inferior fornix until it is wet by the patient´s tears. The patient is then asked to blink and then keep the eyes opened without blinking until a dark spot is noted in the tear film as showed by the arrow. The break up time is measured in seconds. Normal value is > 10 seconds. Values below 10 seconds mean early evaporation of tears due to insufficient lipid layer in the tear film.

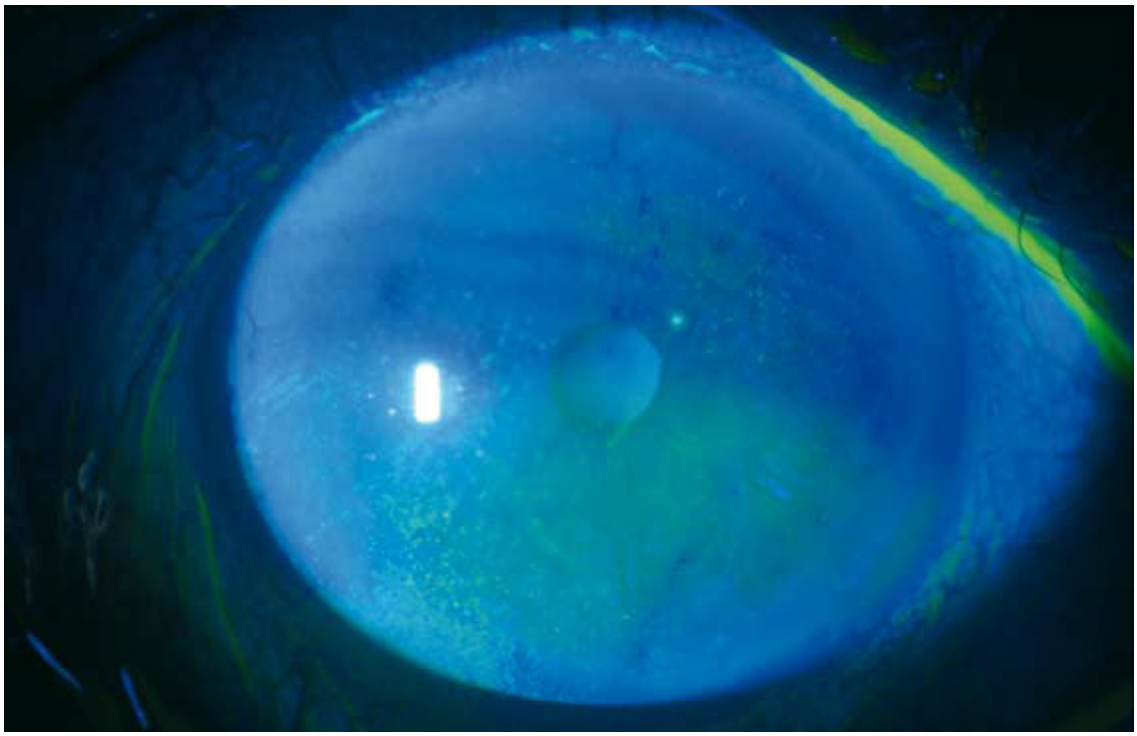


Figure 8. Fluorescein staining is evaluated after performing the tear film break-up time. Areas of epithelial breaks are noted as positive staining with fluorescein dye.

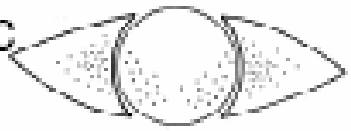
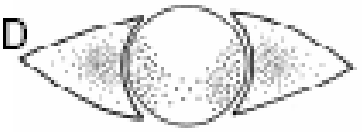
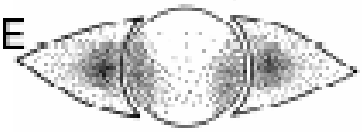
PANEL	GRADE	CRITERIA
A 	0	Equal to or less than panel A
B 	I	Equal to or less than panel B, greater than A
C 	II	Equal to or less than panel C, greater than B
D 	III	Equal to or less than panel D, greater than C
E 	IV	Equal to or less than panel E, greater than D
>E	V	Greater than panel E

Figure 9. The Oxford scheme is used to evaluate fluorescein staining of the ocular surface. The size of the area affected in the corneal and nasal and temporal conjunctival area, as well as the density of staining are evaluated. Grade 0 is considered normal while Grade 4-5 signify severe affectation of the ocular surface.

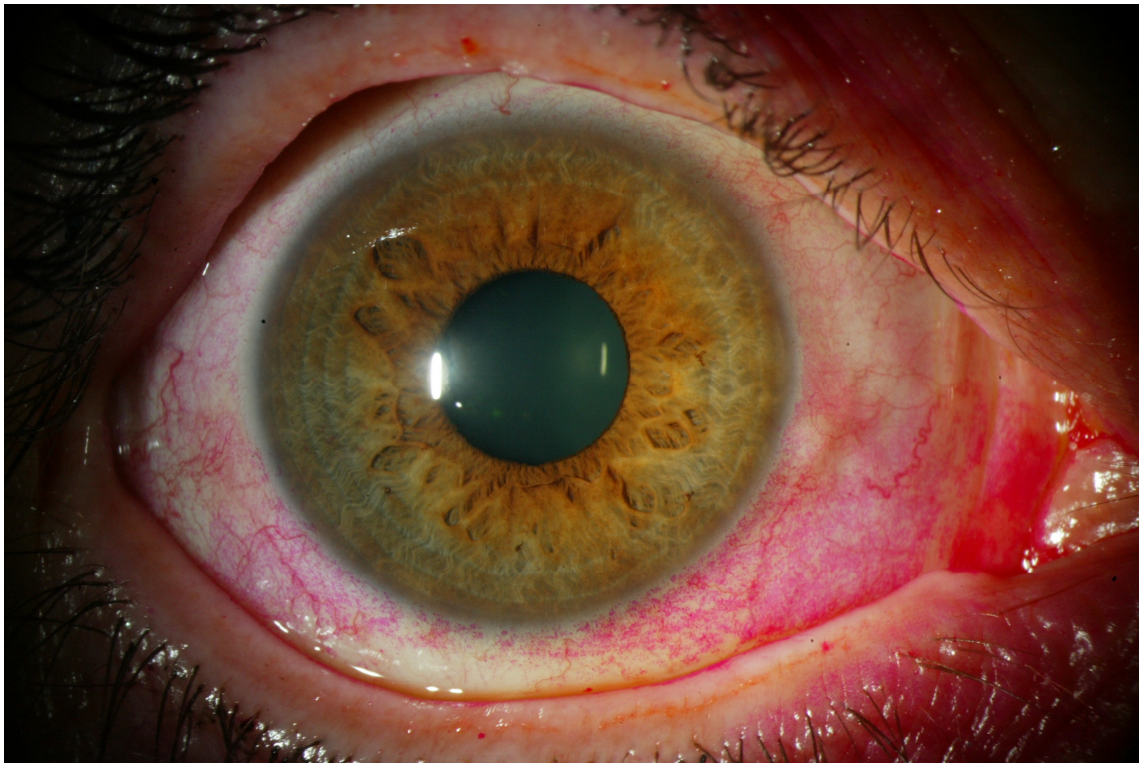


Figure 10. Rose Bengal staining evaluates the presence of devitalized cells in the ocular surface. It is a more sensitive test than fluorescein staining in predicting ocular surface affectation due to dry eye.

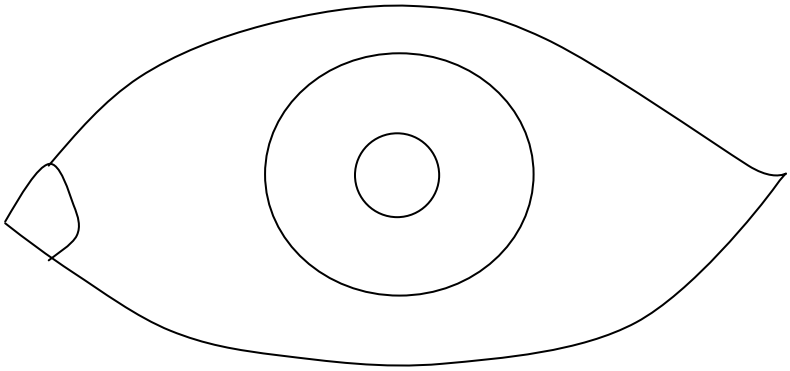
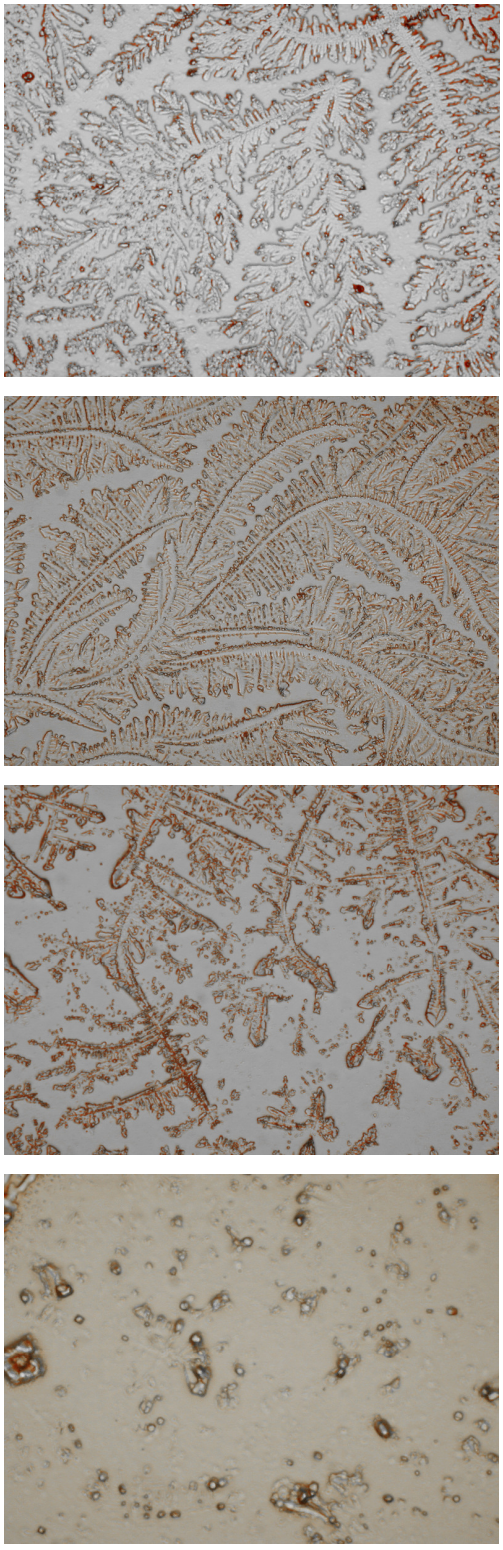
Nasal conjuntival Area	Corneal Area	Temporal conjunctival Area
A	**B**	**C**
		
Overall Score = A + B + C		
0 1 2 3 ☐ ☐ ☐ ☐	**0 1 2 3** ☐ ☐ ☐ ☐	**0 1 2 3** ☐ ☐ ☐ ☐
Scale of 0 – 3 to grade conjunctival staining per area 0 = no staining 1 = presence of some staining 2 = well-defined areas of staining 3 = total staining		

Figure 11. Van Bijsterveld Scheme of conjunctival staining using Rose Bengal. A higher overall score signifies worse affectation of ocular surface due to dry eye.

Figure 12 a. Tear Ferning Test evaluates the mucin layer of the tear film. About 2-3 microliters of tears are collected from the lateral canthal area using a capillary tube without anesthesia. The sample is smeared on a clean microscope slide and allowed to dry at room temperature. Ferning patterns were evaluated using Roland´s classification.



Type I consists of large, monogenous ferns and uniform, closely branching arborisation

Type II ferns are smaller and sparsely distributed

Type III has scarce arborisation with empty spaces

Type IV is complete absence of arborisation and clusters of mucus may be present.

Figure 12 b Ferning patterns based on Rolando´s classification

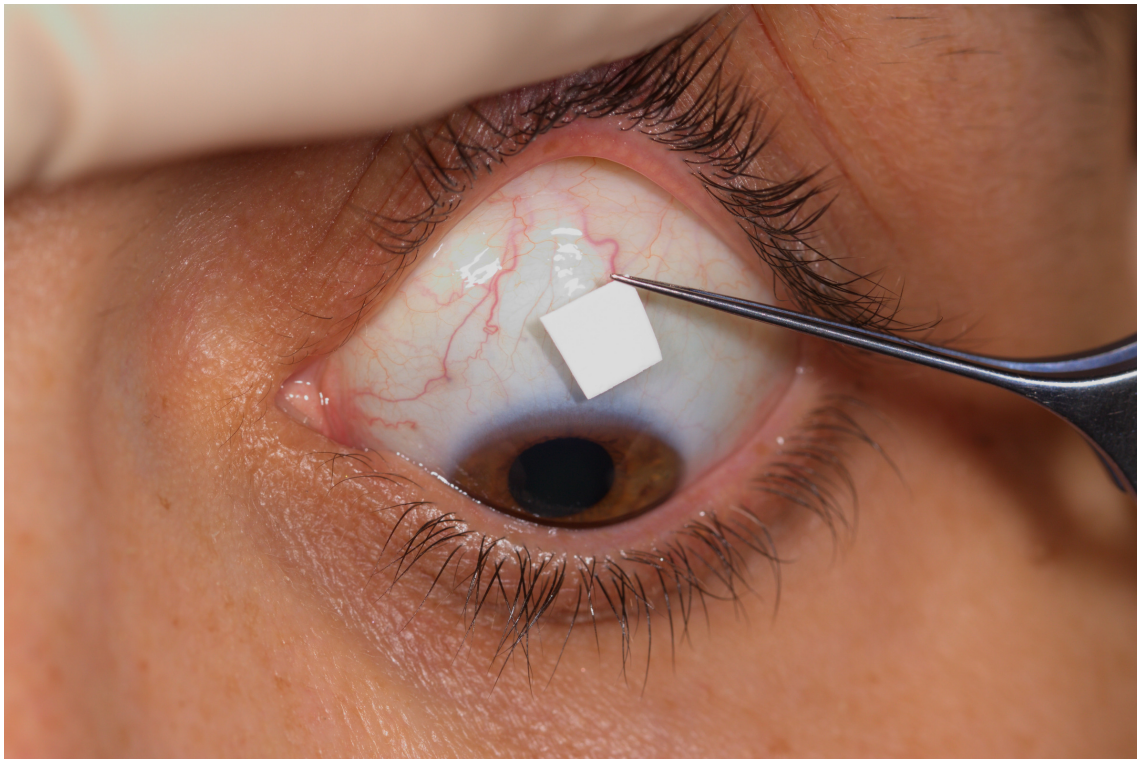


Figure 13. Impression cytology consists in applying a nitrocellulose filter paper on the area of interest. It may be performed on the cornea or the conjunctiva as can be seen on this image. Anesthetic drops are instilled prior to performing this test. The filter paper is applied on the conjunctiva and pressure is applied for 3-5 seconds and lifted with fine forceps. The filter paper is then immersed in a fixative and sent to the laboratory for a series of staining procedures prior to evaluation.

	0	1	2	3	4
Schirmer's test I (in mm/5 min)	Normal	Variable	≤10	≤5	≤2
TFBUT	Normal	Variable	≤10	≤5	Immediate
Fluorescein staining (Oxford)	Normal	None to mild	Variable	Marked central	Severe punctuate erosions
Rose Bengal staining (van Bijsterveld)	Normal	None to mild	Variable	Moderate to marked	Marked

TFBUT= Tear film break-up time

Table adapted from International Dry Eye Workshop (DEWS, 2007b)

Table1.

Dry eye score and the different parameters used to assess the cornea and ocular surface. The parameters chosen are based on the Delphi Panel report.

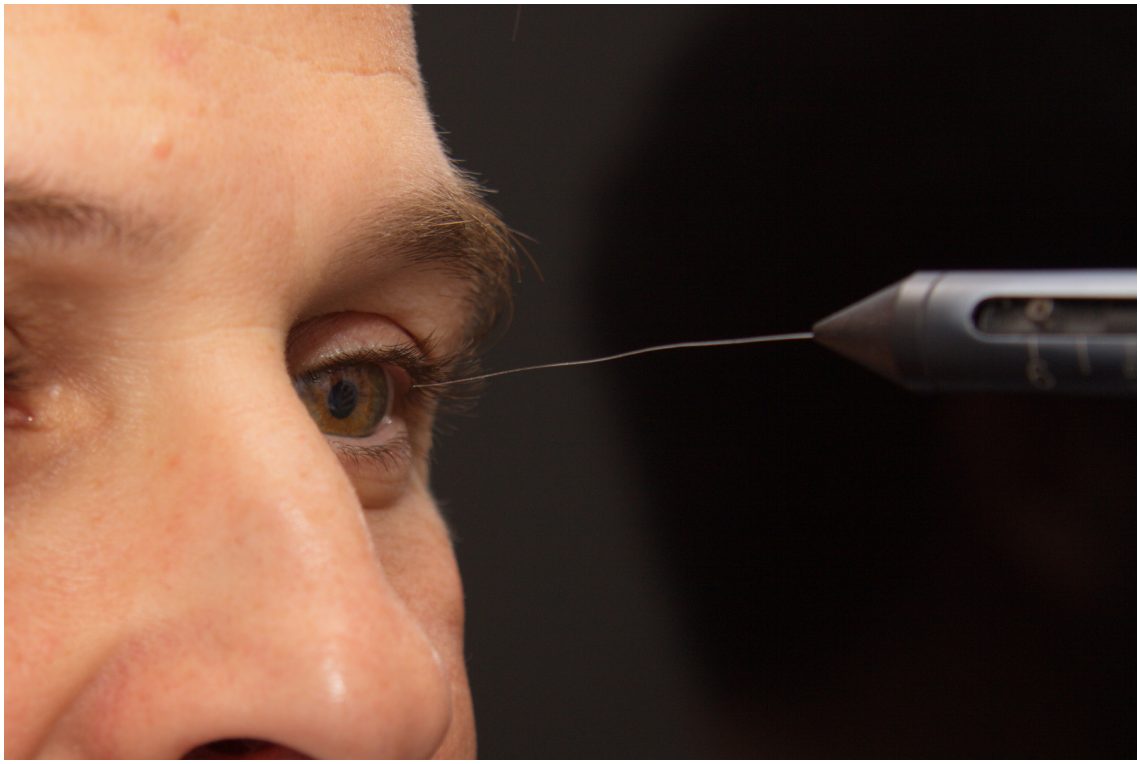


Figure 14. Cochet- Bonnet esthesiometry is at present the most common instrument to measure corneal sensitivity. The tip of a retractable nylon thread measuring about 60 mm is placed on the central part of the cornea. A blink signifies that the patient was able to feel the stimulus. The shorter the length of the nylon thread means lower sensitivity of the central cornea.

Results

Prevalence of ARK

The prevalence of aniridia-related keratopathy (ARK) according to Mackman´s classification was as follows: grade 0 in 12% (3 of 25 patients), grade 1-A in 52% (13 of 25 patients), grade 1-B in 20% (5 of 25 patients), and grade 2 in 16% (4 of 25 patients). Figure 15 shows the different stages of aniridia related keratopathy.

ARK and age

The mean ages of patients based on the degree of ARK were as follows: 12.66 (range: 4 to 22; SD: ± 8.06) for grade 0, 17 (range: 5 to 54; SD: ± 14.42) for grade IA, 22.8 (range: 3 to 47; SD: ± 20.57) for grade IB, and 39.5 (range: 33 to 46; SD: ± 5.58) for grade 2. We found a statistically significant correlation between age and aniridia-related keratopathy (Kruskal-Wallis test: p=0.000) (Figure 16).

ARK and history of ocular surgery

In correlating ARK with history of ocular surgery, we found that 88% (22 of 25) of the patients who had previously undergone any ocular surgery (penetrating keratoplasty, keratectomy, limbal transplant, trabeculectomy, phacoemulsification with intraocular lens implantation and strabismus surgery) presented with some degree of ARK. We found a statistically significant correlation between ocular surgery and the grade of aniridia-related keratopathy (Kruskal-Wallis test: p=0.014).

Schirmer's test I and II

Regarding tear production, we obtained the following results: Schirmer's test I (without anaesthesia) was normal in 33 of 38 eyes (86.8%), with a mean value of 23.03 mm in 5 min (range: 4–30; SD: ± 8.26). Schirmer's test II (with anaesthesia) was normal in 34 of 36

eyes (94.4%), with a mean value of 22.36 mm in 5 min (range 2–30; SD: ± 7.49) Figure 17 shows the frequency distribution in our study population).

TFBUT

When we evaluated the lipid layer of the tear film, TFBUT was reduced (<10 seconds) in 16.7% (4 of 24 eyes). Figure 18 shows the frequency distribution in our study population).

Vital staining

Corneal fluorescein staining (based on the Oxford scheme) was performed in the 48 eyes of 24 patients and was found to be abnormal in 26 of the 48 eyes. We found grade 0 in 22 eyes (45.8%), grade 1 in 17 eyes (35.4%), grade 2 in 5 eyes (10.4%), and grade 4 in 4 eyes (8.3%). None of the patients had grade 3 or grade 5 staining. Figure 19 shows the frequency distribution with respect to oxford scheme. Rose Bengal staining was performed in the 46 eyes of 23 patients and was abnormal in 21 (45.7%). We found grade 0 in 25 eyes (54.3%), grade 1 in 11 eyes (23.9%), grade 2 in 6 eyes (13%), grade 3 in 3 eyes (6.5%), and grade 5 in 1 eye (2.2%). No patients had a score between 6 and 9. Figure 20 shows the frequency distribution of the Van Bijsterveld scheme.

ARK and dry eye severity score

We graded dry eye syndrome by calculating the dry eye score as discussed above; we obtained the following results: 10 of 48 eyes (20.83%) had a dry eye score between 0 and 0.02 (normal), 28 of 48 eyes (58.3%) between 0.021 and 1 (mild), 8 of 48 eyes (16.7%) between 1.01 and 2 (moderate), and 2 of 48 eyes (4.17%) between 2.01 and 3 (severe). It was not possible to perform the ocular surface

tests in one patient due to young age (4 years old). Figure 21 shows the correlation beteweenfrequency distribution of the dry eye score in our study population. We found a statistically significant correlation between the ARK grade and dry eye score (Kruskal-Wallis test: p=0.026). See Figure 22.

ARK and aesthesiometry

The average results of central corneal aesthesiometry (Cochet-Bonnet) performed in the 40 eyes of 20 patients with respect to ARK were: grade 0 (median: 32.50 mm; range: 5–60); grade 1A (median: 50 mm; range: 5–60); grade 1B (median: 10 mm; range: 0–40); and grade 2 (median: 10 mm; range: 5–60). As a result, we found a statistically significant correlation between ARK grade and aesthesiometry (Kruskal-Wallis test; p=0.043). Figure 23 shows the frequency distribution of esthesiometry in our study population.

ARK and corneal thickness

Central corneal thickness was greater than the normal average thickness in all cases (36 eyes of 18 patients), with the following results with respect to ARK grade: grade 0 (median: 627.50 microns; range: 611–644); grade 1A (median: 639.50 microns; range: 602–697); grade 1B (median: 638 microns; range: 590–749), and grade 2 (median: 672 microns; range: 587–1120). However, we did not find a statistically significant correlation between ARK grade and central corneal thickness (Kruskal-Wallis test; p=0.412) Figure 24 shows the frequency distribution of corneal pachymetry in our study population .

Tear ferning pattern

The tear ferning patterns were as follows: grade I in 20% (4 of 20 eyes), grade II in 50% (10 of 20 eyes), grade III in 20% (4 of 20

eyes), and grade IV in 10% (2 of 20 eyes) of patients. This means that 80% of patients had abnormal tear ferning patterns.

We did not find a significant correlation between the number of conjunctival goblet cells and tear ferning pattern test results (Kruskal-Wallis test; p=0.227) or between ARK and ferning pattern grades (kruskal-Wallis test; p=0.707). Figure 25 shows the frequency distribution of the ferning patterns in our study population.

Impression cytology

Conjunctival impression cytology was performed in 26 eyes of 16 patients. Based on Tseng's grading system, 6 eyes (23.1%) had grade 0; 2 eyes (7.7%) had grade 1; 5 eyes (19.2%) had grade 2; 10 eyes had grade 3 (38.5%); and 3 eyes (11.5%) had grade 4 of conjunctival metaplasia. We did not find a significant correlation between the degree of conjunctival metaplasia and aniridia-related keratopathy (Kruskal-Wallis test; p=0.244).

We obtained adequate corneal samples from only 21 of 26 eyes (80.77%) because of advanced corneal degenerative changes, such as nodules in the 5 remaining eyes. We evaluated the presence of goblet cells on the corneal surface in order to diagnose limbal stem cell deficiency presenting clinically as conjunctivalization. Goblet cells were present in 3 of 21 corneas studied (14.3%). We found a weakly positive significant correlation between the presence of goblet cells on the cornea and aniridia-related keratopathy (Kruskal-Wallis test; p= 0.052).

In 4 conjunctival samples, there were fewer than 50 conjunctival goblet cells per mm^2. In this group, 2 eyes (50%) had mild keratinization, and 1 eye (25%) had moderate keratinization. One eye (25%) had no conjunctival keratinization. We did not find a

statistically significant correlation between conjunctival keratinization and ARK grade (Kruskal-Wallis test; p=0.279).

ARK and endothelial cell count

Although technically difficult because of nystagmus, we were able to perform specular microscopy in 13 eyes of 9 patients. Mean endothelial cell density was 2190.84 ± 512.13 cells/mm^2 (range: 1332–2850 cells/mm^2).

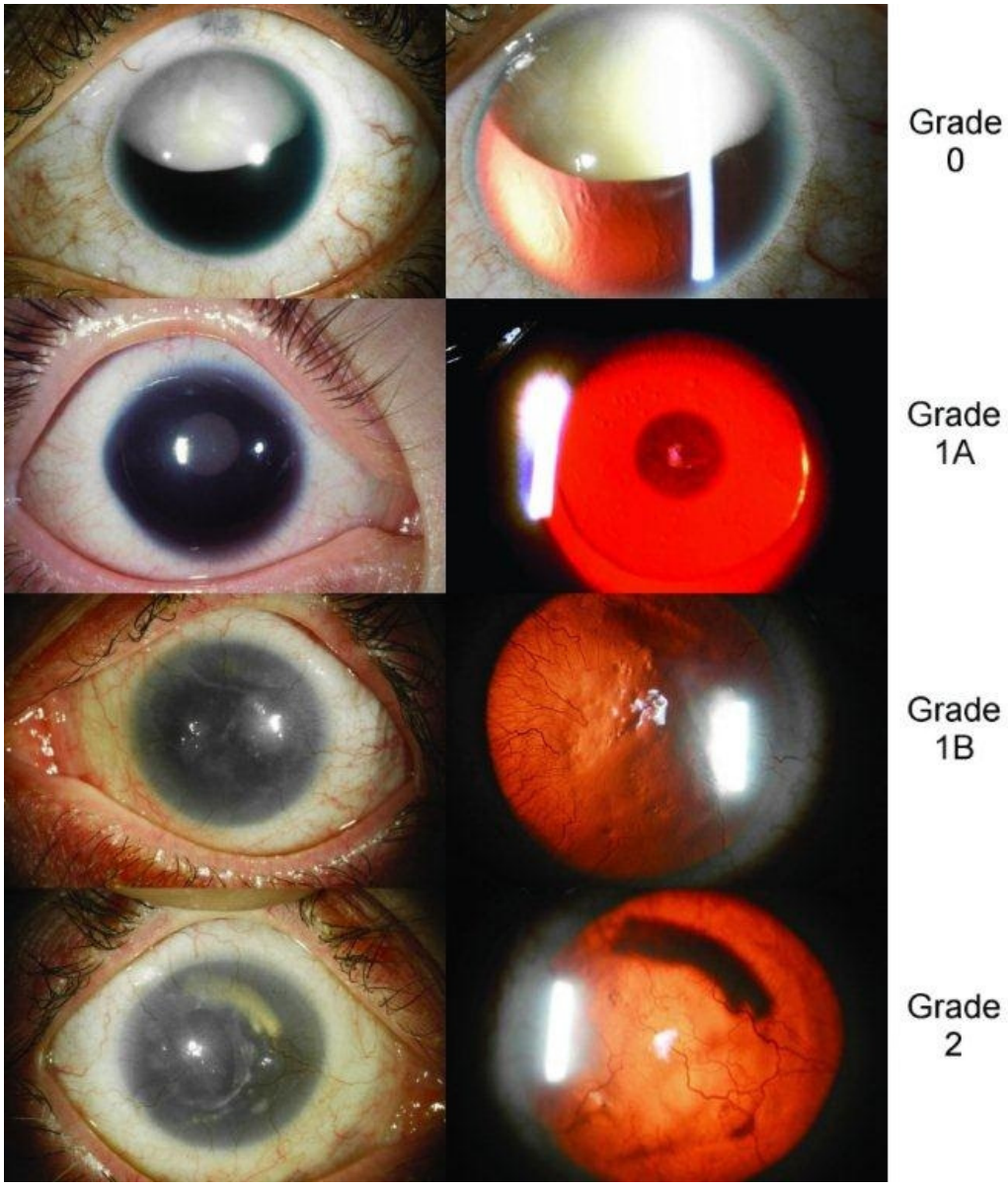


Figure 15. Aniridia-related keratopathy at different stages shown on direct (left column) and retro illumination (right column).

Grade o – peripheral and central cornea not affected

Grade 1A – partial affectation of limbus

Grade 1B – near total affectation of the limbus without central opacification

Grade 2 – 360 degree affectation of the limbus with central corneal opacification

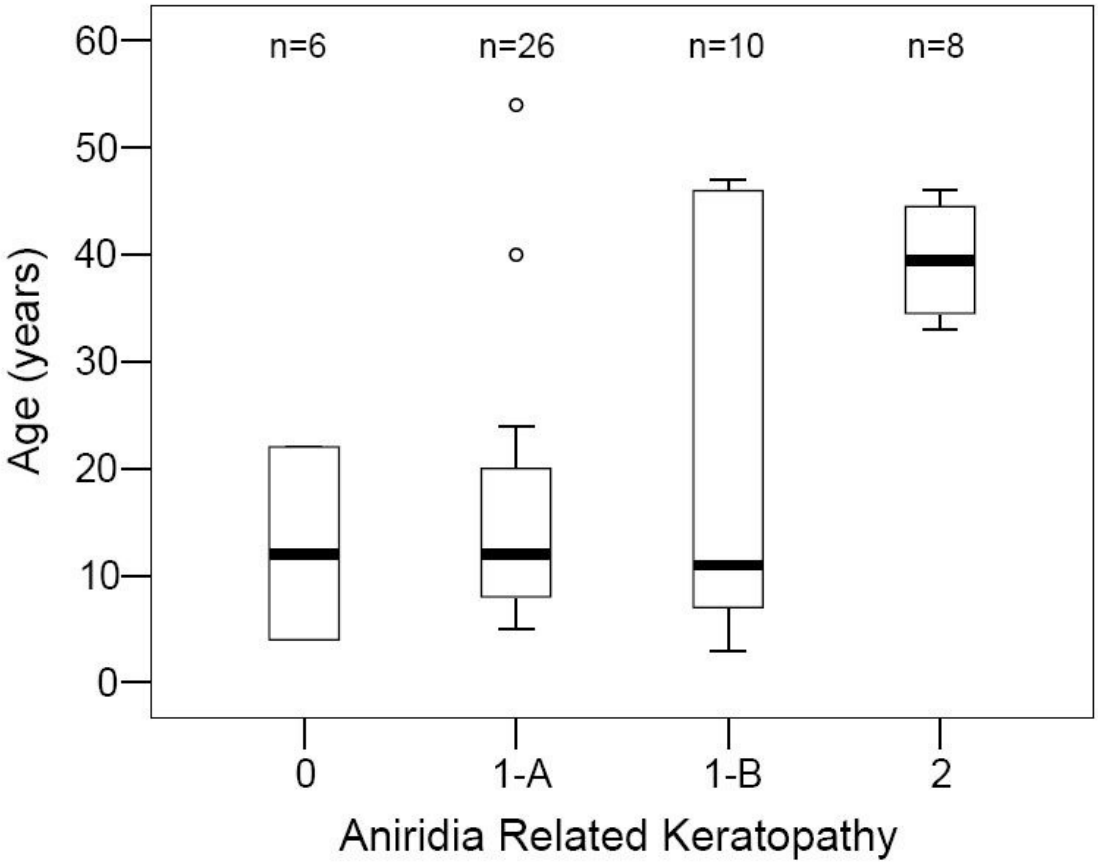


Figure 16. Prevalence of age (years) with respect to the different degrees of aniridia-related keratopathy (ARK) using Mackman's classification: grades 0, 1A, 1B and 2.

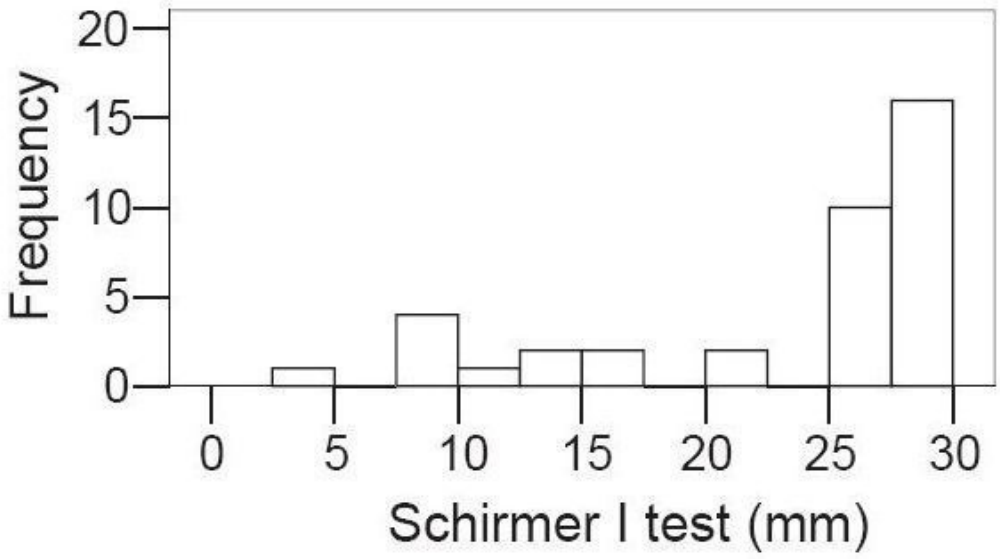


Figure 17. Frequency distribution of Shirmer´s Test I. Normal value > 10 mm in 5 minutes. Our patients in general had normal values.

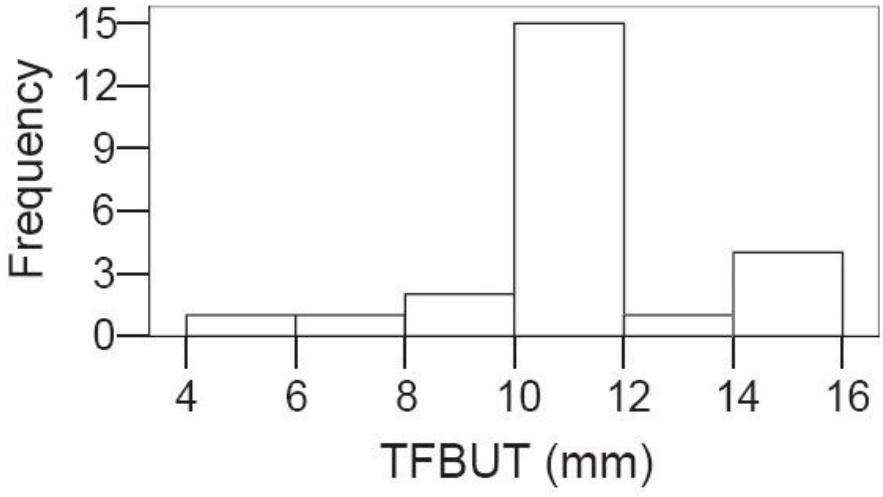


Figure 18. Frequency distribution of TFBUT results. Normal value > 10 minutes. The majority of patients had normal values.

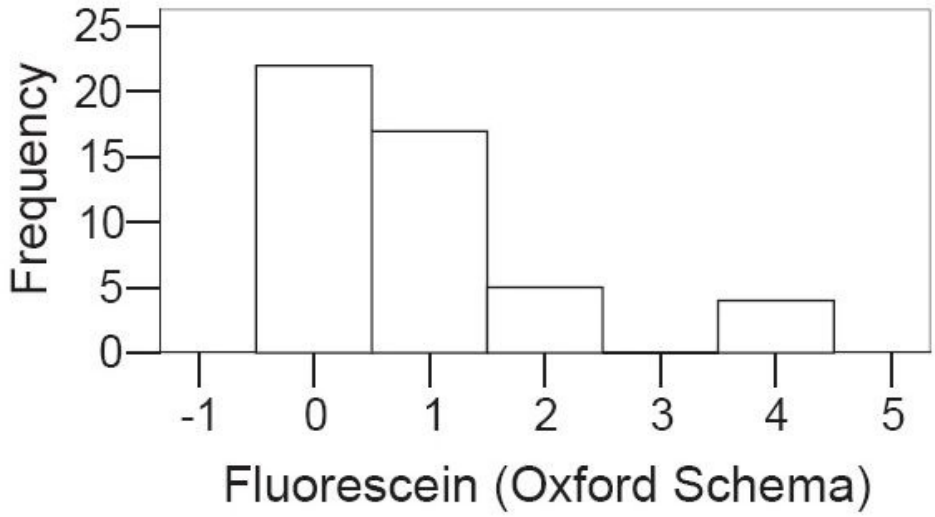


Figure 19. Frequency distribution of vital staining with Fluorescein using Oxford´s scheme. Values > 0 are considered pathologic. More than half of patients (53.2%) had positive staining with fluorescein.

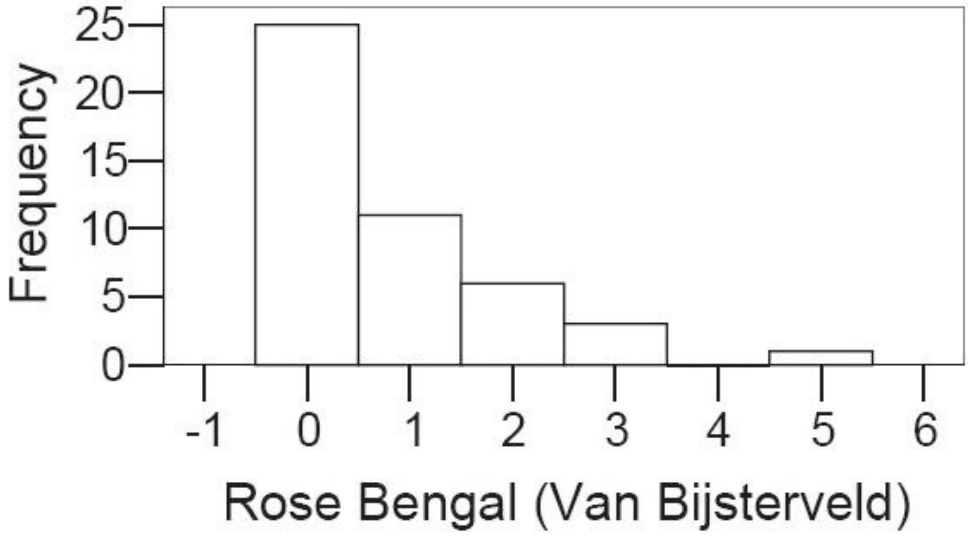


Figure 20. Frequency distribution of vital staining with Rose Bengal. Values> 0 are pathologic. Almost half of the patients (45.7%)had positive staining.

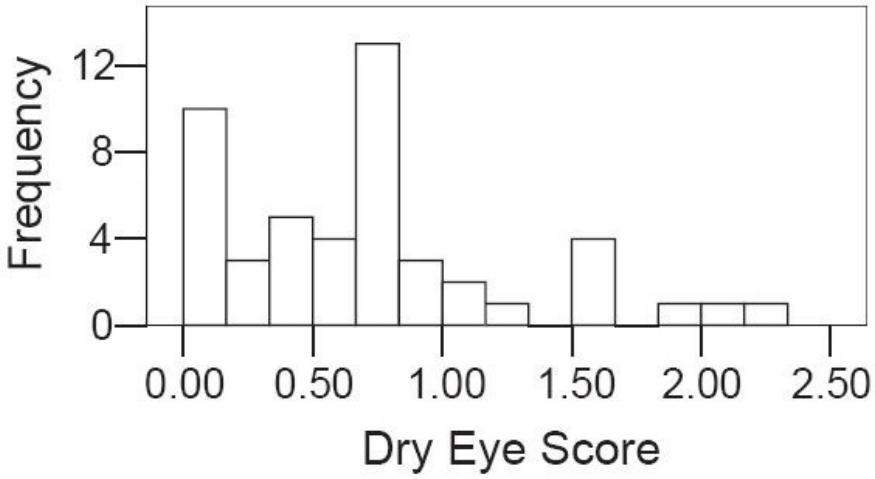


Figure 21. Frequency distribution of dry eye score. 79% of patients had dry eye based on our scoring, most of whom had mild dry eye and a few with moderate to severe dry eye.

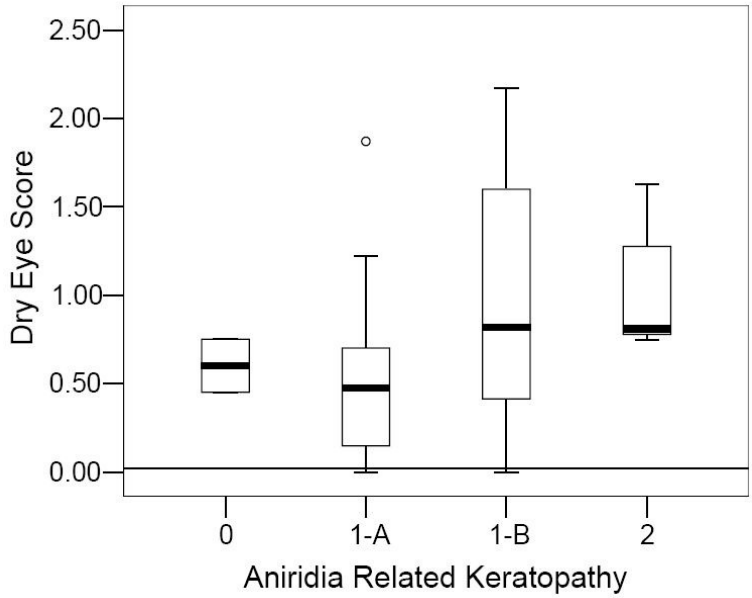


Figure 22. Positive correlation between grading of aniridia related keratopathy and dry eye score was significant. This means that the worse the keratopathy, the worse the dry eye score.

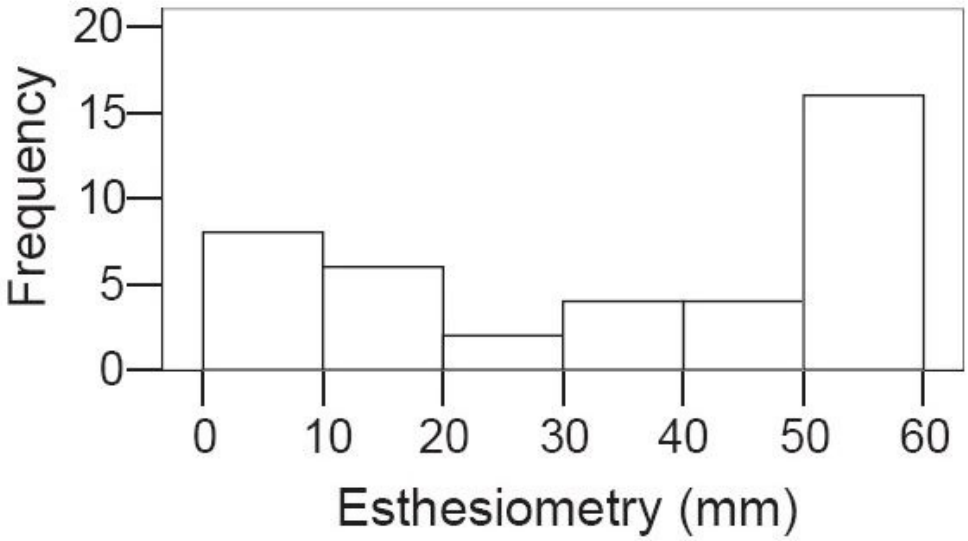


Figure 23. Frequency distribution of esthesiometry results. Normal value is 60 mm. Majority of patients had abnormal values.

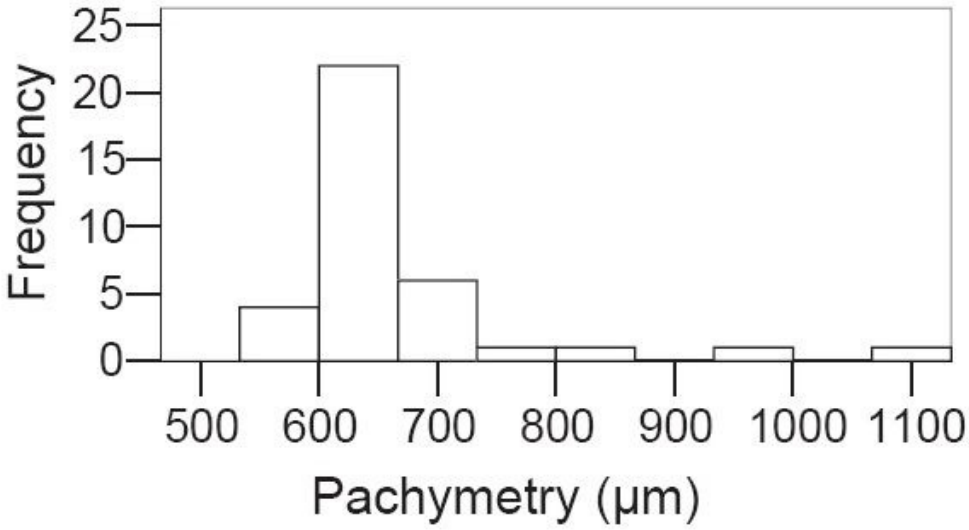


Figure 24. Frequency distribution of ultrasonic pachymetry results. Normal corneal thickness is between 540 to 560 microns. Majority of patients had thick corneas.

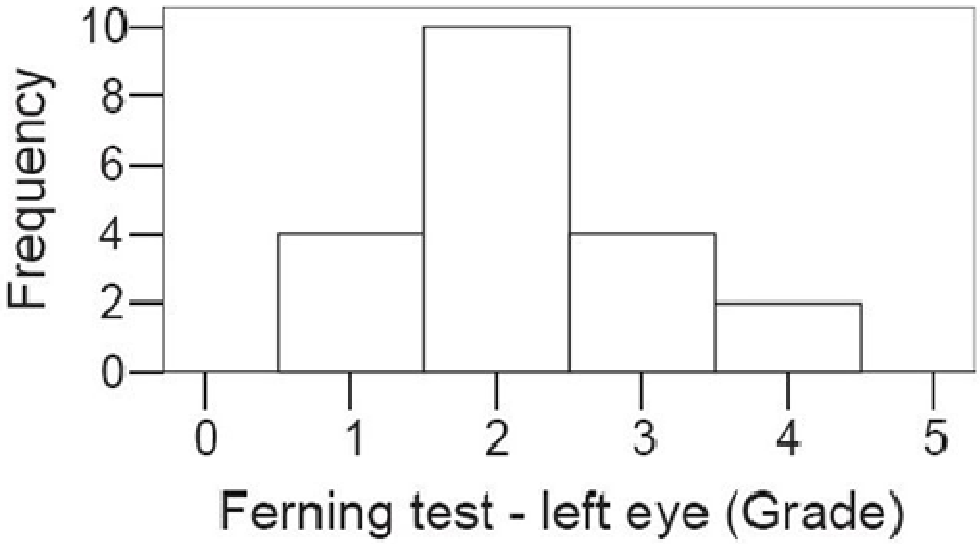


Figure 25. Frequency distribution of tear ferning test. Normal pattern is Grade 1. Majority of patients had abnormal ferning paatern.

Discussion

The PAX6 gene is expressed in the ectoderm during embryogenesis and plays an important role in organizing the development and differentiation of ocular structures, including the cornea, lens, ciliary body, and retina; it is also important in central nervous system development (Walther and Gruss, 1991). It is located in the p13 region of chromosome 11, and a mutation here causes the appearance of aniridia (Nishina et al., 1999; Walther and Gruss, 1991; Ton et al., 1991). A number of studies found no relationship between various mutations and clinical features; in some cases, patients with the same mutation present with different clinical features and degrees of illness (Nishina et al., 1999; Glaser et al., 1994a).

Studies of the characteristics of the ocular and corneal surface in congenital aniridia patients have revealed a pathophysiological mechanism set in motion by dysfunction in limbal stem cells (Dua et al., 2000). The studies demonstrated reduction in desmoglein as well as the beta- and alpha-catenin adhesion molecules, leading to epithelial fragility and subsequent, persistent epithelial erosion and defects (Nelson et al., 1984; Mohan et al., 2002a; Davis et al., 2003). The regulation of matrix metalloproteinase gelatinase B (gelB or MMP-9) depends on the PAX6 gene, and defects in PAX6 lead to fibrin accumulation and infiltration by inflammatory cells; this, in turn, generates stromal opacities and stimulation of neovascular proliferation (Mohan et al., 2002a; Collinson et al., 2004).

Prevalence of ARK

In our study, we found that majority of patients with congenital aniridia had some degree of ARK (Mackman's classification), as evidenced by clinical signs such as peripheral thickening and vascularization of the cornea. This high prevalence is consistent with the findings of other authors (Jastaneiah and Al-Rajhi, 2005; Nishida et al., 1995). In contrast, other studies reveal a lower 20% prevalence of ARK, usually associated with dysfunction in limbal stem cells (Tseng and Li, 1996; Tseng et al., 1998).

ARK and age

We observed that age positively correlates with the degree of ARK. Other studies also emphasize that the severity of ARK increases with age. In a retrospective study of 124 patients with aniridia, the authors found a positive correlation between the stage of keratopathy and age ($p<0.001$), even when using a different classification system for aniridic keratopathy based on 5 stages (0 to 5) (Eden et al., 2010). In another retrospective study in 20 patients (36 aniridic eyes), it was observed that the older the patient, the higher the grade of ARK (Mackman´s classification), with a significant statistical correlation ($p=0.002$) (Jastaneiah and Al-Rajhi, 2005). This correlation is expected since limbal stem cell deficiency, the pathophysiological cause of aniridia-related keratopathy, is a progressive disease.

ARK and history of ocular surgery

Our study suggests a strong relationship between patients with a history of ocular surgery and the degree of ARK ($p=0.014$). Several studies have demonstrated that, due to the extensive fragility of the corneal epithelium, any kind of trauma, such as eye surgery, can affect homeostatic balance (Nelson et al., 1984; Ramaesh et al., 2005; Eden et al., 2010). This can be evidenced through corneal

deterioration and the asymmetry of corneal disease, found in eyes that have undergone ocular surgery (Nelson et al., 1984). This must be taken into account when deciding when to perform surgery in these patients, since risks may outweigh benefits. The same authors found that corneal and ocular surface surgeries in aniridia patients, in the long term (more than five years after surgery), such as penetrating keratoplasty and limbal transplant, have the same visual prognosis as in patients who did not undergo the same surgery. This is explained by the severe fibrotic reaction observed in these cases, in which graft failure, both corneal and limbal, is observed much earlier despite adequate immunosuppression (de la Paz et al., 2008). We therefore suggest a less aggressive approach when deciding the surgical treatment of aniridia-related keratopathy.

Schirmer's test I and II

In assessing the corneal and ocular surfaces, we found that Schirmer's test I and II were normal in almost 90% of the eyes studied, respectively. Several studies also report normal Schirmer's test results in patients with congenital aniridia (Murube and Rivas, 2003; Jastaneiah and Al-Rajhi, 2005; Rivas et al., 2003; Eden et al., 2010), suggesting that these patients do not have aqueous deficiency as the major component of dry eye disease.

TFBUT

We obtained normal results for TFBUT in the majority of our study population. Our results are similar to those reported by Eden et al., where TFBUT was altered in 41% of cases, with no significant correlation between TFBUT and keratopathy (right eye: p=0.186/left eye: p=0.308) (Eden et al., 2010). However, other studies found altered TFBUT (<10 seconds) in most patients, with values ranging from 72% to 80.6% (Jastaneiah and Al-Rajhi, 2005; Rivas et al., 2003). TFBUT testing is an observer-dependent test and may vary

depending on the concentration of fluorescein drops, ambient lighting and atmospheric conditions. Perhaps a better way to assess the lipid layer of the tear film is using the tear film interferometer (Tearscope®, Keeler Inc., USA), since it avoids the use of fluorescein drops. Another parameter to study in this respect is meibomian gland function, which takes into account the ease of expression, quality and level of meibomian gland secretion.

Vital staining

We evaluated corneal staining using fluorescein dye (Oxford scheme) to promptly detect the presence of corneal changes, even in their earlier stages (1A and 1B) (Mackman et al., 1979). In our study, corneal fluorescein staining was abnormal in more than half of the eyes studied according to the Oxford scheme (DEWS, 2007a). Conjunctival staining with Rose Bengal (van Bijsterveld schema) was also performed to stain areas with poor protection of surface epithelium (DEWS, 2007a; van Bijsterveld, 1969). In our study, results were abnormal in almost half of the studied eyes. In a retrospective study, Jastaneiah et al. found that fluorescein and Rose Bengal staining were present in 31 of 34 aniridic eyes with 1B and 2 corneal disease (Jastaneiah and Al-Rajhi, 2005). Rivas et al. also found altered Rose Bengal staining in 30 patients (83%) with congenital aniridia (Rivas et al., 2003). In our study, 45.7% of patients demonstrated positive Rose Bengal staining, while Rivas found abnormal values in 83% of patients. This may be explained by the younger population included in our study, whose mean age was 20.04 (SD ± 15.7), whereas the mean age in the Rivas et al. study was 42 (SD ± 15.3). Here, we stress the importance of using Rose Bengal, or lissamine green as an alternative, since it detects the presence of devitalized tissue, which may be missed when using only fluorescein drops.

ARK and dry eye severity score

We evaluated the severity of dry eye using the dry eye score, Schirmer's test, TFBUT and vital staining. We found that the dry eye score increased as ARK worsened. This agrees with Jastaneiah et al. who also found a statistically significant correlation between severity of corneal involvement and the extent of dry eye disease (Jastaneiah and Al-Rajhi, 2005). This is expected, as the lower the quality and quantity of tears are, the greater the damage to the cornea and ocular surface. Therefore, treatment of dry eye must be based on these parameters in individual cases. If aqueous deficiency is present, abundant tear substitute must be prescribed. If evaporative dry eye is present, lipid-containing tear substitute and dietary supplements containing essential fatty acids must be prescribed and meibomian gland dysfunction must be treated with lid hygiene.

ARK and aesthesiometry

Corneal sensitivity was assessed in using the Cochet-Bonnet aesthesiometer showing a significant correlation between ARK and aesthesiometry results. Our results agree with the Eden et al. study in which corneal sensitivity was tested using a thin cotton-tipped applicator in 63 of 221 aniridic eyes (29%). They found a significant positive correlation ($p=0.001$ for the right eye, and $p=0.005$ for the left eye) between keratopathy and sensitivity (Eden et al., 2010). The decrease in corneal sensitivity could be secondary to neurotrophic keratopathy and/or to the advanced stage of dry eye disease (Benitez del Castillo et al., 2007). Future studies are necessary to clarify these phenomena; corneal sensitivity is now being studied in the context of various types of stimuli (Henderson et al., 2005).(Belmonte et al.,2004). To our knowledge, this is the only study to date confirming Eden's results, offering a more objective and quantifiable method of measuring corneal sensitivity.

ARK and corneal thickness

Regarding central corneal thickness in congenital aniridia patients, we found median values higher than normal for all grades of ARK, which has been already described in other studies (Brandt et al., 2004; Whitson et al., 2005). This contrasts with findings in patients with aetiologically different dry eye syndrome that resulted in reduced corneal thickness (Sanchis-Gimeno et al., 2005; Liu and Pflugfelder, 1999). However, there was no statistically significant correlation between ARK and pachymetry ($p=0.412$), perhaps because of the small number of cases (36 eyes in 18 patients). A number of factors should however be considered. First, most patients with advanced keratopathy would have thicker corneas due to epithelial conjunctivalization. Second, PAX6 mutations cause altered arrangement of collagen fibres resulting from altered metalloproteinases (MMP) that lead to stromal opacities. Since the stroma is the thickest layer of the cornea, this would contribute to increased corneal thickness (Mohan et al., 2002b). The authors are presently performing a parallel study of the histopathological characteristics of aniridia corneal buttons to determine which corneal layer contributes to increased thickness in these patients.

Tear ferning pattern test

We carried out the tear ferning pattern test to assess the mucous layer of the tear film and found that it was abnormal in 80% of our patients.

In previous reports, it was shown that many major sources of tear film mucin exist, even in cases of decreased goblet cell density, for example, lacrimal gland and non-goblet epithelial cells from the conjunctiva (Dohlman et al., 1976). As previously discussed in the ARK and dry eye score section, attention must be paid to the deficiency of the tear film layers in individual cases, and appropriate

treatment for the corresponding deficiency must be provided, whether involving aqueous, lipid or mucin deficiency. To our knowledge, this is the first study describing the use of the tear ferning pattern test in patients with congenital aniridia.

Impression cytology

When considering Tseng´s classification, we found that 73% had conjunctival squamous metaplasia. Jastaneiah et al. found squamous metaplasia in all 23 aniridic specimens (100%), but all had corneal involvement seen clinically according to Mackman´s classification (2 eyes had stage 1A, 21 eyes had stage 1B, and 13 eyes had stage 2). Rivas et al. also found that none of the eyes with congenital aniridia had grade 0 or 1 squamous metaplasia (36 eyes of 18 patients), demonstrating higher degrees of squamous metaplasia in their study. However, Rivas did not include the clinical classification of corneal involvement. Our results demonstrating lower degrees of squamous metaplasia compared to the Jastaneiah and Rivas studies may be explained by our lower mean population age.

Regarding conjunctival goblet cell density, we observed a decrease in the number of goblet cells as squamous metaplasia increased (p=0.000). In our study, we found a decrease in goblet cell density in more than half of the cases which were at stages 0 and 1A according to Mackman's classification. This is in accordance with Rivas et al. who also found significant decrease in conjunctival goblet cell density in 20 of 36 aniridic eyes. However, their study did not take into account the clinical features of aniridic keratopathy. On the other hand, Nishida et al. found a statistically significant increase in conjunctival goblet cell density in 16 aniridic eyes compared with the control group, and all of them had clinical corneal opacification and

vascularization in either the peripheral or entire cornea. Jastaneiah et al. also found statistically significant increased conjunctival goblet cell density with mucinous hyperplasia in 23 eyes. However, their study population demonstrated more advanced degrees of ARK, as most of them were at stages 1B and 2 according to Mackman's classification. Nishida et al. and Jastaneiah et al. may have found increased goblet cell density because their reports include more advanced ARK stages than our study.

Another point of discussion is the lower goblet cell density in the superior bulbar conjunctiva compared with the inferior bulbar area (Kessing et al.,1967). Nishida took samples from the inferior conjunctival epithelium, while Rivas obtained samples from the superior and inferior bulbar conjunctivas. In our patients, we obtained samples from the superotemporal bulbar conjunctiva. It follows that the anatomical site where the samples were taken from may be the cause of the difference in conjunctival goblet cell densities in several reports (Rivas et al., 2003), Lopez-Garcia et al., 2006a), (Lopez-Garcia et al., 2006b), Murube and Rivas, 2003).

In our study, we found 2 eyes with decreased conjunctival goblet cell density (less than 50 cells per mm^2), but with marked keratinization (one mild case and the other moderate). This confirms that conjunctival squamous metaplasia is a dynamic process and that the major histopathological processes (i.e. loss of goblet cells, cellular stratification and keratinization as discussed by Tseng, observed microscopically to grade metaplasia) may overlap and are not mutually exclusive (Tseng, 1985).

Corneal impression cytology demonstrated a weak but significant correlation between the presence of goblet cells on the cornea and ARK. If we consider that limbal stem-cell deficiency

contributes to ARK, as discussed above, the probability of finding conjunctival goblet cells on the cornea increases when assessing eyes with more advanced ARK (grades 1B and 2).

It is therefore important to carry out conjunctival and corneal impression cytology to detect changes early on, estimate the severity and progression of disease, assess treatment efficacy and prevent deterioration in these patients, given that metaplastic changes in response to chronic ocular surface damage take place even in the presence of moist epithelia.

ARK and endothelial cell count

In our study, the mean endothelial cell count was normal in all eyes where we were able to perform the test, measuring 2190.84 cells/mm^2 (SD ± 512.13). Our results are similar to those of Weiss et al., in whose study specular microscopy photographs in 3 eyes of 9 patients with congenital aniridia were obtained (Weiss et al., 1987). Peripheral endothelial cell density and morphology were normal; they found cornea guttata and atypical endothelial cells only in older patients and those who underwent previous ocular surgery. Another study reported endothelial cell counts of 3.397/mm^2 in the right eye and 3.434/mm^2 in the left eye of a 16-year-old patient with normal structure (Whitson et al., 2005). They suggest that endothelial dysfunction does not increase corneal thickness in the presence of a mutated PAX6 gene. Our study is the first to present the results of endothelial cell count in a significant number of patients with this rare congenital disorder. This is quite an unexpected finding since, embryologically, the endothelium is closely related to the iris, lens and angle structures, which are, in general, affected in patients with congenital aniridia and manifested as cataracts and glaucoma. As

previously discussed, the same authors are carrying out a parallel study of aniridic corneal buttons to correlate our clinical findings with histopathological evidence.

Conclusion

Congenital aniridia is a rare ocular disease affecting the cornea and ocular surface. In our study, age, history of ocular surgery and dry eye severity correlated positively with the progression of ARK. Corneal sensitivity was decreased, corneal pachymetry was increased, and corneal endothelial cell count was normal in the majority of cases. The tear ferning pattern test showed decreased mucin production, and impression cytology revealed that squamous metaplasia precedes ARK. Consequently, if the grade of ARK correlates with the severity of dry eye disease, it becomes very important to perform impression cytology for early detection of signs and symptoms of dry eye disease in an attempt to prevent the onset or the progression of ARK and delay visual deterioration in this group of patients.

ARK is a multifactorial disease. Simple and basic tests to evaluate the ocular surface must be performed to determine the proper treatment for each individual case.

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