BioMed Research International

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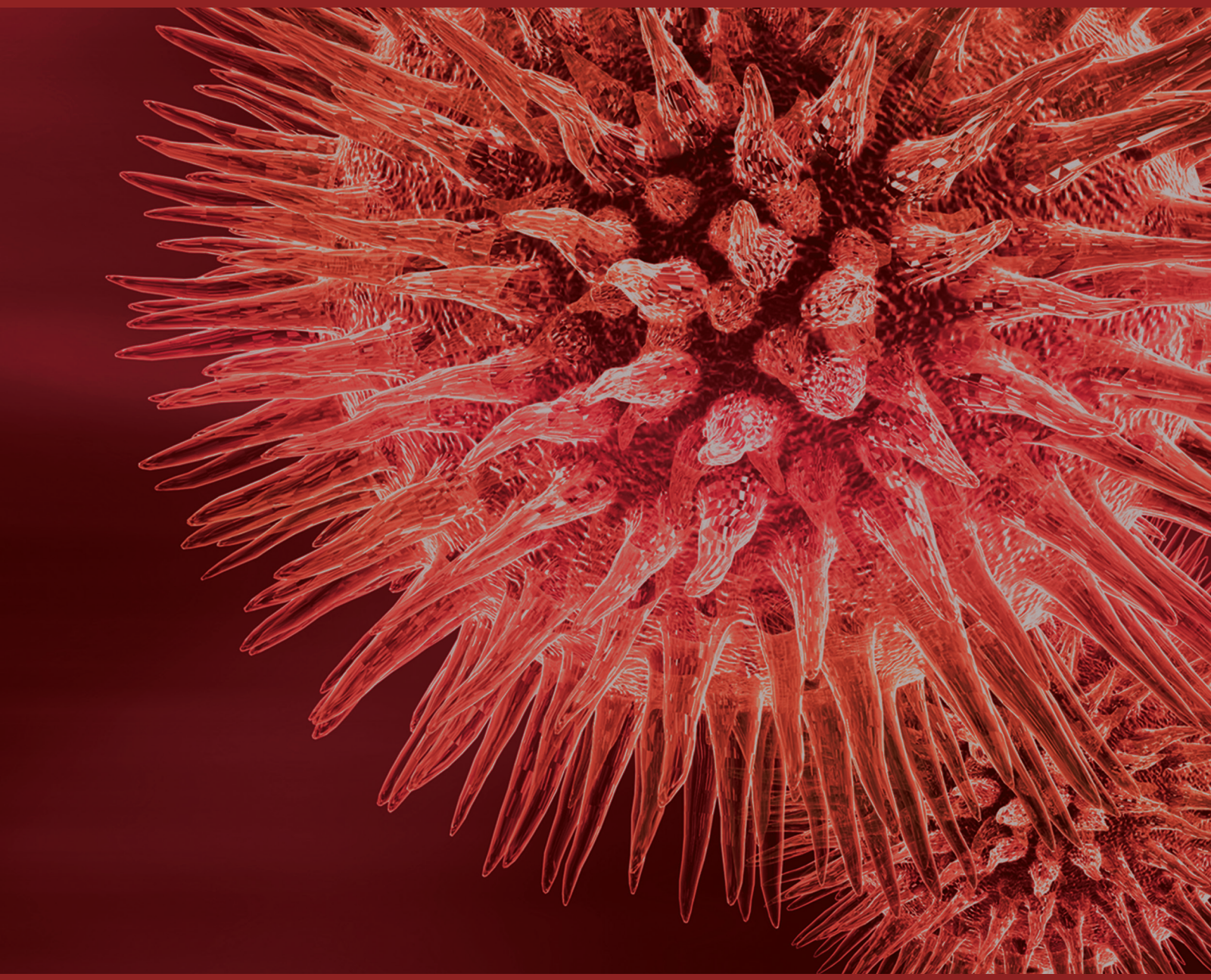
Guest Editors: Alessandro Lambiase, Flavio Mantelli, Marta Sacchetti, Siavash Rahimi, and Giacomina Massaro-Giordano



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Editorial

Rare Diseases of the Anterior Segment of the Eye: Update on Diagnosis and Management

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This special issue is focused on the current approaches used to identify and manage rare diseases of the anterior segment of the eye, which range from congenital to acquired disorders that are caused by ocular or systemic conditions and often have consequences that extend beyond the anterior segment of the eye. Specifically, the groups of Dr. R. Cascella et al. and Dr. G. L. Scuderi et al. provided a complete overview on primary congenital glaucoma and pediatric glaucoma, respectively, from diagnosis to treatment outcomes; the groups of Dr. D. Dobrowolski et al. and Dr. B. E. Ramírez et al. described the use of oral mucosa and stem cell therapy, respectively, for ocular surface reconstruction in rare diseases such as aniridia; Dr. F. Mantelli and colleagues reviewed the diagnosis and management of congenital corneal anesthesia and neurotrophic keratitis; Dr. A. K. Nowinska and colleagues compared different tomographic approaches to evaluate rare corneal dystrophies; the groups of Dr. M. Ołdak and Dr. S. P. Patel explained the pathophysiology of Fuchs endothelial corneal dystrophy and congenital hereditary endothelial corneal dystrophy, respectively; Dr. M. Sacchetti and colleagues provide a guide to diagnose and manage the iridocorneal endothelial (ICE) syndrome; Dr. S. Abdolrahimzadeh and colleagues described a series of rare diseases and ophthalmic alterations that may lead to glaucoma; Dr. M. F. Suárez et al. described how environmental agents can influence a rare corneal disease, climatic droplet keratopathy.

As can be seen from the variety of topics presented in this special issue, the disorders grouped under the umbrella term of rare diseases of the anterior segment of the eye can be considered quite heterogenous and numerous and, in turn, represent a substantial clinical burden on a worldwide basis [1]. In fact, although single disorders are rarely encountered in clinical practice, when taken together, they are not seldom seen. In addition, these diseases often represent a clinical challenge due to the paucity of specific diagnostic criteria and the lack of specific treatments. In fact, rare disorders tend to remain orphan drug indications due to the difficulty of running clinical trials, and surgical approaches are often inadequate due to concomitant anatomical and congenital anomalies.

To make the clinical picture of the rare diseases of the anterior segment of the eye even more difficult to manage, they often have consequences that extend beyond the anterior segment and can severely impair vision. Specifically, while corneal opacities and iris anomalies may directly affect visual function, several diseases such as iridocorneal angle anomalies may induce glaucoma with consequent damage to the optic nerve. In addition, several congenital diseases of the anterior segment of the eye are also associated with facial anomalies and other malformations of intraocular structures, making this group of disorders particularly complex for

the ophthalmologist, who encounters obstacles in patients' management from diagnosis to treatment [2].

The recent advances in translational research, which are reshaping modern ophthalmology, allowed identifying the link between different clinical phenotypes and specific mutations in genes regulating the normal formation and maturation of the anterior segments of the eye [3]. In turn, our ability to understand the pathogenesis of rare and complex ophthalmic diseases has improved and, recently, novel treatments are being proposed for selected disorders of the anterior segment. For instance, biotechnology approaches for the pharmaceutical development of recombinant human molecules as well as novel procedures for ex vivo expansion of limbal stem cells may soon open novel therapeutic perspectives for patients with previously untreatable corneal disorders [4, 5]. Similarly, pre- and posttest genetic counseling plays an essential role in the achievement of an appropriate management of congenital glaucoma, in terms of medical, social, and psychological impact of the disease [6].

Nevertheless, due to the complexity of most of the rare anterior segment disorders of the eye, ranging from corneal disorders such as corneal dystrophies, neurotrophic keratitis, iridocorneal anomalies, and congenital glaucoma, further progress is mandatory to exert a significant impact on the natural history of the diseases [7–10]. Hopefully, the constant growth of novel diagnostic tools, accompanied by a better understanding of the disease pathophysiology, will also foster more preclinical and clinical research in this area of high medical need.

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Research Article

Comparative Study of Anterior Eye Segment Measurements with Spectral Swept-Source and Time-Domain Optical Coherence Tomography in Eyes with Corneal Dystrophies

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Purpose. To compare anterior eye segment measurements and morphology obtained with two optical coherence tomography systems (TD OCT, SS OCT) in eyes with corneal dystrophies (CDs). *Methods.* Fifty healthy volunteers (50 eyes) and 54 patients (96 eyes) diagnosed with CD (epithelial basement membrane dystrophy, EBMD = 12 eyes; Thiel-Behnke CD = 6 eyes; lattice CD TGFBI type = 15 eyes; granular CD type 1 = 7 eyes, granular CD type 2 = 2 eyes; macular CD = 23 eyes; and Fuchs endothelial CD = 31 eyes) were recruited for the study. Automated and manual central corneal thickness (aCCT, mCCT), anterior chamber depth (ACD), and nasal and temporal trabecular iris angle (nTIA, tTIA) were measured and compared with Bland-Altman plots. *Results.* Good agreement between the TD and SS OCT measurements was demonstrated for mCCT and aCCT in normal individuals and for mCCT in the CDs group. The ACD, nTIA, and tTIA measurements differed significantly in both groups. TBCD, LCD, and FECD caused increased CCT. MCD caused significant corneal thinning. FECD affected all analyzed parameters. *Conclusions.* Better agreement between SS OCT and TD OCT measurements was demonstrated in normal individuals compared to the CDs group. OCT provides comprehensive corneal deposits analysis and demonstrates the association of CD with CCT, ACD, and TIA measurements.

1. Introduction

Corneal dystrophy (CD) is a group of inherited, bilateral, symmetric, slowly progressive corneal diseases without any relationship to environmental or systemic factors.

Noninvasive evaluation of anterior eye segment measurements is pertinent for the diagnosis of several corneal dystrophies types as well as other ophthalmic diseases, including glaucoma, keratoconus, and corneal degenerations, and is essential in planning corneal surgical and refractive procedures.

Optical coherence tomography (OCT), first introduced in 1991, is a high-speed, high-resolution, noncontact imaging technique developed for noninvasive cross-sectional imaging in biological systems [1]. The OCT technology has evolved from time-domain (TD OCT) to spectral-domain (SD OCT) and swept-source OCT (SS OCT). Anterior eye segment imaging with the 830 nm light wavelength TD OCT was demonstrated in 1994 [2]. Changing the light wavelength from 830 nm to 1310 nm allowed transscleral imaging with the scleral spur assessment [3]. TD OCT technology has a longitudinal resolution of 18 μm and a transverse resolution of 60 μm. It provides scans at a rate of up to 2048 A scans per sec. SD OCT, introduced in 2002, has an axial resolution of 5.0 μm and a transverse resolution of 15 μm [4, 5]. It scans at 26,000 A scans per sec and provides

an increased signal-to-noise ratio and increased robustness compared with TD OCT [6]. SS OCT uses a monochromatic, tunable, fast-scanning laser source and a photodetector to detect wavelength-resolved interference signals [7, 8]. Commercially available SS OCT was introduced in 2008. It uses a swept-source laser wavelength of 1310 nm, scans up to 30,000 A scans per sec, and has longitudinal and transverse resolutions of 10 μm and 30 μm, respectively. The advantage of SS OCT is the simultaneous acquisition of numerous scans, which provides the possibility of creating a 3-dimensional corneal, anterior eye segment, or gonioscopy views. That feature could be especially important in eyes with corneal opacities to gain the possibility of creating a 3D pattern of the corneal changes.

OCT has been proven to provide reliable measurements of anterior eye segment parameters characterized by good repeatability and reproducibility [9–13]. Most SS OCT morphometry studies are based on normal subjects, with the exception of anterior chamber angle parameters in glaucomatous eyes [14–16] and corneal thickness measurements in keratoconic eyes [17, 18]. Currently, there are no data on anterior eye segment measurements with SS OCT in various corneal dystrophies. Previous papers on OCT imaging focused on describing corneal morphology features in different CDs [19–23]. The SS OCT was proved useful in planning of the phototherapeutic keratectomy to treat granular corneal dystrophy by determining the size, depth, and location of deposits based on the case report study [24]. The authors present a comprehensive, observational, comparative study of corneal thickness, anterior chamber depth, and trabecular iris angle measurements with TD OCT and SS OCT in eyes with corneal dystrophies compared to normal controls. Agreement between the TD OCT and SS OCT measurements is assessed.

2. Material and Methods

The study was conducted in accordance with the ethical standards stated in the 1964 Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Silesia, Katowice, Poland (KNE/0022/KB1/43/I/14). All patients had to sign informed consent before any study procedure.

Fifty healthy volunteers (50 eyes) and 54 patients (96 eyes) diagnosed with various corneal dystrophies (epithelial basement membrane dystrophy, EBMD = 12 eyes; Thiel-Behnke corneal dystrophy, TBCD = 6 eyes; lattice corneal dystrophy TGFBI type, LCD1 = 15 eyes; granular corneal dystrophy type 1, GCD1 = 7 eyes; granular corneal dystrophy type 2, GCD2 = 2 eyes; macular corneal dystrophy, MCD = 23 eyes; Fuchs endothelial corneal dystrophy, FECD = 31 eyes) were recruited for the study.

The inclusion criteria for the healthy subjects group were as follows: best corrected visual acuity of 20/20, refractive error less than or equal to ±3.0 D, and no history of ocular disease or surgery. The mean age of the subjects was 30 ± 7 years; there were 30 women and 20 men. The inclusion criteria for the study group included the clinical diagnosis of corneal dystrophy and no history of ocular surgery. The exclusion criterion was the presence of other ophthalmic or systemic diseases affecting corneal morphology. The mean age of the patients was 49 ± 16 years; there were 39 women and 15 men. 12 eyes of 12 patients with diagnosis of CD underwent keratoplasty procedures; therefore the eyes were excluded from the study group. The healthy subjects and the study group patients were age matched for all CD types, except for FECD. Patients with FECD were on average 15 ± 9 years older. The diagnosis of EBMD and FECD was based on the clinical examination (slit-lamp biomicroscopy and OCT). The diagnosis of all patients with TBCD, LCD1, GCD1, GCD2, and MCD was confirmed with genetic sequencing of TGFBI and CHST6 genes according to the methodology presented in previous author's publications [22, 23]. In the CD group eyes with differentiated severity of the disease were included in the analysis.

Clinical examination consisted of visual acuity, slit-lamp biomicroscopy with photography (magnification 10x; 16x), anterior eye segment time-domain, and spectral swept-source optical coherence tomography.

Anterior segment imaging was performed by one observer. We used two anterior segment optical coherence systems: 1310 nm time-domain OCT (TD OCT; Visante OCT; Carl Zeiss Meditec, Inc., Dublin, California, USA) and 1310 nm swept-source spectral-domain OCT (SS OCT; Casia SS-1000 OCT; Tomey, Nagoya, Japan). During the TD OCT exam, we used anterior segment (16 × 6 mm; 2 × 256 A scans), high-resolution corneal quad scans (10 × 3 mm; 4 × 512 A scans), and an automatic pachymetry map (8 × 128 A scans).

During the SS OCT exam, we used the anterior chamber angle (16 × 6 mm; 64 × 512 A scans) and cornea (10 × 4 mm; 16 × 512 A scans) protocols.

Automated and manual central corneal thickness (aCCT, mCCT), anterior chamber depth (ACD), and nasal and temporal trabecular iris angle (nTIA, tTIA) were measured. The analysis of the measurement results was performed by three observers. ACD was defined as the perpendicular distance from the corneal endothelium at the corneal apex to the anterior lens surface. TIA was defined as an angle measured with the apex in the iris recess and the arms of the angle passing through a point on the trabecular meshwork 500 μm from the scleral spur and the point on the iris perpendicularly opposite [25]. In four eyes from the study group, we could not assess the scleral spur, so they were excluded from the TIA assessment. Corneal morphology assessment was performed and compared between TD OCT and SS OCT. We analyzed the characteristic features, pattern, and location of CD deposits.

Mean values and standard deviation (SD) were calculated for each parameter in the groups with more than 30 eyes (the control group, FECD). Median and range were assessed in the groups with fewer than 30 eyes (EBMD, TBCD, LCD, GCD1, GCD2, and MCD). The values for the parameters were compared between the normal and CDs groups using Student's t-test or the Mann-Whitney U test depending on the sample size. Agreement between pairs of measurements was analyzed with Bland-Altman plots. The 95% limit of agreement (mean

Table 1: Results of automated and manual central corneal thickness (aCCT, mCCT), anterior chamber depth (ACD), and nasal and temporal trabecular iris angle (nTIA, tTIA) measurements by swept-source optical coherence tomography SS OCT and time-domain optical coherence tomography TD OCT. Values were calculated as mean ± standard deviation (SD) or median and range depending on the sample size (<30 or ≥30). BCVA results were presented as range for all groups. EBMD = epithelial basement membrane dystrophy, TBCD = Thiel-Behnke corneal dystrophy, LCD1 = lattice corneal dystrophy TGFBI type, GCD1 = granular corneal dystrophy type 1, GCD2 = granular corneal dystrophy type 2, MCD = macular corneal dystrophy, and FECD = Fuchs endothelial corneal dystrophy.

Parameter	OCT device	Control group	Study group						
CD type			EBMD	TBCD	LCD	GCD1	GCD2	MCD	FECD
Number of eyes		50 eyes	12 eyes	6 eyes	15 eyes	7 eyes	2 eyes	23 eyes	31 eyes
BCVA		1.0	1.0	0.3–0.9	0.1–0.5	0.05–0.8	0.5–0.6	0.05–0.2	0.05–0.4
aCCT [μm]	TD OCT	548.96 ±37.34	545 524–586	600 587–615	583 550–620	550 498–567	538.5 529–548	459 414–492	675.54 ±42.19
	SS OCT	553.96 ±31.91	554.5 518–600	598.5 578–620	587 546–619	546 480–583	528 518–538	453 419–502	682.03 ±41.38
mCCT [μm]	TD OCT	546.94 ±31.13	541.5 517–582	602 587–618	588 528–610	555 490–578	530 520–540	456 417–503	676.03 ±43.13
	SS OCT	550.34 ±31.13	553.5 507–580	599.5 580–625	593 550–621	550 498–576	513 507–519	447 418–508	675.45 ±52.98
ACD [mm]	TD OCT	3.0514 ±0.24	3.085 2.71–3.45	2.9 2.71–3.15	3.13 2.71–3.59	3.11 2.72–3.24	3.22 3.06–3.39	3.05 2.61–3.59	2.30 ±0.35
	SS OCT	2.9718 ±0.25	2.955 2.61–3.38	2.91 2.61–3.05	3.015 2.61–3.59	2.98 2.68–3.05	3.18 3.01–3.36	2.98 2.69–3.59	2.36 ±0.46
nTIA [°]	TD OCT	31.44 ±4.98	31 24–39	27 24–32	30 24–47	33 29–38	33 30–36	31 24–41	21.38 ±4.40
	SS OCT	33.02 ±5.67	33.5 22–42	26.5 22–39	33 26–47	34 32–42	30.5 27–34	32 20–42	19.61 ±4.49
tTIA [°]	TD OCT	29.9 ±5.81	27.5 22–42	27 23–34	29 22–39	32 29–41	30 26–34	30 24–37	19.38 ±5.33
	SS OCT	32.12 ±5.57	31.5 24–44	30 24–36	33 23–40	34 30–40	32.5 29–36	31 24–41	20.29 ±6.01

difference ±1.96 standard deviation) was calculated. A p value of less than .05 was considered statistically significant.

3. Results

Mean, standard deviation, and median and range values of aCCT, mCCT, ACD, nTIA, and tTIA for the control and CDs groups are presented in Table 1.

3.1. Agreement of aCCT, mCCT, ACD, nTIA, and tTIA Measurements. Mean difference in the aCCT measurements by TD OCT and SS OCT was not statistically significant in the control group ($p = .14$) but was significant in the CD group ($p = .04$). The aCCT measured with SS OCT was on average 4.4 μm higher than that measured with TD OCT in the control group and 4.32 μm in the CDs group. The mCCT measurements demonstrated the best agreement between TD OCT and SS OCT with no significant difference in the control group ($p = .12$) and the CDs group ($p = .14$). The ACD measured with SS OCT was on average 0.07 mm lower than that measured with TD OCT in the control group ($p < .001$) and 0.06 mm lower in the CDs group ($p = .01$). The mean difference in the nTIA measurements was significant in both groups, $p = .001$ in the control group and $p = .03$ in the CDs group. The nTIA measured with SS OCT was 1.58° higher than that measured with TD OCT in the control group and 1.8° lower in the CDs group. The mean difference in the tTIA measurements was also significant in both groups with $p < .001$ in the control group and $p < .001$ in the CDs group. The tTIA measured with SS OCT was 2.22° higher than that measured with TD OCT in the control group and 1.97° higher in the CDs group. All data including mean difference, 95% confidence interval, standard deviation, and p value are presented in Table 2. The Bland-Altman plots including the 95% limit of agreement are presented in Figure 1.

3.2. Control Group and CDs Group Measurements Comparison. TD OCT and SS OCT measurements of aCCT and mCCT were significantly different in four corneal dystrophies (TBCD, LCD, MCD, and FECD) compared to the control group.

The aCCT and mCCT measurements were significantly higher in TBCD, LCD, and FECD compared to normal

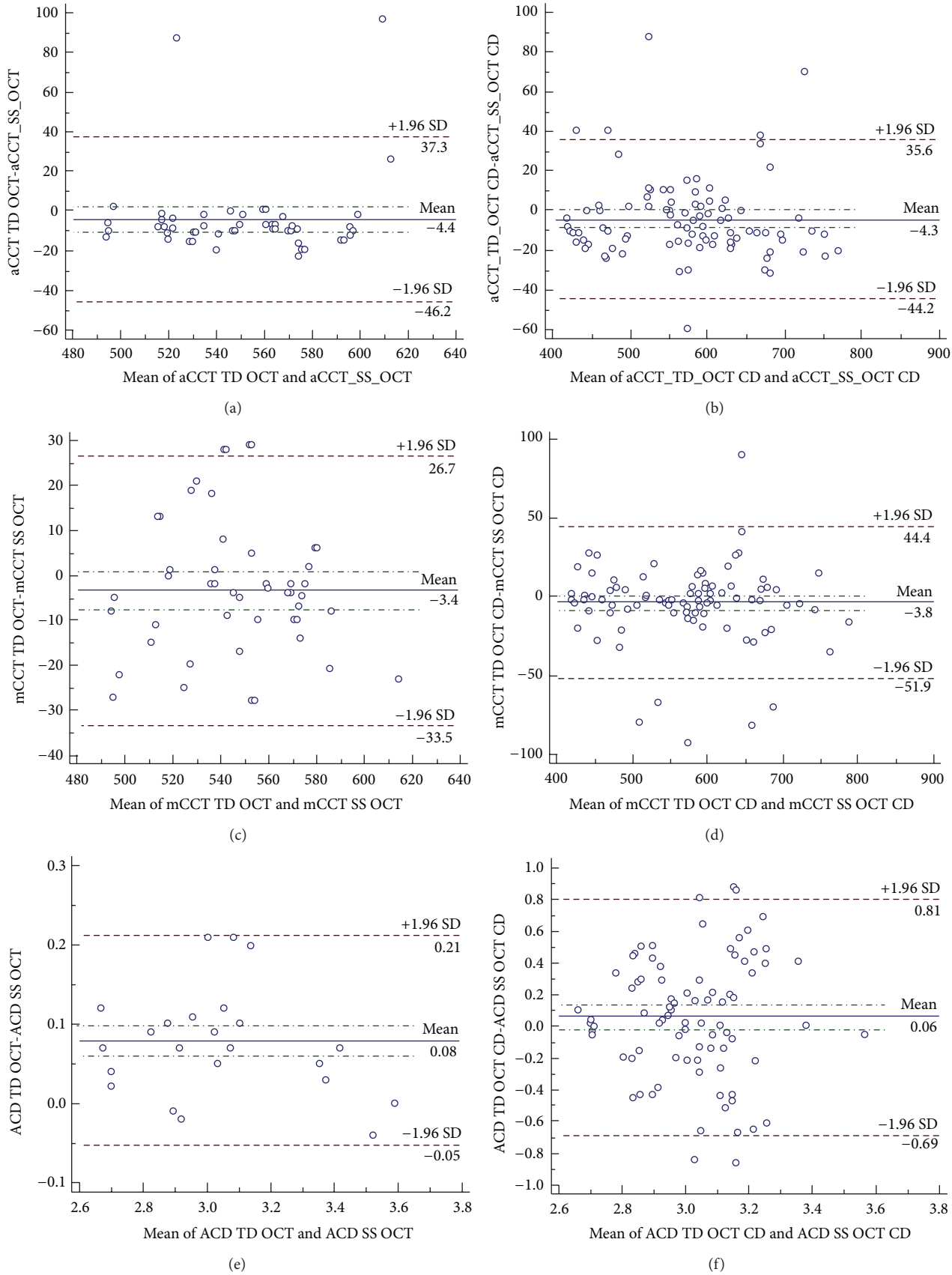


Figure 1: Continued.

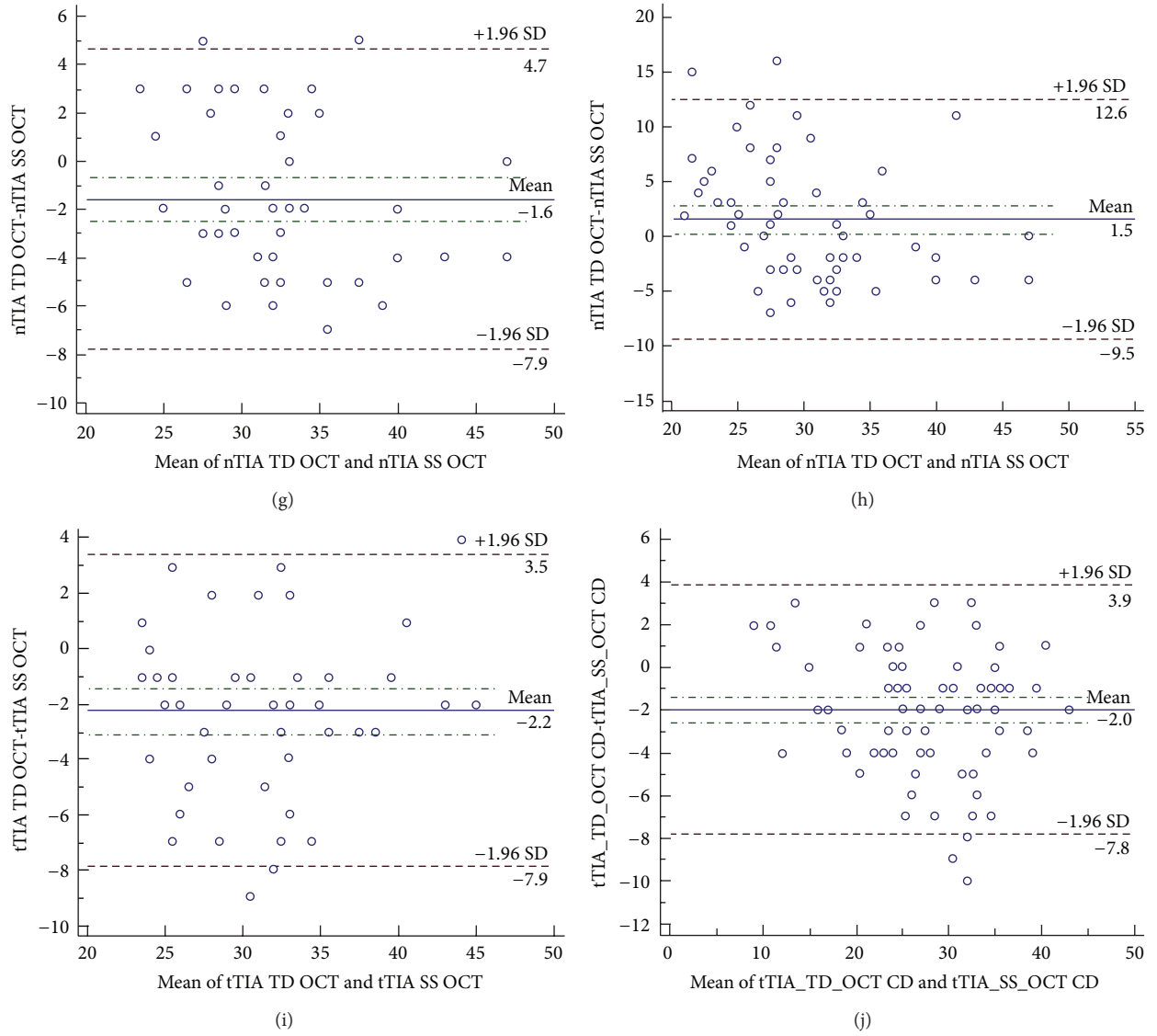
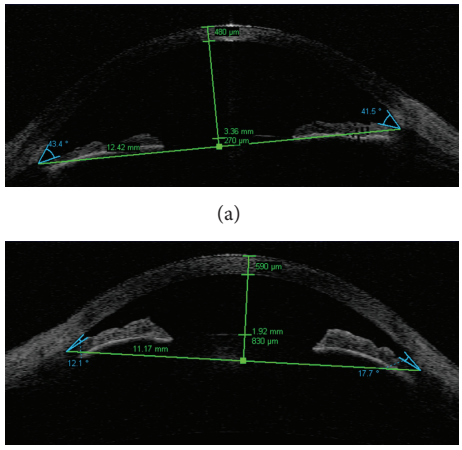


Figure 1: The graphic presentation of Bland-Altman plot comparing measurements of TD OCT and SS OCT in control and corneal dystrophies group (CD group). Dash-dot line: 95% CI: 95% confidence interval of the mean difference; dashed line: ±1.95 SD (standard deviation); aCCT: automated central corneal thickness; mCCT: manual central corneal thickness; ACD: anterior chamber depth; nTIA: nasal trabecular iris angle; tTIA: temporal trabecular iris angle.

Table 2: Bland-Altman plot comparing automated and manual central corneal thickness (aCCT, mCCT), anterior chamber depth (ACD), and nasal and temporal trabecular iris angle (nTIA, tTIA) measurements by swept-source optical coherence tomography SS OCT and time-domain optical coherence tomography TD OCT in control and corneal dystrophies group (CD group). 95% CI:95% confidence interval of the mean difference; SD: standard deviation.

	Control group				CD group			
	Mean difference	95% CI	SD	*p*	Mean difference	95% CI	SD	*p*
aCCT	−4.4	−10.47–1.63	21.29	.14	−4.32	−8.51–0.13	20.35	**.04**
mCCT	−3.4	−7.7–0.97	15.37	.12	−3.75	−8.81–1.31	24.58	.14
ACD	0.07	0.06–0.09	0.06	**<.001**	0.06	−0.01–0.14	0.38	**.01**
nTIA	−1.58	−2.48–−0.67	3.2	**.001**	1.8	0.14–3.47	6.62	**.03**
tTIA	−2.22	−3.04–−0.39	2.91	**<.001**	−1.97	−2.59–−1.36	2.97	**<.001**



(a)

(b)

Figure 2: Anterior segment single 0–180° scan of TD OCT with measured results of following parameters: mCCT, ACD, nTIA, tTIA, ATA, angle to angle distance, and CLR, crystalline lens rise. (a) Control group. (b) FECD. Note the difference between mCCT, ACD, nTIA, and tTIA, which is statistically significant ($p < .0001$).

individuals. In MCD, the analysis revealed lower CCT values compared to the control group.

The mean values ± standard deviation and median values (range) of aCCT and mCCT, ACD, nTIA, and tTIA measured with TD OCT and SS OCT in the control and study group were presented in Table 1.

FECD was the only CD that affected all analyzed anterior eye chamber parameters (Figure 2). The aCCT, mCCT, ACD, nTIA, and tTIA measurements in FECD differed significantly from those for the control group ($p < .001$). The summary of the comparison is presented in Table 3.

3.3. Corneal Morphology Comparison. All corneal characteristic CD features revealed on the SS OCT scans were also visible on the TD OCT scans. That makes both techniques useful for establishing the diagnosis of each corneal dystrophy. The advantage of SS OCT is the simultaneous acquisition of numerous scans, which provides the possibility of creating a 3-dimensional corneal pattern of changes.

All corneal dystrophies deposits were hyperreflective on the TD OCT and SS OCT scans, but the level of increased reflectivity differed and extended from diffuse areas of increased reflectivity in LCD to highly reflective corneal opacities in GCD2. The opacities also differed in shape and pattern depending on the CD type.

No changes in EBMD were distinguishable on either OCT scan. TBCD was characterized by increased reflectivity in the Bowman layer and anterior corneal stroma (Figure 3). The deposits caused the irregularity of the anterior stromal border from the epithelium side forming a sawtooth pattern of hyperreflective material. LCD caused diffuse areas of increased reflectivity in the area of Bowman layer and anterior to midstroma. GCD1 was characterized by focal granular hyperreflective changes in the Bowman layer and anterior to mid corneal stroma. Corneal deposits in GCD2 had the highest reflectivity; there were highly reflective, flat corneal opacities in the anterior stroma accompanied by focal deposits located in the midstroma. MCD caused general increased reflectivity throughout the corneal stroma. The deposits caused the irregularity of the anterior stromal border from the epithelium side and the diffuse areas of hyperreflectivity in Bowman's layer. There was a noticeable flat layer of increased reflectivity in the posterior, peripheral corneal part. FECD caused corneal edema that was characterized by irregularity of the posterior corneal border and corneal epithelial and subepithelial bullae in advanced stages.

4. Discussion

According to the authors of the IC3D classification system (the International Committee for Classification of Corneal Dystrophies), understanding of corneal dystrophies is still evolving due to the development of noninvasive imaging techniques and introduction of genetic testing [26]. OCT provides direct, noncontact, anterior eye segment imaging allowing morphology and morphometry analysis. SS OCT scans 360° around the anterior segment in 2.4 sec showed the depth and extent of the pathologic corneal features. Good repeatability and reproducibility of SS OCT anterior eye segment measurements were proved in normal controls [27–31]. Pachymetric maps made with SS OCT were compared with a rotating Scheimpflug camera, ultrasound pachymetry, specular microscopy, slit-scanning topography, TD OCT, and 830 nm SD OCT with high correlation rates [13, 17, 22–27]. Fukuda et al. revealed that the CCT measured with Scheimpflug camera was significantly larger than that measured with SS OCT, slit-scanning topography, and ultrasonic pachymetry ($p < .001$) [28]. Fukuda et al. revealed that CCT measured with SS OCT was thinner compared with slit-scanning topography ($p < .001$) and ultrasound pachymetry ($p < .001$) [29]. The authors emphasize that CCT values measured with different devices are not interchangeable. Anterior chamber angle parameters such as TIA, TISA 500, 750 (trabecular iris space area at 500, 750 μm from the scleral spur) and AOD 500, 750 (angle opening distance at 500, 750 μm from the scleral spur) measurements repeatability, reproducibility, and agreement between SS OCT and other devices were studied in normal and glaucomatous eyes, but no such studies have been conducted for opaque corneas [14–16, 30, 31]. SS OCT demonstrated the high reproducibility of angle analysis in healthy subjects.

Our study confirms the good agreement of CCT measurements between devices in healthy subjects (aCCT, $p = .4$; mCCT, $p = .12$). The ACD, nTIA, and tTIA measurements differed significantly. ACD values measured with SS OCT were significantly lower (mean difference = 0.07 ± 0.06 mm; $p < .001$). nTIA and tTIA measured with SS OCT were significantly larger (mean difference = 1.58 ± 3.2°; $p = .001$; mean difference = 2.22 ± 2.91°; $p < .001$, resp.). Fukuda et al., who studied agreement of CCT, ACD, and anterior chamber width measurements in 85 normal individuals between the TD OCT and SS OCT prototype, also revealed no statistically significant difference in CCT measurements. ACD measurements were significantly different ($p < .001$); the mean

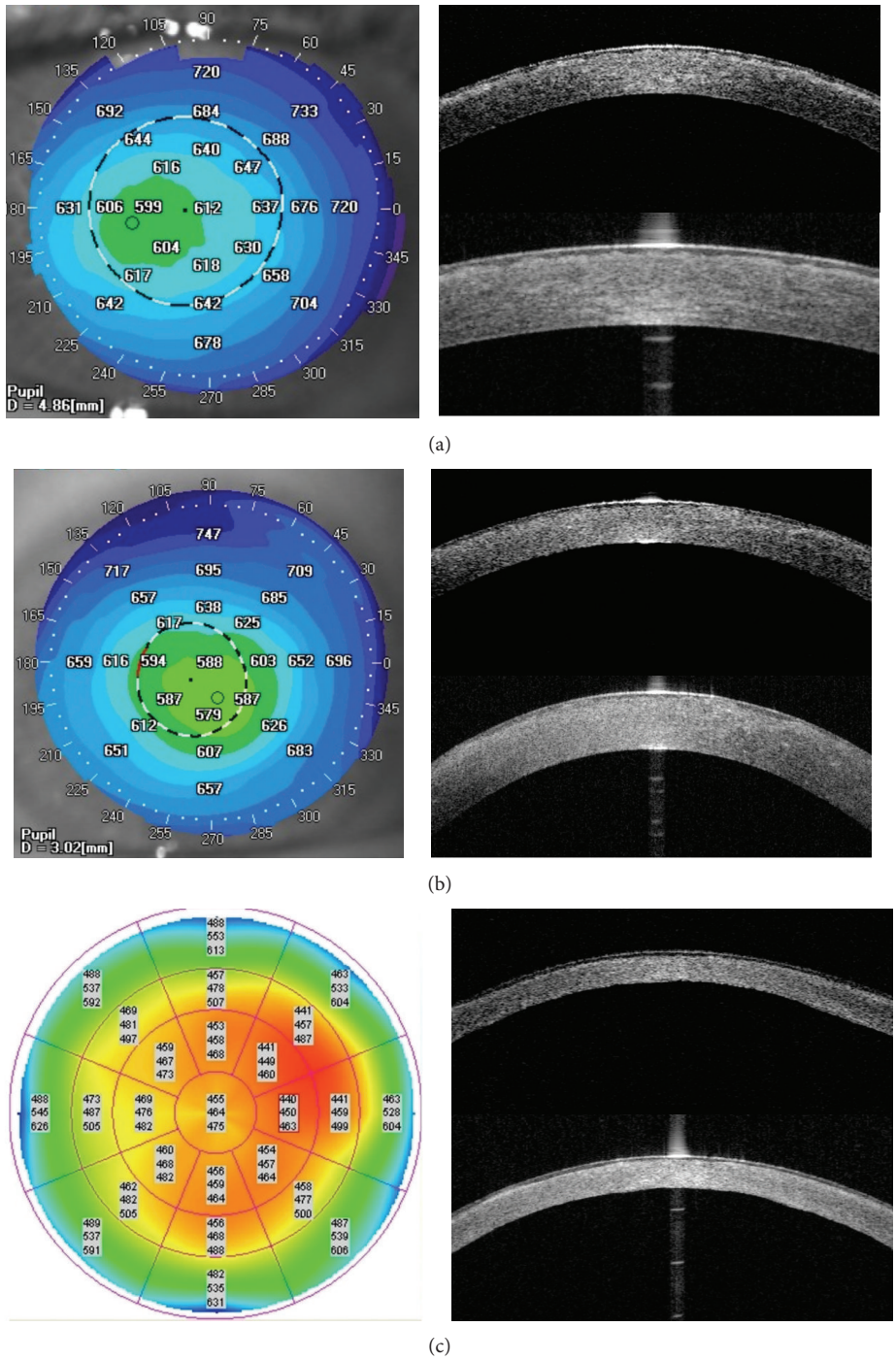
Table 3: Statistical difference of anterior eye segment measurements: automated and manual central corneal thickness (aCCT, mCCT), anterior chamber depth (ACD), and nasal and temporal trabecular iris angle (nTIA, tTIA) measurements by swept-source optical coherence tomography SS OCT and time-domain optical coherence tomography TD OCT comparing different corneal dystrophies and normal eyes. EBMD = epithelial basement membrane dystrophy, TBCD = Thiel-Behnke corneal dystrophy, LCD1 = lattice corneal dystrophy TGFBI type, GCD1 = granular corneal dystrophy type 1, GCD2 = granular corneal dystrophy type 2, MCD = macular corneal dystrophy, and FECD = Fuchs endothelial corneal dystrophy.

Statistical difference	Study group versus normal controls					
	EBMD	TBCD	LCD	GCD1	MCD	FECD
aCCT						
TD OCT	p .89 U 292.5	**p < .001** U 17.5	**p < .001** U 144.5	p .82 U 166	**p < .001** U 6	**p < .001**
SS OCT	p .93 U 295.5	**p < .001** U 48.41	**p < .001** U 170.5	p .24 U 127.5	**p < .001** U 13	**p < .001**
mCCT						
TD OCT	p .72 U 281.5	**p < .001** U 8	**p < .001** U 128	p .85 U 167.5	**p < .001** U 9.5	**p < .001**
SS OCT	p .77 U 284	**p < .001** U 20	**p < .001** U 94	p .84 U 167	**p < .001** U 4.5	**p < .001**
ACD						
TD OCT	p .83 U 288.5	p .24 U 106.5	p .33 U 335	p .55 U 150.5	p .87 U 562	**p < .001**
SS OCT	p .83 U 288.5	p .53 U 126.5	p .30 U 324.5	p .75 U 159	p .53 U 51	**p < .001**
nTIA						
TD OCT	p .93 U 295.5	p .06 U 81	p .69 U 374	p .25 U 128.5	p .98 U 573.5	**p < .001**
SS OCT	p .84 U 289	p .07 U 83.5	p .70 U 375	p .12 U 112	p .49 U 517	**p < .001**
tTIA						
TD OCT	p .68 U 277	p .27 U 109	p .65 U 370	p .11 U 110	p .72 U 545.5	**p < .001**
SS OCT	p .95 U 297	p .30 U 111.5	p .91 U 393	p .23 U 126	p .93 U 568.5	**p < .001**

difference was 0.04 mm smaller compared to our study [28]. Aptel et al. studied CCT, ACD, TIA, TISA 500, 750, and AOD 500, 750 measurements in healthy subjects. The study revealed that ACD measured with SS OCT was significantly larger (mean difference = 0.12 ± 0.08 mm; $p < .001$), and the TIA measured with the SS OCT was significantly lower (mean difference = 4.85° ± 5.30°; $p < .01$). There were nonsignificant differences between the devices for the other parameters ($p > .06$) [30]. The opposite results of the ACD and TIA measurements found by Aptel et al. and our study indicate that there is no systematic difference in ACD and TIA measurements between TD and SS OCT, and the results could vary depending on the device used for measurements.

To date, no comparison of CCT, ACD, and TIA measurements with TD OCT and SS OCT has been published for opaque corneas. Accurate pachymetric and angle measurements in an eye with a corneal opacity are challenging and of great importance in guiding treatment or retreatment in corneal surgeries. Overestimation or underestimation in anterior segment parameters could be misleading in selecting the corneal transplant type as well as deciding the depth of treatment in phototherapeutic keratectomy. The 830 and 1310 nm OCT was proved useful in the selection and planning of surgical procedures to treat GCD by determining the size, depth, and location of deposits [24, 32]. We revealed that only the mCCT showed good agreement between TD OCT and SS OCT in the CDs group ($p = .14$). There were significant differences for other studied parameters. That result should be considered in clinical practice, while planning surgical treatment in CD. It emphasizes the role of corneal manual measurements in establishing the treatment plan.

Our study also indicated the impact of CD on corneal pachymetry and anterior chamber parameters. TBCD, LCD, and FECD caused increased aCCT and mCCT measured with both devices compared to the control group. MCD was characterized by significant corneal thinning, indicated by previous studies [23, 33]. The increase in CCT is the main feature of moderate and advanced FECD, but it is possible that association with other anterior eye segment parameters change is rarely examined. A link of FECD to axial hypermetropia, shallow anterior chamber, and angle closure glaucoma was suggested in the 1990s [34, 35], but another study



(a)

(b)

(c)

Figure 3: Comparison of representative TD and SS OCT corneal scans in CD group. There are no differences in corneal deposits visualization between both OCT systems. (a) TBCD: SS OCT pachymetry map showing the increase of CCT. aCCT of 612 μm. TD OCT high-resolution corneal scan and SS OCT cornea scan showing increased reflectivity in the area of Bowman layer and anterior corneal stroma. The deposits are causing the irregularity of the anterior stromal border from the epithelium side. (b) LCD: SS OCT pachymetry map showing the increase of CCT. aCCT of 588 μm. TD OCT high-resolution corneal scan and SS OCT cornea scan presenting diffuse areas of increased reflectivity in the area of Bowman layer and anterior to mid stroma. (c) MCD: TD OCT pachymetry map indicating corneal thinning with aCCT of 464 μm. TD OCT high-resolution corneal scan and SS OCT cornea scan showing general increased reflectivity throughout the corneal stroma. Note the irregularity of the anterior stromal border from the epithelium side and the diffuse areas of hyperreflectivity in Bowman's layer. There is a noticeable flat layer of increased reflectivity in the posterior, peripheral corneal part.

found no significant difference in ACD between patients and controls [36] and it was not further confirmed with OCT studies. Our study indicated a significant increase in CCT, thus indicating the advanced stage of FECD and the significant decrease of ACD, nTIA, and tTIA in all 31 patients. The significant ACD and TIA change probably is the result of the increase in CCT, which was proved to be one factor associated with narrow ACD and angle closure glaucoma in the Beijing Eye Study 2006 [37].

Regarding CD corneal morphology analysis, our current SS OCT study complements previous findings demonstrated based on TD, SD, and SS OCT [19–23, 32, 33].

The weak part of our study was that including different stages of the CD could affect the outcomes. On the other side, due to the rarity and the individual course of the disease among patients, further division of the study group into subgroups would result in the insufficient number of subjects for statistical analysis.

To conclude, better agreement between SS OCT and TD OCT measurements was demonstrated in normal individuals compared to the CDs group. Our study emphasizes the role of manual measurements in establishing corneal thickness in CDs. OCT provides comprehensive corneal deposits analysis and demonstrates the association of CD with CCT, ACD, and TIA measurements.

Disclosure

All the authors declare no commercial relationships relevant to the subject matter of the paper.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Rare Diseases Leading to Childhood Glaucoma: Epidemiology, Pathophysiogenesis, and Management

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Noteworthy heterogeneity exists in the rare diseases associated with childhood glaucoma. Primary congenital glaucoma is mostly sporadic; however, 10% to 40% of cases are familial. CYP1B1 gene mutations seem to account for 87% of familial cases and 27% of sporadic cases. Childhood glaucoma is classified in primary and secondary congenital glaucoma, further divided as glaucoma arising in dysgenesis associated with neural crest anomalies, phakomatoses, metabolic disorders, mitotic diseases, congenital disorders, and acquired conditions. Neural crest alterations lead to the wide spectrum of iridocorneal trabeculodysgenesis. Systemic diseases associated with childhood glaucoma include the heterogenous group of phakomatoses where glaucoma is frequently encountered in the Sturge-Weber syndrome and its variants, in phakomatosis pigmentovascularis associated with oculodermal melanocytosis, and more rarely in neurofibromatosis type 1. Childhood glaucoma is also described in systemic disorders of mitotic and metabolic activity. Acquired secondary glaucoma has been associated with uveitis, trauma, drugs, and neoplastic diseases. A database research revealed reports of childhood glaucoma in rare diseases, which do not include glaucoma in their manifestation. These are otopalatodigital syndrome, complete androgen insensitivity, pseudotrisomy 13, Brachmann-de Lange syndrome, acrofrontofacionasal dysostosis, caudal regression syndrome, and Wolf-Hirschhorn syndrome.

1. Introduction

Childhood glaucoma is a rare disorder that occurs from birth until teenage years. It usually results in grave visual deterioration [1]. In the pediatric population approximately 8% of blindness has been attributed to the condition [2].

The European Glaucoma Society discriminates *primary congenital glaucoma* (birth to 2 years of age) and *early juvenile glaucoma* (2 years of age to puberty), due to incomplete development of trabecular meshwork, from *secondary childhood glaucoma* [3].

Secondary childhood glaucoma is defined as *associated with congenital ocular anomalies* such as conditions associated with mesodermal dysgenesis of the neural crest, phakomatoses characterized by hamartomas, metabolic disorders, mitotic disorders, and other congenital disorders and *associated with acquired conditions* such as tumors, uveitis, and trauma [3]. Finally, there are rare case reports of diseases that do not normally present glaucoma in their manifestation where the heterogeneity and sporadic nature of the cases make correlations with systemic syndromes and diseases complex and difficult. The purpose of this paper is to review the major conditions associated with secondary childhood glaucoma, providing epidemiological data and notions on pathophysiogenesis, and to assess rare diseases that usually do not include congenital glaucoma among their manifestations.

Table 1: Classification of childhood glaucoma.

Primary congenital glaucoma (trabeculodysgenesis)	
	Secondary childhood glaucoma
Mesodermal dysgeneses of neural crest	Iridocorneal trabeculodysgenesis (i) Aniridia (ii) Axenfeld-Rieger's anomaly (syndrome if systemic associations) (iii) Peter's anomaly (syndrome if systemic associations) (iv) Marfan's syndrome (v) Weill-Marchesani's syndrome
Phakomatoses	Von Recklinghausen's syndrome
	Encephalotrigeminal angiomatosis (Sturge-Weber syndrome and variants such as Klippel-Trenaunay syndrome)
	Oculodermal melanocytosis
Metabolic disorders	Lowe's syndrome
	Homocystinuria
	Mucopolysaccharidoses
Mitotic disorders	Juvenile xanthogranuloma
Other congenital disorders	Trisomy 13 (Patau's syndrome)
	Persistent hyperplastic primary vitreous
	Congenital cataract: (i) In phakic eyes (ii) In aphakic eyes after early surgery
	Rubinstein-Taybi syndrome
	Congenital rubella
Glaucoma associated with acquired conditions	Uveitis
	Trauma (hyphema, angle recession, and ectopia lentis)
	Steroid or drug induced
	Tumors (benign/malignant, ocular/orbital)
	Retinopathy of prematurity

2. Epidemiology

2.1. Primary Congenital Glaucoma. The incidence of childhood glaucoma is 1 in 10,000 to 68,000 live births. A new case of primary congenital glaucoma is diagnosed about once every 5 years in a general ophthalmology center in North America or Western Europe [4–6]. The highest prevalence of childhood glaucoma has been recorded in the Middle East (1 : 2500) [7] and in Slovakia (1 : 1250) [8]. In previous epidemiological studies, the incidence of primary congenital glaucoma was reported as 1 in 10,0003 [9, 10] to 1 in 12,5005 in Western countries. A recent study reported the incidence as 3.31 in 100,000 (1/30,200) in Great Britain and 5.41 in 100,000 (1/18,500) in the Republic of Ireland [5]. Recently, the incidence of primary congenital glaucoma in Spain was reported as 2.85 per 100,000 (1 in 38,000). This is comparable to results in an Australian study where the incidence was 1 in 30,000 births [11]. The presence of consanguinity of parents can increase the incidence by 5 to 10 times [3]. In about 70% of cases, patients' both eyes are affected and it is more common among males [3].

Large epidemiological studies are difficult to carry out as childhood glaucoma presents so infrequently, even if it is the most common glaucoma in the pediatric population [12]. Moreover, since childhood glaucoma appears in the context of systemic diseases, most reports do not specifically describe ophthalmologic anomalies. Therefore, evaluation of the relationship between childhood glaucoma and rare diseases becomes difficult. Table 1 is a classification of childhood congenital glaucoma.

2.2. Mesodermal Dysgeneses of the Neural Crest. The first group of rare systemic diseases leading to secondary childhood glaucoma are those associated with mesodermal dysgenesis of the neural crest [13]. The Axenfeld-Rieger syndrome prevalence is 1–9/1000 000 [14] and glaucoma has been observed in 50% of patients [13]. The prevalence of Peter's syndrome is beneath 1/1000000 [14]. Aniridia has an estimated prevalence between 1 : 40,000 and 1 : 100,000 [15] and the incidence of glaucoma in congenital forms ranges from 6% to 75% [16–18]. Marfan syndrome is a connective tissue disorder with autosomal dominant inheritance and a prevalence of 1 : 5000 [19]. In an international study including 1,000 patients with this syndrome, more than half presented eye manifestations such as myopia (53%), ectopia lentis (54%), and glaucoma (2%) [20]. Weill-Marchesani syndrome is a very rare systemic disease that implicates anomalies of

connective tissue [21] and its prevalence has been estimated at 1 : 100,000 [22]. The main ocular anomalies of Weill-Marchesani syndrome are microspherophakia, ectopia lentis, glaucoma, and myopia. Microspherophakia occurs in 84% of cases, ectopia lentis in 73%, and glaucoma in 80%; severe myopia and cataract are also reported [21].

2.3. Phakomatoses. The second group of rare diseases linked with secondary childhood glaucoma are the phakomatoses characterized by hamartomas. Von Recklinghausen's diseases or neurofibromatosis type 1 is characterized by autosomic dominant inheritance and an incidence of about 1 in 3000 [23]. Glaucoma has been reported in 1/300 patients with this condition [24]. However, a study on 95 patients, with neurofibromatosis type 1, revealed that 23% of 56 patients presented orbitofacial involvement and had ipsilateral glaucoma [25].

Sturge-Weber syndrome has a frequency of approximately 1 in 50,000 births [26]. Glaucoma affects 50% to 70% of patients with this syndrome and its variants (such as Klippel-Trenaunay syndrome) and can be early- or late-onset depending on the age of manifestation [27]. In about 60% of cases the cause is due to anterior chamber abnormalities while in approximately 40% of cases the cause is raised episcleral venous pressure [28]. Oculodermal melanocytosis is a rare condition more frequently encountered in the Asian population with a prevalence of 0.014%–0.034% [29]. Glaucoma is found in about 10% of patients and the pathogenesis may be congenital, developmental or associated with the diffuse characteristic oculodermal hyperpigmentation of the disease which leads to melanocytic glaucoma [30–32]. Phakomatosis pigmentovascularis is a rare combination of oculodermal melanocytosis and Sturge-Weber syndrome (or Klippel-Trenaunay syndrome). In a review of the literature on 281 Asian patients with oculodermal melanocytosis, 9 patients had phakomatosis pigmentovascularis and glaucoma was present in all cases [30].

2.4. Metabolic Disorders. The metabolic disorders leading to childhood glaucoma include mucopolysaccharidoses, Lowe's syndrome, and classical homocystinuria. Lowe's syndrome (oculocerebrorenal syndrome) has a prevalence of 1 in 500,000 [33]. Several cases of secondary congenital glaucoma associated with this disease have been reported; Walton et al. reviewed 7 patients and found that 71% of patients had glaucoma [34]. Classical homocystinuria is caused by cystathionine b-synthase (CbS) deficiency [35–37]. The detection rate of CbS deficiency is 1 in 344,000 [38]. Ectopia lentis, which is often associated with classical homocystinuria [39, 40], occurs in 85% of classical homocystinuria [39, 41, 42]. In a report on 294 patients with mucopolysaccharidoses, 27 eyes of 14 patients had glaucoma and the prevalence of glaucoma was reported from 2.1% to 12.5% [43].

2.5. Mitotic Disorders. The group of mitotic disorders leading to childhood glaucoma includes juvenile xanthogranuloma, the prevalence of which is unknown. It occurs in the pediatric age and 10% of patients present ocular anomalies with occasional glaucoma [44].

2.6. Other Congenital Conditions. The prevalence of trisomy 13 or Patau's syndrome is 1/6500 where glaucoma has been reported among the ocular complications [45].

In a study of 62 cases of persistent hyperplastic primary vitreous, Haddad et al. reported 26 patients where other ocular abnormalities such as leukocoria, microphthalmia, cataract, retinal detachment, glaucoma, and phthisis bulbi were observed [46].

Five to twenty percent of pediatric blindness is attributable to congenital cataract, which occurs in 3-4 per 10 000 births [47]. Although new surgical techniques have been introduced, the incidence of secondary glaucoma following cataract operations in the pediatric age is still high, varying from 6% to 26% [48]. If the surgical procedure is performed before the 9th month of life, the incidence of glaucoma may rise to 50% [48, 49]. Rubinstein-Taybi syndrome as well as congenital rubella appears among congenital disorders associated with glaucoma. Rubinstein-Taybi syndrome has an incidence of 1 in 100 000 newborns [50]. van Genderen et al. reviewed 207 patients with Rubinstein-Taybi syndrome, and 117 had ocular disorders. Congenital glaucoma was described in 31 cases [51]. Epidemiological assessments of ocular disease in large populations of congenital rubella are few. In France just 21 cases of rubella during pregnancy were reported in 2001 and only one child presented congenital rubella syndrome at birth [52]. Various ocular disorders such as retinopathy, cataract, and glaucoma may occur in this condition [53]. In a prospective study by O'Neill in 34 patients with rubella, glaucoma was observed in 29% (11 eyes) and was in the context of microphthalmic eyes in 4 patients [54]. Table 2 is a summary of the epidemiology and percentage of glaucoma in rare diseases leading to secondary childhood glaucoma.

2.7. Rare Diseases. Cases reports of congenital glaucoma have been reported in the context of rare systemic syndromes such as otopalatodigital syndrome type II (OPD II) [55], complete androgen insensitivity [56], pseudotrisomy 13 [57], Brachmann-de Lange syndrome [58], autosomal recessive acrofrontofacionasal dysostosis [59], caudal regression syndrome [60], and Wolf-Hirschhorn syndrome [61] (Table 3).

3. Genetics and Pathophysiology

3.1. Primary Congenital Glaucoma. While most cases of primary congenital glaucoma are sporadic, 10% to 40% are familial and are often associated with consanguinity [62]. Familial forms have autosomal recessive inheritance with variable expression in 40 to 100% of cases [63]. However, the autosomal recessive inheritance modality has been challenged due to the discrepancy between gender distribution, transmission of childhood glaucoma to offspring, and lower predicted afflicted siblings in familial cases [63]. Linkage studies confirm the heterogeneity of primary congenital glaucoma, so these discrepancies can be explained [63, 64].

Primary congenital glaucoma has been associated with 3 chromosomal loci: chromosomes 2p21 (GLC3A), 1p36 (GLC3B), and 14q24 (GLC3C) [62, 63, 65, 66].

Table 2: Epidemiology and percentage of glaucoma in rare diseases leading to childhood glaucoma.

Primary congenital glaucoma 1 in 10,000 to 68,000			
	Secondary childhood glaucoma		
	Disease	Prevalence of disease	Percentage of glaucoma in disease
Mesodermal dysgeneses of neural crest	Iridocorneal trabeculodysgenesis		
	(i) Aniridia	From 1/40,000 to 1/100,000	6–75% [16–18, 24]
	(ii) Axenfeld-Rieger's anomaly (syndrome if systemic associations)	1–9/1000000	50% [13]
	(iii) Peter's anomaly (syndrome if systemic associations)	1/1000000	Not available
	(iv) Marfan's syndrome	1/5000	2% [20]
	(v) Weill-Marchesani syndrome	1/100000	80% [21]
Phakomatoses	Von Recklinghausen's syndrome	1/3000	1% [17, 24], 23% of patients with ipsilateral craniofacial anomalies [25]
	Encephalotrigeminal angiomatosis (Sturge-Weber syndrome and variants such as Klippel-Trenaunay syndrome)	1/50000	50–70% [27] (100% incidence in cases associated with oculodermal melanocytosis [32])
	Oculodermal melanocytosis	14/100000 to 34/100000	10% [29, 32]
Metabolic disorders	Lowe's syndrome	1/500000	71% [34]
	Homocystinuria	1/344000	85% of cases with ectopia lentis [38, 39, 41, 42]
	Mucopolysaccharidoses	1/25000	2.1% to 12.5% [43, 95]
Mitotic disorders	Juvenile xanthogranuloma	Unknown	10% of patients present ocular anomalies with occasional glaucoma [44]
Other congenital disorders	Trisomy 13 (Patau's syndrome)	1/6500	Not available
	Persistent hyperplastic primary vitreous	Unknown	31% of patients present ocular abnormalities with occasional glaucoma [46]
	Congenital cataract	1–6/10000	6–26% following cataract surgery [48]
	Rubinstein-Taybi syndrome	Unknown	15% [51]
	Congenital rubella	Unknown	29% [54]

The mutation of the CYP1B1 gene is involved in 87% of familial and 27% of sporadic cases of congenital glaucoma [63]. Currently 147 mutations of this gene have been found, including point missense, nonsense, frameshift, terminator mutations, deletion, and insertion with significant heterogeneity [67, 68]. CYP1B1 is a drug-metabolizing enzyme of the cytochrome p450 gene family. It is expressed in a large spectrum of tissues including the iris, trabecular meshwork, ciliary body, and anterior uveal tract of the eye [69]. Ocular development is associated with the signalling molecule, metabolized by the gene product, but the procedure still needs clarification [68]. Primary congenital glaucoma is not related to juvenile or adult onset open angle glaucoma linked to the MYOC (TIGR) gene on chromosome 1q25 at locus GLCIA [70], but in some cases a mutation in the CYP1B1 has been found [71].

Alteration of the trabecular meshwork leading to a reduction of aqueous outflow results in childhood glaucoma. It may be a consequence of developmental defects or trabecular structure immaturity [72, 73]. There is no documentation of imperforate Barkan's membrane over the anterior chamber angle, although the trabecular meshwork anomaly was initially described as a "clothed membrane with a shagreened surface" [74–76]. The histopathology of childhood glaucoma is characterized by five different features. One such aspect is an anterior insertion of the iris and ciliary body; thus, the trabecular meshwork is inconsistently exposed to aqueous deflux [76]. The overlap between the ciliary body in its anterior section and the trabecular meshwork in its posterior part leads to poor development of the angle recess. The ciliary muscle, with its longitudinal fibers, passes the internal tip of a poorly developed scleral spur; thus, it obtrudes in the corneoscleral meshwork [77]. The second feature consists in taut and thickened trabecular beams in the meshwork [62]. A third characteristic is a less porous and thicker juxtacanalicular meshwork [76, 78, 79] possibly due to scarce differentiation [73]. Fewer vacuoles in Schlemm's canal endothelium have been described in eyes affected by congenital glaucoma; this may be either an artefact or a consequence of the decrease of aqueous flow [76, 78, 79]. Finally, agenesis

Table 3: Epidemiology and glaucoma in rare diseases that do not usually include congenital glaucoma among their manifestations.

Disease	Secondary glaucoma	
	Prevalence of disease	Percentage of glaucoma in the disease
Rare diseases		
Otopalatodigital syndrome type II	Unknown	One case [55]
Complete androgen insensitivity	1–9/1000000	One case [56]
Pseudotrisomy 13	Unknown	One case [57]
Brachmann-de Lange syndrome	1–9/100000	One case [58]
Acrofrontofacionasal dysostosis with genitourinary abnormalities	Unknown	One case [59]
Wolf-Hirschhorn syndrome	1–9/1000000	One case [61]

of Schlemm's canal has been described; it occurs rarely and probably indicates more severe maldevelopment [73, 80].

Similarity of the angle histopathology between some cases of secondary childhood glaucoma and primary congenital glaucoma suggests that a common pathophysiogenetic mechanism related to angle development may exist.

3.2. Mesodermal Dysgeneses of the Neural Crest. Secondary childhood glaucoma in conjunction with ocular or systemic disorders can be gathered in different subgroups according to their etiology. It can be associated with mesodermal dysgenesis of the neural crest. Several syndromes result from an abnormal development of neuroectodermal cells [13]. In the eye, anterior chamber development follows the migration of neural crest cells forming keratocytes, corneal endothelium, iris stroma, melanocytes, trabecular meshwork, and juxtacanalicular tissue [13]. The gamma of anterior segment dysgeneses, or Axenfeld-Rieger syndrome, is not linked to genetic factors in congenital glaucoma. Mutations in the PITX2 and FOXC1 transcription factor genes account for anterior segment dysgeneses, which are autosomal dominant disorders [81, 82]. A developmental arrest or an abnormal migration of neural crest cells, occurring late in gestation, may lead to chamber malformation resulting in Axenfeld-Rieger anomaly, Peter's anomaly, aniridia or iris hypoplasia, sclerocornea, megalocornea, and congenital glaucoma [13, 83]. These anomalies together with the Marfan and Weil-Marchesani syndromes are linked to glaucoma because of an abnormal anterior chamber angle that effects the drainage of aqueous humour [13].

3.3. Phakomatoses. Several cases of secondary congenital glaucoma have been reported in association with the phakomatoses and hamartomas. Neurofibromatosis type 1, encephalotrigeminal angiomatosis (Sturge-Weber syndrome and variants such as Klippel-Trenaunay syndrome), retinal and cerebellar angiomatosis, and oculodermal melanocytosis are included among these multisystem disorders [3, 84]. Glaucoma onset is seldom encountered in neurofibromatosis type 1 and the pathophysiological mechanisms reported are infiltration of the anterior chamber by neurofibromas, Lisch nodules in the chamber angle leading to secondary angle closure, increase in thickness of the ciliary body and choroid, and developmental angle abnormalities [85–88]. Secondary childhood glaucoma is frequently associated with the Sturge-Weber and Klippel-Trenaunay syndromes and with phakomatosis pigmentovascularis which have the facial naevus flammeus in common. This is due to anterior chamber malformation or raised episcleral venous pressure [30, 89–91]. In phakomatosis pigmentovascularis glaucoma can also be due to an increase in melanocytes which clog the angle. Glaucoma is not strictly part of the manifestations of the other phakomatoses, namely, Von Hippel-Lindau disease, which is characterized by retinal angiomatosis and sclerosis tuberous complex characterized by retinal hamartomas. In these diseases glaucoma has only been reported among the complications of retinal detachment [92].

3.4. Metabolic Disorders. Congenital glaucoma may secondarily occur in the context of metabolic disorders such as mucopolysaccharidoses, Lowe's syndrome, and hyperhomocysteinemia [3]. In these diseases the incidence of secondary glaucoma is attributed to both an anomalous structure of the anterior chamber and engorgement of the trabecular meshwork by extracellular material [3, 29, 84, 93, 94]. The mucopolysaccharidoses are inherited conditions with intra- and extracellular accumulation of glycosaminoglycans (GAG). All subtypes show ocular disorders among which are opacification of the cornea, retinal pathology, and secondary glaucoma [95]. Narrowing of the angle and obstruction of trabecular outflow are secondary to an accumulation of GAG within trabecular cells. [95].

Another condition in this group of metabolic disorders is Lowe's syndrome (oculocerebrorenal syndrome), a rare X-linked, recessively transmitted disease [28] which is characterized by a mutation of phosphatidylinositol 4,5 biphosphate 5-phosphatase, resulting in intracellular accumulation of its PIP2 substrate leading to systemic anomalies involving the kidneys, central nervous system, and the eyes [96]. Classical homocystinuria follows an autosomal recessive mode of inheritance and is a disorder of methionine metabolism [97] due to deficiency of cystathionine b-synthase (CbS) [30–32]. Ectopia lentis represents the most common clinical anomaly leading to diagnosis in most cases [34, 35]. Anterior dislocation of the lens sometimes leads to acute pupillary block with a resulting secondary glaucoma [34].

3.5. Mitotic Disorders. Mitotic disorders such as juvenile xanthogranuloma may present secondary congenital glaucoma as their ocular manifestation [84]. Juvenile xanthogranuloma is due to histiocytic abnormalities that rarely occur in the pediatric age [98, 99]. This disease can damage various ocular structures, but the iris is most frequently affected [100]. The

onset of secondary glaucoma is frequently related to the presence of iris nodules that, due to their vascular nature, may bleed causing hyphema [101].

3.6. Other Congenital Conditions. Secondary childhood glaucoma can also be associated with other disorders such as persistent hyperplastic primary vitreous, congenital cataract, Patau's syndrome, Rubinstein-Taybi syndrome, or congenital rubella [84, 102, 103]. Secondary childhood glaucoma is often associated with congenital cataract and it may occur as postoperative complication of pediatric cataract surgery [48]. Different ocular anomalies are associated with Rubinstein-Taybi syndrome, which results from microdeletions at chromosome 16p13.3 or from mutations in the gene for the CREB binding protein (CBP), located at 16p13.3 [104, 105].

3.7. Acquired Conditions. Uveitis and steroid induced glaucoma are among the major acquired conditions associated with childhood glaucoma. Trauma induced glaucoma can be caused by hyphema, angle recession, and ectopia lentis. Ocular or orbital, benign or malignant, tumors and retinopathy of prematurity can also be a cause. Retinopathies and some drugs can also cause secondary glaucoma and the pathophysiogenetic mechanism is specific to each condition [106–108].

3.8. Rare Diseases. There are some rare diseases, which do not normally present glaucoma in their manifestation.

Kondoh et al. published a case of bilateral glaucoma in a male child presenting otopalatodigital syndrome type II (OPD II) related to a mutation of the gene for filamin A (FLNA). Performing a sequence analysis of the FLNA gene, a missense C to T mutation at position 588, resulting in an Arg196Trp change in the filamin A protein, was found [55]. FLNA gene encodes an actin-binding protein and mutations of the FLNA gene have been found in OPD II, Melnick-Needles syndrome, or frontometaphyseal dysplasia. The association between OPD II, congenital heart defects, and ocular disorder, as presented by Kondoh et al., has not been previously reported. Therefore, additional factors are probably involved in the pathogenetic mechanism behind the OPD-group alterations [55].

Gad et al. reported an association of complete androgen insensitivity with hypertrophic pyloric stenosis and congenital glaucoma in an Egyptian newborn. Performing a sequence of the five exons of the 5a-reductase type 2 gene, no evidence of mutation was obtained while a C to T mutation, which resulted in substitution of the phenylalanine residue by a leucine at position 804, was identified in exon 6. The father's family had a history of glaucoma, ruling out the causative effect of the receptor gene in the pathogenesis of glaucoma and exon 6 of the androgen receptor gene of the mother was normal; therefore, the mutation was considered de novo. It is possible that defective androgen action determined the congenital glaucoma. Moreover, it is interesting to note that CYP1B1 gene, which is the most common early-onset glaucoma gene, is related to steroids. The CYP1B1 gene lies next to the SRD5A2 gene on 2p21 and both the androgen receptor and SRD5A2 genes are expressed in eye structures. However, the consequences of defective signalling pathways on the structure and function of ocular tissues and the correlated gene are still unknown [56].

Sandal et al. reported the only case of congenital glaucoma in a female infant affected by pseudotrisomy 13. Detailed genetic examination, conventional karyotype, and microarray studies are necessary to assess anomalies that are not usually related to this syndrome [57].

Barry Lee et al. first reported a case of buphthalmos and childhood glaucoma associated with Brachmann-de Lange syndrome. Chromosome 3q16 is probably related to Brachmann-de Lange syndrome, while some forms of heritable childhood glaucoma have been mapped to chromosomes 1 and 2. Further genetic studies could be useful to assess a correlation between these disorders [58].

Chaabouni et al. reported the first case of congenital glaucoma in a patient who suffered from acrofrontofacionasal dysostosis with genitourinary abnormalities [59]. As far as we know, this is one of just three case reports in this condition, which has autosomic recessive transmission. The paucity of cases precludes our understanding of the ophthalmological anomalies. Guirgis et al. first reported a case of childhood glaucoma associated with caudal regression syndrome in a 10-week-old infant. The child also had punctal atresia and significant myopia. Embryonic defect of caudal mesodermal development seems to be the cause of caudal regression syndrome [109]. However, goniodysgenesis, caused by neural crest alterations, probably led to childhood glaucoma. A faulty development of surface ectoderm is related to punctual atresia. The embryopathic nature of both caudal regression syndrome and childhood glaucoma, as well as their low incidence, implies that there might be a common cause rather than a coincidence. Furthermore, concomitant punctual atresia validates the notion of embryopathic linkage [110].

Dickmann et al. tried to analyze the relationship between Wolf-Hirschhorn syndrome and ocular defects, specifically to correlate ocular findings with the extension of deletion on chromosome 4, so they investigated a population of 10 patients affected by Wolf-Hirschhorn syndrome [61]. This condition is a genetic disorder that occurs as a consequence of partial deletion of the short arm of chromosome 4; the proximal breakpoint occurs between 4p15.1 and 4p16.3, and the smallest and largest deletion sizes recorded were 2.6 Mb and 20 Mb, respectively [61, 111, 112]. Ocular anomalies appeared in all patients, but just one patient was affected by childhood glaucoma. Comparing genotype with ocular findings, severe ocular abnormalities were associated with large 4p deletions while mild ocular disorders were independent of the deletion size [61]. The only case of childhood glaucoma was observed at birth; it appeared with concomitant corneal clouding, iris coloboma, and cataract. It was associated with a large 4p deletion [61, 113].

4. Management

The signs and symptoms of childhood glaucoma are closely linked to the age of onset and the gravity of the disease;

however, some children are asymptomatic. Corneal alterations can be edema, Descemet's membrane breaks, and opacification. This leads to the typical manifestations of photophobia, epiphora, and consequent blepharospasm. In some cases a misinterpretation of these symptoms delays diagnosis [62]. Improved instrumental technology with optical coherence tomography and ultrabiomicroscopy can provide details on the anterior chamber and ciliary body which is helpful towards diagnosis [114, 115]. The management of childhood glaucoma is also facilitated by new instrumentation to measure intraocular pressure and visual field defects [116–119].

Any type of glaucoma leads to irreversible vision loss in the long term; therefore, the goal of both medical and surgical management is to prevent visual deterioration [62]. Treatment should be carefully tailored, aiming to control intraocular pressure in the course of time. Surgical procedures represent the mainstay of therapy for childhood glaucoma. Although medical therapy alone is rarely effective, it plays an important role as adjunctive treatment to surgery; moreover, it can also be used temporarily before surgery to clear the cornea [62]. Ophthalmologists who use medication should be aware of different risk and benefit profiles in infants and children compared with adults. Allergic reactions and ocular surface toxicity may complicate repeated topical treatment initiated at an early age in childhood [62].

4.1. Medical Treatment. Beta-blockers are usually well tolerated and severe systemic complications are rare in pediatric patients treated with timolol, but bradycardia and bronchospasm can occur especially with the concentration of 0.5%. Cautious initial treatment with low concentrations of timolol is wise. Punctal occlusion after topical timolol determines a decrement of beta-blocker plasma levels, so the use of punctual occlusion after every drop application is advised [62].

Oral carbonic anhydrase inhibitors represent the most efficacious drugs in the reduction of intraocular pressure in childhood glaucoma. Nevertheless it is important to be aware that these can cause decreased appetite and hyperpnea from renal acidosis, dehydration, chronic fatigue, and failure to grow, so prolonged use should be avoided [62]. Topical carbonic anhydrase inhibitors such as dorzolamide and brinzolamide represent a valid alternative to oral administration with less risk of systemic side effects. They reduce intraocular pressure by 25% [120, 121]. Carbonic anhydrase inhibitors and beta-blockers are both suppressors of aqueous production but are used in conjunction in order to yield additive benefit [122]. Brimonidine is a selective alpha-agonist, which reduces intraocular pressure increasing uveoscleral outflow and decreasing aqueous production [123]. Since brimonidine crosses the blood brain barrier, it can lead to nervous systemic toxicity [124]. Brimonidine topical therapy has also been reported to induce bradycardia, hypotension, hypotonia, hypothermia, apnea, and unresponsiveness in children [125–128].

Prostaglandin analogues such as bimatoprost, travoprost, and latanoprost are not effective in pediatric patients. Although in some cases of children affected by primary juvenile glaucoma and glaucoma related to Sturge-Weber syndrome a significant intraocular pressure reduction was found [129, 130], further research is necessary in order to assess the performance of prostaglandins in the treatment of childhood glaucoma.

Miotics such as pilocarpine are not effective for childhood glaucoma. In older children, symptoms of spasm of the ciliary muscle and blurring of vision from induced myopia occur. They can be used for aphakic glaucoma and are adoperated before goniosurgery [62]. Most often childhood glaucoma is better managed surgically rather than medically [62].

4.2. Surgical Treatment. Surgical treatment can be separated into procedures that improve aqueous outflow through the drainage channels (goniosurgery such as goniotomy and trabeculotomy), treatments that by destroying the ciliary body reduce aqueous humor production (cyclodestruction), or surgical procedures that drain aqueous through an alternative way (trabeculectomy, glaucoma drainage devices) [131].

While for primary congenital glaucoma goniotomy represents the procedure of choice, this indication is less certain for other forms of childhood glaucoma [62]. An alternative surgical treatment is trabeculotomy [62]; it appears as a more appropriate choice especially when a cloudy cornea prevents a comfortable visualization of the angle [62]. Filtering surgery may be indicated if goniosurgery procedures are unsuccessful [3]. Trabeculectomy is indicated if an unacceptable elevation of intraocular pressure persists after an adequate number of angle incision procedures [62]. Implant surgery represents a valid alternative for patients with conditions proven to be refractory to goniosurgery or filtering surgery, who are poor candidates to these procedures, or for whom these treatments have been revealed unsuccessful [62]. Long tube drainage device surgery is needed in severe cases of primary congenital glaucoma and, sometimes, in secondary glaucomas [3].

Cyclodestructive procedures can be defined as an intermediate or an additional procedure, useful when primary trabecular surgery has failed [3]. Cyclocryotherapy or both transscleral and endoscopic diode laser cyclophotocoagulation are used for the ablation of ciliary epithelium. The surgeon must consider the anatomic improvement, the long-term visual benefit, and the patient's expectations for the affected eye when making the choice for management strategies [62].

5. Conclusions

There is a wide array of systemic diseases associated with childhood glaucoma. Some conditions like dysgenesis of the anterior chamber associated with neural crest disorders and the phakomatoses are better known. However, there are rare diseases which are occasionally associated with glaucoma. Since the examination of pediatric patients is not always straightforward, ophthalmologists should be updated on the available literature and should be well aware of the conditions, which may lead to visual deterioration due to glaucoma. Treatment should be carefully tailored, in order to control intraocular pressure in the long term. Surgical treatment is

the mainstay of management and medical therapy has an adjunctive role. Furthermore, medication should be carefully monitored due to different risk and benefit profiles in infants and children compared with adults. A multidisciplinary approach to pediatric patients with rare multisystem diseases is highly advisable in both the diagnosis and management of the conditions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Ophthalmic Alterations in the Sturge-Weber Syndrome, Klippel-Trenaunay Syndrome, and the Phakomatosis Pigmentovascularis: An Independent Group of Conditions?

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The phakomatoses have been traditionally defined as a group of hereditary diseases with variable expressivity characterized by multisystem tumors with possible malignant transformation. The Sturge-Weber syndrome, Klippel-Trenaunay syndrome, and the phakomatosis pigmentovascularis have the facial port-wine stain in common. Numerous pathophysiogenetic mechanisms have been suggested such as venous dysplasia of the emissary veins in the intracranial circulation, neural crest alterations leading to alterations of autonomic perivascular nerves, mutation of the GNAO gene in the Sturge-Weber syndrome, PIK3CA mutation in malformative/overgrowth syndromes such as the Klippel-Trenaunay syndrome, and the twin-spotting phenomenon in phakomatosis pigmentovascularis. Other features linked to the port-wine stain and typical to all of the three conditions are glaucoma and choroidal alterations. Glaucoma can be due to malformations of the anterior chamber or high episcleral venous pressure and in phakomatosis pigmentovascularis it can also be associated with angle hyperpigmentation. The choroid can be thickened in all diseases. Furthermore, choroidal melanocytosis in the phakomatosis pigmentovascularis can lead to malignant transformation. Although the multiple pathophysiological mechanisms still require clarification, similarities in ophthalmic manifestations make it reasonable to classify these diseases in an independent group.

1. Introduction

The Sturge-Weber syndrome (SWS) and Klippel-Trenaunay syndrome (KTS) were included in the phakomatoses together with neurofibromatosis, tuberous sclerosis, and von Hippel-Lindau syndrome in 1937 [1]. In support of this hypothesis, and based on histopathological observations, Hogan and Zimmerman [2] in 1962 suggested that the phakomatoses are multisystem hamartoses regardless of the risk of malignant transformation. Since then many authors have included SWS and KTS in the group of phakomatoses whereas others have defined them as "the odd men out" [3–7]. The facial port-wine stain is a characteristic of the SWS, KTS, and phakomatosis pigmentovascularis (PPV). Furthermore, glaucoma and thickened choroid, linked to the port-wine stain, are recurrent ocular findings in all three conditions. Various pathophysiological mechanisms have been proposed, but the clinical similarities, ophthalmic manifestations in particular, make it reasonable to classify these diseases as an independent group.

2. Sturge-Weber Syndrome

The earliest case regarding SWS was reported in 1860 by Schirmer. The patient had bilateral facial nevus as well as unilateral buphthalmos [8]. In 1879, Sturge reported on a case with bilateral facial nevus, vascular deformity, and congenital glaucoma in the right eye and spasms affecting the patient's left side of the body [9]. Then, in the year 1922, the first radiographic evidence of intracranial calcifications was brought forth by Weber [10]. The ophthalmologist van der Hoeve was the first to describe the phakomatoses as a clinical entity of diseases including tuberous sclerosis, neurofibromatosis, and von Hippel-Lindau and Sturge-Weber syndromes [11].

SWS also known as encephalotrigeminal angiomatosis includes naevus flammeus, also known as port-wine stain (PWS), and ipsilateral leptomeningeal angiomatosis as the main features [6]. Estimated frequency is about one in 50,000 live births. This syndrome affects both men and women at a seemingly parallel rate [12].

The pathogenesis of the port-wine stain (PWS) is still not completely understood, but it is linked to progressive ectasia of the superficial cutaneous vascular network [13, 14]. Some authors have suggested that the PWS is related to disorders of neural crest cells [15, 16]; ultrastructural and immunohistochemical studies have demonstrated the absence of perivascular nerves in PWS [14, 17] favouring the hypothesis of an alteration of autonomic nerves surrounding blood vessels which causes deficits of vessel caliber modulation [14, 18]. In the recent years, various authors have proposed that the SWS (and the KTS) should not be classified among other phakomatoses as there is no hereditary pattern or predisposition and the manifestations of both conditions are those of hypertrophy rather than the hyperplasia characteristic to phakomatosis [19], and there is no malignant transformation [11]. In original studies, Parsa elaborated a pathophysiologic mechanism attributing the vascular ectasia in PWS to dysplasia of the emissary veins in the peripheral intracranial circulation resulting in increased retrograde venous pressure within the communicating vessels and the superficial venous plexus of the skin implying that SWS and KTS are products of "acquired venous obstruction rather than neural dysfunction" [20]. Moreover, the author suggested that when venous dysplasia involves the limbs it causes tissue hypertrophy [19, 20]. The presence of combined SWS and KTS has been challenged and it has been advanced that patients diagnosed with KTS who present capillary deformities at a level inferior to the head, in the absence of lymphatic pathologies, are actually afflicted with a variant of SWS [21].

Shirley et al. recently identified a mutation in the GNAO gene, which stimulates the proliferation of cells and inhibits apoptosis by a surge in downstream signaling through the RAS effector pathways [22]. The mutation probably takes place earlier on in SWS with respect to isolated PWS and seems to underlie both of these conditions [22].

Histological evidence has shown that both cerebrovascular and cutaneous lesions correspond to initial localized venous dysplasia during the 4th and 8th week of pregnancy [23]. Some neurologists have suggested that during week 9 the vascular plexus fails to regress in SWS. A lack of ordinary leptomeningeal vessels and blockage due to vascular deformity may lead to hypoxia, ischemia, stasis, and a decline in neuronal metabolism, which is noted particularly when seizures occur [23]. Clinical signs of SWS usually consist of unilateral facial PWS, ipsilateral glaucoma, hemianopia, hemiatrophy, progressive seizures, contralateral hemiparesis, and mental deficiencies [24]. Intracranial lesions appear in the manner of gyriform or "tram-line" calcifications that tend to engage the occipital and parietal lobes, leptomeningeal angiomatosis, neuronal loss, and astrogliosis in brain tissue [25].

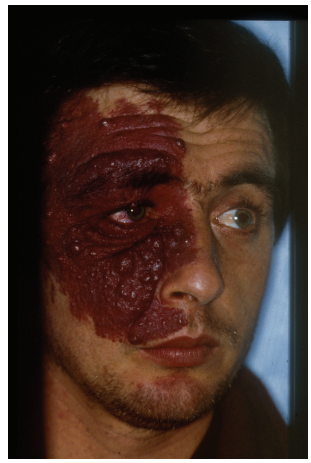

Figure 1: Facial port-wine stain in a patient with Sturge-Weber syndrome.

Stemming from vascular deformities, which affect the face, eyes, and the leptomeninges, the syndrome's manifestations have been divided depending on vascular deformity distribution.

2.1. Cutaneous Signs. The most prominent clinical finding of SWS is unilateral facial PWS from birth (Figure 1).

It is a well-defined macular lesion initially pink in colour with a smooth surface that, unlike hemangiomas, partially blanches with pressure [26]. The lesion develops proportionally with the child and usually gets darker in color [27]. The skin over the PWS can present nodularity or hypertrophy in about 60% of patients above the age of 50 [28] (Figure 2).

PWSs have been commonly described to affect the first sensory distribution of the trigeminal nerve; however, they can even engage the second and the third distribution areas and may be diffuse and bilateral [29]. Waelchli et al. suggested that facial PWSs appear to trace the face's embryonic vasculature as opposed to the trigeminal nerve. Furthermore, they suggest that the prediction of SWS can be based on facial PWS phenotype where those involving the forehead are associated with seizures, neurodevelopmental abnormalities, atypical brain magnetic resonance imaging (MRI), or glaucoma, suggesting diagnosis of SWS [30].

Histopathologic research has shown comparability between SWS related and isolated cases of nevus flammeus.

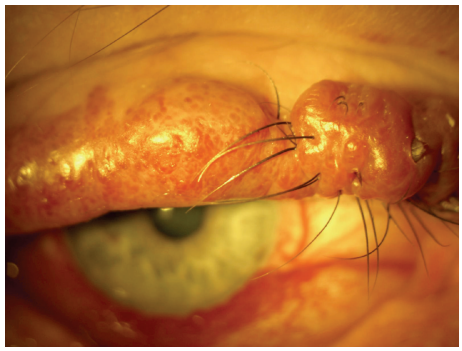

Figure 2: Port-wine stain of the upper lid with nodularity in a patient with Sturge-Weber Syndrome.

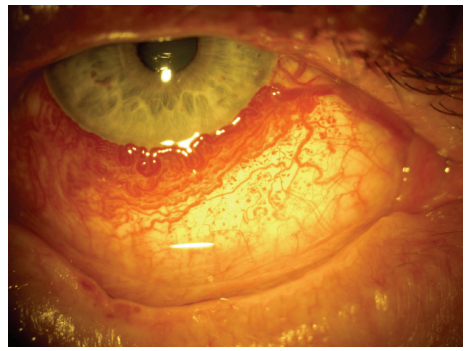

Figure 3: Diffuse conjunctival vascularity in a patient with Sturge-Weber Syndrome.

Principle differences include an increase in dilated thin-walled capillaries and venules mainly situated in the superior segment of the reticular dermis in SWS [31].

2.2. Cerebral Findings. Leptomeningeal angiomatosis often affects the parietal and occipital lobes and is characterized by numerous vessels with thin walls, characteristically enlarged and tortuous, within the leptomeninges. These particular vessels are subject to thrombi [32]. A typical sign of SWS is seizure due to microcirculatory disorders and hypoxia. Seizures that occur in patients who have developed the syndrome by 3 years of age tend to take place on the contralateral side to neurocutaneous signs. These seizures affect 70–90% of patients and get worse with time [33, 34]. Infantile spasms and generalized seizures have also been reported [24]. Further consequential neurological signs to leptomeninges angioma include cephalgy, contralateral hemiparesis, stroke-like episodes, hemianopia, and hemiatrophy. Mental retardation also develops in about 50–60% of patients affected by SWS [35].

MRI is superior to computerized tomography in radiologically outlining typical gyriform parietal and occipital calcifications. Intracranial calcifications are present in roughly 90% of younger patients. Infants, however, may present minimal or total absence of calcifications. The examination of choice to evaluate the extent of vascular deformities is MRI with contrast [35].

2.3. Ocular Manifestations. In as many as 50% of cases the eye is affected. Ocular circulation might be abnormal when skin lesions involve the eyelids. Increased conjunctival vascularity usually produces a pinkish discoloration, which can be diffuse or localized in areas of the bulbar conjunctiva especially in the limbus zone (Figure 3).

Of all SWS related ocular complications, the most common is glaucoma, which affects 50% to 70% of SWS patients [36, 37].

Anterior chamber angle anomalies cause infant glaucoma in roughly 60% of patients while raised episcleral venous pressure triggers glaucoma in 40% of youth and young adult patients [36]. Thus, glaucoma corresponds to two forms depending on the age of development: early-onset (congenital) or later-onset forms. The affected eye presenting glaucoma tends to almost always be ipsilateral to the PWS [37]. The risk of glaucoma is higher when the PWS involves both lids of the eye as opposed to the upper lid only, 72% versus 21%, respectively [36].

There are two major theories on the pathogenesis of glaucoma.

(a) The first is a mechanical pathogenesis linked to a malformation of the anterior chamber angle with consequent increase of resistance to the outflow of the aqueous humor. This is not necessarily associated with flat anterior iris insertion, which is a feature of the congenital form [37].

(b) The second theory involves a high episcleral venous pressure, theorized by Weiss in 1973 [38], according to which the arteriovenous shunts are the cause of high episcleral pressure in patients with episcleral hemangioma. This pathophysiological mechanism is supported by reports of the presence of blood within Schlemm's canal [38, 39]. Also, glaucoma is more pronounced in the presence of a correspondingly greater episcleral hemangioma [40].

Maruyama et al. described a patient with SWS who developed angle-closure glaucoma and showed posterior scleritis with edema of the ciliary body, ciliochoroidal effusion, and anterior rotation of the ciliary body, as well as inflammation of the crystalline lens, which caused closure of the chamber angle [41]. Various disorders can cause ciliochoroidal effusion: venous congestion, ocular inflammation, and other factors or drugs associated with traumatic, idiopathic, neoplastic, and systemic diseases [42, 43]. Acute glaucoma arises as the consequence of ciliary body effusion, which moves forward the iridolenticular diaphragm reducing the amplitude of the anterior chamber angle. This can be observed with ultrasound biomicroscopy, which provides images of the anterior segment, chamber angle, and the ciliary body [44].

Light microscopy has shown clusters of vascular formations in the trabecular meshwork near the scleral spur surrounded by large homogeneous extracellular matrix. Electron microscopy showed that the endothelial layer lining

of Schlemm's canal was associated with basal lamina. The endothelial cells contained several villi and giant vacuoles, which appeared to be transcellular channels [45].

The choroid is the site of the most important vascular alteration associated with SWS. Hemangiomas of the choroid occur in two specific forms: circumscribed forms typically occur in patients with no other systemic disorders and diffuse forms are seen in SWS [46]. In most cases, an increase of well-formed choroidal vessels gives the fundus a consistent bright red or red-orange color [19]. Choroidal hemangiomas usually remain asymptomatic throughout childhood. However, in adolescence or adulthood, the choroid sometimes becomes markedly thickened [47].

Histological features of choroidal angioma in SWS are different from those of circumscribed choroidal angiomas. In SWS, choroidal vasculature does not show proliferation of vessels, pericytes, or endothelial cells [48].

Degeneration or detachment of the overlying retina is a severe complication [49]. Vision loss can consequentially occur following subretinal hemorrhage and serous retinal detachment. Further complications are photoreceptor alteration, cystoid macular oedema, and serous detachment of the neuroepithelium in the macular area [50, 51].

Diagnosing choroidal angioma is achieved through fundus examination with indirect ophthalmoscopy, by which the difference in color of the fundus between fellow eyes can be distinguished and the extension of choroidal involvement can be determined in some cases. Retinography may also aid in the differentiation in color of fellow eyes. Instrumental examinations, important for the diagnosis of choroidal hemangioma, are ultrasonography, indocyanine-green angiography, and enhanced depth imaging (EDI) spectral domain optical coherence tomography (SD-OCT). Ultrasonography confirms the presence of choroidal lesions together with their extension, echogenicity, and characteristics. Indocyanine-green angiography highlights the extension, intralesional vascularity, and possible arteriovenous communications of the choroidal lesions. Finally, EDI SD-OCT allows for extensive assessment of choroidal and any related retinal alterations at the posterior pole [47]. Furthermore, choroidal thickness and morphology and evaluation of the caliber of dilated choroidal vessels can be evaluated with OCT [52].

2.4. Diagnostic Criteria. The main characteristics of SWS include ipsilateral leptomeningeal angiomatosis in the parietal-occipital lobe, unilateral facial PWS, and congenital glaucoma. It is worth noting that these signs are usually manifested only in part.

SWS is divided into four categories:

(1) classic SWS: leptomeningeal and facial angiomas; glaucoma possible;

(2) PWS without evidence of cerebral involvement;

(3) isolated leptomeningeal angioma;

(4) classic form with other systemic associations, such as tuberous sclerosis.

2.5. Treatment of Port-Wine Stains. For cases of facial PWSs, laser treatment ought to be initiated immediately for optimal results. Success of laser treatment is dependent on the location of the deformity. Lesions affecting the central forehead yield better results as opposed to central facial lesions. Unfortunately, PWSs that are left untreated through the years have a tendency to thicken and get darker and, in some cases, nodularity can even develop [33].

A PWS can be eliminated with deep photocoagulation and debulking surgery if there are hypertrophic alterations [19]. However, according to Parsa, this treatment may create a reduction of cerebral venous outflow through collateral vessels of the PWS, thus, potentially worsening cerebral and ocular blood flow anomalies which could lead to cerebral venous deterioration, increase in intraocular pressure, choroidal vessel dilatation, and detachment of the retina due to exudation. According to this author, careful superficial laser treatment would not affect the deep blood circulation or alter the collateral circulation, avoiding these complications [19].

2.6. Treatment of Glaucoma. In SWS related glaucoma, standard management includes lifelong medical treatment as well as surgery. Controlling intraocular pressure (IOP) for optic nerve damage prevention remains the main objective. According to Yang et al. [53] uveoscleral outflow is due to elevated episcleral venous pressure and causes disruption of natural aqueous drainage processes. In a study by Basler and Sowka [40], IOP in about 50% of patients appeared to be successfully controlled by latanoprost in SWS glaucoma. Topical therapy with beta-blockers and carbonic anhydrase inhibitors in conditions of reduced outflow is effective where buphthalmos is not present [54].

However, topical medication in itself is not sufficient in managing SWS-associated glaucoma. Hence, surgery frequently supplements superior long-term management of IOP. The most appropriate surgical approaches in children under four years of age are trabeculotomy and goniotomy although the long-term results have been disappointing [40]. Second-line treatments are filtering procedures: trabeculectomy, posterior lip sclerectomy [39], and trabeculotomy-trabeculectomy [55, 56]. Trabeculotomy eases outflow by overcoming the anterior chamber angle abnormalities, whereas trabeculectomy bypasses the episcleral venous system by creating an alternative outflow. Topical medications are commonly the first-line therapy for patients who develop glaucoma in later stages of life [40]. Filtering procedures may result in more severe problems including bleeding, expulsive choroidal hemorrhage, and a prolonged flat anterior chamber [57, 58].

van Emelen et al. [59] demonstrated the effectiveness of cryocoagulation in combination with topical treatment in a case series of SWS patients with buphthalmos. The efficacy of cyclocryotherapy in addition to trabeculectomy has also been evaluated; however, the complications of extensive cyclocryotherapy are phthisis bulbi and chronic hypotony [60]. The choice of treatment parameters is critical in order to prevent the onset of ocular hypotony. Cyclodestructive procedures have a greater hypotensive effect.

The use of Ahmed valve implantation, which allows the outflow of aqueous with increased performance on the long term, allows us to avoid trabeculectomy and its high risk of intraoperative hemorrhage and suprachoroidal effusion [61]. The Molteno tube has also been used in a small case series in children suffering from SWS. Though the outcomes were not favourable and there was as an elevated complication rate [62].

2.7. Treatment of Choroidal and Retinal Alterations. The principal motive for visual deterioration in patients with SWS-associated choroidal hemangioma is the accumulation of macular subretinal fluid. Treatment is aimed primarily at reducing tumor leakage and also to the destruction of the tumor itself [63]. The most effective and commonly utilized treatment is photocoagulation. Confluent photocoagulation causes destruction of the tumor resulting in reduced leakage [64]. Intense photocoagulative treatment is associated with many complications; thus, some authors have opted for lighter grid treatment [65, 66]. Nonetheless, subretinal fluid recurrence is more frequent with this less intense treatment.

Photodynamic therapy (PDT) is used to lessen subretinal macular fluid. Theoretically, PDT triggers atrophy of the hemangioma vessels and reduces leakage. PDT has been efficient in treating cases of choroidal hemangioma with noteworthy reduction of leakage, even in tumors adjacent to the fovea [66].

External beam radiotherapy (EBR) is used in treating diffuse choroidal hemangiomas associated with exudative retinal detachment [67, 68]. The results are obtained months after the first application and relapse is frequent; thus repeated applications can cause radiation retinopathy, neuropathy, and cataract [67]. In 1960, MacLean and Maumenee recounted the first attempt using brachytherapy coupled with radon seeds to treat circumscribed choroidal hemangiomas [69]. Brachytherapy with Cobalt-60 [70] and Ruthenium-106 [71, 72] showed satisfactory results in reducing exudative retinal detachment in choroidal hemangiomas. Retinal detachment is a rare complication that is surgically manageable, but in one case of exudative detachment intravitreal antivascular endothelial growth factor therapy with pegaptanib showed good results [73].

3. Klippel-Trenaunay Syndrome

The Klippel-Trenaunay syndrome, which was initially illustrated by Klippel and Trenaunay in 1900 [74], is a rare multisystem disorder, which has an incidence of about 1 : 100,000 with no predilection for gender, race, or geographical area and most cases are sporadic [75, 76]. The characteristic triads of congenital anomalies are PWS, varicose veins, and bony and soft-tissue hypertrophy. When this clinical picture is associated with arteriovenous shunting the condition has also been called the Parks-Weber syndrome [77].

In 1960, Pietruschka suggested that the SWS and KTS are one and the same disease [78]. Sharma et al. suggested that the syndromes are closely related and should be named neurocutaneous angioma since there may be different expressions of a disease with a sole pathophysiological mechanism [79].

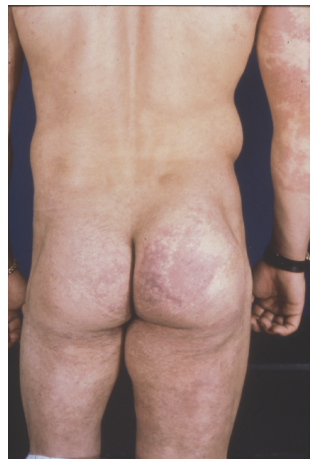

Figure 4: Soft-tissue and bony hypertrophy of the lower limb in a patient with Klippel-Trenaunay Syndrome from [7].

Both conditions share the presence of the PWS. Parsa suggested that the alterations associated with the PWS in KTS arise when venous dysplasia is in the lower extremities (or below the heart level) where venous drainage is poor causing tissue pressure elevation and cellular hypertrophy [19]. Furthermore, lymphatics and veins share a common embryologic origin; thus, lymphatic dysfunction and malformations can occur in the KTS [19]. Kihiczak et al. proposed that KTS may result from vascular and tissue overgrowth due to a specific pathogenic gene [80]. A familial or paradominant inheritance pattern for KTS and a single gene translocation etiology has also been suggested by some authors [81, 82]. Recently, hypermorphic somatic phosphatidylinositol-4,5-bisphosphate 3-kinase and catalytic subunit alpha (PIK3CA) mutations have been found in various patients with malformative/overgrowth syndromes [83]. It has been postulated that the mechanism of malformation and overgrowth during embryogenesis is due to the alteration of multiple signalling pathways including the insulin-like growth factor, vascular endothelial growth factor, and fibroblast growth factor pathways [84].

The condition is commonly seen at birth or early childhood and appears with a PWS, which is present in 98% of patients [85, 86]. Varicose veins are seen more frequently during adolescence and can involve both the deep and superficial venous plexuses; these can be complicated by lymphedema, thrombophlebitis, and ulcers [87]. The cutaneous alteration can be limited to the skin or involve organs such as the colon, liver, spleen, or bladder and can lead to internal hemorrhage [80]. Hypertrophy of soft tissues and bone is more frequently for the lower limbs but any part of the body can be affected with variable extension, which can even be limited to only the fingers or toes or may be severe with massive limb overgrowth [87, 88]. Mental retardation can be encountered especially when patients present hemangiomas of the face and head. Diagnosis of the disease is when at least two signs among the following are present: PWS, varicose veins, soft-tissue, and/or or bony hypertrophy but 63% of diagnosed patients present all three symptoms [89] (Figure 4).

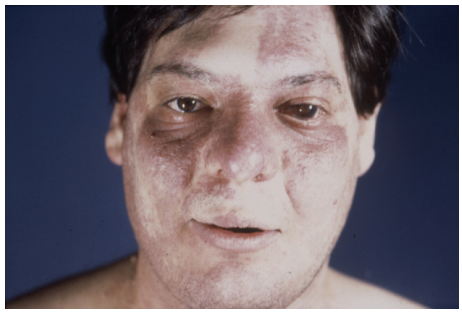

Figure 5: Bilateral facial port-wine stain and glaucoma of the left eye in a patient with Klippel-Trenaunay Syndrome from [7].

3.1. Ophthalmic Features. The most common ophthalmological alterations encountered in the KTS are choroidal hemangiomas similar to those described for the SWS [90]. Glaucoma, also frequently observed, has been associated with anterior chamber malformation [91] or due to raised episcleral venous pressure as in the SWS [38, 39] (Figure 5).

Other ophthalmic alterations have been reported in case studies and consist in: conjunctival telangiectasia, orbital varix, strabismus, oculosympathetic palsy, Marcus-Gunn pupil, iris coloboma and heterocromia, cataracts, persistent fetal vasculature, chiasmal and bilateral optic nerve gliomas, drusen of the optic disk, acquired myelination of the retinal nerve fiber layer, and retinal dysplasia with astrocytic proliferation of the nerve [91–97]. Retinal varicosities have been described and dilated retinal veins have been demonstrated with fluorescein angiography [98].

Very little information is available in the literature regarding the treatment of the ophthalmic manifestations of the KTS but this overlaps with the management of glaucoma and choroidal alterations described for the SWS.

4. Phakomatosis Pigmentovascularis

Phakomatosis pigmentovascularis was first described by Ota et al. in 1947 [99]. Toda in 1966 and Hasegawa and Yashara in 1979 described further variants [100, 101]. It is a neurocutaneous condition where a naevus flammeus is found in association with pigmentary nevi involving the eye (ocular melanocytosis) or the face and body (oculodermal melanocytosis) [99]. PPV has been described prevalently in Asian patients [99, 101, 102] although rare cases in other races have been described [103]. The pigmentary nevi can be nevus spilus or Mongolian spots [99, 104, 105]. Nevus spili are brown, polymorphous spots secondary to epidermis basal layer hyperpigmentation without involvement of the underlying derma [7]. Mongolian spots are formed by an accumulation of melanocytes, which are filled with melanin in the mid and deep derma. They are patchy areas of variable morphology with a colour that can range from bluish to brownish [7]. Ocular melanocytosis or nevo di Ota is when the skin along the ophthalmic, maxillary, and more rarely the mandibular branch of the trigeminal nerve is involved and if the hyperpigmentation only involves the eye it is termed melanosis oculi. 30% of patients have hyperpigmentation of ocular structures [106]. The nevus flammeus or PWS is similar to that occurring in the SWS and KTS [13]. Indeed, PPV has been reported most frequently with SWS and KTS syndromes or SWS alone [103, 107–111].

Neural crest alterations have been long held as a principal cause of many of the manifestations of phakomatoses such as neurofibromatosis [112]. Indeed, research in neurofibromatosis type 1 has shown retinal microvascular alterations [113] even overlying choroidal nodules typical of the diseases [114] suggesting the hypothesis that choroidal and retinal thinning in NF1 could be caused by altered innervation of perivascular vessels due to neural crest cell abnormalities [114]. Furthermore, in PPV oculodermal melanocytosis, Mongolian spots and dermal melanocytosis are derived from aberrant migration of neural crest cells [99, 104]. This theory is based on altered vasomotor regulation, which leads to the formation of phakomas and the naevus flammeus [104, 115]. It is interesting that the association of PPV with neurofibromatosis and Lisch nodules has been described on several occasions [116, 117]. Progress in genetic research has led us to consider that the association of melanocytosis and PWS occurs due to the twin-spotting phenomenon. It is believed that this was produced by somatic recombination where there are two genetically distinct cell clones within an area of cells, which are normal. Therefore, there is a mosaic distribution of alterations and the condition is sporadic without familial transmission [118].

PPV has been classified by various authors [99–101, 104, 106]. Happle divided this group of conditions into 3 distinct types: phakomatosis cesioflammea, phakomatosis spilorosea, and phakomatosis cesiomarmorata [119]. The most frequent type is cesioflammea in 77% of cases followed by spilorosea in 13% of cases and rarely cesiomarmorata; finally, 8% remain unclassified [120]. Phakomatosis cesioflammea features dermal melanocytosis (blue spot) and nevus flammeus, which can be associated with nevus anemicus, focal alopecia, glaucoma, ungueal hypoplasia, and limb asymmetry [118, 121]. Phakomatosis spilorosea involves nevus spilus (speckled freckled nevus) and a telangiectatic nevus with a light pink color (lighter with respect to the naevus flammeus). These can be combined with other symptoms such as hemiparesis, seizures, lymphedema, and limb asymmetry [118]. Finally, the rare phakomatosis cesiomarmorata is the combination of nevus caesius (Mongolion spot or blue-gray nevus) and cutis marmorata telangiectatica congenita, which can be associated with blue sclera, leg hyperplasia, and asymmetric cerebral hemispheres [118].

Ocular manifestations in PPV encompass hyperpigmentation of the conjunctiva, sclera, episclera, iris, trabecular meshwork, and the choroid [118, 122]. Furthermore, melanocytosis of the corneal stroma and pigmentary deposits on the lens have been described [106, 123]. Iris mammillations are also found in association with melanosis oculi [124–126]. These are protuberances, which can have a smooth, villiform, conical, or stellate appearance that can partially or completely cover the iris on its surface giving it a dark and smooth appearance [126]. At times, these mammillations can be confused with Lisch nodules typical of neurofibromatosis

type 1, but Lisch nodules have an irregular distribution on the iris face and are variable in number and dimensions [126, 127]. Ten percent of patients with oculodermal melanocytosis present glaucoma and the mechanism can be due to angle hyperpigmentation or the increase in aqueous outflow resistance due to melanocytes [128]. Congenital or developmental glaucoma [106, 123, 129] has been suggested due to abnormal neural crest development, which leads to anomalous anterior chamber angle [122]. In nine cases described by Teekhasaenee and Ritch, all patients, with both melanocytosis involvement and episcleral vascular involvement for 360 degrees, developed congenital glaucoma [122]. The authors concluded that there is high risk for congenital glaucoma when there is extensive ocular involvement. Partial involvement of the globe, however, predisposes to high IOP, which can develop later in life. Naevus flammeus has been reported to be more strongly associated with glaucoma than oculodermal melanocytosis and the mechanisms involved are those described in the section regarding glaucoma in the SWS.

Patients with oculodermal melanocytosis have a greater risk to develop melanoma of the uvea, 1 in 400 white patients with oculodermal melanocytosis with respect to 6 per million for the general population [130]. In PPV, melanocytosis of the fundus has frequently been described and choroidal melanoma can develop as shown in 3 of 6 patients described by Shields et al. in 2011 and 5 patients by Tran and Zagrafos [118, 131]. Ocular melanocytosis is not always readily visible on external examination and careful fundus examination of patients with PPV, in order to monitor for uveal melanoma, is strongly advised by some authors [70, 118]. Ultrasound biomicroscopy is also advised for evaluation of the ciliary body [132].

There is very little information in the available literature on the management of ophthalmic conditions in the phakomatosis pigmentovascularis. Filtering surgery in association with antimetabolites has been reported [118]. However, similar to reports in the SWS, there is a high risk of suprachoroidal hemorrhage during surgery.

5. Conclusions

Our present knowledge on the multiple pathophysiological mechanisms involved in SWS, KTS, and the PPV does not allow us to determine whether these rare conditions should be included among the phakomatoses. It would seem reasonable to embody these diseases in a group of their own as there may be diverse manifestations of the same spectrum, which would explain the similarities in some clinical aspects. The complex pathophysiology and the role of the neural crest, venous dysplasia, and novel mutations require further clarification.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Diagnosis and Management of Iridocorneal Endothelial Syndrome

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The iridocorneal endothelial (ICE) syndrome is a rare ocular disorder that includes a group of conditions characterized by structural and proliferative abnormalities of the corneal endothelium, the anterior chamber angle, and the iris. Common clinical features include corneal edema, secondary glaucoma, iris atrophy, and pupillary anomalies, ranging from distortion to polycoria. The main subtypes of this syndrome are the progressive iris atrophy, the Cogan-Reese syndrome, and the Chandler syndrome. ICE syndrome is usually diagnosed in women in the adult age. Clinical history and complete eye examination including tonometry and gonioscopy are necessary to reach a diagnosis. Imaging techniques, such as in vivo confocal microscopy and ultrasound biomicroscopy, are used to confirm the diagnosis by revealing the presence of "ICE-cells" on the corneal endothelium and the structural changes of the anterior chamber angle. An early diagnosis is helpful to better manage the most challenging complications such as secondary glaucoma and corneal edema. Treatment of ICE-related glaucoma often requires glaucoma filtering surgery with antifibrotic agents and the use of glaucoma drainage implants should be considered early in the management of these patients. Visual impairment and pain associated with corneal edema can be successfully managed with endothelial keratoplasty.

1. Introduction

Iridocorneal endothelial (ICE) syndrome is a rare disorder (ORPHA64734 available at http://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=en&Expert=64734) characterized by proliferative and structural abnormalities of the corneal endothelium, progressive obstruction of the iridocorneal angle, and iris anomalies such as atrophy and hole formation [1]. The consequences of these changes are cornea decompensation and glaucoma, which represent the most frequent causes of visual function loss in patients with ICE syndrome [2]. The ICE syndrome comprises a spectrum of clinical entities: progressive essential iris atrophy, Cogan-Reese syndrome, and Chandler syndrome [3].

In 1903 Harms extensively described a rare ocular condition characterized by iris atrophy and glaucoma, known as "progressive essential iris atrophy" [4, 5]. Five decades later, Chandler described a rare, unilateral ocular condition characterized by iris atrophy associated with corneal endothelial alterations, corneal edema, and glaucoma [6]. Subsequently, it was suggested that this "Chandler syndrome" and the "progressive essential iris atrophy" are two different forms of the same disease [6, 7]. When Cogan and Reese described a similar condition associated with iris nodules, a third clinical entity was identified and subsequently named "iris nevus" or "Cogan-Reese syndrome" [8–10]. Subsequent studies confirmed that these clinical entities show similar history and clinical findings and share the same pathogenic mechanisms characterized by an abnormal proliferation of corneal endothelium and the unifying term of "iridocorneal endothelial syndrome" was suggested by Yanoff [1, 3, 7, 9, 11].

ICE is sporadic in presentation; it is usually unilateral and typically affects adult patients (more often women in the

third to fifth decade) and eventually severely compromises the visual function if not properly treated [1]. Even when they are promptly treated, surgical interventions for these conditions have variable success rates and the management of ICE syndrome represents a challenge for ophthalmologists.

2. Etiology

The etiology of ICE syndrome is still largely unknown; however, a series of possible triggering events has been described and the debate on ICE syndrome's etiology is still ongoing after more than a century.

Inflammation in patients with the ICE syndrome was mentioned in few early reports and more than one author described the onset of uveitis in these patients [10, 12]. Scheie and Yanoff reported clumps of chronic inflammatory cells in the iris and vitreous in one eye examined histopathologically, and Shields and colleagues observed anterior chamber inflammation in 3 cases [1, 10]. Patel and colleagues also mentioned that an occasional macrophage was observed on the corneal endothelium in 2 cases [13]. Similarly, Eagle Jr. and colleagues described a mild chronic iridocyclitis in 10 out of 16 consecutive patients diagnosed with Cogan-Reese syndrome [3]. This experience is in line with the report of the group of Alvarado, who described 16 out of 25 patients with ICE syndrome with a red eye or a mild uveitis before disease onset and also documented photographically the presence of keratic precipitates in one of these patients [12].

It was the group of Alvarado that first postulated that the endotheliopathy responsible for the development of this syndrome could have a viral origin [12]. In fact, they noted that the endothelial alterations observed in ICE syndrome patients are similar to those observed in viral disorders. In line with this hypothesis, ICE syndrome diseases are usually monolateral acquired disorders, suggesting that affected patients had one eye primarily affected with a virus during the postnatal age and the other eye protected by immune surveillance established a few weeks after the first infection. The seldom described bilateral occurrence of ICE syndrome could be explained by the simultaneous infection of both eyes [12]. In addition, HSV-DNA was detected in the aqueous humor of patients with idiopathic corneal endotheliopathy suggesting a viral origin of these disorders. Nevertheless a direct proof of a relationship between ICE syndrome and herpes simplex keratitis occurring in the same eye has not been demonstrated [12, 14]. However, the first eight years of studies and experiments by using ultrastructural methods and viral cultures to confirm the hypothesis of a viral origin for the ICE syndrome was a total failure. Nevertheless, they did not lose faith in their hypothesis and later decided to use a new method that seemed advantageous for the detection of viral DNA: the polymerase chain reaction (PCR). This "new" technique finally allowed them to detect herpes simplex virus- (HSV-) DNA in corneal tissue and aqueous humor samples from patients with ICE syndrome [12]. Specifically, the authors found HSV-DNA in more than 60% of the tested samples. They also evaluated the possible presence of different viruses' DNA to explain the HSV-negative samples; however, both herpes zoster and Epstein-Barr viruses were not detected. To further prove their results, the authors also performed PCR to detect viral DNA in samples from healthy subjects and from patients affected by different corneal disorders, including bullous keratopathy and keratoconus, and all were negative. It is interesting to underline that the authors also performed the PCR test on the unaffected eye of a patient with ICE syndrome that was also negative [12]. Other authors have shown that Epstein-Barr virus may also play a role in the disease development [15].

The pioneering work of Alvarado and colleagues strongly suggests that HSV may have a relevant etiologic role in the development of ICE syndrome. However, it may not be the only cause or predisposing factor and a lot has yet to be learned on the disease etiology, which remains partially unknown today.

3. Pathogenesis

The pathogenic mechanisms behind the clinical alterations observed in ICE syndrome have been identified in an abnormal proliferation of the corneal endothelium [16–18].

Corneal endothelium is a single layer of uniform, hexagonal cells localized at the inner surface of the cornea into the anterior chamber of the eye. The corneal endothelium lays on a basement membrane, the Descemet membrane. Corneal endothelial cells have an embryological derivation from neural crests. In postnatal age they are postmitotic and, in normal conditions, do not divide. In adult age, corneal endothelial cell density is approximately 3000 cells/mm^2 and it slightly decreases with age [19]. The function of corneal endothelium is to actively maintain corneal transparency through the regulation of fluid, nutrients, and solute transportation between aqueous humor and the cornea structures [20].

In 1978 Campbell and colleagues proposed the "membrane theory" to explain the pathogenesis of ICE syndrome. Specifically, they hypothesized that in the ICE syndrome corneal endothelial cells are primarily affected and show proliferative and structural abnormalities and the ability to migrate into the surrounding tissues [7]. This hypothesis was supported by the evidence obtained by studies with specular microscopy that showed morphologic changes in size and shape of endothelial cells, resembling epithelial cells, also at the earliest stages of all ICE syndromes [16, 17, 21–23]. Moreover, histopathologic studies of eyes with ICE syndrome showed altered corneal endothelial cells with morphological characteristics resembling an epithelial-like phenotype, named "ICE-cells" in 1985 by Sherrard and colleagues [17, 18]. The other observations supporting this hypothesis derive from histologic studies that demonstrated the presence of a membrane composed of endothelial-like cells with a basement membrane obstructing the anterior chamber angle and covering the iris [1, 24].

It was through transmission electron microscopy studies that it was finally confirmed that the endothelial cells of affected patients are abnormal as they develop unique characteristics of epithelial cells [25]. Specifically, electron microscopic examination of these cells has evidenced desmosomes, intracytoplasmic filaments, filopodia, and microvilli [12, 13,

25, 26]. It is worthy of note that corneal edema observed in patients with ICE syndrome had been explained, before these electron microscopy studies, only by a reduction in number of endothelial cells. However, we now know that this is not the case, as the corneal edema is rather caused by the altered endothelial cell function caused by multiple abnormalities of the endothelial cell barrier. In line with the hypothesis of an abnormal endothelial function rather than a reduced number of endothelial cells, Bourne and Brubaker have also shown that before chronic edema develops, the endothelial barrier is actually more impermeable than in healthy subjects [27]. This observation also correlates very well with the hypothesis that the whole disease pathogenesis may be related to reparative activities induced by the injury of endothelial cells caused by a viral infection or by inflammation. In fact, the formation of microvilli and filopodia is well known during the wound-healing process in animal models of endothelial damage as well as in humans whose endothelial cells have been inadvertently damaged by argon or YAG laser [28]. Thus, the presence of these endothelial abnormalities in ICE syndrome may simply reflect the fact that the endothelium is engaged in reparative activities. Unfortunately, however, this activation is later followed by cell damage and loss of function, necrosis, and a continued decrease in cell density, which may be explained, once again, by a viral/inflammatory etiology of the disease [29].

Immunohistochemistry studies showed the presence of vimentin and cytokeratins (CK) in ICE-cells [18, 30, 31]. Levy et al. demonstrated that ICE-cells expressed a profile of differentiation markers (CK5 and CK19, but not CK3, CK8, and CK18) that resembles that of normal limbal epithelial cells suggesting that ICE syndrome may result from ectopic embryonic ocular surface epithelium [32]. Alternatively, these findings are consistent with a metaplastic stimulus resulting in a profound change in the phenotype of normal corneal endothelial cells [31, 32].

Regardless of the etiologic trigger, the final result of all these cellular alterations is that the abnormal endothelial cells in ICE syndrome migrate posteriorly beyond the Schwalbe line to obstruct the iridocorneal angle and into the anterior chamber to cover the iris, where they form an abnormal basement membrane that eventually contracts triggering pupil shape anomalies, iris atrophic damage, and formation of synechiae between adjacent structures [7].

The angle obstruction also causes an increase of intraocular pressure (IOP) and consequent development of glaucoma in 46% to 82% of patients with ICE syndrome [2].

4. Clinical Presentation

ICE syndrome is usually diagnosed in young adults, most often females, although few cases have been described with early onset in children [33–35].

Patients usually present to the ophthalmologist for a change in the shape or position of the pupil. In other cases, patients refer impairment of visual function that ranges from worsening of visual function in the morning due to the corneal decompensation at early stage to blurred vision and/or halos around lights due to glaucoma to a constant reduction in visual acuity. Alternatively, the first diagnosis of an ICE syndrome is made during a routine ocular examination, following the visualization of the abnormal corneal endothelium and/or following the evaluation of the anterior chamber angle by gonioscopy during clinical investigations for a glaucoma suspect.

Although clinical characteristics of ICE syndrome may aid a correct diagnosis, in some cases with severe corneal edema diagnosis may be difficult [2, 36]. This may be due to difficult visualization of the anterior chamber structures when obscured by the edema.

When the corneal endothelial function is sufficient to guarantee corneal transparency, a careful examination of the corneal endothelium can aid towards the diagnosis of an ICE syndrome: a high magnification slit-lamp examination can show a fine, "hammered-silver" or "beaten-bronze" appearance of the endothelium similar to that typically observed in Fuchs dystrophy (FECD). Changes of corneal endothelium in ICE syndrome may be visualized and further evaluated by specular microscopy and, more recently, by in vivo corneal confocal microscopy [17, 37]. Demonstration of the presence of the "ICE-cells" at specular microscopy allows confirming the diagnosis of ICE syndrome. These ICE-cells are typically abnormal, rounded, large, and pleomorphic, with specular reflex showing a typical "light-dark reversal" consisting in a dark surface, with occasional central light spot, and intercellular light borders [17, 21, 23]. The four morphological appearances of these cells described by Sherrard et al. coexist with other cell types giving rise to the four basic ICE variants: (i) disseminated ICE, with ICE-cells scattered throughout an endothelium that appears otherwise essentially normal; (ii) total ICE, with ICE-cells totally replacing the normal endothelium; (iii) subtotal ICE(+), with ICE-cells replacing a variable portion of the endothelium and the remaining being composed of very small cells; and (iv) subtotal ICE(−), with ICE-cells replacing a variable portion of the endothelium and the remaining being composed of enlarged cells [17].

In vivo confocal microscopy (IVCM) is a noninvasive, high resolution imaging technique that represents a useful diagnostic tool in ICE syndrome, also in patients with corneal edema.

It allows the study of all corneal structures at cellular level, providing in vivo images of all corneal cells layers comparable to ex vivo histochemical techniques. In vivo confocal microscopy in patients with ICE syndrome will reveal the presence of "ICE-cells" as pleomorphic epithelial-like endothelial cells with hyperreflective nuclei and cell boarders appearing brighter than cell surfaces [38]. Different "epithelial-like" presentations of endothelial cells have been described at IVCM: one type of abnormal endothelium with quite regular size and shape, a second cell type more irregular in size and shape, similar to the epithelial wing cells on IVCM, and a third, highly irregular cell pattern resembling a surface corneal epithelium. It has been hypothesized that these different observations may be related to the stage of the disease [21, 39, 40].

Nevertheless, although the visualization of these cells can allow making a diagnosis, it is usually considered mandatory to confirm the clinical diagnosis by gonioscopy: in fact,

the anterior chamber angle abnormalities are common to all the ICE syndrome subtypes and include broad-based iridotrabecular synechiae that gradually progress until a complete angle closure develops, if not properly treated. While the visualization of these angle alterations is not generally difficult, it must be kept in mind that the membrane obstructing the trabecular meshwork may be initially difficult to visualize by gonioscopy, and the patients' condition may be confused with a more common open-angle glaucoma. This is especially true if corneal edema is believed to be secondary to the intraocular pressure increase rather than to endothelial pump function insufficiency.

The use of ultrasound biomicroscopy (UBM) may represent a useful tool for the detection of changes of the anterior chamber angle structures in ICE syndrome, especially in the presence of corneal edema that does not allow gonioscopy visualization [41]. In addition, combining UBM with gonioscopy evaluation of peripheral anterior synechiae (PAS) may allow a better characterization of the extent (gonioscopy) and shape (UBM) of PAS in ICE syndrome. Zhang and colleagues reported UBM analysis of 21 eyes with ICE syndrome and observed the presence of PAS in all patients associated with a decrease of anterior chamber depth when compared to normal subjects. The authors demonstrated that UBM was more effective in revealing both PAS and iris atrophy than clinical evaluation at slit-lamp biomicroscopy and gonioscopy alone. Four patients also showed angle closure in the fellow eye at UBM examination. In addition, UBM may identify specific features of the different clinical forms of ICE syndrome [42]. Specifically, in patients with progressive iris atrophy, UBM showed marked iris atrophy, and PAS were less pronounced than in patients with Cogan-Reese syndrome. UBM in Chandler syndrome showed the presence of marked corneal edema with Descemet's folds while PAS were less evident. Patients with Cogan-Reese syndrome showed more extensive, often "arborized" PAS. The more severe extent and height of PAS observed by UBM in progressive iris atrophy and Cogan-Reese syndrome support the evidence of a more severe glaucoma observed in these ICE subtypes than in Chandler's syndrome [1, 42].

In any event, a strict follow-up for glaucoma must always be performed in patients with ICE syndrome, by periodical measuring of intraocular pressure, gonioscopy, and visual field and retina examinations [43, 44].

As previously mentioned, once an ICE syndrome diagnosis is made based on these common clinical features, there are at least three different clinical subtypes of the ICE syndrome (Table 1). Wilson and Shields described a series of 37 patients with ICE syndrome showing essential iris atrophy (22%), Chandler syndrome (57%), and Cogan-Reese syndrome (22%) to characterize this condition and describe the clinical course over 12 years [35]. Most of the clinical differences that allow making a differential diagnosis between them are based on the different level of iris involvement and type/severity of iris abnormalities (Table 1).

Progressive iris atrophy is characterized by marked iris atrophy and hole(s) formation, which can be of two distinct subtypes: stretch holes, resulting from iris thinning on the other side of the direction of pupillary distortion, and melting holes, in which the iris tissue vanishes without previous signs due to tissue ischemia (Figure 1). Gonioscopy may show the presence of PAS causing variable degrees of angle closure and consequent intraocular pressure increase.

In Chandler syndrome, the iris alterations are minimal and the disease is more often diagnosed at early stage by clinicians starting from the observation of a corneal edema. When diagnosis is late and iris anomalies are more pronounced, areas of iris atrophy can be observed but usually never lead to a full-thickness iris hole (Figure 2). Glaucoma may develop due to angle obstruction and PAS.

Finally, in Cogan-Reese syndrome different degrees of iris atrophy can be observed; however the diagnosis is usually made following the observation of a different feature: the presence of multiple iris nodules, usually pedunculated, surrounded by stromal iris showing loss of crypts and a matted appearance. Iris nodules in Cogan-Reese syndrome may develop late in the course of the disease, they appear as fine, yellowish nodules on the iris surface, and later in the disease course they become brown and increase in number.

Although from all the above it may seem that the diagnosis of the different ICE syndrome is quite straightforward, in clinical practice the progressive course of the disease often leads to a challenging diagnosis with a large percentage of cases appearing as mixed forms in the different stages of the disease. As it will be discussed later in detail, the clinical management including surgical approach is usually not linked to the exact diagnosis of the clinical subtype but to the degree of complications such as corneal edema or glaucoma.

Finally, since bilateral cases of ICE syndrome have been reported and subclinical changes have been also described in the contralateral unaffected eye, a careful examination of the fellow eye should be performed, including slit-lamp evaluation of the anterior segment structures, gonioscopy, tonometry, and endothelium evaluation by specular and/or in vivo confocal microscopy [45–48].

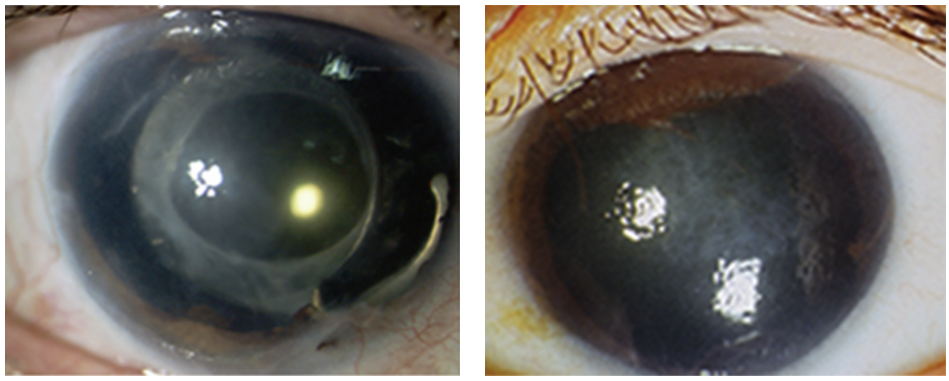
5. Differential Diagnosis

ICE syndrome should be considered in the differential diagnosis for any young adult (especially women) presenting with unilateral iris anomalies, glaucoma, and/or corneal edema [1]. In fact, although the aspect of the ICE syndrome is characteristic, there are different anterior segment diseases that may mimic it and that may be complicated by the same issues such as corneal edema and glaucoma. Among them, corneal endothelial disorders such as posterior polymorphous dystrophy (PPCD) and Fuchs endothelial dystrophy and iris disorders such as Axenfeld-Rieger syndrome, iris melanoma or inflammatory iris nodules, and aniridia should be considered in the differential diagnosis.

Specifically, endothelial cells in PPCD and FECD show epithelial-like changes and express cytokeratins similarly to ICE syndrome [49, 50]. Among them, the FECD is the easiest to diagnose as this disease features similar (but coarser) endothelial anomalies in both eyes and it does not show the anterior chamber, iridocorneal angle, or iris changes

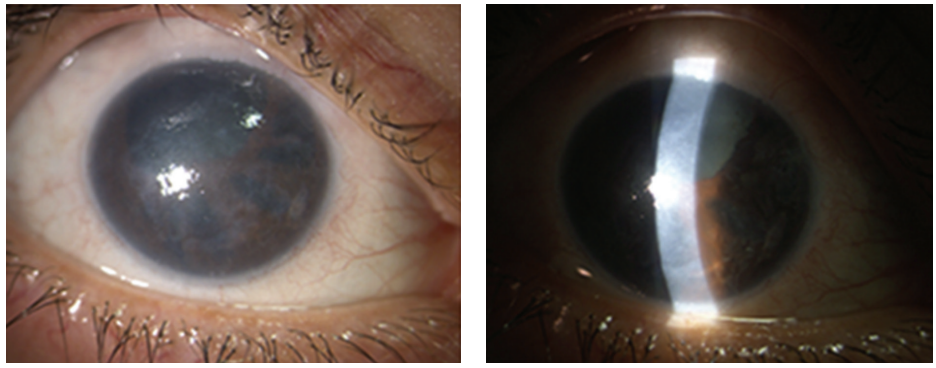
Table 1: Clinical features of the different ICE syndrome subtypes.

	Iris	Pupil	Cornea	Anterior chamber angle
Chandler syndrome	Areas of atrophy (not full-thickness holes)	Corectopia	Early and marked edema, endothelial dystrophy, and ICE-cells at confocal microscopy	Peripheral anterior synechiae
Progressive iris atrophy	Full-thickness hole(s)	Polycoria	Endothelial dystrophy, ICE-cells at confocal microscopy, and corneal edema may occur	Peripheral anterior synechiae
Cogan-Reese syndrome	Nodules and iris atrophy	Changes uncommon	Endothelial dystrophy, ICE-cells at confocal microscopy, and corneal edema may occur	Peripheral anterior synechiae



(a) (b)

Figure 1: Two patients with essential iris atrophy showing extensive iris atrophy and peripheral anterior synechiae (a, b) and corneal edema (b).



(a) (b)

Figure 2: A patient with Chandler syndrome and glaucoma in her right eye showing moderate corneal edema (a), polycoria (b), and peripheral anterior synechiae. She underwent trabeculectomy 7 years earlier. Visual acuity in the right eye was 0.2 decimal units.

always seen in ICE syndrome. Differential diagnosis may be easily reached by IVCM, which will identify the presence of the "ICE-cells" on the corneal endothelium confirming the diagnosis of ICE syndrome [51].

Patients with PPCD, on the other hand, can have several features that strictly resemble the ICE syndrome, making the differential diagnosis more challenging: in fact, in PPCD endothelial metaplasia, pupil abnormalities, iris alterations, corneal edema, and glaucoma caused by angle closure can be observed [49]. Usually the natural history of the disease and a confocal microscopy evaluation can help in making the proper diagnosis, since, instead of the typical "ICE-cells," in PPCD a mixed range of endothelial vesicles and bands are observed. However, probably the most compelling difference

with ICE syndrome is that PPCD is bilateral, and patients also have a typical familiar history since it is an autosomal dominant disease [52].

Another disease that may be very challenging to distinguish from an ICE syndrome is the Axenfeld-Rieger syndrome. In fact, even the pathogenesis of this syndrome is similar as the iris and iridocorneal angle alterations seen in Axenfeld-Rieger syndrome are also caused by a layer of endothelial cells. The only difference is that in Axenfeld-Rieger syndrome they are not secondary to the migration but rather to the presence of a primordial endothelial layer. From a clinical point of view, therefore, the only difference between the two syndromes is that in Axenfeld-Rieger the findings are bilateral and congenital and are often stationary or only have a minor progression over time. Also, at confocal microscopy the corneal endothelium in these patients does not appear altered [53].

Lastly, since in aniridia there are often rudimentary iris stumps and not a complete absence of the iris, this disease may be confused with a late stage progressive iris atrophy. In fact, both can be complicated by glaucoma and corneal clouding. However, in aniridia the corneal clouding is usually caused by a pannus due to a limbal stem cell deficiency and not by endothelial cell dysfunction. In addition, aniridia is a bilateral congenital disease caused by a defect in the PAX6 gene; therefore, additional congenital ocular malformations are often present, including optic nerve hypoplasia, and patients usually have very poor vision and nystagmus [54].

Iris nodules observed in Cogan-Reese syndrome also require differential diagnosis with other conditions showing similar iris changes such as neurofibromatosis, iris melanoma, and sarcoidosis. Von Recklinghausen's neurofibromatosis shows pigmented iris nodules which, differently from Cogan-Reese syndrome's nodules, are bilateral, flatter, and more similar to iris nevi [55]. The presence of intraocular inflammation may help differentiate iris nodules in sarcoidosis form that present in Cogan-Reese syndrome.

Another important differential diagnosis of iris nodules in Cogan-Reese syndrome is the malignant melanoma of the iris. The presence of endothelial changes and cornea edema, PAS, and iris atrophy may correctly orient the diagnosis toward the ICE syndrome [56].

6. Management and Treatment

The HSV hypothesis in the pathogenesis of the ICE syndrome suggests that antiviral treatment may be beneficial in the management of this disease [12]. However, this has yet to be proven and, to date, there is no medical or surgical treatment that can definitely solve any of the ICE syndrome subtypes, and the final therapeutic target is the prevention and management of the visual impairing complications, namely, corneal edema and glaucoma.

The use of hypertonic saline solution instilled as eye drops may be beneficial in the morning to reduce the corneal edema when it is more pronounced.

Topical antiglaucomatous medications are usually the first line of treatment, since a reduction in intraocular pressure can also improve corneal edema. Suppressants of aqueous humor production, including topical beta blockers, alpha agonists, and carbonic anhydrase inhibitors, are preferred and are usually accompanied by miotics although their added value is considered minimal [57]. Since the role of HSV in ICE syndromes has not been completely ruled out, prostaglandins should be used with caution in patients with ICE-related glaucoma as their use has been reported to stimulate recurrence of herpes simplex [58]. High failure rate (from 60% to 88%) of medical treatment for glaucoma is reported in literature and when topical treatments fail or are insufficient, surgical approaches are followed [2, 11]. In a case series of 82 consecutive cases, 37 (45%) required one or more trabeculectomies [59]. Filtrating surgery in ICE-related glaucoma showed a lower success rate than in other types of glaucoma [2, 59, 60]. Studies showed a survival rate of trabeculectomy of about 60% after 1 year and 40% after 2 years of follow-up [59]. These percentages decrease for reinterventions below 20% of success rate [2]. Antifibrotic agents have been proposed to increase the success rate of filtrating surgery in ICE syndrome. The use of postoperative 5-fluorouracile showed failure in 5 out of 9 patients that required additional glaucoma surgery within 1 year [61]. Intraoperative use of mitomycin-C in 10 patients with ICE syndrome and glaucoma showed a good IOP control in 8 out of 10 eyes after a mean of 14.9 months of follow-up [60]. A larger study on 26 patients with ICE-related glaucoma showed a survival rate of trabeculectomy with antifibrotic agents of 73% at 1 year and 44% and 29% after 3 and 5 years [62]. This study also reported that needle and manipulation of the bleb and trabecular flap in these patients did not increase the success rate [62]. The failure of filtering surgery may be due to the progressive growth of the abnormal endothelial membrane extending over the trabecular meshwork and the filtration site [3, 13]. By this point of view, glaucoma drainage implants (GDIs) may overcome the regrowth of the membrane in the filtration site, and studies on the efficacy of GDIs in ICE-related glaucoma showed high success rate of about 70% at 1 year, form 70% to 40% after 3 years, and of 53% after 5 years [62, 63]. In these studies, 20%–50% of patients required replacement or repositioning of the tube, and the authors advise lengthening the tube allowing the possibility of future reposition and keeping the tip of the tube far away from cornea and iris structures [62].

Some studies reported a higher success rate of glaucoma management and surgery in patients with Chandler syndrome, and the authors hypothesize that this different clinical outcome may be due to the less aggressive proliferative endothelial growth observed in these patients [35, 59, 62]. Regardless of the type of surgical approach, cyclodestructive procedures such as cyclophotocoagulation are still very often needed because intraocular pressure control is very challenging in ICE syndrome patients, who are younger than the typical glaucoma patient and, therefore, tend to have a more pronounced cicatrizing response that can lead to the failure of all filtering procedures [2].

In advanced cases of corneal edema with well-controlled IOP, corneal surgery should be considered to improve visual function and reduce pain. Penetrating keratoplasty (PK) was proposed in few small reports with short follow-up, with

variable success rate from 83% to 100% [64–67]. Penetrating keratoplasty was able in improving visual function and pain relief in patients with ICE syndrome; it also allowed a clear media for monitoring of optic disc and visual field changes in patients with associated glaucoma. Long-term results from DeBroff and Thoft reported graft failure in 83% and graft rejection in 2 out of the 6 eyes of patients with essential iris atrophy treated with PK. They also reported the presence of postoperative anterior uveitis resistant to corticosteroid treatment in all eyes [68]. Alvim and colleagues revised the surgical outcome in 14 patients with ICE syndrome followed up to 58 months after PK. They reported early graft failure in 50% of patients due to rejection in 6 patients and endothelial failure in 1 patient. At the end of follow-up, clear graft was reported in 85% of cases, with 6 patients requiring a second PK [69].

In 2007, M. O. Price and F. W. Price Jr. reported the successful use of Descemet stripping with endothelial keratoplasty (DSEK) in 3 pseudophakic patients with ICE syndrome and corneal edema, introducing endothelial surgery in the surgical treatment of ICE syndrome [70]. Endothelial keratoplasty is a surgical procedure that selectively replaces dysfunctional endothelium, sparing the corneal stroma and epithelium. This surgical technique offers several advantages for the treatment of corneal edema in ICE syndrome, when compared with PK. In fact, endothelial keratoplasty provides rapid visual recovery with minimal refractive changes, avoids the use of sutures, and better maintains corneal recipient integrity and innervation [71]. Both deep lamellar endothelial keratoplasty (DLEK) and the Descemet stripping endothelial keratoplasty (DSEK) have been successfully performed in patients with ICE syndrome [70, 72, 73]. The DSEK procedure consists in the replacement of abnormal endothelium and Descemet membrane, while DLEK requires an excision of a posterior lamella of the recipient's cornea stroma. DSEK is simpler and less invasive and allows more rapid visual function recovery compared to DLEK [74]. However, DLEK may offer some advantages in eyes with ICE syndrome characterized by iris anomalies, PAS, and flatter anterior chamber. In a case series of 7 phakic eyes with ICE syndrome, DLEK was successfully performed by Huang and colleagues, who preferred DLEK because the excision of the recipient bed allowed an easier positioning of the donor graft with less manipulation [75]. Recently, a new endothelial keratoplasty, the Descemet's membrane endothelial keratoplasty (DMEK), has been introduced to achieve better visual recovery and to decrease immunologic rejection [76, 77]. However, the efficacy of this surgical approach in patients with complex anterior ocular segment disorders, such as ICE syndrome, has not yet been demonstrated.

Obviously, since all corneal surgery procedures do not completely remove the abnormal endothelium, they are not able to halt the progression of PAS and glaucoma in ICE patients [66].

Lastly, it is worthy of note that iris reconstruction with or without the use of intraocular prosthesis has been proposed in ICE syndrome for both cosmetic reasons and for reducing the visual disturbances of polycoria [78].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pediatric Glaucoma: A Literature's Review and Analysis of Surgical Results

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The purpose of this paper is to review the surgical options available for the management of pediatric glaucoma, to evaluate their advantages and disadvantages together with their long-term efficacy, all with the intent to give guidelines to physicians on which elements are to be considered when taking a surgical decision. Currently there is a range of surgical procedures that are being used for the management of pediatric glaucoma. Within these, some are completely new approaches, while others are improvements of the more traditional procedures. Throughout this vast range of surgical options, angle surgery remains the first choice in mild cases and both goniotomy and trabeculotomy have good success rates. Trabeculectomy with or without mitomycin C (MMC) is preferred in refractory cases, in aphakic eyes, and in older children. GDIs have a good success rate in aphakic eyes. Nonpenetrating deep sclerectomy is still rarely used; nevertheless the results of ongoing studies are encouraging. The different clinical situations should always be weighed against the risks associated with the procedures for the individual patients. Glaucomatous progression can occur many years after its stabilization and at any time during the follow-up period; for this reason life-long assessment is necessary.

1. Introduction

Pediatric glaucoma is a condition characterized by an elevated intraocular pressure (IOP) and optic nerve damage and it can be potential cause of blindness [1].

The most common classification is the following.

(1) Primary congenital glaucoma, including isolated idiopathic developmental anomalies of the angle structures.

(2) Glaucoma arises in a series of systemic diseases some associated with iridocorneal trabeculodysgenesis syndrome such as Axenfeld Rieger syndrome or Peter's anomaly [2], the phakomatoses in particular Sturge-Weber syndrome and its variants [3–5]. It can be associated also with neurofibroamatosis [6, 7], homocystinuria, Lowe's syndrome, mucopolysaccharidosis, juvenile xanthogranuloma [8, 9].

(3) Secondary glaucoma associated with acquired ocular disease.

Depending on the timing of the diagnosis and on features and circumstances of presentation of the glaucoma, aspects that must all be very carefully evaluated, surgery represents the mainstay of treatment, while topical medication may be needed for additional IOP control following surgery or as a temporary measure.

Currently, there are different surgical options, including newer surgical procedures that are improvements of the traditional ones; these in association with the use of antimetabolites and of new viewer systems contribute greatly to the improvement of the prognosis of this disease.

The purpose of this paper is to analyze the surgical options available for the management of pediatric glaucoma and to evaluate their advantages and disadvantages together with their long-term efficacy, all with the intent to give guidelines to physicians on which elements are to be considered when taking a surgical decision.

Table 1: Summary of angle surgery results in patients with primary congenital glaucoma.

Years	Author	Surgical technique	Eyes	Follow-up (years)	End point	Success rate
1953	Barkan	Goniotomy	196	17 yrs	IOP <20 mmHg	80%
1982	Shaffer	Goniotomy	287	15 yrs	IOP <20 mmHg	76.7%
1979	Lunz	Trabeculotomy	86	6.5 yrs	IOP <18 mmHg	89.5%
2007	Yalvac	Trabeculotomy	24	3 yrs	IOP <18 mmHg	92% 1 yr 82% 2 yrs 74% 3 yrs

Table 2: Summary of trabeculectomy surgery in primary congenital glaucoma.

Years	Author	Surgical technique	Eyes	Follow-up	End point	Success rate %
2004	Rodrigues	Trabeculectomy: 30 eyes with MMC* 61 eyes without MMC	91	Patients with MMC 70.2 ± 58.2 months Patients without MMC 43.8 ± 31.3 months	IOP between 15 and 21 mmHg	**
2010	Madhy	Trabeculectomy: 15 eyes MMC and amniotic membrane transplantation 15 eyes trabeculectomy MMC*	30	18 months	IOP between 15 and 21 mmHg	80 60

Intraocular pressure (IOP), mitomycin C (MMC), and millimeters of mercury (mmHg).
*MMC 0.4 mg/mL for 3 minutes.
**The authors did not report values of success rate; they reported P values obtained by Fisher exact test for categorical variables and survival analysis to access the success rate through time. No significant difference on the failure time of the procedure, for both success IOP values considered, was observed between the groups ($P = 0.746$ for IOP <21 mmHg and $P = 0.216$ for IOP ≤15 mmHg).

2. Angle Surgery: Goniotomy and Trabeculotomy

2.1. Goniotomy. The initial approach for primary congenital glaucoma is still angle surgery: goniotomy in the case of a clear cornea or trabeculotomy in the case of a cloudier cornea (Tables 1 and 2). Experienced surgeons have a high rate of success with this technique. Nonetheless, the patient should fit within certain criteria such as the primary congenital glaucoma with no other ocular or systemic anomalies, first diagnosed at least one month after birth but before on year of age, and the corneal diameters need to be less than 14 mm.

Goniotomy was described for the first time by Barkan in 1948 [10] although his pioneer was actually de Vincentis who first performed it in 1893 [11]. The main aims and steps of this procedure have remained the same throughout the years; however, some modifications have been introduced, such as the use of a surgical microscope, the introduction of new goniolens (Koeppe, Barkan, Ritch), and the use of viscoelastic material to protect the cornea and lens.

The surgical steps include entering the anterior chamber, using a goniotomy knife to perform a corneal incision so as to reach the opposite side of the chamber and then incise the trabecular meshwork for 100–110 degrees, visualized using a goniolens. One of the most recently introduced is the Ritch lens; this is a direct lens that allows a 160 degree view of the angle and obstructs only half of the cornea thus making it easier to introduce the goniotomy instrument into the eye while the goniolens is in place. In case of failure it can be repeated.

Barkan in 1953 described the result of his 17 years of work with goniotomy in congenital glaucoma; he reported an 80% success rate in 188 eyes, with postoperative IOP values less than 20 mmHg without medications [12].

In 1982 Shaffer described results in 287 operated eyes. Glaucoma was considered cured if the intraocular pressure remained below 20 mmHg without medication for at least six months and cupping of the optic nerve was either the same or improved. The success rate was 76.7%. However, when the signs or symptoms of glaucoma were present at the birth or above the age of 24 months, the success rate was close to 30%. In contrast the success rate improved to 94% in cases diagnosed between the age of 1 and 24 months after one or two goniotomies [13]. This difference can be explained by the microstructural changes that are detectable in the different age groups; indeed patients over the age of 24 months are known to have less cellularity and more collagenous tissue with respect to younger patients. In the same work he also

Table 3: Summary of trabeculectomy surgery in primary congenital glaucoma and secondary glaucoma including: aphakia, aniridia, juvenile open angle glaucoma, uveitis, and Sturge-Weber syndrome.

Years	Author	Surgical technique	Eyes	Follow-up	End point	Success rate %
1999	Freedman	Mmc trabeculectomy postoperative 5 FU or suture lysis	21	23 months	4< IOP >16 mmHg	52.4
2000	Snir	Trabeculectomy 5 FU or MMC plus postoperative 5 FU	12	25.8 ± 12.2 months	IOP <20 mmHg	58.3
2008	Giampani	MMC* trabeculectomy	114	61.2 ± 26.1 months	5< IOP <21 mmHg	55.26

Intraocular pressure (IOP), millimeters of mercury (mmHg), years (yrs), mitomycin C (MMC), and 5 fluorouracile (5-FU).
*MMC 0.4 mg/mL for 3 minutes.

described that after 15 years of follow-up 3 patients had an increase between 20 and 30 in IOP and therefore required medical therapy [13].

Another study discussed long-term goniotomy complications; in particular the authors described a risk of relapse of glaucoma for at least 15 years. Patients whose symptoms of congenital glaucoma presented at birth were more likely to relapse than those whose symptoms developed in the first few months of life. Furthermore, eyes that required multiple goniotomies in infancy were more likely to relapse than those controlled by a single procedure (Table 3) [14].

Complications include mild and transient hyphema, cyclodialysis, iridodialysis, peripheral anterior synechiae, and cataract formation.

The preoperative presence of angular synechiae of more than 180° of circumference is associated with a failure rate of 100%. Some authors proposed preoperative laser treatment of synechiae with YAG laser [15].

2.2. Trabeculotomy. Trabeculotomy was described for the first time in 1960 by Burian; this surgical technique provided the identification of Schlemm's canal, its radial incision, and incannulation for 360 degrees with a nylon filament. Burian described an elevated success rate [16]; however, potential false passages and the difficulty to control the nylon filament made the modification of the surgical technique necessary.

In 1970 Harms and Dannheim created a scleral superior flap, similar to the flap created for trabeculectomy, so as to have a better identification of Schlemm's canal. He also introduced a new instrument: Harms' trabeculotome that acts for approximately 120° and has two parallel arms that help the surgeon by providing an external guide. If one procedure alone is not enough, it can be repeated [17].

Also Allen and Burian described trabeculotomy using a rigid probe with satisfactory results [18].

No well-designed studies to compare the efficacy of a rigid probe versus the nylon filaments have been conducted as of yet, but the small studies available in literature show a similar efficacy between the two instruments.

In 1979 Luntz reported the surgical results of standard trabeculotomy for the treatment of congenital glaucoma in 86 eyes; he described a success rate of 89.5% (IOP 18 mmHg or less under anesthesia using Schiotz tonometer). The age of the patients ranged from 2 weeks to 12 years and the mean follow-up was approximately 6.5 years [19].

Yalvac et al. reported his surgical results with standard trabeculotomy after 1, 2, and 3 years of follow-up in 24 patients the success rate was, respectively, 92%, 82%, and 74%. The end point was IOP less than 18 mmHg and more than 5 mmHg with a single intervention, stabilization of cup disc ratio, and lack of corneal enlargement [20].

A recent study by Ou and Caprioli reported a success rate ranging from 87% to 92% in case of primary congenital glaucoma presenting before one year of age [21].

In the last years a 360° trabeculotomy with an illuminated microcatheter has been described.

The use of illuminated probes allows for controlled visualization of the position of the catheter while advancing along Shlemm's canal. Dao et al. reported a 75% success rate in achieving 360° cannulation as an initial procedure in children with primary congenital glaucoma [22].

Several studies compared the efficacy of goniotomy and trabeculotomy and concluded that both are successful techniques [23, 24]. There is a general consensus to use goniotomy for initial forms with clear cornea and trabeculotomy for mild severe forms with corneal opacity.

Complications include transient hyphema, choroidal detachments or hemorrhage, and false passage into the eye; less common complications are iridodialysis and rupture of the Descemet membrane [25, 26].

2.3. Trabeculectomy. Trabeculectomy is a filtering procedure that is generally utilized if angle surgery in primary congenital glaucoma fails to work; however, some surgeons prefer to perform it as primary choice in aphakic glaucoma [25].

Although several studies report acceptable results, the success rate is lower than that reported in adult patients and varies from 35 to 50% [26–30]. Several studies report the enhanced success (54% to 85%) of this procedure when used as the primary intervention for congenital glaucoma [31]. Trabeculectomy presents special technical difficulties in childhood because buphthalmic eyes are bigger than usual and the lumbar anatomy is frequently impaired and it can lead to iris and ciliary body incarceration and vitreous loss [30–33]. Furthermore in childhood there is an exuberant fibrotic postoperative response with scar formation which occludes

the filtering site and causes long-term trabeculectomy failure. To obviate this drawback the use of antimetabolites has been introduced. Freedman et al. retrospectively reviewed *21 eyes of 17 patients* (mean age 2.6 years, minimum 1 month maximum 16 years) who underwent mitomycin C (MMC) trabeculectomy, selective use of postoperative 5 fluorouracile (5-FU), and diode laser suture lyses in patients suffering from refractory primary and secondary glaucoma. The follow-up period for all cases was 16.4 ± 11.4 months. The preoperative IOP of 34.1 ± 5.7 mmHg was reduced to the mean values of 16.9 ± 11.2 mmHg at last follow-up, with a mean IOP decrement of 17.2 ± 9.1 mmHg ($P \leq 0.001$). The use of preoperative eye drops (for all cases) was reduced from 2.4 ± 0.6 before operation to 1.1 ± 1.0 after operation ($P \leq 0.001$).

The cumulative success rate was 54.2%. A lower success rate was noted in patients younger than 1 year of age (30%) compared with those older than one year (73%) and in aphakic eyes (2%) compared with the phakic ones (64%) [34]. The reason is unknown. Beauchamp and Parks described a more exuberant healing response which together with a thicker Tenon's layer and lower scleral rigidity and thickness may be considered responsible [30]. Although others have noted poor success of MMC-augmented trabeculectomy in aphakic children, the reasons for frequent failure in these patients are unknown [35]. Susanna Jr. et al. reported an overall success rate (IOP between 4 and 21 mmHg) in 67% of 79 eyes patients (mean age of 76 months and a mean follow-up of 17 months), with devastating complications in 3 eyes [36].

The use of antimetabolites led to the improvement of the prognosis, but at the same time other complications were reported: thin avascular bleb, bleb related endophthalmitis, and long-term hypotonia and bleb leak carry substantial ocular morbidity and the potential for visual loss as well as the need for additional surgery. An increased incidence of postoperative complications is reported in mitomycin trabeculectomy comparing to trabeculectomy without antimetabolites [37–39]. Sidoti et al. reported a significant incidence of endophthalmitis in childhood compared to the rates reported in adults [28].

In the early postoperative period, the most common complications faced by the glaucoma surgeons involve either elevated IOP or hypotonia. Other complications are wound leak, choroidal hemorrhage, malignant glaucoma, cystoid macular edema, encapsulated bleb iris prolapsed, and synechiae [28–40].

Major intraoperative complications in particular expulsive choroidal hemorrhage and intraoperative choroidal effusion are more frequent in eyes with glaucoma secondary to Sturge-Weber syndrome.

The reason is due to vascular anomalies and increased pressure in episcleral veins, ciliary body veins, and choroidal veins that quickly loose fluid from the intravascular to the extravascular space when eyes are opened and intraocular pressure suddenly declines. To prevent this rare but potential catastrophic event the authors have proposed prophylactic intervention such as posterior sclerotomy, prophylactic radiotherapy or laser photocoagulation of the choroidal hemangioma, or electrocautery of the anterior episcleral vascular anomaly, also the reduction of intraoperative hypotonia by a rapid closure of the scleral flap seem to be effective to reduce the risk of expulsive choroidal hemorrhage and intraoperative choroidal effusion [41].

2.4. Glaucoma Drainage Implants. Glaucoma drainage implants (GDIs) provide an alternative option for IOP reduction in complicated pediatric glaucomas that are refractory to angle or filtering surgery. GDIs employ a silicon tube placed in the anterior chamber to shunt aqueous humor to the subconjunctival space where it is connected to a plate positioned in the equator region. A fibrous capsule forms around this plate. The size and the material of the plates vary between the different devices.

The first drainage system utilized was the Molteno valves in 1970 [42]. Afterwards other valves were introduced; the most utilized actually together with Molteno implants are Bearveldt and Ahmed valves [43].

The Ahmed valve is a device with a unidirectional valve restriction flow mechanism, designed to open when IOP is higher than 8 mmHg. This design is highly effective to reduce postoperative hypotonia compared to nonvalved devices which requires a two-step procedure to prevent postsurgical hypotonia and the use of medication to control IOP until the tube opens. Success rate in the different studies varies considerably from 31.3% to 92.7%; this huge difference is due to the different populations enrolled, different devices utilized, and different follow-up lengths (Tables 4 and 5) [4–47]. For instance Al-Mobarak and Khan reported a low success rate of 31% after two years in patients younger than 2 years while Al-Mobarak and Khan reported a success rate of 86% in patients with a median age of 6 years [48]. Yang and Park reported in 38 eyes with congenital glaucoma and 41 with aphakic glaucoma success rates of 92 and 90%, respectively, at 1 year of follow-up which decreased to 42 and 55% after 10 years [49]. Several studies performed on young patients did not show significant differences in postoperative IOP values [50, 51].

A meta-analysis of aqueous shunts by Minckler et al. revealed no advantages to the adjunctive use of antifibrotic agents or systemic corticosteroids [52]. The paucity of evidence that MMC improves outcomes and its potential complications have led to their more limited use [53, 54].

Freedman et al. comparison of GDIs and MMC trabeculectomy shows a major success rate of the former one in both short and long term (87% versus 36% at one year and 53% versus 19% at 6 six years) [55]. These results are confirmed in other studies. Pakravan et al. reported a success rate of 87% (IOP between 5 and 21 mmHg) in aphakic glaucoma treated with Ahmed implants versus 67% in those treated with MMC trabeculectomy 40%. He also reported an increase of complication rate in the MMC trabeculectomy group (40% versus 26.7%) [56].

The complications associated with GDIs are largely reported and seem to be more frequent in childhood compared to adulthood. The most frequent complications are shallows and flats in the anterior chamber, hypotonia, hyphema, choroidal effusion, corneal tube contact, iris abnormalities, and endophthalmitis associated with tube extrusion. Strabismus has been described after GDIs and it is due to the

Table 4: Summary of GDIs implants surgery in patients with primary congenital glaucoma.

Years	Author	Type of valve implanted	Eyes	Follow-up	End point	Success rate %
2004	Al Torbak	Ahmed with corneal transplant	20	30.9 months	IOP between 5 and 21 mmHg without medical or surgery additional intervention	44 at 2 yrs 33 at 4 yrs
2014	Audrey	Bearveldt	45	30 months	IOP <21 mmHg	93.3 at 3–9 months 86.7 at 12–18 months 86.7 at 24–30 months
2014	Reza Razeghinejad	Ahmed	33	32 ± 18.3 months	IOP <21 mmHg	97 1 yr 85 2 yrs 56 5 yrs

Intraocular pressure (IOP), years (yrs), and millimeters of mercury (mmHg).

Table 5: Summary of GDIs implants surgery in patients with primary congenital glaucoma and secondary glaucoma including: aniridia, peter's anomaly, neovascular glaucoma, aphakic glaucoma following congenital cataract extraction, microphthalmia glaucoma after trauma, glaucoma following retinal detachment surgery, and juvenile glaucoma.

Years	Author	Type of valve implanted	Eyes	Follow-up	End point	Success rate %
1997	Eid	Molteno single or double plaits, Soker, Beraveldt	18	47.3 ± 25.1 months	6< IOP <21 mmHg	44.4
2007	Autrata	Molteno or Bearveldt	76	7.1 ± 6.5 yrs	7< IOP <21 mmHg	91 at 1 yr 82 at 2 yrs 76 at 3 yrs 71 at 4 yrs 67 at 5 yrs
2008	O'malley	Ahmed, Bearveldt, Molteno	79	5.5 yrs (mean)	IOP <21 mmHg	92–90 at 1 yrs 42–55 after 10 yrs
2009	Al Mobarak	Ahmed	42	11.1 ± 5.5 months	IOP <22 mmHg	73.8 at 1 yr 63.3 at 2 yrs
2009	Khan	Ahmed	31	11.8 ± 5.6 months	IOP <22 mmHg	90.9 at 2 yrs 58.4 at 2 yrs

Intraocular pressure (IOP), years (yrs), and millimeters of mercury (mmHg).

placement of the implant in the vicinity of the rectus muscle insertion [57–61].

In Sturge-Weber syndrome higher rate of expulsive choroidal hemorrhage, choroidal effusion, and prolonged flat anterior chamber are described. Hamush et al. reported choroidal detachment in 3 eyes of 11 patients [62]. Amini et al. reported 3 cases of choroidal detachment in 9 patients with Sturge-Weber related glaucoma who underwent Ahmed valve implantation [63]. Budenz et al. reported a serous choroidal detachment in two patients of 9 patients (20%) which underwent Baerveldt valve implant resolved spontaneously without the need to drain, the self-limiting nature of this complacence was due to prophylactic posterior sclerotomies performed, in more than one quadrant [64].

2.5. Deep Sclerectomy. Deep sclerectomy (NPDS) involves the unroofing of Schlemm's canal under a scleral flap, with concurrent removal of juxtacanalicular trabecular tissues and creation of a trabeculodescemetic window that provides the resistance to aqueous drainage, in order to prevent hypotonia. This procedure is technically more demanding so its use is not generalized and it is even more limited in childhood. Only few studies are reported in literature [65, 66]. A prospective study conducted in Saudi Arabia by Al-Obeidan et al. regarding NPDS as first surgical choice in 120 patients with PCG reported a success rate of 82.4% [67].

NPDS is particularly suitable in complicated glaucoma such as Sturge-Weber syndrome where hypotonia and choroidal effusion can have catastrophic consequences. Audren et al. did not report any cases of choroidal effusion of NPDS in 12 eyes of nine patients (follow-up 26 months) with glaucoma related to Sturge-Weber syndrome. So it may be considered theoretically safer than trabeculectomy and GDI and could be regarded as an alternative when possible [68].

3. Conclusions

Management of congenital glaucoma is still a challenge for physicians, starting from diagnosis passing through the timing and choice of the most appropriate surgical approach and ending with the long-term follow-up. Today diagnosis is facilitated with ultrasound biomicroscopy and optical coherence tomography of the anterior segment which are imaging techniques which can show the angle and surrounding structures with great detail [69, 70]. Intraocular pressure generally is measured under anesthesia; however, most anesthetic drugs lower it. Generally ketamine is avoided since it raises the values of intraocular pressure. Various instruments can be used to measure intraocular pressure in children like the rebound tonometer, the pneumotonometer, and Perkins applanation tonometer. In particular the I-care tonometer has shown to be promising in measuring IOP in awake young children [71]. Its validity and limits are well known in adult patients [72]. New surgical techniques and improvements of the traditional ones are available to manage pediatric glaucoma, which, however, remains still a challenging and niche type of surgery, probably also due to its low worldwide incidence. The different clinical situations should always be weighed against the risks associated with the procedures for the individual patients.

It is generally accepted that angle surgery remains the first choice in mild cases and both goniotomy and trabeculotomy have good success rates.

Trabeculectomy with or without MMC is preferred in refractory cases, in aphakic eyes and in older children. GDIs have a good success rate in aphakic eyes.

NPDS is still rarely used to date for long-term follow-up are available but medium term results of on-going studies are encouraging. There is also a paucity of information on sight-threatening complications. De Silva et al. evaluate the risk of glaucomatous progression in 19 patients (mean follow up of 33 years) evaluating at each control: elevation of the disk, IOP, progression of optic disk cupping, and visual fields.

Goniotomy was the first surgical approach in 27 eyes, 2 of these eyes needed a second goniotomy. Nine eyes (30%) were treated with trabeculectomy: 2 eyes with MMC 1 eye with β-irradiation, 1 eye secondary trabeculectomy with MMC after 19 years, and 2 eyes requiring transscleral cyclodiode laser. A total of 9 eyes (30%) showed progressive PCG, with a reduction of visual acuity >0.2logMAR units and/or progression of optic disk cupping >0.2 cup/disk ratio as a consequence of elevated IOP (>21 mmHg). The authors have found that the glaucomatous progression can occur many years after its stabilization and at any time during the follow-up period.

The authors hypothesized that the increased visual impairment with long-term follow-up is partly a consequence of sight threatening PCG progression or ocular-comorbidity that can occur at any point in the follow-up period [73].

These results underscore the importance of a life-long assessment and necessity of monitoring the progression of damage. Standard achromatic perimetry is currently the gold standard for detecting visual field loss in glaucoma and to monitoring disease progression over time. Despite advances in imaging of the optic nerve and the retinal nerve fiber layer none of the available methods has been proven to be helpful in the clinical setting for longitudinal assessment of glaucoma [74, 75].

Considerable progress has been made in the in the diagnosis and management of congenital glaucoma but potentially it remains cause of blindness, for this reason innovative studies are required to improve the existing medical and surgical options and to experiment newer.

Conflict of Interests

No conflicting relationship exists for any author.

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Review Article

Congenital Corneal Anesthesia and Neurotrophic Keratitis: Diagnosis and Management

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Neurotrophic keratitis (NK) is a rare degenerative disease of the cornea caused by an impairment of corneal sensory innervation, characterized by decreased or absent corneal sensitivity resulting in epithelial keratopathy, ulceration, and perforation. The aetiopathogenesis of corneal sensory innervation impairment in children recognizes the same range of causes as adults, although they are much less frequent in the pediatric population. Some extremely rare congenital diseases could be considered in the aetiopathogenesis of NK in children. Congenital corneal anesthesia is an extremely rare condition that carries considerable diagnostic and therapeutic problems. Typically the onset is up to 3 years of age and the cornea may be affected in isolation or the sensory deficit may exist as a component of a congenital syndrome, or it may be associated with systemic somatic anomalies. Accurate diagnosis and recognition of risk factors is important for lessening long-term sequelae of this condition. Treatment should include frequent topical lubrication and bandage corneal or scleral contact lenses. Surgery may be needed in refractory cases. The purpose of this review is to summarize and update data available on congenital causes and treatment of corneal hypo/anesthesia and, in turn, on congenital NK.

1. Introduction

The cornea is the tissue with the richest innervation in the human body. Marfurt et al. [1] showed that around 70 nerve bundles enter the cornea at the corneoscleral limbus and then give rise through repetitive branching to a moderately dense midstromal plexus and a dense subepithelial plexus. It is well known that the trigeminal nerve is responsible for providing sensitivity to the cornea but also for providing a trophic support through the release of neurotrophic factors that play a fundamental role in maintaining its anatomical integrity, transparency, and function.

The ophthalmic branch of the trigeminal nerve has 2 reflex arcs: a motor arc that regulates eyelid movements (i.e., blinking) and an autonomic arc that regulates the secretion of goblet cells and lacrimal and meibomian glands. The integration of these two reflex arcs is responsible for the production, maintenance, and stability of the preocular tear film, which is also responsible for providing a trophic support to the cornea. Therefore, the impairment of corneal sensory innervation is overall devastating as it triggers a detrimental loop in which a reduction in trophic support to the tissue is accompanied by an aberrant reduction in the lacrimation reflex and in blinking, with a consequent damage to epithelial cells, which are also burdened by a parallel deficiency in spontaneous epithelial repair [2–5].

Patients suffering decrease or absence of corneal sensitivity develop a clinical condition called neurotrophic keratitis (NK), also known as neurotrophic keratopathy or neuroparalytic keratitis: regardless of the underlying cause, NK is a rare degenerative disease of the cornea caused by an impairment of corneal sensory innervation, characterized by decreased or absent corneal sensitivity (hypo/anaesthesia), resulting

in spontaneous epithelial breakdown and reduced corneal healing [6].

NK can be caused by systemic, ocular, congenital, or iatrogenic diseases that lead to a damage to the fifth cranial nerve.

2. Aetiopathogenesis

Although a wide range of ocular and systemic diseases may cause neurotrophic keratitis, one common insult is always present: a lesion of the fifth (trigeminal) cranial nerve or its ophthalmic branch [6].

The most common causes of neurotrophic keratitis are viral infections (herpes simplex and herpes zoster keratoconjunctivitis) [7, 8], followed by surgical interventions to the trigeminal nerve or for acoustic neuroma [9]. In fact, neurosurgical procedures can cause an insult and consequent damage to the trigeminal nucleus, root, or ganglion, or also directly to the ophthalmic branch of the nerve [10, 11]. Toxicity from chronic use of topical ocular medications may also cause nerve damage and result in corneal hypo/anaesthesia [12, 13]. Neurotrophic keratitis has also been associated with systemic diseases such as diabetes mellitus [14, 15]. A complete list of all known causes of NK is provided in Table 1.

Generally speaking, the aetiopathogenesis of corneal sensory innervation impairment in children recognizes the same range of causes as adults, although they are much less frequent in the pediatric population. In fact, diseases such as uncontrolled diabetes and advanced multiple sclerosis and leprosy are very unrealistic in children, and even herpes simplex infection, which may occur in children, needs a long history of recurrences before inducing damage to the corneal nerves. In addition, it must be considered that corneal and refractive surgery, as well as abuse of local anesthetics [23, 24], chronic topical glaucoma therapy [25, 26], and chronic contact lens wear [27], which are amongst the most common iatrogenic causes of NK in adults, play a very limited role in childhood.

On the other hand, some extremely rare congenital diseases could be considered in the aetiopathogenesis of NK in children. Among them, the rare congenital corneal anesthesia could be a cause of corneal ulceration and scarring in children. Typical onset is up to 3 years of age, more commonly between the age of 8 and 12 months. It may occur as an isolated disease or associated with systemic diseases such as familial dysautonomia (Riley-Day syndrome), Goldenhar syndrome, Möbius syndrome, and congenital insensitivity to pain. Typically, the congenital corneal anesthesia occurs bilaterally; one-sided cases, however, are also described. The cornea may be affected in isolation or as part of a sensory deficit of the 1st and 2nd (and possibly 3rd) branch of the trigeminal nerve [5, 6].

3. Congenital Causes of the Disease

Only scattered NK cases have been reported, highlighting the rarity of NK development in congenital syndromes that are rare *per se*. Those include the following congenital diseases, all considered rare diseases.

Table 1: Aetiopathogenesis of neurotrophic keratitis.

Infections	Herpes simplex Herpes zoster Leprosy [16]
Corneal pathologies	Dystrophies (i) Lattice (ii) Granular
Iatrogenic injury	Contact lens wear Surgeries Laser-assisted in situ keratomileusis [17] Corneal incision [18] Lamellar and penetrating keratoplasty
Topical medications	Anaesthetics Timolol Betaxolol Trifluridine Sulfacetamide Diclofenac sodium [19]
Toxic substances	Chemical burns Exposure to oleoresin capsicum pepper spray [20] Exposure to hydrogen sulfide (H2S) [21]
Cranial nerve V palsy	Trigeminal neuralgia surgery Neoplasm Aneurysm Facial trauma [10] Congenital: (i) Riley-Day syndrome (ii) Möbius corneal hypoesthesia (iii) Goldenhar syndrome (iv) Familial corneal hypesthesia (v) (Familial trigeminal anesthesia) (vi) Congenital insensitivity to pain with anhidrosis
Systemic diseases	Diabetes Vitamin A deficiency Multiple sclerosis
Miscellaneous	Increasing age Adie's syndrome [22]

3.1. Familial Dysautonomia (Riley-Day Syndrome). The Riley-Day syndrome belongs to the hereditary sensory and autonomic neuropathies (HSAN) and according to the numerical classification of four distinct forms of HSAN that was proposed by Axelrod and Gold-von Simson it is also known as HSAN III [28]. It is an inherited autosomal recessive disorder that affects the development and function of nerves throughout the body, with a prevalence of less than 1 on 1,000,000. This condition is almost exclusive to individuals of Eastern European Jewish ancestry (Ashkenazi Jews), and the incidence is 1 in 3,600 live births [28]. The carrier frequency in this population is about 1/30. If both parents are carriers, there is a 25% chance with each pregnancy to give birth to an affected child. Worldwide, there have been approximately 600 diagnoses recorded since the discovery of the disease, with approximately 350 of the affected individuals still living [29].

Corneal ulcerations have been reported in up to 50% of patients [30]. However, it must be noted that this disease is characterized by congenital alacrima, resulting in severe dry eye rather than NK, and it has never been reported that the corneal lesions in affected patients are NK ulcers: there is only one Riley-Day syndrome patient reported in the scientific literature with a corneal epithelial defect caused by "loss of corneal sensation and hypolacrimation" [31]. Therefore, it cannot be determined whether this lesion was a NK PED or a dry eye epithelial damage. Even if we assume that this was a case of NK, the impact of Riley-Day syndrome on the overall NK prevalence can safely be considered negligible.

3.2. Goldenhar-Gorlin Syndrome (Oculo-Auriculo-Vertebral Spectrum). This syndrome was first described by Canton in 1861 and Von Arlt in 1881, but only in 1952 Goldenhar-Gorlin provided a comprehensive description. It is a rare congenital defect (prevalence 1–9 in 100,000) which manifests with a number of craniofacial abnormalities that affect the structures embryologically derived from the first and second branchial arches and usually involve the face (hemifacial microsomia) and ears (microtia). In 50% of cases other anomalies to heart and eyes (epibulbar dermoid) can be found. Most cases are sporadic with 1-2% cases transmitted as autosomal dominant (in this case the most likely affected region is 14q32 and the male to female ratio is 2 : 1). It affects between 1/5000 and 1/25000 live births [32]. Overall, the ophthalmic anomalies involve approximately 66% of cases, with epibulbar dermoids (28–35%), blepharoptosis (10%), superior palpebral coloboma (20%), and microphthalmia (18%). Corneal manifestations are thought to be related to both decreased tear production and neuroparalytic keratitis, possibly due to aplasia or hypoplasia of the trigeminal nuclei, as can be retrieved from case reports involving a few children or young adults [33–35]. In addition, an isolated involvement of the facial nerve can be observed in approximately 20% of cases.

The series published by Mansour et al. [36] does not mention NK as one of the ocular manifestations of this syndrome in 57 consecutive patients. In fact, only one case of corneal anesthesia associated with epithelial punctate keratopathy with this disease has ever been reported [37]. The typical asymmetric facial presentation of this syndrome suggests that this could be a case of exposure keratopathy; however, even if we assume that this was a case of NK, the impact of Goldenhar-Gorlin syndrome on the overall NK prevalence can safely be considered negligible.

3.3. Möbius Syndrome. This extremely rare disease was first described by the ophthalmologist P.J. Möbius in 1888, who described a few patients with the concomitant presence of a nonprogressive congenital paralysis of the facial and abducens nerves. The actual prevalence is unknown; however, only approximately 300 cases have been reported in the literature to date with no differences in gender [38]. Most cases of Möbius syndrome are isolated sporadic cases with no notable family history. It is estimated that there are, on average, 2 to 20 cases per million births. In a study conducted on the Dutch population in 1996, an incidence of 1/189000 live births was found [39]. It must be noted that in this syndrome it is not the trigeminal nerve, but the facial nerve, that is involved. Specifically, the Möbius syndrome is caused by an abnormal development of the 7th cranial nerve (facial) in all patients and of the 6th cranial nerve (abducens) in 75% of the cases. Only in a minority of cases other cranial nerves, including the trigeminal nerve, can be involved. Therefore, it is more likely that a corneal lesion, in the context of this syndrome, is caused by exposure keratopathy (by facial paresis) and not by NK. While it is theoretically possible that a subset of patients affected by Möbius syndrome with trigeminal involvement have corneal hypesthesia, NK cases have not yet been described and, therefore, there is no impact of this syndrome on the overall NK prevalence. On the other hand, other ophthalmic anomalies have been described in this syndrome, including congenital ptosis, epicanthus, and hypertelorism.

3.4. Familial Corneal Hypesthesia (Familial Trigeminal Anesthesia). The prevalence of this condition is unknown, and only scattered case reports have been published in scientific literature. However, these reports do not differentiate between isolated trigeminal anesthesia and corneal hypesthesia associated with the other syndromes listed above [40]. Therefore, the impact of familial trigeminal anesthesia on the overall NK prevalence can safely be considered negligible.

3.5. Congenital Insensitivity to Pain with Anhidrosis (CIPA). The exact prevalence of this rare condition is unknown. According to scientific literature only 52 cases of CIPA have ever been reported worldwide, of which only 14 had corneal involvement [41]. Considering the extreme rarity of this condition, even if we assume that the cornea was involved in all cases during the patients' life, the impact of CIPA on the overall NK prevalence can safely be considered negligible.

However, the early diagnosis and prompt treatment of this rare condition are mandatory to prevent corneal complications such as scarring and perforation.

Regardless of the cause behind the damage to corneal sensory innervation, once NK develops, the corneal epithelium is the first target of the disease, which begins with dystrophic changes of the epithelial cells and, later, with frank epithelial defects that have a poor tendency to spontaneous healing. Epithelial damage of increasing severity is observed according to the stage of the disease. Progression of the disease can lead to corneal ulceration, infection, melting, perforation, and, ultimately, loss of sight [6].

4. Clinical Staging of the Disease

According to the Mackie classification, it is possible to classify neurotrophic keratitis into three stages (1 to 3 in order of increasing severity) [6].

(i) *Stage 1* is characterized by punctate keratopathy and/or corneal epithelial hyperplasia and irregularity, which may be associated with superficial neovascularization and stromal scarring. In addition dry eye signs

may be observed, including vital dye (such as rose bengal) staining of the inferior palpebral conjunctiva and decreased tear film break-up time.

(ii) *Stage 2* is characterized by a persistent corneal epithelial defect (PED), typically oval or circular in shape, with smooth and rolled edges. An area of poorly adherent opaque and oedematous epithelium is typically found rolled-up around the margin of the epithelial defect. As this loose epithelium can easily detach spontaneously, a rapid enlargement of the defect is often observed. Oedema of the corneal stroma may also be present, and it is not uncommon to observe also an inflammatory reaction in the anterior chamber.

(iii) *Stage 3* often ensues if stages 1 and 2 are not treated appropriately. In this stage the corneal stroma is involved and a corneal ulcer is observed. Corneal ulceration tends to progress to perforation and/or stromal melting if not promptly and properly treated. Corneal melting and perforation can be also iatrogenically caused by inappropriate use of topical steroids or by secondary infections of the nonhealing ulcer.

5. Clinical Diagnosis of the Disease

The medical history and clinical findings are crucial for making a proper diagnosis. Anamnesis often highlights systemic diseases, previous neural surgeries, recurrent corneal herpetic infections, trauma, and topical drug abuse. Other characteristic observations include a disproportion between clinical signs as observed at the slit lamp and symptoms as referred by the patient, who is often completely asymptomatic. In fact, corneal sensitivity is a key information to orientate the diagnosis. Obviously, considering all this, it is clear that making a diagnosis of congenital corneal anesthesia and NK is extremely challenging in young children, unless the diagnosis of a well-known syndrome causing corneal anesthesia is confirmed. In fact, in adult, collaborative patients, a qualitative assessment of corneal sensitivity reduction can be easily measured using a piece of twisted cotton, but a quantitative evaluation using a Cochet-Bonnet or no-contact gas esthesiometer is preferred and should be mandatory. Cochet-Bonnet esthesiometry is the most widely used method and it is performed by touching the central and peripheral cornea with a nylon thread that can be elongated up to 60 mm (the longer the thread, the lighter the touch on the cornea) and by recording the patient's response. All the different quadrants of the cornea have to be tested separately because in some cases, including herpes simplex and zoster keratitis, the impaired corneal sensitivity may be only sectorial [6]. This test is difficult to perform, if possible at all, in young children, who are not collaborative and are not able to tell to the ophthalmologist at which length of the Cochet-Bonnet thread they feel the corneal sensation upon touching. The use of in vivo corneal confocal microscopy to visualize the altered subepithelial nerve plexus is also not feasible in young children.

Slit lamp examination can be of great help, especially in children, to evidence the characteristics corneal lesions according to disease severity. Iris atrophy may be a sign of previous herpes infection or previous intraocular inflammation, which sometimes is observed in stage 2 NK patients [42]. There may be optic nerve pallor or swelling due to an intracranial tumor that causes trigeminal compression and impairment; therefore, a complete visit including dilated fundus oculi examination must always be carried out; in the case of visualization of optic nerve head changes it is always important to exclude glaucoma by intraocular pressure testing and visual field examination [43, 44].

Additionally, a clinical evaluation of the different cranial nerves' function can help localize the cause of the decreased corneal sensation. For instance, an isolated dysfunction of VII and VIII cranial nerves may indicate an acoustic neuroma or damage from surgical resection of the lesion, while the paresis of cranial nerves III, IV, and VI may indicate an aneurysm or cavernous sinus pathology that can also affect the trigeminal nerve.

Lastly, the eyelids also need to be carefully examined for both diagnostic and prognostic reasons. In fact, eyelids that do not close properly not only indicate a cranial nerve VII palsy and help in the disease diagnosis but also can worsen the clinical picture and accelerate progression towards stage 3 disease for epithelial exposure by lagophthalmos [6].

Corneal anaesthesia is the hallmark of NK and, therefore, the presence of ocular symptoms such as burning, foreign body sensation, photophobia, and dry eye easily orientates the diagnosis towards other ocular surface diseases. Infective, toxic, or immune corneal ulcers also differ from NK as they are typically accompanied by ocular inflammation and stromal infiltrates. To exclude the presence of an infective ulcer, a microbiologic examination needs to be performed (in the presence of a nonhealing corneal lesion, not only if infiltrates are present, microbiologic exams should always be performed). In order to exclude toxic corneal ulcers, all topical treatments must be discontinued. In order to exclude immune corneal ulcers, a thorough systemic evaluation for immune disorders should also be carried out.

Since superficial corneal vascularisation and epithelial defects can also be seen in limbal stem cell deficiency an additional examination that can be performed in doubtful cases is impression cytology, a minimally invasive technique that allows the direct visualization and identification of the corneal or conjunctival epithelial phenotype by Periodic Acid Schiff staining or immunostaining for cytokeratins [6].

6. Prognosis of the Disease

The prognosis of neurotrophic keratitis depends upon a wide range of factors, including the specific cause behind corneal sensitivity impairment, the degree of corneal hypo/anesthesia, and the association with other ocular surface diseases such as dry eye, exposure keratitis, and limbal stem cell deficiency. The prognosis of congenital NK is usually poor as no medically effective therapy is currently available and due to the chronic and degenerative nature of the

disease most patients are likely to end up developing disease complications or associated ocular surface alterations.

While the presence of associated ocular surface diseases may differently affect the prognosis, it is well known that usually the more severe is the corneal sensory impairment, the higher is the rapidity of disease progression towards corneal melting, perforation, and sight loss due to anatomical loss of the eye or permanent loss of corneal transparency [6].

Nevertheless, even in patients that do not have a complete corneal anesthesia or associated diseases such as secondary bacterial infection, and sometimes even despite early and appropriate therapy, neurotrophic keratitis may still progress to stage 3 (corneal ulcer) disease. When a neurotrophic corneal ulcer develops, it always requires prompt action in order to stop the stromal lysis and prevent perforation. Unfortunately, even when corneal perforation is avoided, once a corneal ulcer develops a permanent decrease in visual acuity from corneal scarring and astigmatism can still be the final outcome of the disease [6].

7. Prevention

Given the wide range of underlying pathologies observed in NK, no consistent approach to prevention is realistic. The most important preventive approach is the prompt identification of stage 1 patients who can be addressed with intense and continuous ocular lubrication with preservative free compounds. The prevention of disease progression with the use of therapeutic contact lenses is also a valid approach for small persistent epithelial defects.

8. Treatment

NK treatment largely depends on disease stage and severity. In fact, the therapeutic approach for stage 1 disease is mostly aimed at preventing epithelial breakdown, while treatment for stages 2 and 3 is mostly aimed at facilitating healing of the corneal lesion in order to prevent irreparable consequences on visual function. More specifically, in the presence of a persistent epithelial defect, treatment aims at preventing stromal involvement with corneal ulcer formation, while more advanced cases, with the presence of a corneal ulcer and stromal melting, require immediate attention to stop the stromal lysis and prevent corneal perforation. This is often obtained by surgery.

Although pharmacological treatments for NK are being intensively studied, with currently ongoing phase 2 clinical trials evaluating growth factors and neuropeptides in both Europe and USA [31, 45], none are yet available. However, the use of preservative-free artificial tears may help improve the corneal surface at all stages of disease, and continuous lubrication with preservative-free compounds needs to be recommended, especially in children, not only to stage 1 patients. The use of preservative free topical antibiotic eye drops is often encouraged and recommended in children to prevent superinfections in eyes with NK at stages 2 and 3; however, it must also be kept in mind that NK lesions are non-infective and, therefore, the use of topical antibiotics is only preventive. When ocular inflammation is present, especially in the case of hypopyon visualization in the anterior chamber, topical steroids have been proposed for NK and may be highly necessary; however, their use is very controversial and must be extremely cautious since steroids may increase the risk of corneal melting and perforation by inhibiting stromal healing. Topical nonsteroidal anti-inflammatory drugs may also inhibit the healing process and should be avoided since their added benefit to control anterior chamber inflammation would be minimal. Regardless of the concomitant use of topical steroids, in the event of stromal melting, the use of topical collagenase inhibitors such as N-acetylcysteine [46] and systemic administration of tetracycline or medroxyprogesterone has been suggested and is usually considered a valid therapeutic option [47]. Nonpharmacological treatments for NK include therapeutic corneal or scleral contact lenses in the event of PED to promote corneal epithelial healing and postpone severe corneal complications [48]. Prolonged therapeutic contact lens use may increase the risk of secondary infections and the concomitant use of topical antibiotics is mandatory [49].

Surgical treatments should be avoided, especially in children, as they are all burdened by a reduction in visual function. Therefore, surgery is usually reserved for refractory cases or for cases that show rapid disease progression towards stromal thinning despite all the best therapeutic efforts. Partial or total tarsorrhaphy is the most simple and widespread procedure used to promote corneal healing in the presence of a neurotrophic PED. Surgical tarsorrhaphy may be performed easily and the tarsorrhaphy opening may be enlarged a few weeks after corneal healing; however, opening a tarsorrhaphy prematurely often results in an early disease recurrence. Alternatively to the tarsorrhaphy, the use of botulinum A toxin injection of the eyelid elevator muscle has been proposed to cover the PED and promote healing [50].

Amniotic membrane transplantation (AMT) is a surgical technique for ocular surface reconstruction that carries relatively good results and can be considered in the management of refractory neurotrophic corneal ulcers. AMT is relatively easy to perform and is effective in promoting corneal epithelial healing, reducing vascularisation, and reducing ocular surface inflammation. A multilayer AMT has also been proposed for treating deeper neurotrophic corneal ulcers [51].

The conjunctival flap is a more invasive procedure that should be restricted only to very severe cases with impending perforations. In fact, although this surgical procedure is very effective as it is able to restore ocular surface integrity and provide metabolic and mechanical support for corneal healing, it also severely compromises visual function and the esthetic outcome is also very poor: in this procedure, the deep corneal ulcer (or corneal perforation) is covered by a pedunculated conjunctival flap secured in place by fine sutures that provides vascular support to the nonhealing cosmetic cornea. The main goal of this procedure is to preserve the anatomical integrity of the eye, but it does obviously severely impact visual function since the flap vascularizes [52, 53].

In the presence of a small perforation (less than 3 mm) a less invasive approach can be used, by the application of cyanoacrylate glue on the lesion, followed by the application of a soft bandage contact lens or AMT. Larger defects require

a conjunctival flap or lamellar keratoplasty; however, it must be kept in mind that the success rate of corneal transplants in NK patients is very low due to the lack of trophic support, with consequent poor wound healing and risk of PED/ulcer recurrence [54].

In children with congenital corneal anesthesia or other forms of disease that may cause NK, a prompt diagnosis and a life-long monitoring and therapy are important to prevent amblyopia and permanent visual damage. The main goal of therapy is to prevent the formation of epithelial defects. An intensive lubrication is essential. The wearing of safety glasses to avoid self-inflicted injuries is also generally recommended in young children.

If persistent epithelial defects or frank corneal ulcers develop, a fast and aggressive therapy is necessary in order to avoid a corneal perforation. In persistent epithelial defects, soft highly hydrophilic contact lenses can be used together with prophylactic application of antibiotic unpreserved eye drops.

When ulcers develop, amniotic membrane transplantation may be considered in children. Tarsorrhaphy and a protective ptosis by Botulinum toxin injection in the levator palpebrae can be considered in more severe cases [55, 56], but the amblyogenic potential of all these treatments must always be considered in young children.

9. Conclusions

The diagnosis of congenital corneal anesthesia is a real challenge for ophthalmologists because the typical diagnostic tests, such as Cochet-Bonnet corneal esthesiometry, are often not feasible in young children. Therefore, it is mandatory for the ophthalmologists to be aware of the rare systemic disorders that can cause congenital corneal anesthesia and NK, including the Riley-Day, Möbius, and Goldenhar syndromes. Once corneal anesthesia is suspected or a corneal lesion is observed in the affected children, the clinical management is also a real challenge. In fact, although a number of promising clinical trials are ongoing to test therapeutic interventions for NK, there is currently no medication available to restore corneal sensitivity and cure the disease. All current medical treatments, from intense ocular lubrication to therapeutic contact lens and to autologous serum eye drops, are useful to prevent or slow down progression in patients diagnosed with stage 1 or early stage 2 disease. However, once a large epithelial defect or a frank neurotrophic corneal ulcer is present, surgery is often needed to prevent further disease progression and avoid corneal perforation. Unfortunately, surgical interventions such as tarsorrhaphy, amniotic membrane transplantation, or conjunctival flap inevitably cause a decrease in visual function, which can result in amblyopia when performed in young children.

Disclosure

Flavio Mantelli is an employee of Dompé US; Alessandro Lambiase and Marta Sacchetti are consultants for Dompé Farmaceutici S.p.a.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Climatic Droplet Keratopathy in Argentina: Involvement of Environmental Agents in Its Genesis Which Would Open the Prospect for New Therapeutic Interventions

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Climatic droplet keratopathy (CDK) is a degenerative corneal disease of unknown etiology. We described CDK for the first time in Latin America in the Argentinean Patagonia (El Cuy). A deeper knowledge of CDK pathogenic mechanisms will provide new therapeutic strategies. For that reason we investigated the prevalence of CDK in El Cuy and its existence in other 3 provinces with similar climate. Patients eyes were examined, habits throughout lives were inquired about, and serum ascorbate (sAA) was determined. All individuals work outdoors for most of the day. All regions had normal O_3 levels. Individuals from regions 1, 2, and 3 had very low consumption of vegetables/fruits and low sAA levels. Conversely, region 4 individuals had balanced diet and higher sAA concentrations. CDK was only found in region 3 where individuals had partial deficiency of sAA and did not use eye protection. No CDK was found in regions 1 and 2 where individuals had similar work activities and dietary habits to those in region 3 but wear eye protection. No disease was found in region 4 where individuals work outdoors, have balanced diet, and use eye protection. To summarize, the CDK existence was related not only to climate but also to the dietary habits and lack of protection from sunlight.

1. Introduction

In 1898, Baquis [1] described for the first time an acquired degenerative disease of the human cornea (colloid degeneration of the cornea), potentially disabling and being characterized by a slow progression to corneal opacity. The general clinical features of this disease, which occurs in individuals who inhabit certain arid regions of the world [2–5], were reported by Klintworth [6]. The first descriptions of the disease were given by Baquis in 1898 [1] and also in 1935 by Lugli [7]. In 1937, Zanettin found climatic droplet keratopathy (CDK) in fishermen from the Dahlak Archipelago of the Red Sea and described a severe form which leads to blindness [8]. Since then, the disease has been described in different parts of the world [9–33] and more recently in Argentina [34]. The severity of this disease can be classified in three stages, according to the portion of cornea involved and the clinical aspects:

(i) Grade 1: it is characterized by multiple small translucent subepithelial deposits, located near the temporal and/or nasal limbus, best seen with backscattered slit-illumination and high magnification. A prelimbal fringe of clear cornea is also frequently observed. At this stage, the visual acuity is not compromised.

(ii) Grade 2: the opacity extends over the lower two-thirds of the cornea, giving a fuzzy appearance. The indemnity of the superior cornea, protected by

the eyelid, suggests an etiologic factor contributing to chronic corneal exposure to ultraviolet radiation (UVR) and other stresses.

(iii) Grade 3: it is characterized by the presence of clusters of golden subepithelial droplets of different sizes, about 1 mm diameter, which cover the cornea as the disease progresses. In advanced cases, areas of the anterior stroma with vascularization, opacification, or fibrosis may be seen. In general, the visual acuity is severely affected by this stage.

In addition to the findings related to the cornea, the solar radiation that chronically reaches the bottom and most exposed part of the iris could play an important role in inducing depigmentation or atrophy in superficial layers, as previously observed in some patients with CDK [35].

Although globular deposits in the anterior layer of the corneas with CDK were described many years ago using optical and electron microscopy [36, 37], more recently, these anomalies have been further characterized by us using *in vivo* confocal microscopy (IVCM) [38].

Although some components in the corneal droplets have been identified [39, 40], the exact composition still remains unknown. A few years ago, Fujii et al. prepared and characterized a polyclonal antibody against peptides containing D-β-Asp [41, 42], and the same authors later showed that CDK and pinguecula samples were immunoreactive for peptides containing D-β-Asp and advanced glycation end products (AGEs) [43, 44].

Tears contain many identifiable proteins [45], with the variation in their composition possibly defining biomarkers that could lead to a better understanding of the underlying pathology [46]. As CDK is an ocular surface disease and the analysis of the tear proteins may result in further understanding of this disease we studied tears glycoproteins in CDK patients using glycopeptide capture techniques and proteomics. Our results suggest that the enzymatic glycosylation may also be involved in the formation of deposits in CDK, since altered levels of N-glycosylation of certain proteins were observed in the tears of patients with CDK [47].

We have also investigated matrix metalloproteinases (MMP) and their inhibitors, TIMP, in patients with CDK, because these molecules control the degradation of the corneal epithelium and stroma. We showed increased levels of gelatinases and proinflammatory cytokines, as well as decreased expression of TIMP-1 in tears and biopsies of patients with CDK. Similar results were obtained when corneal epithelial cells were exposed to UVR *in vitro* [48, 49]. This data suggests that the pathogenesis of this disease is partly driven by a significant inflammatory response with the poor antiproteolysis shield making the cornea more vulnerable to increased levels of MMP.

The present study was conducted in order to investigate if nutrition, work activity, and eye protection from solar radiation are involved in the development of CDK. We carried out this research by studying individuals who inhabit a region of Argentinean Patagonia where we have found patients with this corneal disease (El Cuy department in the Argentinean Patagonia) and in other three Argentinean regions with climatic conditions similar to those of El Cuy department.

Figure 1: Map of the four regions included in this study.

Even though this disease has received different denominations over the years, CDK being the most common name, we shall try to convince the scientific community that, based upon the results of this paper, a more accurate name for this corneal pathology would be environmental droplet keratopathy, rather than climatic droplet keratopathy.

2. Materials and Methods

2.1. Geographical and Climatic Characteristics of the Regions. Figure 1 shows the different regions studied.

Region 1. Santa Catalina department, in the province of Jujuy, is located in the Altiplano region of Puna, at an average height of 3700 meters above sea level, between 65°53′–66°45′ west longitude and 21°47′–22°15′ south latitude. It is located about 2250 km from the Atlantic Ocean with its northern limit being Bolivia. This region has a cold arid climate (Bwk; Köppen climate classification); see World Map of Köppen-Geiger climate classification direction insert: http://koeppen-geiger.vu-wien.ac.at/shifts.htm (accessed January 9, 2015).

The soil is clayey-sandy and covered by low shrub or steppe vegetation. It presents a high diurnal range of temperature, with very low annual rainfall (100–200 mm) and scarce clouds. The region suffers the constant action of strong winds as also found in Patagonia, with the average temperature being below 18°C.

Region 2. Quebrachos department, in the south of the province of Santiago del Estero, is located between 200 and 500 meters above sea level, between 63°13′–63°28′ west longitude and 28°59′–29°45′ south latitude. The region has a subtropical climate (Csa/Csb; Köppen climate classification) with an annual average temperature of 21.5°C and extreme variations of up to a maximum of 45°C. The rainfall is greater during

the summer months, with a maximum of 500 mm and a minimum of 300 mm. The strongest winds occur in winter with a mean speed of approximately 75 km/h. The vegetation is typical of the Chaco/Santiagueño native vegetation with the presence of quebracho, carob, mistol, chañar, and so forth.

Region 3. El Cuy department is located in the center/west of the province of Río Negro, 750 meters above sea level, between 67°54′–69°04′ west longitude and 38°56′–40°25′ south latitude. It is located 280 km from the border with Chile and 300 km from the Atlantic Ocean. This region has cold semiarid climate (Bsk, Köppen climate classification) with annual rainfall being less than 190 mm and a very low relative humidity. The region experiences hot dry summers (often exceptionally hot) and cold winters, with great diurnal temperature variations.

Region 4. General Roca department is located in the north of the province of Río Negro. This region lies between 66°56′–68°00′ west longitude and 38°56′–39°07′ south latitude, 370 meters above sea level and 400 km from the Atlantic Ocean and 340 km from the border with Chile. This region has a cold semiarid climate (Bsk, Köppen climate classification), but due to the intervention of man and water provided by irrigation canals it is a green valley with numerous farms. It is protected from winds by the edges of the plateau, which act as walls.

2.2. Type of Study and Individuals. This investigation was approved by the Institutional Review Board of the Catholic University of Córdoba and the Institutional Research Ethics Committee of Health, Ministry of Health of the province of Córdoba, Argentina (recorded in the RePIS), and carried out in accordance with the principles of the Statement of Helsinki.

Nonprobabilistic consecutive patients older than twenty years, who live during their entire life in any of these regions of Argentina and who agreed to take part in the study after reading a summary of the research project and signed a written consent, were examined by specialists in ophthalmology. The study sample was composed of 89, 134, 125, and 113 individuals for regions 1, 2, 3, and 4, respectively.

2.3. Ophthalmologic Examination. All subjects received a thorough ophthalmologic examination that included detailed examination of the anterior eye segment using a slit lamp (Slit-Lamp 100/16, Carl Zeiss, Oberkochen, Germany; AO Slit Lamp 11665, American Optical Co., Buffalo, USA; Led Slit Lamp XL-1, Shin-nippon, Ohira Co., Niigata, Japan). Many individuals also received a test of visual acuity with a Snellen chart and Landolt rings or E for illiterate individuals, objective refraction determination using an autorefractometer (RK1, Canon Inc., Tokyo, Japan). Three representative cases of patients with different grades of CDK were also studied using *in vivo* confocal laser scanning microscopy (IVCM) as previously described [38].

2.4. Lifestyle. All patients completed a questionnaire related to diet, work activity, and the wear of eye protection (sunglasses, hats) during their entire life.

2.5. Ascorbate Serum Concentration. The levels of serum ascorbate (sAA) were studied in twenty randomly selected participants from each region (age and gender matched) by high performance liquid chromatography (HPLC) using an LC-18 column (25 cm high × 4.6 mm diameter with a particle size of 5 μm) as previously described [50]. For region 3 only CDK patients were studied.

2.6. Levels of Ozone. The total O_3 column concentration average in a period of 10 years for each of the regions was obtained using the values of ozone from the website of the National Aerospatial Agency (NASA, USA): Total Ozone Mapping Spectrometer (TOMS-NASA), http://ozoneaq.gsfc.nasa.gov/tools/ozonemap/. For each year, four measurements were obtained on March 21, June 21, September 21, and December 21 in order to determine the annual average. The total O_3 column concentration was measured in Dobson units (DU), considering that normal values range between 230 and 300 DU.

2.7. Statistical Analysis. The data were analyzed using different statistical tests such as Chi-Square test and analysis of variance (ANOVA) followed by post hoc least significance difference. The level of statistical significance was set at $p < 0.005$.

3. Results

Table 1 presents the data for the average age, gender distribution, and prevalence of CDK in individuals living in the four Argentinean regions. As it can be seen CDK was only observed in 25 individuals (8 women and 17 men) living in region 3 (20% prevalence). Grade 1 was observed in 15 individuals (60%), grade 2 in 7 individuals (28%), and grade 3 in 3 patients (12%). No cases were found in individuals younger than 38 years old.

In Figure 2, IVCM oblique images of three representative patients with different grades of the disease clearly show the increase in hyperreflective dot-like deposits at the subepithelial layer as the disease progress.

Values of pterygium and pinguecula are summarized in Table 2. Pinguecula prevalence was significantly higher than pterygium for regions 1, 2, and 3, but not for region 4 ($p < 0.0005$). When we compared the prevalence of pterygium among the four regions there was no significant difference ($p = 0.2457$), whereas the prevalence of pinguecula was of significant difference ($p < 0.0001$).

In the same region where we found CDK there was the highest prevalence of the other two diseases. The pterygium and pinguecula prevalence were significantly higher in region 3, only when they were compared to values from region 4 ($p = 0.0688$ and $p < 0.0001$, resp.).

The principal labor activities for the different regions are summarized in Table 3. The occupation in region 1 is sheep and domestic camelid breeding, whereas for region 2 it is goat rearing, logging, and coal production. Individuals in region 3 are mainly dedicated to sheep farming, sheep shearing, and, in some cases, the manufacture of wool, while individuals in

Table 1: Demographic data and prevalence of CDK in the four Argentinean regions.

	Region 1 (n = 89)	Region 2 (n = 134)	Region 3 (n = 125)	Region 4 (n = 113)
Ages, Mean ± SD	45.38 ± 17.24	55.88 ± 18.34	54.83 ± 13.93	65.16 ± 11.15
(range)	(20–88)	(48–86)	(20–88)	(41–87)
Gender (F/M)	(39/50)	(48/86)	(52/73)	(36/76)
CDK prevalence	0%	0%	20%	0%
n (F/M)	0	0	25 (8/17)	0

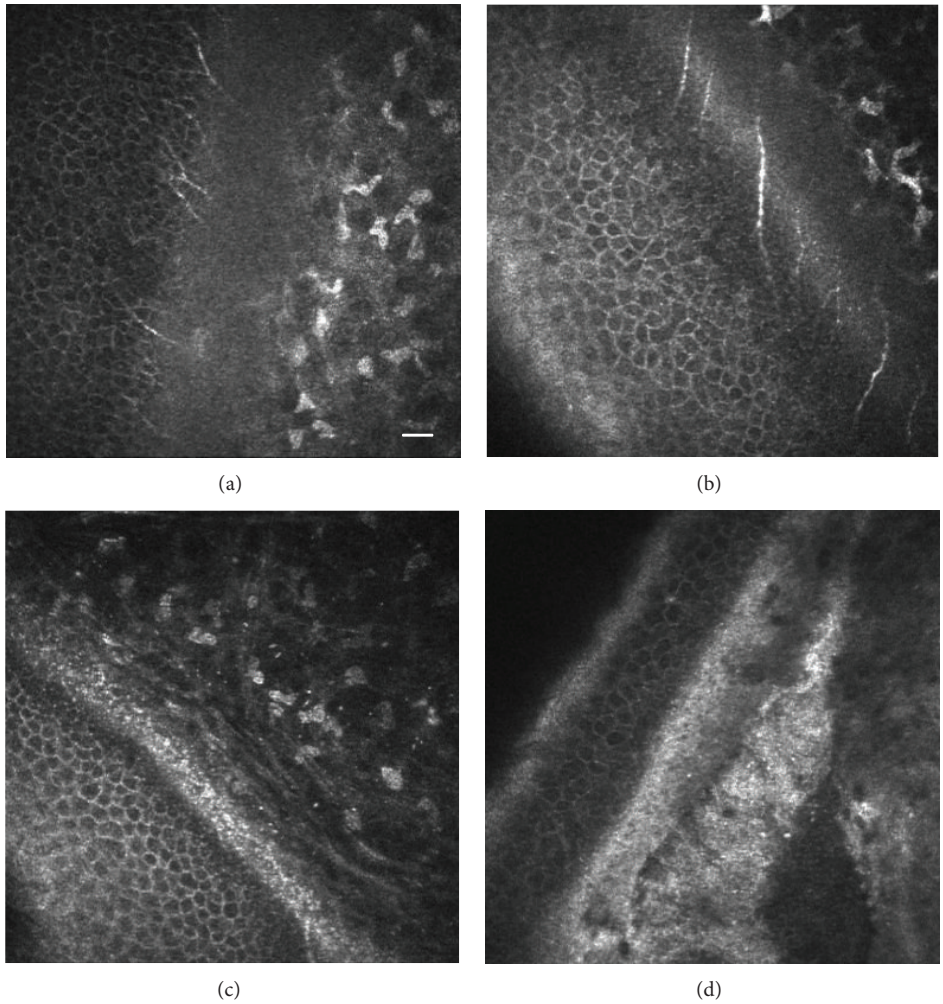

Figure 2: *In vivo* confocal microscopy showing oblique sections of the human cornea where the epithelium, Bowman's layer, and the anterior stroma can be observed. (a) Normal cornea without hyperreflective subepithelial deposits, (b) (grade 1) dot-like deposits at the level of Bowman's layer, (c) (level 2) showing an increase in the density of the hyperreflective dot-like deposits in Bowman layer and superficial stroma, and (d) (grade 3) hyperreflective and nonreflective condensation deposits in the Bowman layer and superficial stroma. Bar = 50 microns.

region 4 work on tasks related to the cultivation, harvesting, and packaging of pears, apples, and plums.

The main dietary habits of individuals in region 1 are the consumption of meat, quinoa, corn, and potatoes; in region 3 sheep meat is ingested two or three times a day, with small amounts of milk taken sporadically. Vegetables and fruit are exception items in the diet of the inhabitants of these regions. The diet of individuals in region 2 consists of some vegetables (potatoes, zucchini), some meat, and a scarce amount of fruits. In contrast, individuals inhabiting region 4 have a balanced diet (meat, vegetables, cereals, and fruit) (Table 3). In all regions, individuals manifest drinking yerba mate infusion (a local infusion).

Dietary deficiency in foods rich in AA was reflected in the low sAA levels found in the individuals living in regions 1, 2, and 3 (0.27 mg/dL ± 0.13, 0.31 mg/dL ± 0.11, and 0.21 mg/dL ± 0.09, resp.). These values differed significantly ($p < 0.001$) from individuals in region 4 (0.72 mg/dL ± 0.44).

Table 2: Percentages of pterygium and pinguecula in the four regions of Argentina.

	Region 1 ($n = 89$)	Region 2 ($n = 134$)	Region 3 ($n = 125$)	Region 4 ($n = 113$)
Pterygium				
(+/−)	(12/77)[a]	(21/113)	**(21/104)**[c]	(10/103)
(%)	(13.48)	(15.7%)	**(16.44%)**	(8.8)
Pinguecula				
(+/−)	(54/35)[b]	(47/87)	**(81/44)**[d]	(15/98)
(%)	(60.67)	(35.1%)	**(64.75%)**	(13.3)

[a]For regions 1, 2, and 3 pterygium prevalence was significantly lower than pinguecula ($p < 0.0005$).
[b]The prevalence of pinguecula among the four regions was significantly different ($p < 0.0001$).
[c]Pterygium prevalence was significantly higher in region 3 versus region 4 ($p = 0.0688$).
[d]Pinguecula prevalence was significantly increased in region 3 versus region 4 ($p < 0.0001$).

Table 3: Main work activity, nutrition, and use of eye protection.

	Region 1 (% of individuals)	Region 2 (% of individuals)	Region 3 (% of individuals)	Region 4 (% of individuals)
Source of livelihood	Sheep and camelid farming 72/89 (80.9%)	Goat rearing, deforestation, and charcoal production 104/134 (77.6%)	**Sheep farming and shearing** 117/125 (93.6%)	Fruit pickers 80/113 (70.8%)
Feeding intake	Meat, quinoa, corn, potatoes, and little milk (100%)	Meat, potatoes, zucchini, and few fruits (100%)	**Sheep meat and a little milk** (100%)	Meat, vegetables, cereals, and fruits (100%)
Eye protection	Winged hats 74/89 (83.1%)	Sunglasses 110/134 (82.1%)	**Almost none** 113/125 (90.4%)	Hat and sunglasses 88/113 (77.9%)

As can also be observed in Table 3, more than 80% of individuals in region 1 use winged hats, whereas in region 2 sunglasses are worn. In region 3, most individuals do not wear glasses or hats, while in region 4 the majority of people studied use hats and/or sunglasses.

The twenty-five CDK patients manifested they worked outdoors in sheep farming, never used eye protection, had a very restricted diet as previously shown during their life, and had the lowest AAs concentration. As can be seen in Table 3 the majority of individuals from this region who do not suffer CDK have the same work activity and habits.

When the total O_3 column concentrations (Dobson units) measured on March 21, June 21, September 21, and December 21 of each of the last ten years from each region were analyzed no significant differences at annual average were found (data not shown). We then added all the values corresponding to the last 10 years and obtained that the mean ± SD of total O_3 column concentrations for regions 1, 2, 3, and 4 was 252.6 ± 14.5, 272.4 ± 16.7, 288.3 ± 10.2, and 273.7 ± 5.8, respectively. These results rule out any thinning of the ozone layer in the four studied regions.

4. Discussion

CDK has been defined as a rural disease in which the clinical presentation and severity of corneal injuries can vary significantly depending on the region and its weather. More severe forms of CDK have been described in arid regions with high temperatures, such as those of the islands of the Red Sea [8, 29], than in cold regions such as Labrador and the Arctic Circle [23].

Even though CDK is a well-differentiated clinical entity, with bilateralism being the rule and often asymmetry, a differential diagnosis with other entities is needed, such as secondary spheroidal degeneration, gelatinous drop-like dystrophy, corneal edema, band-shaped corneal degeneration, Salzmann's nodular degeneration, climatic stromal proteoglycan keratopathy, Vogt's limbal degeneration, superficial corneal dystrophies, and hypertrophic peripheral corneal degeneration. The combined use of slit lamp biomicroscopy and IVCM facilitate the diagnosis of this disease [35, 38].

Our hypothesis about the genesis of CDK is that in the cornea of those people chronically exposed to unfavorable environmental conditions (high exposure to UVR, lack of vegetation/shade, dry climate with windy conditions, airborne particle bombardment, partial AA nutritional deficiency, lack of eye protection, and genetic factors) an oxidative stress and inflammatory processes lead to a progressive degradation and accumulation of proteinaceous material in Bowman's membrane and the superficial stroma [51]. We have recently shown elsewhere that, in patients with CDK, a hypersensitive reaction occurred in the cornea with the initial participation of important proinflammatory components of the innate immune system [52]. We have previously shown a lack of correlation between genetic ancestry (as represented by haploid genetic systems) and the incidence of CDK in Argentina [53]. Also, in previous unpublished studies we have investigated the variation at the blood and HLA-DRB1 alleles groups between patients and relevant controls. Although

we found that the expression of 0 Rh+ and DRB1*14 was higher in CDK patients there were not any significant associations between these alleles and CDK. Since ALDH3A1 gene encodes a protein that protects the mammalian cornea from harmful UVR, we are currently studying genetic variation at this locus in the population that suffers from CDK.

Although the eye depends on the energy from visible radiation to carry out its fundamental physiological processes, it can also be damaged by this radiation as well as by UVR. Eye diseases in which sunlight is implicated are called ophthalmoheliosis, with these conditions representing important eye health hazards in many communities worldwide. However, the interpretation of clinical examination is complicated, because of the difficulty in measuring the quantity and exact wavelength of light to which an individual has been exposed, as well as the length of time over which an injury has been progressing [54].

Acute or chronic corneal exposure to UVR induces altered proteins, DNA fragmentation, free radical generation, lipid peroxidation, and so forth, resulting in actinic keratosis or keratopathies affecting primarily the epithelium and the anterior stroma. Other studies have shown that exposure to UVR is responsible for the development of cortical pterygium and cataracts [55].

The high content of AA, some proteins, and nucleic acids in the corneal epithelium function as a filter, by absorbing up to 77% of UVR of dangerous wavelengths [54, 56–58]. This vitamin is synthesized by different mechanisms both in the animal kingdom and in plants. However, bats, guinea pigs, and primate anthropoids, including humans, have lost the ability to synthesize AA as a result of mutations of L-gulono-γ-lactone oxidase (GLO) genes, and this powerful antioxidant must be incorporated in the diets of these animals (see [59]). Tears also have among different components that prevent oxidative stress a constant level of AA which is maintained by the lachrymal gland and not by the cornea [57, 60]. Our group, as others, working with guinea pigs fed on different doses of AA and exposed to UVR, have shown that cornea damage produced by radiation is related to the dose of AA consumed [59, 61, 62]. In humans, corneal haze after photorefractive keratectomy has only been reported in Norway, when the sun is visible 24 hours/day [63], but the administration of a dietary supplementation with AA during pre- and postoperative periods can reduce its incidence [64].

As mentioned above, the development of CDK has been associated with overexposure to UVR [65]. However, association does not necessarily mean causation. To infer causality, it is necessary to conduct field studies to assess the individual dose-response to UVR. The values of UVR can be calculated at ground level using radiometers or computer programs that manipulate different variables such as ozone, latitude, date, time, and cloudiness, among others [66]. The estimation of UVR dose reaching the eyes is also very important in order to be able to determine its harmful effects. However, getting these values requires the installation of equipment with constant evaluation by qualified staffs, which have to simultaneously record climatic factors, such as clouds and winds. Given that CDK is a chronic disease of slow evolution, we should had made all these measurements for many consecutive years in the four extremely isolated Argentinean regions we investigated, which was certainly an unfeasible task. For these reasons, in the present investigation we did not calculate the UVR values at ground level for any of the four Argentinean regions. Instead, we determined the total O_3 column concentration for these regions during 10 years using the website of the National Aerospatial Agency (NASA, USA), and all the O_3 values found during this extended period of time were within the normal range, excluding the possibility of any thinning in the O_3 layer in these Argentinean regions which could have increased the amount of UVR reaching the ground surface and affect the individuals corneas.

Our findings also allowed reaching conclusions about the role played by labor activity, diet, and the use of appropriate eyes protection on the genesis of CDK. We observed CDK only in people who worked in sheep farming during their entire life in region 3 of Argentina (characterized by a dry, sunny climate, with sandy and arid soil sparsely covered by small shrubs that only allowed the development of highly adapted plant species, which could be exploited by cattle, but was not suitable for growing vegetables or fruits). Surprisingly, we did not find CDK in region 1 or 2 (provinces of Jujuy and Santiago del Estero), which had similar climate and soils and where individuals have similar eating habits and work activity to those of region 3.

The questionnaire about food consumption clearly indicated a dietary deficiency of rich vitamins foods, especially AA, in individuals from regions 1, 2, and 3. Those answers were corroborated by the low sAA concentrations found in blood samples. In contrast, in region 4 there was a greater agricultural activity, mainly fruit cultivation, using Río Negro water for irrigation. Thus, individuals from this region had a much more balanced diet, as their dietary intake includes meat, vegetables, and fruits, with the consequent higher concentrations of sAA. These results are consistent with other investigations which clearly demonstrate the importance of AA in corneal protection against the damaging effects of UVB [59].

Other observations of our work are related to the use of protective eyewear for UVB (winged hats, visors, or sunglasses), commonly used by individuals from regions 1, 2, and 4, where no CDK was found, but not in region 3, where we found a 20% prevalence of this disease. It should also be borne in mind that regions 1, 2, and 3 are characterized by the presence of extensive areas devoid of shadows.

Based upon our results, which clearly demonstrate the existence of CDK only in one out of four Argentinean regions with similar climates, it should be worth considering that a proper name for this corneal pathology should be environmental droplet keratopathy or environmental proteinaceous corneal degeneration, rather than climatic droplet keratopathy because its genesis is not only related to the climate. The prevalence of this disease is high in this area of the Argentinean Patagonia, but many individuals living in this region, in spite of having the same work activity, nutrition, and eye protection, do not suffer CDK. This implies that beside harsh environmental conditions and lack of protection from the detrimental effect of UVR other factors (genetics) could contribute to the onset of this disease.

In addition, when we studied old adult sheep that graze in the same region of Patagonia where we found this human disease (and therefore were exposed to the same environmental conditions as patients with CDK) it was observed that, despite having superficial corneal abrasions, these sheep did not suffer from this corneal degenerative disorder as observed in humans [67] and our unpublished data. This was probably due to the fact that sheep, unlike humans, are able to synthesize AA from the grass they eat.

The results of the pinguecula and pterygium prevalence (ophthalmoheliosis) were as follows: In people inhabiting region 4 there was the lowest number of pinguecula and pterygium cases, whereas the highest prevalence of both diseases was in region 3 (Table 2).

Classically, it has been postulated that pterygium is in part the result of conjunctival inflammatory process secondary to a sustained exposure to UVR. The high prevalence of this disease in region 3 may be related to the fact that these individuals spend long hours each day outdoors with sheep, being consequently exposed to UVR and high winds without any eye protection. This is reinforced by the findings of lower percentages of pterygium in regions 1, 2, and 4, where individuals usually wear hats/goggles for sun protection.

In summary we present new and convincing results that contribute to the increasing knowledge about the genesis of this human corneal degenerative disease which open the prospect for a new therapeutic strategy in the prevention as well as progression of CDK. Since the only treatment in advanced cases is a corneal transplantation, which in different impoverished regions of the world is not an available option, the implementation of simple preventive measures such as proper eyes protection and adequate diet with normal levels of AA would contribute to eradication of this disease.

5. Conclusions

CDK was only found in high prevalence in individuals inhabiting one out of four Argentinean regions (region 3: El Cuy department). The feeding pattern of individuals from regions 1, 2, and 3 was characterized by a very low consumption of vegetables and fruit with a high intake of meat. In contrast, individuals from region 4 generally had a balanced diet (meat, vegetables, cereals, and fruits). Low serum AA levels were found in individuals from regions 1, 2, and 3 (0.27 mg/dL ± 0.13, 0.31 mg/dL ± 0.11, and 0.21 mg/dL ± 0.09, resp.), where the intake of fruits and vegetables is low. These values were lower ($p < 0.001$) than those determined for region 4 (0.719 mg/dL ± 0.446). All the individuals work outdoors during the greater part of the day. The primary work activity is cattle breeding, with the exception being region 4, which has fruit collectors. No CDK was found in regions 1 and 2, which have similar climate and soils. Here, individuals have habits and work activities comparable to those of region 3 but use eye protection. There were no cases of this disease either in region 4 where outdoor work also predominates but individuals have a balanced diet without AA deficit and use eye protection. We also found lower percentages of pterygium in regions 1, 2, and 4, where individuals usually wear hats/goggles for sun protection.

CDK was clearly associated with a poor ingestion of fruits and vegetables rich in AA, low levels of AA in sera, and lack of eye protection from UVB, and not merely with the climate. The O_3 values determined during the last ten years in all the regions were within the normal range excluding the possibility of any thinning in the O_3 layer in any of these regions.

Based upon our results, it should be worth considering that a proper name for this corneal pathology should be environmental droplet keratopathy or environmental proteinaceous corneal degeneration, rather than climatic droplet keratopathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Julio Alberto Urrets-Zavalía and Horacio Marcelo Serra equally contributed to this work.

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Clinical Study

Stem Cell Therapy for Corneal Epithelium Regeneration following Good Manufacturing and Clinical Procedures

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Objective. To evaluate outcomes of cultivated limbal epithelial transplantation (CLET) for management of ocular surface failure due to limbal stem cell deficiency (LSCD). *Design*. Prospective, noncomparative, interventional case series and extensive comparison with recent similar studies. *Participants*. Twenty eyes with LSCD underwent CLET (11 autologous; 9 allogeneic) and were followed up for 3 years. Etiologies were divided into 3 prognostic categories: Group 1, chemical injuries (7 eyes); Group 2, immune-based inflammation (4 eyes); and Group 3, noninflammatory diseases (9 eyes). *Intervention*. Autologous and allogeneic limbal epithelial cells were cultivated on amniotic membranes and transplanted. Evaluations were based on clinical parameters, survival analysis, and in vivo confocal microscopy (IVCM). European Union Tissues/Cells Directive and good manufacturing procedures were followed. *Main Outcome Measures*. Improved clinical parameters, absence of epithelial defects, and improved central corneal epithelial phenotype. *Results*. Success rate was 80% at 1-2 years and 75% at 3 years. Autografts and allografts had similar survival. Success rate was significantly lower in prognostic Group 1 (42.9%) than in Groups 2-3 (100% each). All clinical parameters improved substantially. By IVCM, 80% of cases improved in epithelial status. *Conclusions*. CLET improved corneal epithelium quality, with subsequent improvement in symptoms, quality of life, and vision. These results confirm that CLET is a valid therapy for ocular surface failure.

1. Introduction

Corneal epithelial stem cells reside throughout the entire circumference of the corneoscleral limbal niche that includes the limbal crypts of the palisades of Vogt. They are responsible for the maintenance of a healthy corneal epithelium, in part, by replacing aged or damaged epithelial cells during normal cell turnover [1–3]. A deficiency or lack of corneal epithelium renewal due to limbal epithelial stem cell depletion or dysfunction results in the so-called limbal stem cell deficiency (LSCD) syndrome, which is a difficult and complex disorder to manage when it is complete and severe. The hallmark of the LSCD phenotypic endpoint is the replacement of the corneal epithelium by conjunctival epithelium. With the loss of the limbal epithelial stem cells, the limbal conjunctival epithelial cells invade the superficial cornea. In LSCD, the unstable ocular surface causes recurrent corneal epithelial breakdown or nonhealing ulceration and vascularization associated with chronic inflammation. These surface alterations result in pain, photophobia, decreased vision, and eventual corneal blindness [1, 4–10]. This syndrome engenders a high risk for corneal graft failure as the donor graft (or artificial cornea) does not have epithelial limbal cells. Likewise, amniotic membrane transplantation, although useful in partial LSCD cases, is inadequate for total LSCD, requiring the addition of limbal tissue [11].

Logically, the definitive treatment for medically irreversible total and/or severe LSCD is the transplantation of limbal tissue or limbal epithelial cells. This approach repeatedly has been shown to help ocular surface regeneration, and consequently it improves the prognosis of a subsequent corneal graft [1, 4–10].

Other types of cells are being tested at present and some have reached the human clinic. For instance, the use of autologous conjunctival cells cultivated ex vivo on amniotic membrane has been recently published [12]. Nonocular mucosal epithelial cells such as those from the oral mucosa have provided non-stem cells resources for use in humans [13, 14], and there are ongoing clinical trials with this approach. Stem cells derived from nonocular mucosal or nonepithelial sources have not yet reached the human clinic, although there are clinical trials already registered on the potential use of mesenchymal stem cells in LSCD (https://www.ClinicalTrials.gov/ Identifier: NCT01562002). Finally, the exciting possibility of using induced pluripotent stem cells as a source of limbal epithelial stem cells with translational potential has just begun [15].

The encouragement for limbal epithelial transplantation was initiated with whole limbal transplantation, first performed in the treatment of severe unilateral ocular surface disease due to LSCD in 1989 by Kenyon and Tseng [16]. At present, whole limbal transplantation is performed through a limbal biopsy, usually of the 4–6 o'clock hour region from the healthy eye in unilateral LSCD cases [17]. In cases of bilateral LSCD, limbal transplant tissue is usually derived from a living relative or a cadaveric donor. At present, these procedures are performed mainly in the United States of America (USA). The so-called "Cincinnati procedure" combines conjunctival limbal allograft and keratolimbal allograft from a living relative of the patient or the same modified technique in which tissues are autologous [18].

A novel surgical technique for unilateral LSCD was recently published by Sangwan et al. [19]. This technique, called "simple limbal epithelial transplantation," extracts a small autologous biopsy of whole limbal tissue from the contralateral healthy eye. The biopsy tissue is divided into 8–10 pieces, placed on top of fresh human amniotic membrane already glued by fibrin to the scraped corneal bed in the diseased eye. A modification of this technique has just been published to make this procedure available in the USA by using cryopreserved amniotic membrane (American Food and Drug Administration [FDA] approved) in a double layer that sandwiches the limbal cells [20].

An alternative to whole limbal tissue transplantation is to use stem cell therapy, where stem cells and other cells resident in the niches are isolated and expanded. For corneal epithelium reconstruction, the technique is called "cultivated limbal epithelial transplantation" (CLET), first reported successful in two cases by Pellegrini et al. in 1997 [21]. In this procedure, the donor limbal tissue is cultivated in vitro, allowing the cells to proliferate. The required amount of tissue is fairly small (1-2 × 1-2 mm limbal biopsy), which decreases the risk of LSCD in the donor eye in unilateral cases. It also makes it possible, at least in theory, to remove the small biopsy even if the contralateral donor eye has some limbal damage. When the disease is bilateral and the LSCD is complete, the transplant must be allogeneic. Because the source of the expanded cadaveric tissue can be small and the cultured limbal stem cells may have unique immunoprotective properties [3, 22], patients subjected to CLET require lower doses of immunosuppressive therapy for a shorter period of time than those undergoing whole limbal transplantation.

Currently it is not clear if autologous and allogeneic CLET have similar success rates. There are also very few reports that compare CLET with other means of transplanting limbal tissues. The only report comparing CLET with whole donor limbal transplantation suggests that CLET is superior to conventional techniques [23]. These data are based on each procedure being performed separately on each eye of the same patient. Currently there are no studies that compare CLET with simple limbal epithelial transplantation.

Since that first report in 1997 [21], the long-term restoration capacity of the ocular surface by CLET has been demonstrated in many reports, and it is more widely used at present. The CLET procedure is now approved in the European Union (EU), provided that it follows the EU Tissues and Cells Directive regarding good manufacturing procedures (GMP). Further, each transplantation medical institution must receive permission from each national regulatory agency. In the USA, there is no FDA approval for the use of GMP facilities to process and cultivate the autologous or allogeneic pieces of limbus. Consequently, there are not many reports on the long-term efficacy of CLET procedures that follow the most stringent regulations and GMP that, at least in the EU, equate cell therapy to any other drug therapy.

We report herein the long-term outcome of both autologous and allogeneic stem cell therapy by CLET. The study was performed in compliance with the EU Tissues and Cells Directive for reestablishing the corneal epithelium phenotype in cases of ocular surface failure due to total or severe LSCD caused by three groups of diseases: chemical injuries, immune-based inflammation, and other less severe diseases. As there are few studies published following these requirements we have extensively compared our results with others using similar approaches.

2. Methods

2.1. Patients. Twenty eyes of 19 Caucasian patients diagnosed with LSCD were treated by CLET (Table 1). The protocol was approved by the institutional review board of IOBA-University of Valladolid. Additional approval was obtained from the University of Valladolid Ethics Committee. Informed consent was granted by all patients after full explanation of all procedures. All experiments and procedures were conducted in accordance with the GMP and good clinical practice norms and the EU Tissues and Cells Directive. The Declaration of Helsinki and the provisions of "National Organic Law 15/1999 of 13 December on the Protection of Personal Data" (BOE number 298, 14-12-1999, pp. 43088–43099) were followed in terms of patient rights, identification, data management, and statistical analysis. As this study was not a clinical trial but rather a clinical series study, it was not registered in the clinical trial databases.

Table 1: Preoperative data and outcomes at 12 months of 20 eyes with ocular surface failure due to limbal stem cell deficiency syndrome (LSCD) subjected to cultivated limbal epithelial transplantation (CLET).

Eye number/gender/age	LSCD etiology/group*/grade†	Cell source	OSDI I/F	NEI-VFQ-25I/F	Visual potential‡	BCVA I/F	Ciliary hyperemia (0–4) I/F	Central corneal epithelial opacity (0–4) I/F	Corneal neoves-sels area (0–4) I/F	Corneal neovessels length (0–4) I/F	Central corneal epithelial irregularity (0–3) I/F	SPK (Fluor) (0–5) I/F	PED (0–4) I/F	Epithelial phenotype (IVCM) I/F	One-year final outcome *Comments*
1/M/47	Chemical injury/1/T	Auto-	77.0/39.5	68.8/68.8	4	0.05/0.001	3/2	4/4	4/4	3/4	3/3	0/2	0/0	Mixed/Conj	**Failure month 3** *2nd CLET (number16)*
2/F/71	Multiple surgeries/3/S	Auto-	45.0/25.0	71.4/74.5	0	0.00001/0.00001	3/0	1/0	3/0	2/0	3/1	3/1	1/0	Mixed/corneal	**Success**
3/F/79	Multiple surgeries/3/T	Auto-	28.0/20.0	68.8/74.4	0	0.00001/0.00001	1/0	3/2	3/1	2/1	3/1	4/0	0/0	Conj/corneal	**Success**
4/M/69	Multiple surgeries/3/T	Auto-	44.0/9.4	87.8/89.7	0	0.00001/0.00001	2/0	3/2	4/2	3/2	2/1	3/0	2/0	Conj/mixed	**Success**
5/F/63	Stevens Johnson/2/S	Allo-	65.0/50.0	71.6/71.6	1	0.05/0.06	3/1	2/0	2/2	2/2	3/1	3/1	1/0	Mixed/corneal	**Success** ***Failure month 35***
6/M/62	MMP/2/S	Allo-	4.1/0.3	91.9/91.9	1	0.8/0.8	3/0	1/0	4/0	4/0	2/1	3/0	0/0	Mixed/corneal	**Success**
7/M/66	Multiple surgeries/3/S	Auto-	47.0/67.8	81.2/84.2	0	0.001/0.001	3/1	3/2	4/2	4/3	3/2	3/0	1/0	Mixed/corneal	**Success**
8/M/36	Chemical injury/1/S	Auto-	31.0/29.1	80.6/80.6	1	0.25/0.6	2/1	3/1	2/1	4/2	3/1	2/0	0/0	Conj/corneal	**Success**
9/F/34	Stevens Johnson/2/T	Allo-	98.0/89.5	40.7/57.4	1	0.05/0.2	3/2	2/1	1/0	1/1	2/1	4/1	1/0	Conj/corneal	**Success**
10/F/63	Stevens Johnson/2/T	Allo-	78.0/30.0	52.1/76.9	1	0.5/0.8	3/2	2/0	4/3	3/1	2/1	2/1	1/0	Conj/corneal	**Success**
11/M/27	Postinfectious keratitis, PKP/3/T	Auto-	46.0/22.7	69.1/72.8	4	0.001/0.001–0.4 after 2nd surgery	4/1	4/3	3/1	2/1	3/2	2/0	0/0	Conj/mixed	**Success** *PKP + cataract (month14) + valve*
12/M/52	Postinfectious keratitis/3/T	Auto-	62.5/5.0	93.0/95.3	3	0.05/0.2	1/0	3/2	4/2	4/2	3/2	2/0	0/0	Conj/mixed	**Success** *Refused PKP*
13/M/36	Chemical injury/1/S	Auto-	8.3/8.3	79.6/74.7	3	0.2/0.5	1/0	3/1	2/1	3/2	3/1	0/0	0/0	Conj/corneal	**Success**
14/F/54	Postinfectious keratitis, PKP; RD surgeries/3/T	Auto-	28.0/18.8	81.9/84.7	4	0.0001/0.001–0.01 after 2nd surgery	2/1	4/2	4/3	3/2	2/1	4/1	0/0	Conj/mixed	**Success** *PKP + cataract (month 30)*
15/M/37	Congenital aniridia/3/S	Allo-	98.0/56.8	16.2/28.1	4	0.001/0.01	3/1	3/2	3/2	3/2	3/1	4/1	2/0	Conj/mixed	**Success** *Refused further surgeries*
16/M/48	Chemical injury; previous CLET (number 1)/1/T	Allo-	33.0/22.7	66.6/64.3	3	0.001/0.001	3/2	3/3	4/4	4/4	2/1	2/2	0/0	Conj/Conj	**Partial success** *Refused PKP*
17/M/52	Chemical injury/1/T	Allo-	52.0/50.0	12.6/37.7	4	0.001/0.001	3/1	4/4	3/3	3/3	3/3	3/3	0/0	Conj/Conj	**Failure month 4**
18/F/33	Chemical injury, PKP, severe ocular allergy/1/T	Allo-	65.0/22.7	60.9/73.0	4	0.62/0.01	4/2	4/4	4/4	4/4	3/3	2/3	0/0	Mixed/Conj	**Failure month 9**
19/F/49	Congenital aniridia/3/T	Allo-	47.7/63.6	35.3/33.1	2	0.02/0.05	3/0	2/1	4/1	3/2	3/1	2/0	0/0	Mixed/corneal	**Success**
20/M/53	Chemical injury/1/S	Auto-	31.3/9.4	63.7/96.3	1	0.5/0.8	2/1	3/1	1/1	1/2	3/1	4/0	2/0	Conj/corneal	**Success**
Mean (SD)	—	—	49.5 (25.8)/34.3 (23.4)	64.7 (22.9)/71.5 (19.4)		0.15 (0.25)/0.20 (0.31)	—	—	—	—	—	—	—	—	—
Median (IQR)	—	—	—	—		.	3.0 (1.0)/1.0 (1.3)	3.0 (1.3)/2.0 (1.3)	3.5 (1.3)/2.0 (2.0)	3.0 (2.0)/2.0 (1.3)	3.0 (1.0)/1.0 (1.0)	3.0 (1.3)/0.5 (1.0)	0.0 (1.0)/0.0 (0.0)	—	—

*Group 1: chemical injuries, Group 2: immune-based inflammatory diseases, Group 3: noninflammatory diseases; †T: total, S: severe; ‡visual potential: 1, improvement with CLET only (corneal opacity was only superficial); 2, improvement with one surgery different from corneal transplant after CLET (i.e., cataract removal); 3, improvement with subsequent corneal transplant after CLET (corneal opacity was full thickness); 4, improvement with subsequent corneal transplant plus another surgery (cataract removal unless otherwise specified) after CLET, and 0: no possibility of improvement (i.e., due to irreversible retinal pathology). Auto-, autologous; Allo-, allogeneic; BCVA, best corrected visual acuity; BCVA values 0.01, 0.001, 0.0001, and 0.00001 equivalent to counting fingers, hand motion, light perception, and no light perception, respectively; Conj, conjunctival; Fluor, fluorescein staining; IQR, interquartile range; IVCM, in vivo confocal microscopy; MP, mucous membrane pemphigoid; NEI-VFQ-25, National Eye Institute-Visual Function Questionnaire; OSDI, ocular surface disease index; SPK, superficial punctate keratitis; PED, persistent epithelial defect; PKP, penetrating keratoplasty; and SD, standard deviation.

However we have now registered a clinical trial that has just finished recruitment (https://www.clinicaltrials.gov/).

LSCD diagnosis was confirmed at the initial visit based on (1) clinical signs under slit lamp examination (SLE), including absence of the normal appearance of the limbal area and at least 3 of the following corneal signs: central epithelial irregularity ≥2 (range 0–3), central epithelial opacity ≥2 (range 0–4), superficial neovascularization (area and length of neovessels) ≥3 (range 0–4), and recurrent or persistent epithelial breakdown, all as defined below; (2) conjunctival-like or mixed epithelial cell phenotype in the central cornea (as defined below) observed by in vivo confocal microscopy (IVCM). All patients had already been through medical therapies intended to quiet their ocular surface and reverse as much as possible any treatable limbal dysfunction.

Grading of LSCD was made by clinical evaluation based on parameters previously published [24, 25]. We selected only patients with total LSCD, in which the cornea was completely vascularized and opacified, or severe LSCD, in which there were recurrent or persistent epithelial defects, peripheral corneal conjunctivalization involving more than 2 quadrants, and central cornea opacification. Moderate cases of LSCD with sectorial conjunctivalization involving less than 2 quadrants, none of which reached the central cornea, were not considered for this study.

The final outcome of CLET strongly depends on the etiology of LSCD, among other factors. Thus, the candidate eyes were grouped into one of the following three etiological categories at the initial visit: Group 1 had chemical injuries (only included at a minimum of 12 months after the acute trauma and after other therapies had failed); Group 2 had immune-based inflammatory diseases (i.e., cicatrizing conjunctivitis such as Stevens-Johnson syndrome, mucous membrane pemphigoid, atopic keratoconjunctivitis, rosacea-related keratoconjunctivitis); and Group 3 had other noninflammatory conditions such as sequelae from multiple surgeries, chronic sequelae of infectious keratitis already sterile, congenital aniridia, contact lens wear-related, and so forth.

Although limbal stem cells may have immunosuppressive properties [3, 22], immune rejection has been reported after allogeneic CLET [26]. Thus, to prevent any possible immune rejection, patients who were to receive allogeneic CLET were started at the initial visit on oral immunosuppressive therapy with one of the following drugs: 1.5–2.0 g/day of mycophenolate mofetil, 3–5 mg/kg/day of cyclosporine A, or 1-2 mg/kg/day of azathioprine. The immunosuppressive treatment was maintained for 12 months after CLET, after which the drug was tapered and discontinued in the next 3 months. We closely monitored potential side effects at each visit, by phone if necessary and by blood pressure and blood/urine workup every 1-2 months.

Five to 7 days after the initial visit, unilateral LSCD cases who agreed to undergo autologous CLET underwent a limbal biopsy in the contralateral healthy eye. The limbal cells were grown in culture for 3–5 weeks, and the CLET procedure was then performed using the harvested cells. Bilateral cases had allogeneic CLET from cadaveric donors between 4–6 weeks after the initial visit so that the elapsed times between that visit and CLET were similar in all patients.

Patients were then evaluated 24 hr. after CLET, weekly for the first month, monthly until the sixth month, and every other month up to the first year. After the first year, patients were evaluated clinically every 6 months for 2 more years, reporting clinical and photographic outcomes up to three years after CLET.

Prior to the initiation of these procedures, all patients were screened for the mandatory transmittable diseases: human immunodeficiency virus, human T-cell leukemia-lymphoma virus, hepatitis C and B virus, and syphilis. All allogeneic tissue donors (cadaveric limbus and human amniotic membrane) were similarly screened.

2.2. Cultivation and In Vitro Expansion of Limbal Epithelial Cells. Cells destined for CLET were cultured at the University of Valladolid Institute of Molecular Biology and Genetics Cell Processing Unit that was licensed and accredited by the Spanish Agency of Medicines and Sanitary Products (AEMPS). The institute operates under GMP regulations and holds a protocol registered as PEI 09-137 by the AEMPS.

Donor human amniotic membranes and cadaveric limbal rings came from a registered and accredited tissue bank (Blood and Tissue Community Center, Oviedo, Asturias, Spain) and were screened for transmittable diseases as described above. Allogeneic limbal rings were obtained within 7 days of death from corneal donors who were under 60 years old. Autologous limbal biopsies were marked by the surgeon with a stitch in the upper right corner to ensure consistency when plating the explant to ensure the same way up; they were sent from the operating room to the cell production unit at 4–10°C in culture medium (Figure 1).

Human amniotic membranes were prepared using our standardized protocol. After thawing the membranes previously stored at −80°C, they were washed with phosphate buffered saline (PBS) (Life Technologies-Gibco, Madrid, Spain) and treated with trypsin (Life Technologies-Gibco) for 15 min at room temperature. This was followed by gentle cell scraping to separate the epithelial cells from the underlying stroma. Once deepithelialization was complete, the amniotic membrane stroma was washed twice in PBS to remove cellular debris. It was then attached to the bottom of a 35 mm cell culture dish so that the basement membrane faced upwards to serve as a substratum for the limbal epithelial cells.

Limbal tissues were processed during the 4 hr. following arrival (Figure 1). Either the autologous limbal explant or one piece of allogeneic limbal tissue (2 × 2 mm each) was cultured on top of the denuded amniotic membrane. The culture was performed initially under a drop of fetal bovine serum (FBS) (Life Technologies-Gibco), in standard conditions of 37°C, 95% humidified air/5% CO_2 gas mixture. After 24 hr., 3 mL of the following culture medium was added: DMEM/F12 media (1 : 1 mixture) (Life Technologies-Gibco), 5% FBS (Life Technologies-Gibco), 50 μg/mL hydrocortisone (Sigma Aldrich, St Louis, MO, USA), 0.5 ng/mL cholera toxin (Gentaur, Kampenhout, Belgium), 5 ng/mL insulin-transferrin-selenium (ITS) (Sigma Aldrich), 0.5% dimethyl sulfoxide (Sigma Aldrich), 2.5 ng/mL human epidermal growth factor (Life Technologies-Gibco), and 0.5 mg/mL gentamicin

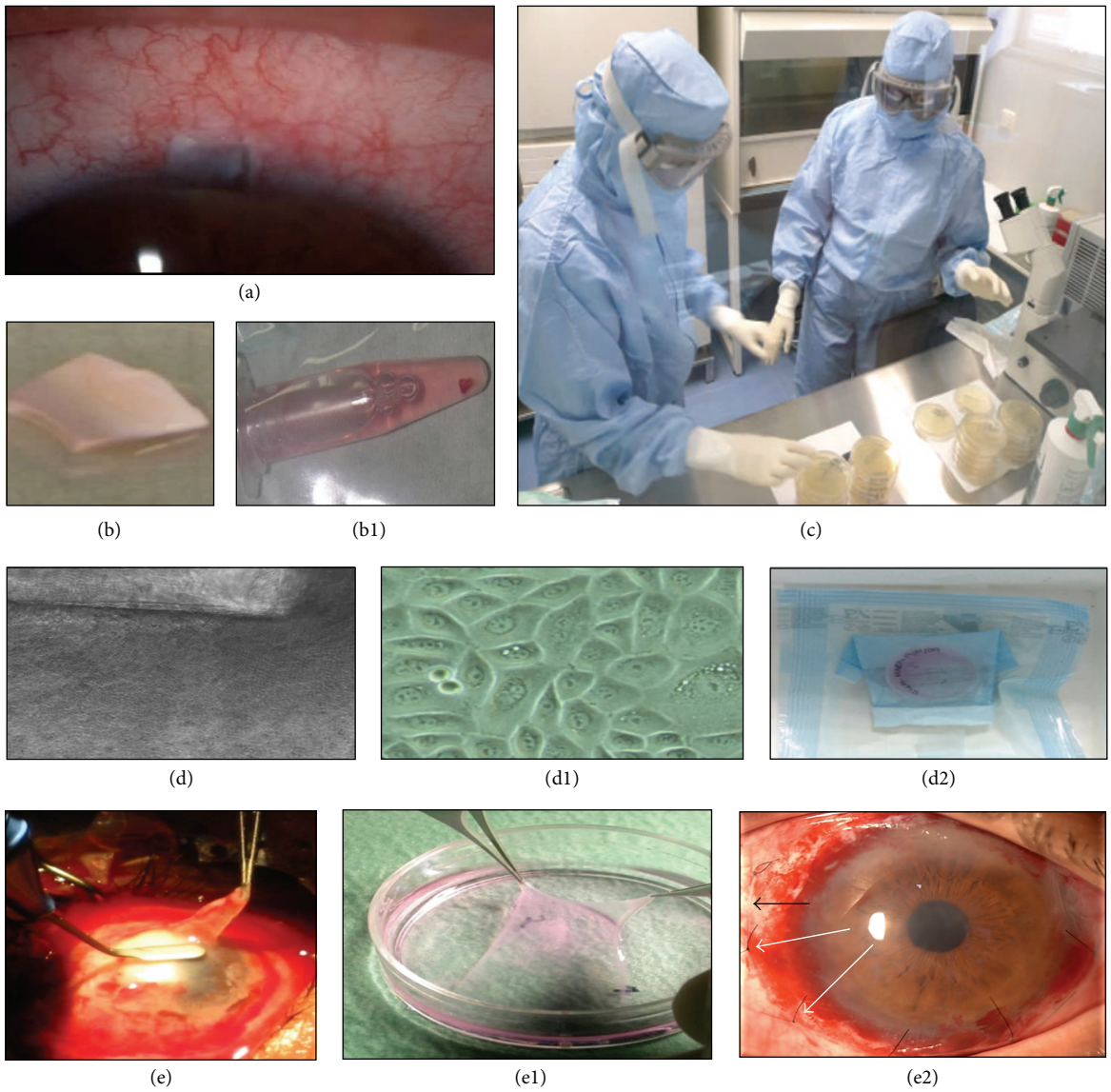

Figure 1: Limbal biopsy, limbal epithelial cultivation, and cultivated limbal epithelial transplantation (CLET). (a) Healthy donor eye 24 hr. after a 2 × 2 mm limbal biopsy; (b) the biopsy tissue was placed in an Eppendorf tube with culture medium (b1); (c) the biopsy was processed in a good manufacturing practice-cell processing unit within the next 4 hr. and for the next 4-5 weeks; and (d) the explant was placed on denuded human amniotic membrane, as viewed by contrast phase microscopy. Limbal epithelial cells began outgrowth from the explant at 1-2 weeks (d1). The explant was then removed, and the outgrowth was maintained until reaching confluence at which time it contained approximately 250,000 cells. The cell product was then sent to the medical center for CLET (d2). (e) Superficial keratectomy in the diseased contralateral eye (Case 1); (e1) human amniotic membrane with epithelial limbal cells confluent on top is removed from culture dish; (e2) the complex of amniotic membrane-limbal stem cells is placed on top of the previously denuded corneal and sclerolimbal surface; the amniotic membrane limit is observed (black arrow) with cells facing down and sutured (white arrows). A scleral lens is then applied.

(Life Technologies-Gibco). The culture medium was changed every 3 days.

Limbal explants were kept in culture until a cellular outgrowth front of approximately 2 mm was present (1-2 weeks), and then the explants were removed to avoid stromal cell contamination and to allow further cell proliferation in the area previously occupied by the explant, as previously described [27]. Intermediate and final culture media were checked for sterility. The final product had to be negative for the tested microbiologic agents (aerobes, anaerobes, fungi, and mycobacteria) by using a validated test (Bact-ALERT) that inactivates antibiotics and thus eliminates the possibility of masked contamination. Additionally the final product had to be up to 80% confluent, which meant an average of 250,000 cells per product. All of these procedures, as well as the characterization of the cultures, were previously established in our regular cell culture laboratory [27] and then transferred to the GMP clean room. Further characterization of the cultures was not possible due to the scarceness of the tissues because of the limited amount of biopsy material.

When cultivated cells were ready for CLET, the final cellular products were sent to the medical institution within 4 hr. of the surgery (Figure 1).

For limbal cells grown out of cadaveric explants, an additional culture was plated outside the GMP facility in parallel to the one planned for clinical use. In all cases, most cells (>70%) were highly positive for the limbal progenitor cell phenotype markers p63, CK15 and weakly positive for the corneal differentiated epithelium phenotype markers cytokeratins CK3/CK12 (data not shown). More in depth staining and complete characterizations were achieved before transferring this technique from the cell culture lab to the GMP clean room and thus not repeated [27, 28].

2.3. Surgical Procedures: Limbal Biopsy and CLET. All operations were performed by the same surgeon (coauthor José M. Herreras) and were carried out in a standard manner. Limbal biopsies were performed under topical anesthesia: 0.1% tetracaine chlorohydrate and 0.4% oxybuprocaine chlorohydrate solution (Colircusí Anestésico Doble, Alcon Laboratories, Ft. Worth, TX, USA), following the standards for aseptic procedures. Biopsies measuring 2 × 2 mm were obtained from regions of the corneoscleral limbus (Figure 1) where the palisades of Vogt were better defined by SLE and by IVCM at the initial visit [29].

For the CLET procedure, retrobulbar anesthesia was achieved with 3 cc of 5% lidocaine (Lidocaine Braun, Braun Medical SA, Melsungen, Germany). First, a conjunctival peritomy was performed and tissues were recessed leaving bare sclera. Fibrovascular pannus, if present, was scraped and removed from the recipient cornea extending to the limbal area, allowing a gentle 360° limbal peritomy to be performed. The scraped surface was polished with a diamond bur, and bleeding vessels were cauterized. Then the CLET graft was carefully lifted from the culture dish and placed with the epithelial limbal cells facing the recipient ocular surface so that cells were in immediate and close contact with the tissues (cornea and limbal areas) to be repaired. The graft was then sutured to the perilimbal episclera, 2–4 mm posterior to the limbus, with 8 interrupted 10-0 nylon stitches (Figure 1). Topical eyedrops (see below) were then applied and an 18–22 mm diameter bandage contact lens was set in place, and the eye was patched for 24 hr.

Twenty-four hours after surgery, each patient was evaluated and topical treatment with the fixed combination of 1% prednisolone acetate and 0.3% tobramycin (Tobradex, Alcon Laboratories) was prescribed 4 times per day for 6 weeks. Then, 1 mg/mL dexamethasone (Maxidex, Alcon Laboratories) was instilled 4 times a day and slowly tapered in the next 3 months. The contact lens was removed after complete disappearance of the amniotic membrane at 2 to 8 weeks, and the stitches were also removed at that time.

2.4. Evaluation Endpoints. Table 1 shows the summary of all evaluation endpoints performed at the initial visit and at 12 months for each of the 20 eyes subjected to CLET. At 24 and 36 months, only clinical evaluations were performed.

2.4.1. Clinical Examination and Photographs. LSCD-related symptoms and the impact on each patient's life were evaluated with two self-administered questionnaires at the initial visit and 12 months after CLET. First, ocular surface-related symptoms caused by LSCD were evaluated with the ocular surface disease index (OSDI), where scores >12 indicate abnormal symptomatology, with >32 meaning severe symptoms [30]. Visual function-related aspects of the quality of life were evaluated with The National Eye Institute 25-Item Visual Function Questionnaire (NEI-VFQ-25) where higher scores on a scale 0 to 100 indicate better function [31].

Best corrected visual acuity (BCVA) was determined with a Snellen visual chart. Counting fingers, hand motion, light perception, and no light perception were converted to Snellen equivalents, 0.01, 0.001, 0.0001, and 0.00001, respectively, as published so that a mean value could be calculated [32]. One or more line changes in BCVA at 12 months after CLET were considered as improvement or worsening. However, BCVA improvement is never the primary goal of CLET, as this technique intends to reconstruct the corneal epithelium and will not necessarily affect stromal or endothelial pathologies for which other visual rehabilitation techniques may be needed after CLET. To avoid misinterpretation by the patient, the potential dependence of the visual prognosis on the surgical procedures (Table 2) judged to be necessary to restore vision after CLET was explained at the initial visit.

SLE (Topcon SL-8Z, Topcon Corp., Tokyo, Japan) with fluorescein corneal staining and photographs of the graft-treated eye were performed at each visit. In autografts, the biopsy site was also monitored. Two of the authors (Beatriz E. Ramírez and Margarita Calonge) evaluated the same parameters of LSCD independently, and in case of disagreement, the average score was recorded. Photographs were taken with diffuse light using the program IMAGENet (Fuji Fujifilm Finepix S1 Pro, Fuji Photo Film Co., Ltd., Tokyo, Japan). The following clinical characteristics were evaluated (Table 3): ciliary hyperemia, central corneal epithelial opacity, central corneal epithelial irregularity, corneal epithelial integrity including superficial punctate keratitis and persistent epithelial defects, and corneal superficial neovascularization based on the extension or area involved and neovessel length.

2.4.2. In Vivo Confocal Microscopy. IVCM examination of the central cornea with the Heidelberg Retinal Tomograph HRT-3 and Rostock Cornea Module (HRT3, Heidelberg Engineering GmbH, Heidelberg, Germany) was performed at the initial visit and at 12 months after CLET to evaluate the phenotype of the epithelium covering the central cornea. Optical sections from the central cornea were taken at the basal and superficial layers of the epithelium. Basal cell morphology was classified as "corneal-like," having regular, hexagonal cells with a cell diameter <20 μm, or "conjunctival-like," having closely packed round or irregularly shaped cells with a cell diameter of >20 μm and occasional goblet cells. In some cases, the basal cell morphology was classified as "mixed," with both phenotypes present [24, 33].

Table 2: Prognostic classification on the potential for visual recovery in patients suffering from limbal stem cell deficiency and scheduled for cultivated limbal epithelial transplantation (CLET).

Visual prognosis	Ocular media opacity	Surgeries judged to be necessary to recover full potential vision
Grade 1	Corneal opacity restricted to anterior cornea (epithelial and anterior stroma)	One surgical procedure: CLET only
Grade 2	Corneal opacity restricted to anterior cornea (as Grade 1) plus another noncorneal reason for visual loss (e.g., cataract)	Two surgical procedures: CLET+ noncorneal surgery (e.g., cataract removal most likely)
Grade 3	Full thickness corneal opacity	Two surgical procedures: CLET+ corneal transplant
Grade 4	Full thickness corneal opacity plus another noncorneal reason for visual loss (e.g., cataract)	Three surgical procedures: CLET+ corneal transplantation-corneal surgery (e.g., cataract removal)
Grade 0	Any grade of corneal opacity plus noncorneal irreversible visual loss (e.g., irreversible retinal pathology, advanced glaucoma)	No potential for gain: CLET performed to avoid globe removal

IVCM was also performed in the limbal area to select the site for biopsy in cases of autologous CLET. The area where more and better defined palisades of Vogt were found was selected as the site for biopsy. In some cases, the wounding and healing of the biopsy site were closely followed, as published elsewhere [29].

2.4.3. Definition of an Overall Success, Partial Success, and Failure. As visual acuity by itself is a poor endpoint to evaluate the success of CLET and associated therapies, we made an integral evaluation of each patient through primary and secondary outcomes. Each patient was evaluated with this same protocol at 12 months after CLET.

Primary outcomes were as follows: (1) improvement in visual-related aspects of the quality of life evaluated with the NEI-VFQ-25 or improvement in ocular surface symptoms, evaluated by OSDI; (2) improvement by at least one step in at least 3 of the 4 following clinical parameters evaluated by SLE: ciliary hyperemia, central corneal epithelial irregularity, central corneal epithelial opacity, and superficial punctate keratitis; (3) complete absence (Grade 0) of persistent epithelial defects; and (4) presence of a more corneal-like phenotype in the central cornea as assessed by a change from the conjunctival phenotype to either corneal or mixed phenotype or from a mixed phenotype to a corneal phenotype as evaluated by IVCM [24, 33].

Secondary outcomes were as follows: (1) BCVA improvement of one line or more and (2) amelioration measured by at least a one-step decrease in the superficial corneal peripheral neovascularization area or neovessel length.

The outcome was considered successful only when all four primary outcomes were achieved. The outcome was considered partially successful when the patient presented only two of the primary outcomes or when one primary outcome in addition to one secondary outcome was achieved. Failure meant that only one or none of the primary outcomes were met.

After the first year, patients were evaluated only clinically by SLE every 6 months, until the end of follow-up, 3 years after CLET.

2.5. Statistical Analysis. Quantitative characteristics were expressed as means ± standard deviations (SD), and qualitative variables were described in percentages. The median and interquartile ranges (IQR) were used to summarize distributions of ordinal variables. Normality assumptions were checked by the Shapiro-Wilk test. The Wilcoxon signed-rank test was used to evaluate improvement of the subjective questionnaires OSDI and NEI-VFQ-25 and the change in quantitative and ordinal clinical variables at 12 months after CLET. Differences between the means of two independent groups were tested by Student's t-test or the nonparametric alternative, Mann-Whitney U test, if the normality assumption was not valid. Relationships between two qualitative variables were evaluated by Fisher's exact test. To compare the success rates for the three predefined prognostic groups and the type of CLET, the test for equality of proportions was used. Kaplan-Meier survival analysis was applied to estimate transplant survival. The log-rank test was used to compare the univariate survival curves of autologous and allogeneic type. Statistical analysis was performed using R Statistical Software version 3.1.0 (Foundation for Statistical Computing, Vienna, Austria) by a licensed statistician (coauthor IF).

3. Results

The demographic data and initial and final clinical data collected at 12 months for each case are presented in Table 1. CLET was performed in 20 eyes (12 males, 8 females) of 19 patients (age, 51.6 ± 14.5 years; range, 27–79 years). There was no significant difference in age between males and females (t-test, $p = 0.3039$). All cases were followed up to 3 years, although follow-up of Case 1 logically ended when he had a second CLET at month 13 (Table 1).

The three prognostic groups had the following etiologies: Group 1, composed of chemical injuries, had 7 eyes (35% of cases, 4 autografts, 3 allografts); Group 2, composed of immune-based inflammatory diseases, had 4 eyes (20%, 3 with Stevens-Johnson syndrome, 1 with mucous membrane pemphigoid, all allografts); and Group 3, composed of non-inflammatory diseases, had 9 eyes (45%, 4 with sequelae from

Table 3: Grading of ocular surface clinical characteristics.

	Ciliary hyperemia [50]	Central corneal epithelial opacity [50]	Central corneal epithelial irregularity [24]	Corneal epithelial integrity		Corneal superficial neovascularization [50]	
				Superficial punctate keratitis*	Persistent epithelial defect area [24]	Area	Length
Grade 0	White conjunctiva	None	Normal/absent		None	None	None
Grade 1	Widening of the vessels	Mild	Mild		≤1/4	≤1/4	1 mm
Grade 2	Mild hyperemia	Moderate	Moderate		>1/4 and ≤1/2	>1/4 and ≤1/2	2-3 mm
Grade 3	Moderate hyperemia	Severe with faint pupil	Severe		>1/2 and ≤3/4	>1/2 and ≤3/4	4-5 mm
Grade 4	Intense hyperemia	Severe with no visible pupil	N/A		>3/4	>3/4	≥6 mm
Grade 5	N/A	N/A	N/A	>Grade 4	N/A	N/A	N/A

*Modified from [51].

multiple surgeries, 3 with postinfectious keratitis, and 2 with congenital aniridia; 7 autografts, 2 allografts). Twelve eyes had total LSCD and 8 had severe LSCD. The time between disease onset and CLET was 77.2 ± 88.9 months (range, 6–321 months).

The time required for limbal cell expansion and cultivation on human amniotic membrane was 24.7 ± 5.8 days. Cultures from a cadaveric source required 26.1 ± 6.0 days, and cultures from an autologous source required 23.5 ± 5.7 days; however, this difference was not statistically significant (t-test, $p = 0.4251$).

Eleven eyes (55%) with unilateral disease had autologous CLET. Four cases belonged to prognostic Group 1, and 7 cases were in Group 3. The remaining 9 eyes (45%) received allografts: 3 eyes in Group 1, 4 eyes in Group 2, and 2 eyes in Group 3 (Table 2). There was no significant difference in the ages of the autologous and allogeneic recipients (t-test, $p = 0.4929$) or gender distribution (Fisher's exact test, $p = 0.3618$). Of the 9 eyes that received allogeneic CLET, two had unilateral disease. Case 18 had unilateral chemical injury but also had bilateral perennial allergic conjunctivitis, and for this reason we did not use her contralateral eye as donor. Another allograft with unilateral disease was Case 16, who like Case 1 had unilateral chemical injury. He initially received an autologous CLET; however, after the 12-month mandatory follow-up, he received an allogeneic CLET so as not to compromise the healthy eye with a second biopsy. Four eyes in Group 2 had allografts (immune-based diseases are always bilateral) and most cases in Group 3 (7 of 9) had autografts. Thus there was no independence between the source of transplanted cells (autografts versus allografts) and the etiology of the disease (Fisher's exact test, $p = 0.0468$).

There were no intraoperative or postoperative complications during either biopsy harvesting or CLET. No episodes of immune rejection were recorded in the eyes that had received allografts. All biopsies were extracted from the superior limbal area as the palisades of Vogt in this area are the richest source of limbal stem cells [2, 5, 8, 29].

Oral immunosuppression was used in all 9 allograft patients because immune rejection has been reported to occur in 23.8% of cases in a series of allogeneic CLET who were nonimmunosuppressed even though the patients were given high doses of systemic steroids [26]. Mycophenolate mofetil (1.5–2 g/day) was used in Cases 5 and 6; cyclosporine A (3–5 mg/kg/day) was prescribed in Cases 9, 10, 16, 17, and 18; and azathioprine (1.5–2 mg/kg/day) was used in Cases 15 and 19. The drugs were well tolerated in all cases, and no withdrawals or discontinuations were necessary. Mycophenolate mofetil had to be lowered from 2 g/day to 1.5 g/day in Case 5 due to asthenia; cyclosporine A was also lowered from 5 to 3 mg/kg/day in Cases 17 and 18 due to mild elevation in blood pressure. There were no episodes of graft rejection.

There was one adverse event. Case 1 developed a severe infectious conjunctivitis (*Staphylococcus aureus*, culture positive) 9 weeks after surgery. Although the infectious process was successfully controlled with topical antibiotics, the autograft started to deteriorate and was considered a failure at month 3. Due to the nature of this patient's job, we had recommended to him that he take extreme eye protective measures. He was noncompliant in this recommendation, which created a risk factor that probably resulted in the eye infection.

3.1. Overall Success/Failure Rate and Survival Analysis. The overall success was 80% after both the one- and two-year follow-up periods. The rate decreased to 75% after 3 years. At 12 months after CLET, when we performed a thorough evaluation, 16 of the 20 eyes achieved the four primary outcomes (Table 1) while 4 eyes (20%, 3 complete failures, 1 partial success) were considered failures. All failures were from Group 1 (chemical injuries). At one year, the success rate was clearly lower in prognostic Group 1 (4 out of 7 eyes, 42.9%) than in Groups 2 and 3 (100%). Among these failures, Case 1 had autologous CLET, and Cases 16, 17, and 18 had allogeneic CLET. Therefore the success rate at one year for autografts was 90.9% (10 of 11), and for allografts it was 66.7% (6 of 9). There was no significant difference in the success/failure rates between the autologous and allogeneic CLET procedures (equality of proportions, $p = 0.4315$) (Figure 2).

Even in the failed Cases 1, 17, and 18, there was subjective improvement as shown by lower OSDI scores and higher NEI-VFQ-25 scores (Table 1). Case 1 had another CLET (Case 16) in which the final outcome was graded as a partial success. This patient elected not to try to rehabilitate his chemically injured right eye any further as he remained mostly asymptomatic, and his left eye had full vision. Failed Case 17 (Figure 3) also chose not to do any additional procedure.

Case 18 failed after 9 months. After completing the 3-year follow-up, she entered a clinical trial on cell therapy that has finished recruitment (https://www.clinicaltrials.gov/ Identifier: NCT01562002).

All 3 failures and the partial failure after 12 months belonged to prognostic etiology Group 1. The success rate of this group, 42.9%, was significantly lower than for Group 2, the autoimmune-based cases (100% success, equality of proportions, $p = 0.0096$), or Group 3, the miscellaneous group (100% success, equality of proportions, $p = 0.0096$). None of the 3 failed cases in Group 1 deteriorated any further after the CLET failure.

Only one eye failed after the first year (month 35) after CLET (Case 5, Stevens-Johnson syndrome, prognostic Group 2), showing recurrence of epithelial barrier breakdown. This patient was also recruited at month 37 for the cell therapy clinical trial mentioned above.

Survival curve analysis (Figure 2) showed a probability of success (Kaplan-Meier) at 1 year and 3 years after CLET of 0.80 (confidence interval [CI] 95%, 0.643–0.996) and 0.75 (CI 95%, 0.582–0.966), respectively, for all cases. Autologous CLET had the same survival probability, 0.9091 (CI 95%, 0.0867–0.7541), after both 1 and 3 years. Allogeneic CLET had a 1- and 3-year survival probability of 0.667 (CI 95%, 0.420–1.00) and 0.556 (CI 95%, 0.31–1.00), respectively. The difference in survival between autografts and allografts was not significant (log-rank test, $p = 0.0949$).

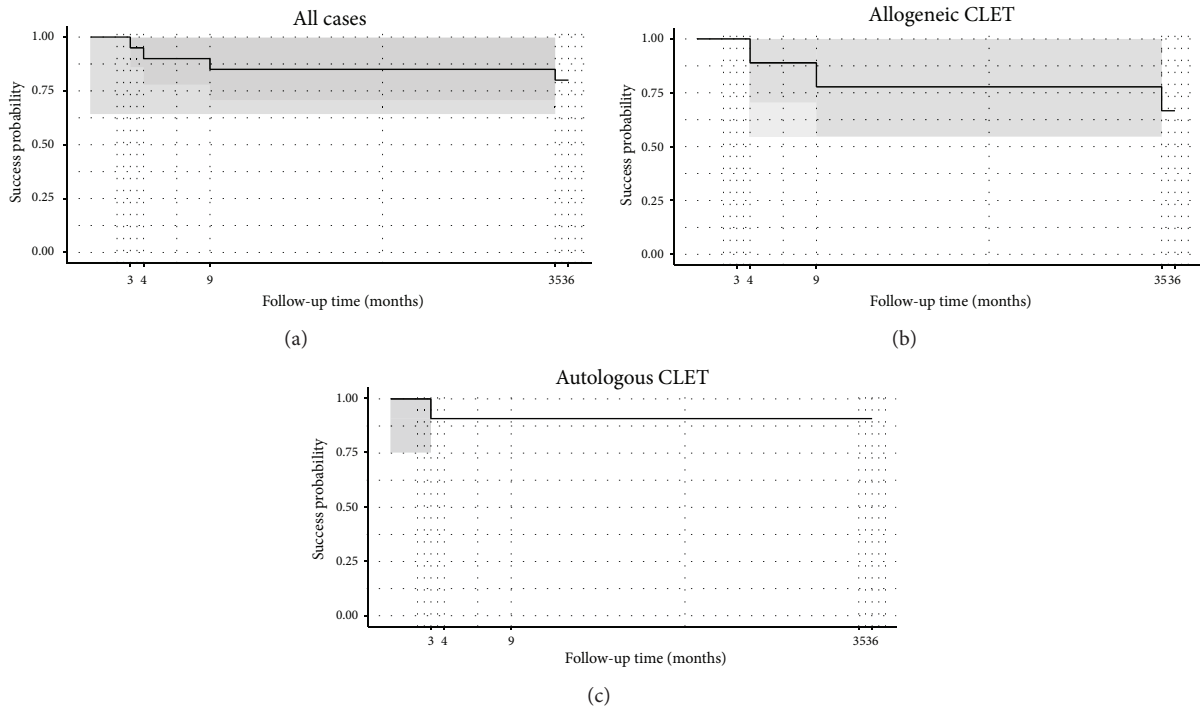

Figure 2: Kaplan-Meier survival (success) curves of the 20 cases undergoing cultivated limbal epithelial transplantation (CLET) according to follow-up time (maximum of 36 months) (a) and separated by the origin of cells, allogeneic CLET (b) and autologous CLET (c). Shaded areas represent the confidence bands. Survival analysis showed a probability of success at 1-2 years and at 3 years after CLET of 0.80 (confidence interval [CI] 95%, 0.643–0.996) and 0.75 (CI 95%, 0.582–0.966), respectively, for all cases (a). Allogeneic CLET had a 1-2-year and a 3-year survival probability of 0.667 (CI 95%, 0.420–1) and 0.556 (CI 95%, 0.31–1), respectively (b). The survival probability for autologous CLET was 0.9091 (CI 95%, 0.0867–0.7541) after 1, 2, or 3 years (c). The difference in survival between autografts and allografts was not significant (log-rank test, p value: 0.0949).

3.2. Clinical Outcome

3.2.1. Quality of Life and Ocular Surface Symptoms. The OSDI score for ocular surface symptoms at the initial evaluation was 49.5 ± 25.8 (Table 1), indicating that the symptoms were severe [30]. All cases showed a reduction in OSDI score following the first month after CLET (36.1 ± 25.2; Wilcoxon signed-rank test, $p = 0.001$). The improvement was maintained one year after CLET (34.3 ± 23.4, Wilcoxon signed-rank test, $p = 0.0035$; Table 1). The scores were not influenced by the source of donor cells (autologous versus allogeneic) (t-test, initial visit $p = 0.0954$, final visit $p = 0.1420$).

The visual-related quality of life score after CLET, measured by the NEI-VFQ-25, improved from 64.7 ± 22.9 at the initial evaluation to 71.5 ± 19.4 at the 1-year follow-up (Wilcoxon signed-rank test, $p = 0.0057$, Table 1).

Autograft cases started with significantly higher NEI-VFQ-25 values than allografts at the initial visit (76.9 ± 9.2 versus 49.8 ± 26.1, resp., t-test, $p = 0.0147$). The autograft cases also ended with significantly higher scores than the allograft cases at the final visit (81.5 ± 9.4 versus 59.3 ± 22.0, resp., t-test, $p = 0.0176$). However, when the increased percent of NEI-VFQ-25 score after CLET was compared for the two graft procedures, the difference between autografts and allografts was not significant (16.0 ± 20.0% versus 18.6 ± 26.0%, resp.; Mann-Whitney U test, $p = 0.8788$). Therefore, it is not possible to say that autografts improved visual-related quality of life issues more than allografts did.

3.2.2. Parameters Evaluated by SLE. By SLE, complete reabsorption of the amniotic membrane was observed at 4 weeks after surgery in 12 eyes (60%), between 5 and 7 weeks in 7 eyes (35%), and in 8 weeks in one eye (5%).

Hyperemia was one of the signs that patients verbalized as having improved the most. It went from 3.0 ± 1.0 (median ± IQR) at the initial evaluation to 1.0 ± 1.3 at month 12 (Wilcoxon signed-rank test, $p = 0.0001$), improving in all 20 eyes (Table 1). At the initial visit, hyperemia was significantly greater (Mann-Whitney U test, $p = 0.0170$) in those patients that would later have allogeneic CLET, 3.0 ± 1.0, versus those who would receive an autograft, 2.0 ± 1.5.

The preoperative central corneal epithelial opacity score was 3.0 ± 1.3 (median ± IQR), and it improved significantly to 2.0 ± 1.3 (Wilcoxon signed-rank test, $p = 0.0003$) at one year (Table 1). For all three failed cases, the initial opacity score was at the maximum value of 4 and did not change after CLET (Table 1). It also remained unchanged in the partial success case (Case 16). For the remaining 16 eyes, central corneal epithelial opacity scores decreased. Considering only successful cases at the end of the 12 month follow-up, corneal central opacity was significantly greater in cases with

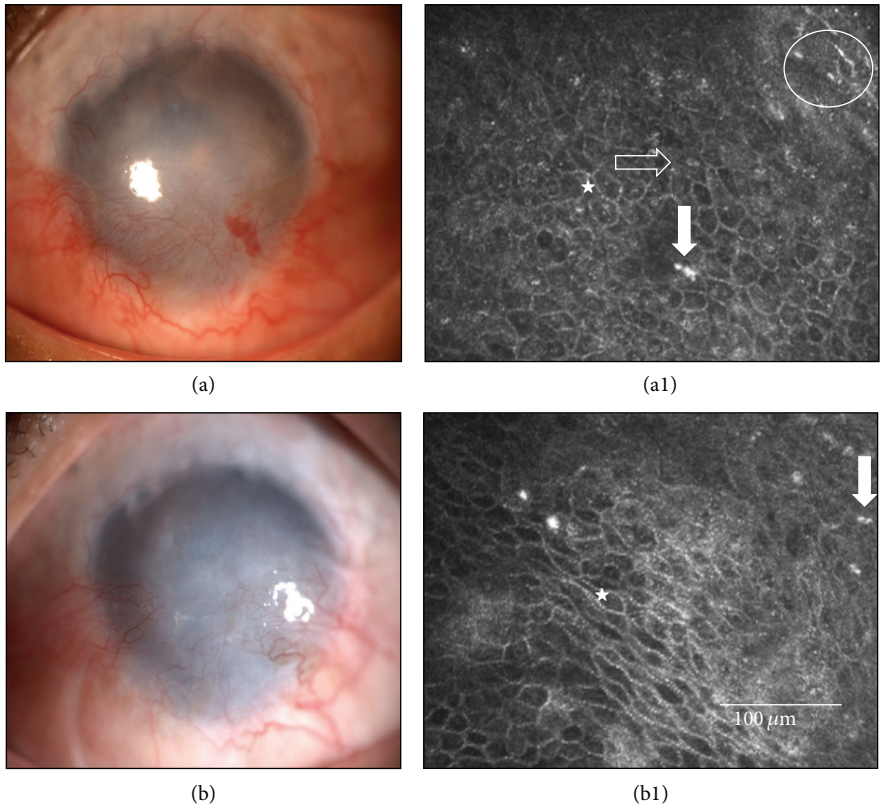

Figure 3: Case 17 (Table 1) before and after cultivated limbal epithelial transplantation (CLET). This 52-year-old male suffered a chemical injury in his right eye 5 years earlier. He developed total limbal stem cell deficiency and showed at the initial visit an opaque and vascularized cornea (a) with a conjunctival-like phenotype at in vivo confocal microscopy (IVCM) in central cornea (star), with goblet cells (horizontal arrow), inflammatory cells (vertical arrow), and Langerhans cell (circle), (a1). He received an allogeneic cultivated limbal epithelial transplantation (CLET). (b) After 12 months and although his symptoms and ciliary hyperemia had improved, this case was considered a CLET failure as his epithelial phenotype in central cornea (b1) was still conjunctival-like (star), with inflammatory cells (vertical arrow), evaluated by IVCM.

autografts, 2.0 ± 1.0, compared to those with allografts (0.5 ± 1.0, Mann-Whitney U test, $p = 0.0479$).

Epithelial irregularity in the central cornea was significantly reduced from the initial score of 3.0 ± 1.0 to 1.0 ± 1.0 at the 12-month follow-up visit (Wilcoxon signed-rank test, $p = 0.0002$). For 17 of 20 eyes (85%), it decreased by a single step. For one eye, Case 13, it decreased by two steps. For the 3 cases that failed, the epithelial irregularity remained at the maximum pre-CLET level (Table 1). These results were independent of the source of cells (autologous or allogeneic) (Mann-Whitney U test, initial visit $p = 0.2321$, final visit $p = 0.7419$).

Superficial punctate keratitis, measured by corneal fluorescein staining, had a median pre-CLET score of 3.00 ± 1.25 that diminished at 12 months after CLET to 0.5 ± 1.0 (Wilcoxon signed-rank test, $p = 0.0010$). It increased in 2 of the 3 failed cases at 12 months and remained the same in the other failure and in the partially successful case. It was one of the parameters that improved in all successful cases, except for Case 13, where it was unchanged, as there was no initial corneal fluorescein staining (Table 1).

In the 12-month follow-up visit, corneal fluorescein staining was greater in those eyes that had received allografts, 1.0 ± 1.0, compared to those with autografts, 0.0 ± 0.5 (Mann-Whitney U test, p value = 0.0288). Considering only successful cases, there was tendency for greater staining in eyes that had received allografts compared to eyes that had received autografts, but the difference was not significant (Mann-Whitney U test, p value = 0.0814).

In agreement with the corneal staining, persistent epithelial defects also improved significantly (Wilcoxon signed-rank test, $p = 0.0115$). It was present in 8 cases before CLET and improved to total closure in successful cases. None of the failed cases had preoperative epithelial defects, however (Table 1). The source of cells, autografts versus allografts, did not affect this parameter (Mann-Whitney U test, initial visit $p = 0.8965$, at the final visit all cases equal 0).

Corneal neovascularization decreased in area from 3.5 ± 1.3 at the pre-CLET assessment to 2.0 ± 2.0 after 12 months (Wilcoxon signed-rank test, $p = 0.0009$) (Table 1). Similarly, the length decreased from 3.0 ± 2.0 to 2.0 ± 1.3 at the 12-month follow-up (Wilcoxon signed-rank test, $p = 0.0043$). For the three failed cases (Figure 3) and for the partially successful case, the values remained unchanged at 12 months from the initial high values. It also remained unchanged in Case 5 that failed at 35 months after CLET.

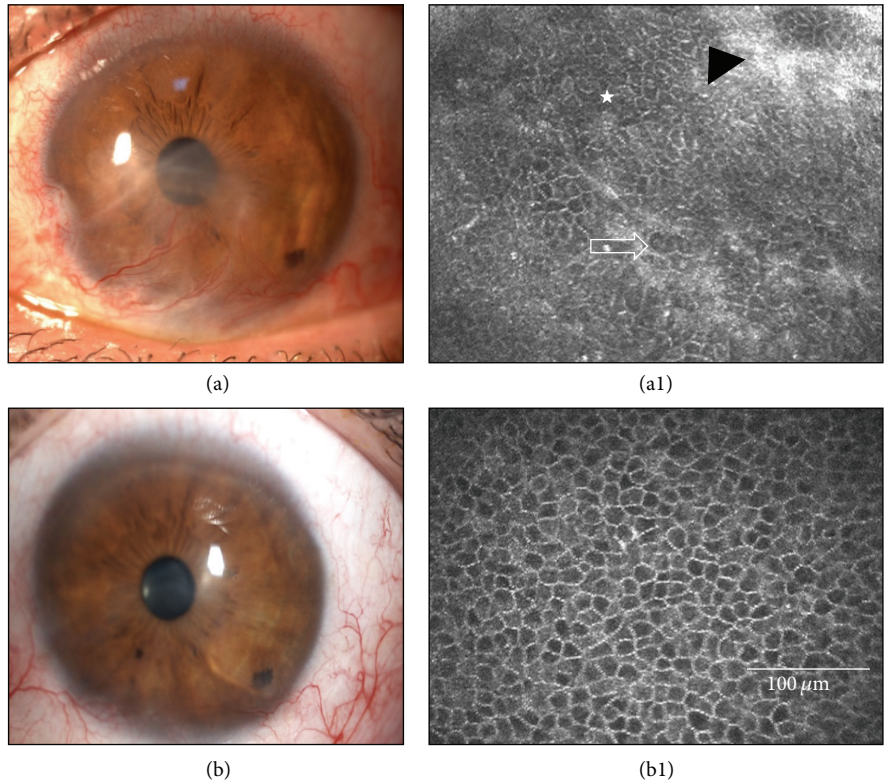

Figure 4: Case 8 (Table 1) before and after cultivated limbal epithelial transplantation (CLET). This 36-year-old man had limbal stem cell deficiency due to a unilateral chemical burn, with vascular pannus invading the visual axis (a). (a1) In vivo confocal microscopy (IVCM) shows a typical conjunctival-like epithelial phenotype in his central cornea (star), with goblet cells (arrow) and fibrosis (black arrowhead). This case was graded preoperatively in terms of visual prognosis as Grade 1, meaning that only cultivated epithelial transplantation (CLET) would be required for visual rehabilitation. After 12 months, this case was considered successful as all clinical signs improved (b) and symptoms decreased and IVCM showed an epithelial corneal phenotype (b1).

Case 20, although successful, had mild neovascularization that remained unchanged in area but increased one step regarding neovessel length (Table 1). Corneal neovessel scores were unaffected by the nature of the cells (auto- or allogeneic) transplanted (Mann-Whitney U test, initial visit neovessel area $p = 0.7427$ and neovessel length, $p = 0.66$; final visit neovessel area $p = 0.4589$ and neovessel length $p = 0.8099$).

3.3. IVCM Determination of Epithelial Phenotypes in the Central Cornea. Evaluation of the central corneal epithelial cell phenotypes by laser IVCM was the most objective primary endpoint [24, 33]. Before CLET, 13 eyes (65%; Cases 3, 4, 8, 9–17, and 20) had a conjunctival epithelial phenotype in the central cornea (Figures 3–5). One year after CLET, 6 of the 13 cases (Cases 3, 8–10, 13, and 20) improved to the corneal-like epithelium phenotype (Figure 4), and 5 eyes (Cases 4, 11, 12, 14, and 15) evolved to a mixed phenotype (Figure 5). The partially successful case (Case 17) and one of the 3 failed cases (Case 17) maintained the conjunctival phenotype. The remaining 7 eyes (Cases 1, 2, 5–7, 18, and 19) were classified in the initial examination as having the mixed epithelium phenotype. One year after CLET, 5 of these eyes (71.43%) changed to the corneal phenotype (Cases 2, 5–7, and 19). The 2 remaining cases (Cases 1 and 18) worsened to the conjunctival phenotype and were consequently considered failures. The type of transplant, autograft or allograft, had no influence in these results (Fisher's exact test, initial visit $p = 0.6424$, final visit $p = 0.3359$).

In summary, at the end of the first year after CLET, 80% of the cases had improved epithelial status in the central cornea. Of these cases, 68.8% improved from conjunctival to corneal phenotype. Of the total number of cases, the epithelial status of 10% remained unchanged and 10% worsened.

3.4. BCVA, Visual Potential, and Visual Rehabilitation. In successful cases, BCVA increased from 0.15 ± 0.24 at the initial visit to 0.25 ± 0.33 at 12 months after CLET (Wilcoxon signed-rank test, $p = 0.0059$). However, when all cases were analyzed, the increase in BCVA, from 0.15 ± 0.25 initially to 0.20 ± 0.25 at 12 months, was not significant (Wilcoxon signed-rank test, $p = 0.0914$). These results were not affected by the source of donor cells (autologous or allogeneic CLET) (Mann-Whitney test, initial visit $p = 0.1779$, final visit $p = 0.2022$).

In 10 successful cases at one year after CLET (Cases 5, 8–10, 12–15, 19, and 20), BCVA improved one line or more (Table 1). These cases represented 50% of the total 20 eyes and 62.5% of the 16 successful eyes. These 16 eyes were the only

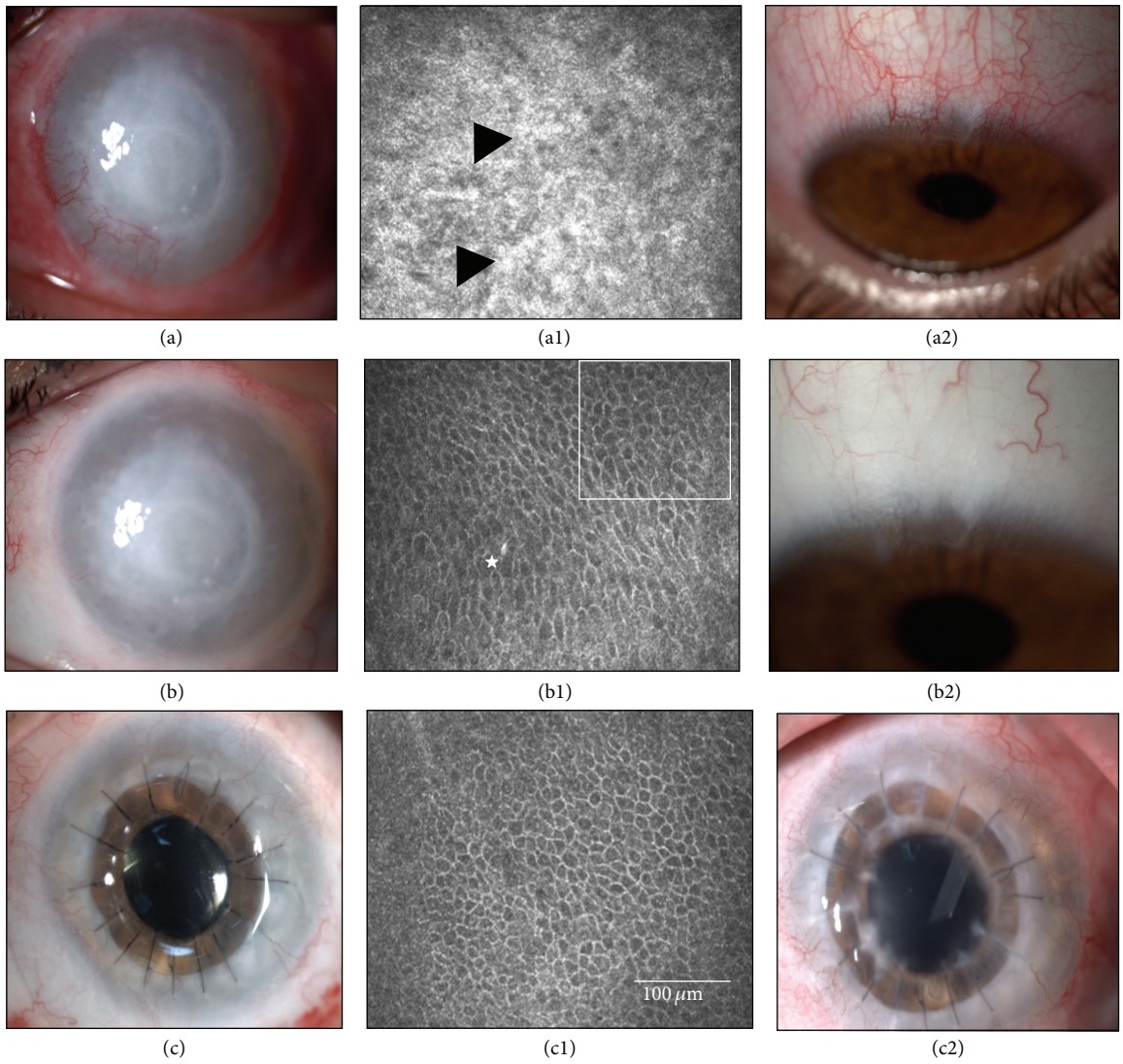

Figure 5: Case 11 (Table 1) before and after cultivated limbal epithelial transplantation (CLET). This 27-year-old male had a total limbal stem cell deficiency due to an early failed penetrating keratoplasty 7 years before. It was performed 5 years after a contact lens-related *Acanthamoeba* keratitis (a). (a1) In vivo confocal microscopy (IVCM) in the central cornea showed intense fibrosis (black arrows) and a conjunctival epithelial phenotype. (a2) Limbal cells for cultivated limbal epithelial transplantation (CLET) were obtained from his contralateral healthy eye, the biopsy site of which is shown 3 months after biopsy. (b) Twelve months after autologous CLET, corneal neovascularization had almost vanished and IVCM showed a mixed epithelium phenotype (b1), conjunctival phenotype (star), and corneal phenotype (square). (b2) Limbal donor site 12 months after biopsy. (c) Fourteen months after CLET, a penetrating keratoplasty and cataract removal were performed, followed 12 months later with a compact and clear graft. IVCM showed a corneal phenotype (c1). (c2) The corneal transplant was still successful after 2 years (3 years after CLET) although an Ahmed valve was implanted 10 months after corneal transplant to treat his elevated intraocular pressure.

ones with any probability to improve (Table 2, prognostic Grade 1, 2, 3, or 4). The visual prognosis was Grade 0 in the remaining 4 eyes for which the retinal pathology was irreversible. Five of the 10 cases (Cases 5, 8, 9, 10, and 20) were previously assigned a visual prognosis of Grade 1, meaning that CLET alone, without further intervention, was likely to improve vision (Table 2, Figure 4). Two more of those 10 eyes (Cases 12 and 13) were assigned Grade 3, meaning that a corneal transplant would be needed. Unexpectedly, they improved sufficiently to refuse any further rehabilitation. The predictions in Cases 11 (Figure 5) and 14, Grade 4, were accurate. Case 14 required a full thickness corneal transplant and cataract removal. Although the patient was very satisfied with the result, only a modest gain in BCVA was achieved. This patient had been warned in advance about the potential for a poor visual result regardless of any rehabilitation. She had undergone three previous surgeries due to retinal detachment, and the affected eye had 45-degree exotropia before CLET. Case 15 decided not to have any further rehabilitation as he had gained comfort, and due to moderate to severe

nystagmus, it was unlikely that his vision would improve. The visual potential prediction in Case 19 was also adequate with an improved BCVA of 0.2 with a mild nystagmus.

BCVA remained unchanged in 8 cases (Cases 2–4, 6, 7, 11, 16, and 17; 40% of the total 20 eyes and 37.5% of the 16 successful eyes). Four of the successful eyes (Cases 2, 3, 4, and 7) were given a visual potential of Grade 0, as all of them were previously affected by irreversible retinal pathologies. Thus no change in BCVA was expected. The central cornea status of successful Case 6 (visual potential Grade 1) improved, but his already good BCVA did not improve, which was attributed to cataract progression. In one more successful eye (Case 11, Figure 5), as predicted based on the assigned visual potential of Grade 4, BCVA only improved from hand motion to 0.4 after corneal transplantation and cataract removal. Partially successful Case 16 had a predicted visual potential of Grade 3, and he refused to pursue a corneal transplant because of the improved comfort after CLET and the full vision with his fellow eye. Case 17 (Figure 3), with a predicted visual potential of Grade 4, failed, and no further action was considered.

Vision became further impaired in 2 of the 3 cases (Cases 1 and 18) considered as failures. This represents 10% of the total 20 eyes. In Case 18, BCVA diminished dramatically, not because her corneal status became further impaired (Table 1), but rather due to intense progression in her incipient cataract. The rapid development of the cataract could have been due to the large amount of steroids that she needed, because in addition to eyedrops, she also required both inhaled and oral steroids for her severe asthma. This patient subsequently enrolled in the ocular surface cell therapy clinical trial previously mentioned.

4. Discussion

With this study, we have added to the existing body of data that supports the safety and efficacy of CLET procedures performed under GMP rules for the management of ocular surface failure due to LSCD. Overall, our major findings are that (1) 80% of the cases were successful at 1 and 2 years after transplantation and 75% remained successful at 3 years, (2) the efficacies of autologous and allogeneic limbal cell culture were similar to one another, and (3) among the three prognostic groups, those with chemical burns had the least satisfactory outcomes.

We also showed the feasibility of mild immunosuppression for a year. There were no adverse effects and no episodes of immune rejection [26]. Whether or not immunosuppression is absolutely necessary is yet controversial due to the reported immunoprotective properties of limbal stem cells against inflammatory challenges of the ocular surface [3, 22].

This study also clarifies the need for strict and well-predefined success criteria, which has been stressed by almost all authors working in this field. We adopted a modified combination of the criteria used by Shortt et al. [24]. These or a different arrangement of evaluation outcomes should be agreed upon so that studies from different parts of the world could be more comparable and so that multicenter studies and clinical trials could be a reality [34].

The intention of CLET is to improve the damaged or diseased corneal epithelium phenotype and integrity and subsequently to improve corneal barrier function. In support of that goal, this study also confirms laser IVCM as an excellent minimally invasive imaging technique to visualize with great detail the quality of the corneal epithelium before and after CLET. Although it may not be necessary in routine clinic evaluations, it proved to be a powerful tool in the evaluation of the corneal epithelium in our study and in clinical trials where objective endpoints are required [24, 33].

Since the first two patients undergoing successful autologous CLET for unilateral chemical burns in 1997 [21], many more reports have been published using this transplantation technique. Not surprisingly, these reports have utilized different sets of patients, cell product preparation protocols, surgical techniques, evaluation criteria endpoints, and follow-up periods. There are many relevant reviews focused on different aspects of corneal stem cell transplantation that are beyond the scope of this publication, which focuses mainly on clinical reports. In general, CLET success rates are above 60% as reviewed by Baylis et al. in 2013 [6] and earlier by Shortt et al. in 2007 [35].

Our study can be better compared to those performed, as ours was, after the EU Tissues and Cells Directive and GMP rules became mandatory in the EU in 2006. In our series of 20 cases, the success rate was 80% at both 1 and 2 years and 75% at 3 years. Nevertheless, the obvious differences in patients and protocols prevent full and totally reliable comparisons among different studies.

The first study published in Europe in compliance with European regulations and GMP rules was by Shortt et al. in 2008 [24] (Moorfields Eye Hospital-University College of London, London, United Kingdom). They reported 10 cases with 6-month success of 60%, evaluated clinically by corneal impression cytology and by IVCM. They had the same proportion of chemically injured eyes, 40%, as our series, with no cases of immune-based cicatrizing ocular surface diseases. In contrast, 20% of our cases were immune-based cicatrizing diseases. Their series, as ours, included both autograft and allogeneic CLET cases. Although no statistical analyses were done, these authors reported better results for allografts (70%, 7 of 10 cases) than for autografts (33%, 3 of 10 eyes). We had a slightly better prognosis for autografts but the difference in outcomes between autografts and allografts was not statistically significant. The fact that their allograft tissues derived from donor cadavers were larger than the autologous tissue might have influenced their results. In contrast, we used the same amount of tissue for each type of transplant. However, it is difficult to say if the amount of tissue starting for the in vitro expansion could have a significant effect on the outcome. Vision improved in 70% of Shortt et al.'s, 10 patients, whereas it did so in 50% of our patients. In the subset of our cases that were considered successful, 62.5% of the eyes showed vision improvement.

Preparation of the cell product by Shortt et al. [24] was similar to ours. They also used amniotic membrane but prepared the limbal cells in a suspension culture system, while we used an explant culture system. We have ample experience in both techniques and are now considering changing to

the cell suspension technique. Currently in our laboratory, cell suspensions achieve more confluent primary cell cultures, in less time, with more cells and with less fibroblast contamination, although both techniques have the same cell viability and proliferative capacity and same electron microscopy characteristics (unpublished data). These results, however, are derived from expanded cadaveric tissues sent for research purposes and thus are not suitable for clinical uses. The tissues were from very old donors (around 80 years old) and had longer elapsed times between enucleation and culture (data not shown). It is possible that the differences between cell suspension- and explant-derived cells will not be the same with the cadaveric tissues used for clinical purposes. Cadaveric tissues suitable for clinical applications are always derived from donors who are younger in age (less than 60 years old in our series) and for which there is less time between enucleation and usage. These factors are reported to be influential in clinical outcomes [36]. In contrast to cadaveric tissues, autologous tissues are always fresher (processed within 4 hr. in our cases) and not available for research. Shimazaki et al. compared the explant technique without the use of feeder cells or air lifting to the explant techniques with 3T3 feeder cells and airlifting and with the cell suspension technique [37]. They found similar in vitro characteristics among the culture techniques, but the clinical results were best with cells derived by suspension culture, in agreement with other authors [21, 25]. This is one of the many aspects that needs careful evaluation in future well-designed studies.

The surgical technique and medical management used by Shortt et al. [24] were quite similar to ours, although they placed their cell product with cells facing externally, while we did just the opposite. These authors also relied on IVCM as the primary objective measure of success, and in fact we followed their parameters. We decided not to perform impression cytology, because in agreement with other authors [25, 38], it is painful, unnecessary, and risky. Impression cytology requires stripping off 3-4 layers of corneal epithelium, a procedure that unnecessarily stresses the new, healthier tissue. Further, impression cytology does not add any relevant information to the clinical diagnosis [38]. IVCM is better and more safely serves as an objective method to evaluate the corneal epithelium [24, 33]. In any case, neither IVCM nor other more invasive procedures are necessary to establish the success or failure of CLET in the routine clinical environment.

Shortt et al. recently reported the 3-year results on LSCD for Stevens-Johnson syndrome and congenital aniridia [34], diseases that we have classified in prognostic Groups 2 and 3, respectively. They found positive results at one year and a deterioration of the tissues thereafter. Our two congenital aniridia cases and two of the three Stevens-Johnson cases, however, remained stable. The third Stevens-Johnson case failed at 34 months. Immune-based cicatrizing disorders are very difficult to treat, and they have a poor prognosis [37]. Although most authors have claimed stability after one year, this is not yet fully known, especially with respect to the different etiologies. There are no published large case series on these nonchemical burn eyes as they are rare diseases. Our results will need corroboration as it would be very useful to know how the duration of CLET success depends on the etiology of each disease.

Two years after the first European study by Shortt et al. [24] that followed the institution of GMP regulations, Kolli et al. [39] (Royal Victoria Infirmary-Newcastle University, Newcastle Upon Tyne, United Kingdom) attempted to attribute the success or failure of treating LSCD with CLET solely to the cell product. They recruited a strictly uniform group of 8 patients with unilateral total LSCD due to chemical burns. They treated the cases by autologous CLET, using amniotic membrane and xeno-free products under GMP rules. Based on clinical evaluation, they reported a 100% success rate with a mean follow-up of 19 months. Vision improved in 5 of the 8 eyes, a success rate similar to our own (62.5%). In our study, we had only 4 chemical burn cases treated with autologous CLET (Cases 1, 8, 13, and 20, Table 2), which were the most comparable with Kolli et al. [39]. While one case failed, vision improved in the other 3 cases.

Using fibrin-cultured autologous CLET, Rama et al. [25] reported on 107 cases followed up for a mean of about 3 years. Their success rate was 68.2% after one graft, with a final successful clinical outcome of 76.6% after regrafting 11 eyes. This success rate is very similar to ours, 75% at 3 years, even though most of their cases were chemical/thermal injuries and they only performed autografts. Full evaluations were done at 1 year, as we did, and grafts that were considered successful remained stable. Failed cases did not worsen as compared with baseline, as we also observed. Their judgment for success/failure was based on clinical grounds, and like us, they stopped doing corneal impression cytology for the same reasons that we did not do them.

The main difference with our protocol and that of Rama et al. is the stem cell final product. They used a fibrin-derived matrix instead of amniotic membrane like we used. Additionally, Rama et al. used clinical grade-certified 3T3-J2 cells, a mouse embryonic 3T3 fibroblast cell line that was lethally irradiated, as a feeder layer in contrast to our use of denuded amniotic membrane. In contrast to our use of the explant technique and the subsequent outgrowth of primary cell cultures, they used primary cell cultures that were trypsinized to transfer the cells to the fibrin-based substrate. Additionally, their cell cultures were transplanted 24–36 hr. after transfer to the transport container while ours were used within the next 4 hr. In summary, different etiologies, different cell sources (always autologous for Rama et al.), and different cell product protocols prevent reliable comparisons. Nevertheless, despite all the significant differences, our final success rates were fairly similar. This perhaps indicates that different protocols may work equally well as long as viable, stem cell-like cells are transplanted, as they showed in their series. How all of the possible variables affect the final outcome remains unknown, and it would be extremely difficult to test each of the variables independently. Thus we advocate reaching an agreement on all possible variables in all centers willing to participate in multicenter studies.

In 2010, Pauklin et al. [40] (University of Duisburg-Essen, Essen, Germany) published a series of LSCD cases for 32 total CLET eyes and 12 partial ones. The mean follow-up time was approximately 2 years, and their success rate, based

on clinical grounds, was similar to ours, between 68% (full corneal stability) and 84% (clear central cornea). Grafting was significantly more successful in eyes treated with autologous CLET (77% of 30 eyes) than with allogeneic CLET (50% of 14 eyes, cadaveric or living related donors), in disagreement with our results and others [24, 41]. Perhaps the better prognosis of the etiologies undergoing autologous CLET could explain their better results. While they did not state that they adhered to the GMP standards, their cell product was fairly similar to ours, although they used intact amniotic membrane while we used denuded membranes. It seems as though they must have applied the cells facing externally because they placed a second amniotic membrane as a patch. Interestingly, they provoked a pharmacological eyelid ptosis to protect the graft, while we used a large contact lens.

In 2011, Sangwan and his group [42] (L. V. Prasad Eye Institute, Hyderabad, India) published a 10-year (2001–2010) retrospective study on xeno-free autologous CLET, including the largest series reported, 200 unilateral total LSCD cases due to chemical burns. Their cell product, prepared without clear reference to GMP adherence, was a limbal cell monolayer on denuded amniotic membrane, as ours; however, they did not plate the whole explant, but rather, shredded it first to obtain primary cultures. They reported 71% success rate, evaluated on clinical grounds, with a mean follow-up of 3 years, very similar to our series and as reported by most authors. Failures occurred mainly within the first year. They also reported a visual gain of two lines in 60.5% of eyes with no further surgery, similar to our 50–62.5% with visual gain. They used sutures or fibrin glue and did concomitant symblepharon surgery in 45% of cases and keratoplasty in 5% of them. Although understandable in clinical practice, performing more than one surgery certainly increases postoperative inflammation, and that can affect limbal cell survival. In fact, the same group later reported a worse prognosis when penetrating keratoplasty was performed at the same time as autologous CLET than when CLET was done first and keratoplasty at least 6 weeks after [43]. We waited a year to schedule our two penetrating keratoplasty cases, as did most authors, including Basu et al., who later waited at least one year after CLET before the next surgery [44]. We strongly recommend avoiding any surgery other than CLET in the context of prospective clinical studies and trials so as not to mask results from CLET.

These authors also reported similar results with autologous CLET irrespective of whether the limbal biopsy was taken from a healthy section of the affected eye or from the contralateral eye [45]. Several groups have reported that CLET can be safely repeated [25, 42, 46, 47]. We repeated one case in our series that ended up as partial success (Case 16), but with great alleviation of symptoms.

Sejpal et al. [47] also have significant experience in CLET for pediatric patients, mostly due to chemical/thermal injuries. The success rates for these cases were similar to those reported for adults. Similarly good results for pediatric CLET in 26 ocular burn cases were reported by Vajpayee et al. [48]. We had no pediatric cases, as the main cause for LSCD in children, chemical injuries, is exceedingly rare in our geographic area.

Prabhasawat et al. [41] (Siriraj Hospital-Mahidol University, Bangkok, Thailand) published a series of 19 LSCD cases (13 total, 6 partial) managed with CLET. Whether or not GMP rules were followed was not stated. They used clinical observations and impression cytology to evaluate success/failure, and their series had etiologies similar to ours. They reported final success of 73.7% with a mean follow-up of 26 months, although they included some cases followed up for only 6 months. They used limbal sections ranging from 2×2 mm to 2×2 cm from contralateral eyes. The epithelium was detached from the limbal explants with dispase and seeded on amniotic membrane with a final yield of 2–4 layers of epithelium. This suggests that limbal cells may have been differentiated as what happens when stem cells become multilayered [49]. In two cases at the time of surgery, a 6-mm central disk was punched out of the amniotic membrane upon which the cells were cultured, which would have eliminated a significant number of transplanted cells. They also removed symblepharon in some patients at the same time of grafting, even using mitomycin C, which is unusual and can mask results. They stated that the autografts were less successful (66.7%, 8 of 12) than the allografts (85.7%, 6 of 7). These results were similar to Shortt et al. [24] but contrary to Paulkin et al. [40] and us. Perhaps the fact that 83.3% of their autografts and 42.9% of their allografts were for chemical burns could explain this difference. Additionally, they had more symblepharon cases within the eyes that had received autografts. While the final success rate was similar to ours, differences in surgical management and cell products make comparisons difficult.

In 2013, Qi et al. [26] (Shandong Eye Institute, Qingdao, China) published, without any statement on GMP policy, a series of 42 eyes undergoing allogeneic CLET. They centered their work on the incidence of immune rejection, describing the clinical characteristics. They reported 23.8% immune rejections, all in eyes with chemical/thermal burns and occurring between 1 and 6 months. The outcome for the remaining 32 eyes (76.2%) was successful. This success rate was similar to ours, although our failures were not immune rejection-related, probably because our patients had systemic immunosuppression. This immune rejection rate cannot be taken as the rate under nonmedicated circumstances, as their patients received high, immunosuppressive doses of systemic steroids. They also had frequent use of topical steroids and 1% cyclosporine eyedrops. We, as others, strongly prefer using oral nonsteroidal immunosuppression rather than high doses of oral steroids that can produce a high frequency of undesirable and severe side effects.

The most recent series reported in the EU was in 2014 by Zakaria et al. [38] (Antwerp University Hospital, Antwerp, Belgium) in which 15 patients had total and 3 had partial LSCD, with etiologies similar to ours. Autologous ($n = 15$) or allogeneic ($n = 3$, 2 from HLA-matched living related donors and 1 from cadaveric donor) xeno-free CLET was performed and followed up for 22 months. The clinically judged overall success rate was 67%, worse in chemical burns but similar to our 75% at 3 years. Contrary to our results, these authors did not see a significant reduction in pain or photophobia. This could be due to the great difficulty in

properly assessing clinical symptoms in patients and/or the fact that they used clinical scales for pain and photophobia to measure symptoms, while we used the OSDI and NEI-VF-25 assessments. They cultivated explants on top of amniotic membranes and followed the same technique as ours to prepare the host bed. However, they applied the composite graft, for example, amniotic membrane and cells facing up, with tissue fibrin glue. They then placed a second amniotic membrane on top and tucked it under the conjunctiva before suturing it. A big difference is that, at the time of surgery, the composite graft contained both the cultured cells and the original limbal biopsy, which we removed when outgrowth was seen. They do not state if they operated under GMP rules, although they probably did as it is mandatory in the EU. They started offering corneal transplantation one year post-op, as we did. We also agree with these authors in not performing corneal impression cytology because the cell pick-up was low and the procedure risked disrupting the transplanted epithelium.

5. Conclusion

In conclusion, we showed that limbal cell expansion and culture can be successfully achieved following our protocol using GMP conditions and following EU regulations. Both autologous and allogeneic CLET significantly improved the quality of corneal epithelium in patients with ocular surface failure due to LSCD. It enabled subsequent improvement in symptoms, increasing the quality of life in 75% of the patients after 3 years. These results confirm other reports that CLET is a successful treatment for ocular surface failure due to LSCD, although in some patients it seems to be insufficient. The merits of having a predefined prognostic schema like ours (Table 2) or one similar to it seem self-evident. It provides documentable guidelines for different surgical and medical treatments and sets reasonable physician and patient expectations for outcomes based on the current status of the eye. Thus we encourage the routine use of it or ones like it. In our study, visual improvement was achieved in those eyes previously classified in our schema as having the potential to gain vision with only CLET. Symptoms were greatly alleviated in all successful patients. Our study also affirms that laser IVCM is a good, minimally invasive, technique to assess the main evaluation endpoint, corneal epithelium restoration in the central cornea, in clinical studies and trials. Finally, we demonstrated that a strict but ample clinical composite score coupled with an objective imaging technique is ideal tool for use in future clinical trials.

Finally, consensus in cell preparation protocols and patient-related issues (pre-, intra-, and postoperative) should be sought to coordinate multicenter clinical studies and trials that help answer many of the still remaining questions about this otherwise overall successful transplantation technique. Needless to say, clinical efforts must be paralleled with research efforts so that these complex and difficult blinding diseases can be better treated.

Disclosure

This study was presented in part as a poster at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, Seattle, WA, USA, May 5–9, 2013.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The funding organizations had no role in designing or conducting this research.

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Review Article

The Genetics and the Genomics of Primary Congenital Glaucoma

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The sight is one of the five senses allowing an autonomous and high-quality life, so that alterations of any ocular component may result in several clinical phenotypes (from conjunctivitis to severe vision loss and irreversible blindness). Most parts of clinical phenotypes have been significantly associated with mutations in genes regulating the normal formation and maturation of the anterior segments of the eye. Among the eye anterior segment disorders, special attention is given to Glaucoma as it represents one of the major causes of bilateral blindness in the world, with an onset due to Mendelian or multifactorial genetic-causative traits. This review will point out the attention on the Primary Congenital Glaucoma (PCG), which is usually transmitted according to an autosomal-recessive inheritance pattern. Taking into consideration the genetic component of the PCG, it is possible to observe a strong heterogeneity concerning the disease-associated loci (*GLC3*), penetrance defects, and expressivity of the disease. Given the strong PGC heterogeneity, pre- and posttest genetic counseling plays an essential role in the achievement of an appropriate management of PCG, in terms of medical, social, and psychological impact of the disease.

1. Introduction

The sight is one of the five senses allowing an autonomous and high-quality life. Hence, sight defects may turn out to be really restricting for the life quality, representing a discriminating or even a life-threatening issue, especially in developing countries. As a matter of fact, about the 75% of information we receive about the world around us relies on the ability of the eyes to receive the light and transform it into a "picture" [1].

Given the critical importance of the sight organ, it is not surprising that alterations or malformations of any ocular component may result in several clinical phenotypes, ranging from conjunctivitis to Cataract until the severe vision loss and the irreversible blindness. Ocular clinical syndromes may develop owing to environmental agents (infections acquired during pregnancy or later in the life, aging processes, and systemic disorders), which can affect the ocular structures at the developmental or mature stages. However, most parts of clinical phenotypes have been significantly associated with mutations in genes regulating the normal formation and maturation of both posterior and anterior segments of the eye [2]. This review will be particularly focused on the genetics and genomics of the anterior segment disorders.

2. Genetic and Phenotypic Overview on the Eye Anterior Segment Mendelian Disorders

The clinical manifestations characterizing the anterior segment disorders reflect both phenotypic and genotypic heterogeneity, with multiple overlapping symptoms and associated

genes [3]. Firstly, it is important to distinguish the anterior segment disorders characterized by a Mendelian or simple genetics from the disorders developed in consequence of complex or multifactorial traits. In particular, while the Mendelian diseases are due to causative rare mutations in specific genes (causative gene) and are inherited according to Mendel's laws, multifactorial diseases are caused by an interplay between several genetic and environmental triggering factors [2, 4]. In the large majority of cases, the genetic variants, associated with multifactorial diseases, are not rare mutations but rather polymorphisms, located on susceptibility genes and reported at high frequencies in normal/nonaffected population. These genetic markers are not sufficient to cause the disease but they only represent risk factors, determining a higher susceptibility to a particular disease. As mentioned below, it is interesting to notice that the presence of modifier genes, polymorphisms, or mutations may also be responsible for the variable expressivity occurring in Mendelian and complex diseases [5, 6]. In addition, the recognition of the distinctive features related to each anterior segment disorder can be complicated by different factors [5] such as

(i) the presence of extraocular (systemic) clinical manifestations, as facial dysmorphisms, dental dysplasia, redundant preumbilical skin, and short stature;

(ii) heterogeneous inheritance patterns (mainly autosomal-dominant and/or autosomal-recessive inheritance patterns);

(iii) developmental abnormalities occurring during embryogenesis and maturation of the eye anterior segment tissues (leading to congenital diseases);

(iv) foreign events, such as inflammations, infections, and trauma.

Among the most studied Mendelian diseases, it is important to mention Axenfeld-Rieger syndrome (ARS, #180500, #601499, and #602482), a rare disorder which affects 1 : 200.000 individuals and follows an autosomal-dominant inheritance pattern. The ARS etiopathology is characterized by an abnormal development of the anterior chamber, the cornea, and the iris. Clinical hallmarks of the disease include hypoplasia, corectopia, polycoria, posterior embryotoxon, and peripheral anterior synechiae (iris strands). Facial, dental, umbilical, and skeletal defects are also usually present as extraocular manifestations [5, 7].

Another Mendelian disorder affecting the anterior segment of the eye is referred to as Peters' Anomaly (#604229). It presents a heterogeneous inheritance, mostly autosomal-recessive, although autosomal-dominant and sporadic cases have been reported. The disease is caused by an incomplete separation of the cornea from the iris or the lenses, resulting in a variable central corneal opacity among patients. Systemic abnormalities reported in patients with Peters' Anomaly are short stature, cleft lip palate, growth, and mental delay [5, 8].

The complete or partial absence of the iris results in a pathological phenotype known as Aniridia (#106210). It affects 1 : 50.000–100.000 newborns worldwide and it is inherited in autosomal-dominant pattern. Aniridia is characterized by a reduced visual acuity and photophobia. Suddenly, it is associated with behavioral problems, developmental delay, and difficulties in detecting the odors [5, 9].

Concerning the disease phenotypes associated with malformations of the sclera and the cornea, it is important to mention the Sclerocornea and the Megalocornea. Sclerocornea (#18170) is a congenital disorder characterized by the absence of separation between sclera and cornea, which presents, as a consequence, a flat curvature. The malformation results in a nonprogressive corneal opacification (peripheral, sectoral, or central) [5, 10]. Megalocornea (#309300) instead is generally inherited in an X-linked recessive pattern and it consists of an enlargement of the corneal diameter (>13 mm) at birth, a deep anterior chamber, and normal IOP [5, 11].

Among the anterior segment disorders, particular attention has been paid to Keratoconus and Glaucoma, since both of the diseases are characterized by the presence of both Mendelian and multifactorial traits which give rise to different forms of Keratoconus and Glaucoma.

Keratoconus (#148300) is a noninflammatory degenerative disorder of the cornea affecting 1 in 2.000 individuals. The disease is due to the bulging and distortion of the corneal curvature and surface, which lead to the loss of visual acuity. A number of environmental factors have been associated with the development of Keratoconus, especially long-term contact lens wear, chronic eye rubbing, and atopy of the eye. However, the observation of familiarity transmission and studies on monozygotic and dizygotic twins have demonstrated that Keratoconus is characterized by a significant genetic component. In fact, the disease showed to be mostly transmitted in an autosomal-dominant inheritance pattern, although reduced penetrance and autosomal-recessive transmission have been reported [12].

Concerning Glaucoma several types of diseases can be distinguished, depending on the presence of causative Mendelian or multifactorial characters. In multifactorial Glaucoma, the clinical phenotype originates from the interaction between genetic/molecular (*LOXL1* genetic polymorphisms/retinal ganglionic cell death) and environmental (aging, diet, stress, and life-style) factors [13–15]. Although the hereditary component of the disease remains poorly understood, it is known that it can affect both genders, with higher risk for male subjects, advanced age people, and African descendants [16]. This form of Glaucoma is quite similar to several multifactorial, chronic, and age-related degenerative diseases, such as Osteoporosis, Osteoarthritis, Alzheimer's Disease, Coronary Artery Disease, Cataract [17]. In fact, multifactorial Glaucoma is a chronic neurodegenerative process, with an onset in the middle age and a slow progressive course, without particular signs or symptoms. Subjects affected by Glaucoma show a reduction of the visual field, blurry vision, decreased sensitivity to color, and contrast [16]. Over the multifactorial form, it is important to remark the existence of Glaucomas which are characterized by Mendelian inheritance (mainly autosomal-recessive and

autosomal-dominant). Further details about clinical and genetic profile of Mendelian disease will be hereafter given.

Over the phenotypic manifestations of the anterior segment diseases, it is important to take into consideration the genetic background of these disorders. In fact, traditional linkage and positional cloning approaches identified a number of "modifier genes" associated with the development, inheritance pattern, variable expressivity, and phenotype of the anterior segment disorders. Such genetic modifiers consist of mutations or polymorphisms in specific genes, involved in the pathological mechanisms responsible for the disease phenotype [5, 13]. However, the identification of disease-causative genes led to the observation that not only is more than one gene mutation accountable for the same clinical condition, but also variable expressivity and incomplete penetrance can occur in patients carrying the same mutation. In addition, Genome Wide Association Studies (GWAS) allowed the identification of other genetic modifiers, especially common genetic variants associated with the individual susceptibility to eye anterior segment disorders. Each disease-associated individual variant confers a low risk when taken alone, while a set of low-risk variants provide a higher predictive value of the overall susceptibility to the disease [5]. The genes involved in the pathogenesis of these disorders include transcription factors in the majority of cases, although some genes code for transporters and glycosylation proteins. Given their function, mutations in the disease-causative genes will obviously have an impact on the downstream gene regulation, the integrity of the mature protein, and its final interaction with the cellular compartments [18]. The common low-risk variants instead are mostly located in noncoding regions (>80%) near the causative genes and essentially affect the overall gene expression patterns (regulation of RNA splicing and transcription activities) [5]. The variability of the gene expression profile can also be altered by epigenetic events (DNA methylation, histone acetylation/deacetylation, and structural chromatin modification), which act on the DNA transcription rather than on the primary DNA sequence [19]. In particular, DNA methyl transferases (DNMTs) were found to be differentially expressed in tissues and related cells of the eye anterior segment, such as the cornea, conjunctiva, anterior lenses, and trabecular meshwork [19]. Altered methylation profiles have been associated with few anterior segment disorders (such as Pterygium, #17800) [20]. Moreover, experiments on human cells and animal models demonstrated that histone modifications (HDA3) and miRNAs (miR29, miR204, miR146a, and miR24) play a role in the development of anterior segment diseases (as the Primary Open Angle Glaucoma, #137760) [21]. However, further research may help to study and discover epigenetic pathways concerning the etiopathogenesis of the congenital disorders affecting the eye anterior segment.

Independently of the phenotype/genotype features of the disorders, it is important to underline that a pathological phenotype does not result from the disruptive effect of one single gene mutation, but from the alteration of the network of genes to which the mutated gene specifically belongs. As a matter of fact, the high phenotypic and genotypic heterogeneity of the anterior segment disorders of the eye is actually the final outcome of the cross-talking between the disease-causative genes and the individual surrounding environment [5].

2.1. The Genes Causative of the Eye Anterior Segment Disorders. To date, a number of causative genes have been involved in the pathogenesis of the eye anterior segment disorders, some of which will be briefly described in the following paragraphs.

Both genes *PITX2* and *FOXC1*, located at 4q25 and at 6p25, respectively, code for two transcription factors (Pituitary Homeobox 2 and Forkhead Box C1). Linkage analysis has discovered that specific types of mutations in these genes are associated with specific pathological phenotype. In fact, intragenic mutations and/or deletions in *PITX2* and *FOXC1* have been reported in ARS syndrome with or without systemic anomalies. Copy Number Variation (CNV) has been found in ARS and Peters' Anomaly, while missense mutations have been identified in patients affected by Peters' Anomaly [3, 22].

PAX6 was the first gene to be associated with human anterior segment disorder. The gene is mapped on 11p13 and encodes for the transcription factor Paired Box 6. The mutational spectrum of the gene includes nonsense, splicing, insertion, and deletion mutations, resulting in the Aniridia disease. However, some missense mutations in *PAX6* have been reported in Peters' Anomaly and other ocular defects [18, 22].

B3GALTL is located on 13q12.3 and is responsible for the beta-1,3-glucosyltransferase (B3Glc-T) enzyme production, which is involved in the glycosylation process of proteins (posttranslational addition of sugar molecules). Deletion and splicing mutations have been reported in patients with Peter Plus Syndrome (PPS, #261540, Peters' Anomaly with variable systemic anomalies) [5, 22].

SOX2 is mapped on 3q26.3-q27 and codes for the transcription factor SRY-like box 2. It is especially involved in the development of the eyes, so that *SOX2* mutations have been associated with Sclerocornea and other ocular diseases [18].

CHRDL1 is located on Xq23 and encodes for the ventroptin protein, which works as bone morphogenic protein antagonist. Genomic techniques have associated mutation in *CHRDL1* with the X-linked form of Megalocornea [18].

VSX1 is situated on 20p11.21 and produces the transcription factor Visual System Homeobox 1. It is functionally involved in the regulation of the events occurring during craniofacial and ocular development. Missense mutations were found to be involved in the pathogenesis of Keratoconus, particularly concerning the autosomal-dominant form with variable expressivity and incomplete penetrance [12].

Other genes related to the above-mentioned and other eye anterior segment disorders (not described in this review) include *FOXE3*, *BMP4*, *BMP7*, *LAMB2*, *COL4A1*, *FGFR2*, *CYP1B1*, *LTBP2*, and *MYOC* [22].

In particular, this review will be focused on the genetic components underlining the congenital form of Glaucoma, a rare pathological condition affecting the anterior chamber drainage structures of the eye.

3. Focus on a Specific Disorder of the Anterior Segment of the Eye: The Glaucoma

As mentioned above, in the context of the eye anterior segment disorders special attention is given to Glaucoma. It represents one of the major causes of bilateral blindness in the world and several types of diseases have been classified to date, according to presence of Mendelian or multifactorial genetic-causative traits. In particular, Glaucoma consists of a pathological condition characterized by the progressive death of Retinal Ganglion Cells (RGCs), degeneration of the optical nerve, and vision loss as final result. The real explanation for RGCs death is not yet clear, although the level of IOP is known to play a fundamental role [13]. In particular, the IOP depends on the homeostasis between the aqueous humor produced in the ciliary body (and secreted in the posterior chamber) and its drainage through the trabecular meshwork in the anterior chamber angle. The balanced production and outflow of aqueous humor are constant and generate a positive pressure within the eye (~15 mmHg). Malfunctioning and/or malformations of the trabecular meshwork may result in the elevation of IOP over the limit ranges (>21 mmHg) and consequently the development of Glaucoma. Trabecular meshwork disruption can depend on a number of factors, such as mechanical and oxidative stress, aging, and genetic mutations [23]. The improper drainage of aqueous humor determines the increase of IOP, which in turn generates a mechanical stress and strain on the lamina cribrosa (the exit points of the optic nerve fibers in the sclera). The resulting stress blocks the transport of neurotrophic factors to the RGCs that finally die by apoptosis [24].

Glaucoma can be classified into primary, secondary, and primary congenital. The primary form is a nonsyndromic condition and it does not originate from any previous anterior segment abnormality, trauma, or inflammation. According to the compromised regions, two types of primary Glaucoma can be distinguished: Primary Closed-Angle Glaucoma and Primary Open-Angle Glaucoma. In the first condition, the drainage of aqueous humor is clogged by the closure of the angle between the iris and the cornea, while in the second form the fluid meets high resistance because of a malfunctioning of the trabecular meshwork [23].

The secondary Glaucoma instead is linked to the presence of ocular injuries or systemic conditions (for example, diabetes and long-term corticosteroid use) [23]. Even in this case, Closed-Angle and Open-Angle subtypes can be distinguished. On this subject, the Pseudoexfoliation Syndrome (PEXS) represents one of the major causes of secondary Open-Angle Glaucoma (accounting for the 25% of all Open-Angle Glaucomas). PEXS is described as an age-related systemic disease, which leads to the development of the secondary Glaucoma because of the accumulation of exfoliation material in the trabecular meshwork and consequently the increase of IOP [14].

This review will point out the attention on the Primary Congenital Glaucoma (PCG) which is a rare form representing the 1–5% of all cases of Glaucoma [25]. PCG generally presents an autosomal-recessive inheritance pattern, so that it affects only the 25% of newborns from parents carrying PCG-associated mutations. However, the incidence of PCG is highly heterogeneous in relation to the population, the geographic region, and the prevalence of consanguineous relationships. In fact, the incidence rate is estimated to be 1 : 2.500 in Saudi Arabia and Slovakia Gypsy populations (because of highly frequent consanguineous relations), in contrast with the lower incidence rate recorded among Western populations ranging from 1 : 18.500 and 1 : 30.000 [25, 26].

On the basis of the age of onset, it is possible to classify three subtypes of PCG: neonatal or newborn when presented at the birth or within the 1st month of life; infantile, diagnosed from 1st month to 2 years; and late-onset, recognized after 2 years. Usually, in 70–80% of cases, PCG affect both of the eyes (bilateral form) [27].

The clinical hallmarks of PCG diagnosis include IOP > 21 mmHg, optic cupping, epiphora, corneal edema and Haab striae, globe enlargement (buphthalmos), photophobia, blepharospasm, and hyperlacrimation. The PCG phenotype is due to a trabeculodysgenesis phenomenon, that is, an abnormal development of anterior chamber leading to the enlargement of the trabecular meshwork bundles and consequently reduction of the trabecular area available for the aqueous humor outflow [28]. In addition, the humor drainage is reduced because of the interference of immature iris, ciliary body, and anterior chamber angle structure which appear superimposed on the trabecular meshwork and compromise the aqueous humor outflow. The final result of these anterior segment structural defects and the altered aqueous humor drainage is the elevation of IOP and the enlargement of the entire ocular globe [25].

4. Genetics of PCG

Taking into consideration the genetic component of the PCG, it is possible to observe a strong heterogeneity in terms of disease-associated loci, penetrance defects, and expressivity of the disease among the different populations. Concerning the disease-associated loci, the Human Genome Organization established a specific nomenclature for Glaucoma-associated genetic loci. In fact, "GLC" stands for the general name of genes involved in Glaucoma; "1, 2, and 3" indicate the type of primary Glaucoma (Open-Angle, Closed-Angle, and congenital/infantile Glaucoma, resp.); "A, B, C, and D" refer to the progressive genes mapped for each Glaucoma type [26]. This review will focus particularly on *GLC3* loci, since these refer to the primary congenital Glaucoma.

4.1. GLC3 Loci. To date four PCG loci have been classified under *GLC3* subgroup, namely, *GLC3A*, *GLC3B*, *GLC3C*, and *GLC3D*.

The first locus to be associated with PCG was *GLC3A*, mapped on 2p21 chromosomal region. At this locus, about 147 mutations have been found in the gene *CYP1B1*, coding for the homonymous protein (cytochrome P450, family 1, subfamily B, and polypeptide 1) [29, 30]. *CYP1B1* consists of 3 exons and 2 introns, of which the first exon is a noncoding region, while the second and third exons are responsible for the production of CYP1B1 protein [25]. The *CYP1B1* mutations can be missense, nonsense, insertions, and

deletions and result in the disruption of enzymatic activity and functionality of CYP1B1 protein [25, 31].

The mutations showed an autosomal-recessive inheritance pattern and are the most common variations identified among PCG patients, especially in consanguineous cohorts. However, a high variable distribution of *CYP1B1* mutations has been reported among the different worldwide populations, with 90–100% found in Saudi Arabia and Slovakia Gypsy populations, 14–30% among USA and European populations, and 15–20% reported in Japanese and Chinese populations [31–33]. Interestingly, in families carrying *CYP1B1* mutations cases of incomplete penetrance and variable expressivity have been observed. In fact, patients harboring the same mutations showed a different degree of disease severity, age of onset, or even lack of the disease phenotype [25, 26].

The pathogenetic role of the CYP1B1 protein in PCG has to be yet clarified, although a possible involvement in the metabolic pathways required for eye anterior chamber development is suggested, particularly for the trabecular meshwork formation [26]. On this subject, a recent study conducted on *Cyp1b1*$^{-/-}$ mice demonstrated that *Cyp1b1* deficiency was responsible for the increased oxidative stress and ultrastructural defects in the TM tissue of early-life animals. In particular, the absence of CYP1B1 protein (due to *Cyp1b1* silencing) hampers the proper removal of Reactive Oxygen Species (ROS) and, consequently, compromises the development and differentiation of TM tissue. In addition, the increased oxidative stress in *Cyp1b1*$^{-/-}$ mice caused the decrease of the Periostin (Postn) production, which results in the loss of the mechanical strength and structural integrity of the TM tissue [34].

GLC3B and *GLC3C* loci have been associated with the PCG, although no gene has been identified in both regions to date. In particular, *GLC3B* is located at 1p36.2–1p36.1 while *GLC3C* maps on 14q24.3–14q31.1 [25, 26].

Concerning the *GLC3D* locus (14q24), autosomal-recessive null mutations have been reported at this position and located in the *LTBP2* gene. *LTBP2* codes for the Latent Transforming beta Binding Protein 2, a matrix protein playing a role in tissue repair processes and cell adhesion. The role of the PCG still remains to be explained. However, it has been demonstrated that *LTBP2* is expressed in the trabecular meshwork and ciliary body and PCG-causative null mutations have been found in consanguineous Pakistani, European Gypsy, and Iranian families [26, 35].

4.2. Other PCG-Associated Genes. Families affected by PCG carried mutations in *MYOC* gene, independently of the presence or absence of *CYP1B1* mutations. *MYOC* is a 3-exon gene, located at 1q24.3–1q25.2 chromosomic region, and codes for the glycoprotein myocilin. The function of the protein is unknown, while its expression has been reported at high level in the trabecular meshwork and the ciliary body. Mutations in *MYOC* have been found to be a leading cause of aqueous outflow obstruction through the trabecular meshwork and of the consequent IOP elevation. A number of *MYOC* mutations have been associated with the juvenile- and adult-onset primary Glaucoma, characterized by an autosomal-dominant/autosomal-recessive inheritance pattern with high penetrance.

Concerning the role of *MYOC* in PGC, possible interactions with *CYP1B1* and/or other unknown loci have been hypothesized to work as genetic modifiers to determine an earlier onset of the disease in patients harboring mutations in *MYOC* and *CYP1B1*, with respect to the patients negative for *MYOC* or *CYP1B1* mutations [25, 26, 36].

Another putative PCG-associated gene is *FOXC1*, a protein expressed in periocular mesenchyme cells, producing ocular drainage structures as iris, cornea, and trabecular meshwork. Interestingly, a deletion in *FOXC1* has been detected in PCG and other ocular and nonocular defects, highlighting a possible involvement of *FOXC1* in the pathogenetic pathway of the disease [25, 26].

Mutations in *BMP4* gene (14q22-q23) have been identified in patients affected by PCG and other disease phenotypes. *BMP4* codes for the bone morphogenetic protein 4 and it is expressed in different tissues, among which is the ocular vesicle, and in the optic cup. However, further research is needed to clarify the role of *BMP4* in PCG pathogenesis [26].

5. The Genetic Counseling in the Management of PCG

As PCG is a severe disease characterized by a particular genetic base and familiarity, genetic counseling represents a useful tool for the disease management (clinical features of the disease, prevision of the disease progression, and medical complications) [37]. Genetic counseling is a communication activity oriented to help the patient and/or their family in receiving medical information concerning the genetic features associated with PCG. In particular, the genetic specialist has to explain the recurrence risk of PCG in relation to the presence of positive/negative familiarity and to the frequency of PCG in the general population. The construction of familiar pedigree may be of help for clarification of the genetic model of inheritance of the disease. In fact, although the majority of cases of PCG appear to be sporadic cases or to follow an autosomal-recessive inheritance pattern, in rare cases some families reported an autosomal-dominant inheritance model. After the clarification of the genetic base of the disease, the recurrence risk and familiarity for PCG have been assessed, and the specialist should suggest and give details about the possibility of performing a genetic test for the detection of PCG-causative mutations. In particular, as illustrated before, mutations in *CYP1B1*, *LTBP2*, and *MYOC* have been found to be causative of PCG among different worldwide populations [25]. However, the specialist has to put in evidence the variable expressivity and penetrance defects occurring in presence of causative mutations. In fact, it is important to clarify to the patient that the positivity for PCG-associated mutations does not always correspond to the development of the effective pathological phenotype and the disease may manifest at later age. In case of presence of PCG-associated mutations, the genetic specialist should suggest to the patient a constant follow-up of his own clinical conditions. This aspect is strongly important in case of siblings

of children positive to *CYP1B1* mutations, where there is a 25% higher risk of PCG development because of the variable expressivity of the disease phenotype [25, 38].

To help understand the pros and cons of genetic tests, it is very important to perform the informative counseling prior and after the test (pre- and a posttest). In this context the explanation of biological aspects and technical approaches related to the genetic test may help in understanding limits of negative results, false-negatives, and the need of further investigations.

Unfortunately, some cases may result negative to the analysis of PCG-causative known mutations, so that undiscovered mutations in other unidentified genes may be possible (e.g., unknown genes mapped on *GLC3B* and *GLC3C* loci). Social and psychological implications of the lack of a responsible gene involve the impossibility to perform genetic tests for the prediction of the disease risk in families with a positive-PCG history. This aspect may appear as a "sword of Damocles" and may affect deeply the couple perspective in matter of family construction.

6. Conclusion

The visual loss is due to interruption/aberration/malformation of the normal functioning of the ocular structures. Early-onset ocular diseases usually have Mendelian inheritance, while common adult-onset disorders are inherited as complex traits. To date, several genetic and molecular studies have provided insights into the biological processes underlying many ophthalmic disorders. In this context, GWAS identified different genetic variants contributing to a number of common ocular complex disorders.

Glaucoma is a heterogeneous group of disorders causing irreversible blindness worldwide. As previously described, most forms of Glaucoma present a significant genetic component, characterized by the different causative or susceptibility genes identified through traditional (genetic linkage) or novel (GWAS) approaches. Among the different forms of Glaucoma, we focused our attention on the PCG, that is, the single most common childhood Glaucoma.

PCG is a rare disease affecting the structures of the anterior chamber of the eye anterior segment. Although rare, it is still the most common Glaucoma in infancy and causes a disproportionately high percentage of childhood blindness worldwide. It displays a strong heterogeneity in terms of disease phenotype and genotype. Genetically speaking, PCG presents a familiarity trend, with higher frequency in populations where consanguineous relationships are common (Saudi Arabia and Slovakia Gypsy populations). To date, several mutations in *CYP1B1*, *LTBP2*, and *MYOC* have been associated with the development of the disease, although the expressivity phenotype and the penetrance are variable. Given these data, pre- and posttest genetic counseling has an essential role for an adequate management of PCG, in terms of medical, social, and psychological impact of the disease.

It is important to remark that the eye has been at the forefront of translational gene therapy largely because of the availability of appropriate disease targets and its suitable anatomic features. These advantages have further fostered the research that culminated in the establishment of various clinical trials for the gene therapy of ocular diseases. In this perspective the identification of modifier genes should be encouraged as well as the analysis of the interaction between genes and environmental factors. The challenge for the future is to classify patients in relation to their genetic background, in order to provide personal treatment and management.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Fuchs Endothelial Corneal Dystrophy: Strong Association with rs613872 Not Paralleled by Changes in Corneal Endothelial *TCF4* mRNA Level

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Fuchs endothelial corneal dystrophy (FECD) is a common corneal endotheliopathy with a complex and heterogeneous genetic background. Different variants in the *TCF4* gene have been strongly associated with the development of FECD. *TCF4* encodes the E2-2 transcription factor but the link between the strong susceptibility locus and disease mechanism remains elusive. Here, we confirm a strong positive association between *TCF4* single nucleotide polymorphism rs613872 and FECD in Polish patients (OR = 12.95, 95% CI: 8.63–19.42, $\chi^2 = 189.5$, $p < 0.0001$). We show that *TCF4* expression at the mRNA level in corneal endothelium ($n = 63$) does not differ significantly between individuals with a particular *TCF4* genotype. It is also not altered in FECD patients as compared to control samples. The data suggest that changes in the transcript level containing constitutive *TCF4* exon encoding the amino-terminal part of the protein seem not to contribute to disease pathogenesis. However, considering the strong association of *TCF4* allelic variants with FECD, genotyping of *TCF4* risk alleles may be important in the clinical practice.

1. Introduction

Fuchs endothelial corneal dystrophy (FECD) affects approximately 4% of the population over the age of 40 and is the most common genetic disorder of the corneal endothelium. The disease usually has a late onset and presents clinically during the fifth or sixth decade of life [1]. It is characterized by thickening of Descemet's membrane and deposition of extracellular matrix in the form of guttae [2]. Patients with FECD have a reduced density of corneal endothelial cells. As the cells regulate corneal hydration and maintain its transparency, their loss may eventually progress to corneal oedema and vision loss [1]. For the end-stage disease transplant surgery represents the only definitive treatment. FECD is a leading indication for corneal transplantation and Descemet's Stripping Automated Endothelial Keratoplasty (DSAEK) is considered a standard procedure for these patients [3].

Genetic basis for FECD is complex and heterogeneous. Early-onset form of endothelial dystrophy with some of the phenotypic features of FECD occurs rarely and displays an autosomal dominant mode of inheritance with mutations in the *COL8A2* gene (1p34.3, MIM* 120252). The more common late-onset FECD begins usually after the age of 40 years and both familial and sporadic FECD cases have been described. Familial late-onset FECD is inherited in autosomal dominant fashion. However, the majority of late-onset FECD cases are sporadic with a negative family history. It is much more common and severe in females than in males (3-4 : 1) [4], which contradicts a strictly autosomal dominant transmission but follows multifactorial inheritance. Higher

Table 1: Loci, genes, and genetic variants related to late-onset FECD.

Chromosome	Locus/gene/variant	Late-onset FECD
1p35.1	rs760594	F
5q12.3	rs1301475	F
5q33.1–q35.2	FCD3	F
7q22.3	rs257376	F
8p21.3	rs2466216	F
8p21.1	rs9797	F
8q21.13	rs1380229	F
9p22.1–p24.1	FCD4	F
10p11.22	*ZEB1*	F/S
10q24.2	rs1889974	F
13pter-q12.13	FCD1	F
15q22.2	rs235512	F
15q22.31	rs352476	F
15q25.3	*AGBL1*	F/S
17q25.3	rs938350	F
18q21.1	*LOXHD1*	F/S
18q21.2	*TCF4*	F/S
18q21.2–q21.32	FCD2	F
20p13	*SLC4A11*	F/S
20p12.2	rs674630	F
Xq28	rs1990383	F

F: familial, S: sporadic.

risk of cornea guttata was independently associated with older age, female sex, and a thinner central corneal thickness [5] as well as genetic variants [6].

To date, four different genes *ZEB1* (10p11.22, MIM*189909), *AGBL1* (15q25.3, MIM*615496), *LOXHD1* (18q21.1, MIM*613072), and *SLC4A11* (20p13, MIM*610206) as well as four causal loci on chromosomes 5q33.1–q35.2 (FCD3), 9p22.1–p24.1 (FCD4), 13pter-q12.13 (FCD1), and 18q21.2–q21.32 (FCD2), together with a number of susceptibility loci, have been implicated in the pathogenesis of FECD (summarized in Table 1) [7, 8]. A strong association has been established between *TCF4* gene (18q21.2, MIM*602272) variants and FECD. A genome-wide association study has identified a common biallelic deep intronic *TCF4* single nucleotide polymorphism (SNP, rs613872; NG_011716.1:g.50559C>A) as a highly significant risk factor for FECD [6]. In addition to that, a separate study reported a trinucleotide expansion (CTG18.1) in the intron region of *TCF4,* which is highly prevalent in FECD individuals [9]. The role of these associations in FECD development remains poorly understood. In the study we set out to analyze whether the presence of *TCF4* rs613872 risk allele affects the expression of *TCF4* in corneal endothelial cells, which would provide a functional link between a risk factor and disease mechanism in FECD.

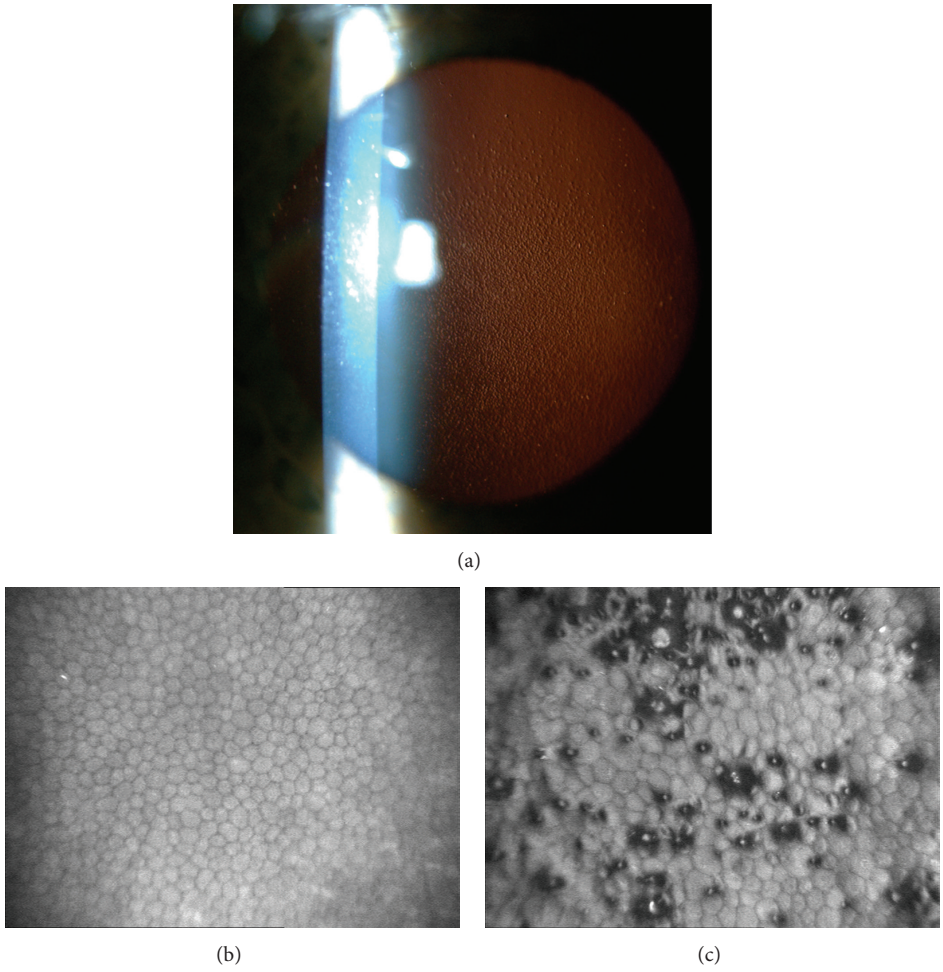
2. Patients and Methods

The study conformed to the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee. Patients were recruited from the Department of Ophthalmology, Medical University of Warsaw, and gave informed consent prior to participation. The diagnosis of FECD was based on the visualization of "guttae" and stromal oedema by slit-lamp examination, confocal microscopy *in vivo* (IVCM, Confoscan 4, Nidek Technologies), and anterior segment optical coherence tomography (CASIA Cornea/Anterior Segment OCT SS-1000, Tomey) (Figure 1).

2.1. Genotyping. Genomic DNA was isolated from peripheral blood samples of sporadic, unrelated FECD patients (n = 252; 187 females and 65 males) with a standard salting-out procedure. Control DNA samples (n = 323), representative of the background population of central Poland, came from the repository of the Department of Medical Genetics [10]. The *TCF4* rs613872 was genotyped using ABI Custom TaqMan SNP Genotyping Assay (Applied Biosystems) and the real-time PCR system (Viia7, Thermo Fisher Scientific). The accuracy of genotyping was confirmed by Sanger sequencing in selected subjects.

2.2. RNA Isolation and Quantitative Real-Time PCR. Corneal endothelial cell monolayers attached to Descemet's membrane were obtained from FECD patients (n = 40) during endothelial keratoplasty (DSAEK) or excised from donor corneoscleral buttons (n = 23) that were not used for transplantation. The FECD patients and controls included in the RNA studies derived from the group genotyped at rs613872 (FECD group: TT n = 3, TG n = 31, GG n = 6; control group: TT n = 14, TG n = 6, GG n = 3). The specimens were submerged in the RNAlater storage solution (Ambion, Austin, TX, USA) and frozen immediately. Total RNA was extracted using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was generated from 500 ng of RNA with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Gene expression was measured by real-time PCR (Viia7, Thermo Fisher Scientific) using PCR primers (Oligo, Warsaw, Poland) and probes (Roche Universal Probe Library) designed with the ProbeFinder software, version 2.50 (Roche). The analyzed coding region of *TCF4* is present in both TCF4-A and TCF4-B isoforms. It is located in the amino-terminal part of the protein, close to the activation domain 2 (AD2). For *TCF4* quantitative PCR (qPCR), the primer pair 5′-GCACTTTCCCTAGCTCCTTCT-3′ and 5′-GCATAGCCAGGCTGATTCAT-3′ and probe 25, for *RPL13A* (60S ribosomal protein L13a) qPCR the primer pair 5′-CTGGTGCTTGATGGTCGAG-3′ and 5′-GTTGATGCCTTCACAGCGTA-3′ and probe 77 were used. Expression values for *TCF4* were calculated using the modified double delta Ct (ΔΔCt) method and absolute quantification and normalized to *RPL13A* [11].

2.3. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) was analyzed with the χ^2 test in the patient and control groups. The odds ratios (ORs) with 95% confidence intervals (95% CI) and p values were calculated with the Web-Assotest program (http://www.ekstroem.com/assotest/assotest.html). Differences in gene expression between



(a)

(b)

(c)

Figure 1: Characteristic features of FECD. (a) Slit-lamp photography shows the presence of pathological guttae, focal excrescences of Descemet's membrane at the level of corneal endothelium in a patient with FECD. (b) Confocal microscopy image of the corneal endothelium in a control subject demonstrates a regular mosaic of the endothelial monolayer with bright cell bodies and dark, hexagonal cell boundaries. (c) Confocal microscopy in a patient with FECD reveals pleomorphism and polymegathism of the endothelium and typical guttae as dark bodies with a central bright reflex.

Table 2: Genotype distribution and allele frequency of *TCF4* rs613872 in patients with FECD and control subjects.

TCF4 rs613872 genotype	Genotype counts, *n* (%)				OR dominant model (95% CI)	Allele counts, *n* (%)		χ^2 statistic, *p*
	TT	TG	GG	Total		T	G	
FECD patients	46 (18.25%)	170 (67.46%)	36 (14.29%)	252	12.95 (8.63–19.42)	262 (51.98%)	242 (48.02%)	156.7 <0.0001
Controls	240 (74.30%)	74 (22.91%)	9 (2.79%)	323		554 (85.76%)	92 (14.24%)	

the groups were analyzed with a two-sided unpaired *t*-test (Statistica, StatSoft, Poland). Pearson's correlation was used to assess the degree of the relationship between gene expression and age or sex. $p < 0.05$ was considered to indicate statistical significance.

3. Results

Both the patient and control groups were genotyped for the *TCF4* SNP rs613872. In FECD patients the distribution of the TT, TG, and GG genotypes showed a significant deviation from Hardy-Weinberg equilibrium ($\chi^2 = 31.1$, $p < 0.0001$), while in control subjects the genotype distribution remained in the Hardy-Weinberg equilibrium ($\chi^2 = 1.3$, $p = 0.265$). Allele G was significantly more prevalent in patients with FECD than among control subjects (Table 2). The odds ratio (OR) for two copies of the risk allele (GG homozygotes versus TT homozygotes) was 20.87 (95% CI: 9.42–46.24), whereas the OR for one copy of the risk allele G (TG heterozygotes versus TT homozygotes) was 11.99 (95% CI: 7.90–18.19). Testing the association between FECD and the *TCF4* genotype under dominant, additive, or recessive models, we found that the most plausible model was the

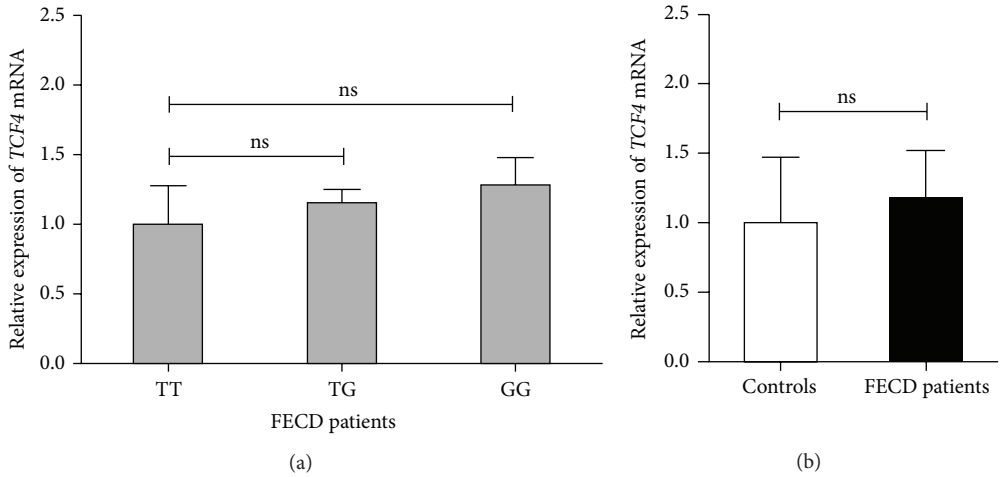


Figure 2: Expression of *TCF4* in corneal endothelial cells. The amount of *TCF4* mRNA was quantified by real-time PCR in relation to RPL13A. (a) Expression of *TCF4* in FECD patients with respect to a different *TCF4* genotype at rs613872 (TT $n = 3$, TG $n = 31$, and GG $n = 6$) is shown; ns: nonsignificant. (b) Average values of *TCF4* mRNA expression in control samples (white bars) and FECD patients (black bars) are shown; ns: nonsignificant.

dominant one whereas the additive and recessive models could be formally rejected as indicated by the p values for model fit ($p < 0.0001$). The dominant model (combining the GG and TG into one category) conferred the highest OR of all the tested models (OR = 12.95, 95% CI: 8.63–19.42, $\chi^2 = 189.5$, $p < 0.0001$).

Expression of *TCF4* was not age or sex dependent. There were no differences in *TCF4* mRNA levels in FECD patients with a particular *TCF4* SNP rs613872 genotype (Figure 2(a)) but also when *TCF4* genotypes of patients and controls were analyzed together (data not shown). No increase in the amount of *TCF4* mRNA transcripts was observed in FECD patients as compared to the control group (Figure 2(b)).

4. Discussion

Our data are in accord with previous studies showing that the presence of a *TCF4* risk allele at rs613872 is much more common in Caucasian patients with FECD and strongly predisposes to the development of the corneal dystrophy [6, 12–16]. Indian FECD patients do not show a distinct association with this *TCF4* variant, which may be explained by a small sample size studied [17]. In Chinese FECD patients no association could be observed as the population is not polymorphic at this genomic position [18–20]. In these FECD patients, *TCF4* genetic variants adjacent to rs613872 (e.g., rs17089887 in both Indian and Chinese subjects) were strongly associated with FECD, which suggested the presence of significant disease-causing changes in the nearby regions of these alleles [17, 19, 20]. All the relevant SNPs in the respective populations are in strong linkage disequilibrium with a newly identified CTG18.1 trinucleotide repeat expansion in the *TCF4* gene [9, 17, 20, 21], which confers a transethnic association with FECD.

TCF4 is now widely recognized as a major contributor to FECD. One may assume that patients carrying *TCF4* risks alleles and thus having an increased susceptibility to FECD should more often have follow-up ophthalmic examinations. Diagnosis of cataract in these patients might be an indication for an earlier cataract surgery before a full-blown, clinically significant FECD develops and the patients require a combined procedure of corneal transplantation and cataract surgery.

TCF4 genetic variants are strongly associated with FECD but so far little is known about a possible involvement of *TCF4* gene products in the development of FECD. Hypothesizing that the strong genetic association may indicate a causal relationship and the *TCF4* genetic variant may represent a tissue-specific regulatory element, *TCF4* mRNA studies in corneal endothelial cells were performed. Analyzing more than 60 different corneal endothelium and Descemet's membrane complexes, we have not found any significant differences in the expression of *TCF4* at mRNA level in FECD patients as compared to control samples. There were no differences in *TCF4* expression between the carriers and noncarriers of the *TCF4* risk allele neither in the group of FECD patients alone nor when FECD patients and controls were pooled together. The data suggest that changes in the transcript level containing constitutive *TCF4* exon encoding the amino-terminal part of the protein seem not to contribute to disease pathogenesis.

Recently, unaltered *TCF4* expression was also reported in another group of FECD patients [22]. In the absence of distinguished differences in the amount of mRNA, transcript activity still may be altered or posttranslational mechanisms may affect protein structure, level, or cellular distribution. A novel and so far the only identified link between *TCF4* susceptibility and FECD disease mechanism is the formation of toxic poly(CUG)n RNA and missplicing events in patients with *TCF4* repeat expansion. In corneal endothelial cells of these patients the *TCF4* repeats are actively transcribed and seem to preferentially accumulate into poly(CUG) RNA foci.

A protein found to be immobilized in RNA foci is MBNL1, a splicing regulator. Consequently, differential splicing events were found in corneal endothelium of FECD patients [23].

Our study confirms and extends the association of *TCF4* with FECD by testing a novel previously not analyzed population. It advances our knowledge on the role of *TCF4* in the development of FECD. Genotyping of *TCF4* risk alleles might be considered as a diagnostic procedure as the genetic results have an important predictive value and are of clinical utility.

Conflict of Interests

The authors declare no conflict of interests regarding this paper.

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Review Article

SLC4A11 and the Pathophysiology of Congenital Hereditary Endothelial Dystrophy

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Congenital hereditary endothelial dystrophy (CHED) is a rare autosomal recessive disorder of the corneal endothelium characterized by nonprogressive bilateral corneal edema and opacification present at birth. Here we review the current knowledge on the role of the *SLC4A11* gene, protein, and its mutations in the pathophysiology and clinical presentation of CHED. Individuals with CHED have mutations in *SLC4A11* which encodes a transmembrane protein in the SLC4 family of bicarbonate transporters. The expression of SLC4A11 in the corneal endothelium and inner ear patterns the deficits seen in CHED with corneal edema and hearing loss (Harboyan syndrome). *slc4a11*-null-mouse models recapitulate the CHED disease phenotype, thus establishing a functional role for SLC4A11 in CHED. However, the transport function of SLC4A11 remains unsettled. Some of the roles that have been attributed to SLC4A11 include H^+ and NH_4^+ permeation, electrogenic Na^+-H^+ exchange, and water transport. Future studies of the consequences of SLC4A11 dysfunction as well as further understanding of corneal endothelial ion transport will help clarify the involvement of SLC4A11 in the pathophysiology of CHED.

1. Introduction

Congenital hereditary endothelial dystrophy (CHED) is a rare disorder of the corneal endothelium with an early onset of corneal edema. Original classifications of CHED described two forms [1]. CHED1 was an autosomal dominant disease and presented with progressive corneal clouding beginning within the first few years of life. CHED2 was an autosomal recessive disease and presented with corneal clouding at birth or in the immediate newborn period. Recent genetic analyses and review of clinical findings now confirm that the previously termed autosomal dominant CHED (originally CHED1) is a form of posterior polymorphous corneal dystrophy with early and severe corneal edema [2]. It is thus no longer considered in the category of CHED (i.e., the entity CHED1 is now classified with posterior polymorphous corneal dystrophy). Based on the 2015 update to the International Classification of Corneal Dystrophies, the term "CHED" now exclusively refers to autosomal recessive CHED (originally CHED2) [3]. This review adopts this new classification and discusses only the autosomal recessive disease, henceforth called "CHED." Mutations in SLC4A11, a transmembrane protein in the family of bicarbonate transporters, are present in the majority of CHED cases studied. This review focuses on the role of SLC4A11 in the pathophysiologic mechanisms and clinical presentation of CHED.

2. Mutations in *SLC4A11*

The first clue to the origin of CHED came from the use of mapping techniques that enabled researchers to link

autosomal recessive inheritance of CHED to genetic markers within the 20p13 chromosomal locus [5, 22–25]. This assignment eventually led to the identification of mutations in the *SLC4A11* gene as the genetic basis of CHED [5]. *SLC4A11* encodes a membrane protein that was originally termed "BTR1" (Bicarbonate Transporter Related protein 1): a name that reflects its membership in the SLC4 family of bicarbonate transporting membrane proteins [26]. Despite the name, the disparate amino acid sequence of SLC4A11 segregates it from the family. Perhaps, as a consequence, robust HCO_3^- transport is not among the numerous molecular actions that have been attributed to SLC4A11 [17, 27]. At the time of writing, mutations in *SLC4A11* have been described in more than one hundred individuals with CHED (Figure 1) and signs of CHED have been recapitulated in several strains of *slc4a11*-null mice [18, 19, 28]. However, there is still much to be learned about the molecular action and the role of SLC4A11 in the healthy and diseased cornea.

3. Clinical Phenotype and Molecular Expression

The *SLC4A11* gene, which encodes at least three products (SLC4A11-A, -B, and -C), is expressed in a wide variety of cell types including corneal endothelium, spiral ligament fibrocytes of the inner ear, and various renal epithelia [5, 18, 19, 21, 29]. The molecular expression pattern of SLC4A11 correlates with the observed clinical phenotype for autosomal recessive CHED.

3.1. Corneal Endothelium. Disease of the corneal endothelium is the hallmark of CHED with corneal edema and opacification presenting at birth or shortly thereafter. The degree of opacification varies from mild to severe with a bluish-gray ground glass appearance (Figure 2). The opacification does not typically progress or regress. The cornea is uniformly thickened 2-3 times normal. Nystagmus of varying degrees and amblyopia may be present in individuals with more severe opacification. Photophobia, epiphora, and inflammation are not prominent features. (In contrast, the previously termed CHED1, now posterior polymorphous corneal dystrophy, presents with progressive corneal edema and opacification, typically not present at birth. Photophobia and epiphora are more common.) Primary disease of the corneal endothelium is the culprit for edema in CHED. The normal hexagonal endothelial mosaic is altered or absent. When endothelial cells can be visualized by specular or confocal microscopy, the cells are attenuated and fibrotic. Thickening of the endothelial basement membrane, Descemet's membrane, is visible by slit lamp examination. Two of three mouse models with disruptions in *slc4a11* have also replicated the corneal defects seen in humans [29–31]. These mice have progressive corneal swelling, doubling the thickness of the cornea, and thickening of Descemet's membrane [30, 31]. In these mouse models, the endothelium is present but the endothelial cells of older mice are swollen, distorting the hexagonal array, and exhibit vacuolization indicating cell distress [30, 31].

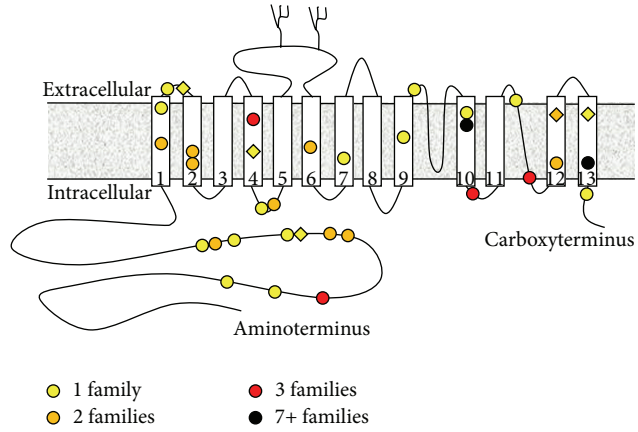

Figure 1: Cartoon of SLC4A11 protein topology showing the location of CHED-linked missense mutations. Approximately half of the individuals identified with CHED-linked mutations in SLC4A11 carry homozygous nonsense, frameshift, deletion, or splice-site mutations (not shown) that are predicted to result in the loss of active SLC4A11 protein. The other half carry homozygous missense mutations (colored circles) that are predicted to alter the protein sequence of SLC4A11 protein, noting the site of residues that are presumably important for the correct folding and activity of SLC4A11. Six individuals with CHED carry compound heterozygous (CH) mutations in SLC4A11: the location of missense mutations that have only been described in these individuals, and not in homozygous form, is marked with colored diamonds. The color of the circles and diamonds denotes the number of cases in which the mutation has been observed. Moving from amino- to carboxyterminus, the single cases (yellow) are R125H, E143K, S232N (CH with R329X), R233C, T262I, T271M, G394R, E399K, T401K (CH with L473R), L473R (CH with T401K), R488K, C611R, G709E, H724D, T754M, R804M, M856V (CH with S213P), and L873P. Sites mutated in two instances (orange) are R209W, S213L (plus CH S213P/M856V), A269V, C386R, G417R, G418ID, S489L, T584K (plus CH T548K/R112X), T833M, and L843P (both instances in CH form with frameshift mutations). Sites mutated in three instances (red) are A160T, G464D, P773L (including CH P773L/R112X), and V824M. Note that homozygous inheritance of A160T has also been observed in one unaffected individual and thus may not be the exclusive cause of CHED in these individuals [4]. Sites mutated in seven or more instances (black) are R755Q/R (including five instances of R755Q, one CH case of R755Q/R875X, and four instances of R755W) and R869C (seven instances). References: [4–16].

3.2. Inner Ear. In the mouse inner-ear, slc4a11 is expressed in the vestibular labyrinth and in fibrocytes underlying the stria vascularis [29, 30]. The stria vascularis is responsible for formation of endolymph and vestibular labyrinth for transduction of signals for hearing and balance. As may be expected from this expression pattern, high-frequency hearing loss is also a feature of CHED. Harboyan syndrome describes the condition of sensorineural hearing loss in the setting of CHED [32]. Although it has been described as an entity separate from CHED, a recent study suggests that some degree of hearing loss may develop in all individuals with time [33, 34]. Given the progressive nature of hearing loss in Harboyan syndrome, it may be missed in some individuals if tested at too young age. To date, the hearing loss has

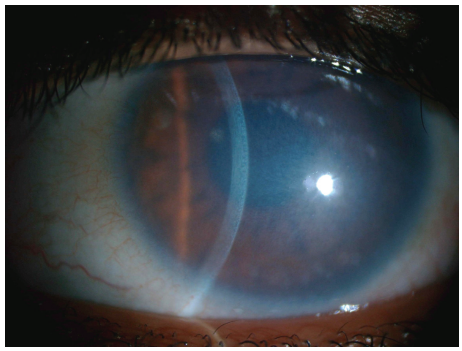

Figure 2: Clinical photograph of CHED demonstrating bluish-gray ground glass appearance. The slit beam highlights the uniform thickening of the cornea. Note the lack of corneal vascularization and inflammation. (Photograph courtesy of Arif O. Khan, Division of Pediatric Ophthalmology, King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia.)

never been documented in the prelingual period. Progressive hearing loss is also recapitulated in mice with disrupted *slc4a11*, even in the strain of mouse that did not exhibit any CHED-like ocular signs [29, 30].

3.3. Kidney. Slc4a11 is expressed in mouse kidney and has been detected in the proximal tubules, the thin descending limb of the loop of Henle, and the collecting ducts [30, 35]. Two mouse models of CHED with disruptions of *slc4a11* have a urine concentrating defect resulting in decreased urine osmolarity and corresponding decrease in all urine electrolyte concentrations [30, 31]. Interestingly, there is only one report evaluating kidney function in one human with CHED and no defect was found. The older age of this individual (55 years old) suggests that any potential progressive defect would have been detected if present compared to evaluation in a younger individual [36].

3.4. Trabecular Meshwork. There are several case reports of glaucoma presenting in patients with CHED. However, to date, there are no reports on the expression of SLC4A11 in the trabecular meshwork and aqueous outflow pathways of the eye. Review of published cases suggests a tenuous association. One series reported three cases of glaucoma with CHED [37]. The first case included a family history of congenital glaucoma and the subject also had partial aniridia with ectropion uveae. In the second case, iris vascularization was noted as well as heavy vascularization of the anterior stroma upon histological examination of the excised cornea. In the third case, the subject had partial aniridia. A subject in another case report of CHED with glaucoma also had iris hypoplasia [38]. Review of these published cases suggests the involvement of more global anterior segment dysgenesis in addition to CHED. Faced with the potential diagnosis of glaucoma in CHED, careful examination should be performed to consider the diagnosis of posterior polymorphous corneal dystrophy (formerly autosomal dominant CHED1) for which glaucoma is a more common codiagnosis (15%) [3]. While the common origin of the corneal endothelium and anterior chamber angle from neural crest would support the association of glaucoma and CHED, SLC4A11 expression has not been demonstrated in aqueous outflow structures of the eye.

4. The Molecular Actions of SLC4A11

SLC4A11 dysfunction clearly plays a key role in CHED pathogenesis, but little is known of its normal function. Several molecular actions have been assigned to SLC4A11, yet which of these are of physiological or pathophysiological importance remains to be determined (Figure 3). The first study to address the action of SLC4A11 reported that human SLC4A11 expressed in the HEK293 human-kidney-derived cell line formed Na^+ and H^+ (or OH^-) channel [27]. Na^+ and/or H^+/OH^- transport mediated by SLC4A11 has subsequently been observed in other mammalian cell lines [20, 31] and in cultured bovine corneal endothelial cells [17]. SLC4A11 has also been noted to enhance NH_4^+ permeability in HEK293 cells [31]. Intriguingly, SLC4A11 can also act like an aquaporin, enhancing cellular water permeability [21]. An initial report that SLC4A11 was an electrogenic Na^+-coupled borate cotransporter (therefore renamed "NaBC1") [27] is controversial as others have been unable to detect evidence for any borate-dependent action of SLC4A11 [17, 20, 21, 31]. With all of these observations on SLC4A11 action but no consensus, the possibility remains that SLC4A11 has another function, yet to be determined.

5. The Role of SLC4A11 in the Cornea

Immunohistochemical studies reveal the presence of SLC4A11 in the corneal endothelium of humans, rats, and mice [18, 21, 28, 29]. In the corneal endothelium of mice, slc4a11 is present exclusively in the basolateral membrane [18, 21]. The purpose of the endothelial layer is to prevent stromal edema by countering the osmotically driven movement of water from the aqueous humor into the collagen matrix of the stroma. The endothelial cell layer is leaky due to its high paracellular permeability. Thus, rather than prevent the movement of water into the stroma, endothelial cells draw water out of the stroma coupled to secretion of ions from the stroma to the aqueous humor. This "pump-leak" mechanism, recently reviewed in [39], is represented in Figure 4. The usefulness of SLC4A11 to normal corneal function has yet to be fully elucidated, in part because its *in vivo* action is uncertain. If SLC4A11 functions like an aquaporin to mediate H_2O flux, then it could promote transcellular (stroma to aqueous humor) water flux in concert with aquaporin 1 [21]. If SLC4A11 functions in Na^+-H^+/OH^- transport, then it could support the "pump" mechanism by modulating intracellular pH, volume, or membrane potential [17, 20].

6. The Contribution of SLC4A11 Dysfunction to CHED

Humans harboring *SLC4A11* mutations (or mice with a disrupted *slc4a11* gene) exhibit contrasting corneal endothelial

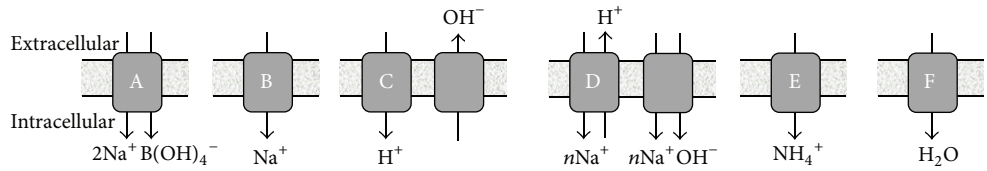

Figure 3: Suggested molecular actions of the SLC4A11 protein. A: Electrogenic sodium/borate cotransporter [17]. B: conductive sodium permeation pathway [17]. C: conductive proton influx permeation pathway, which is thermodynamically equivalent to a hydroxyl efflux pathway [17, 18]. D: coupled electrogenic nNa/H exchange, which is equivalent to electrogenic nNa-OH cotransport ($n > 1$) [19, 20]. E: NH_4^+ permeation pathway [20]. F: H_2O permeation pathway [21].

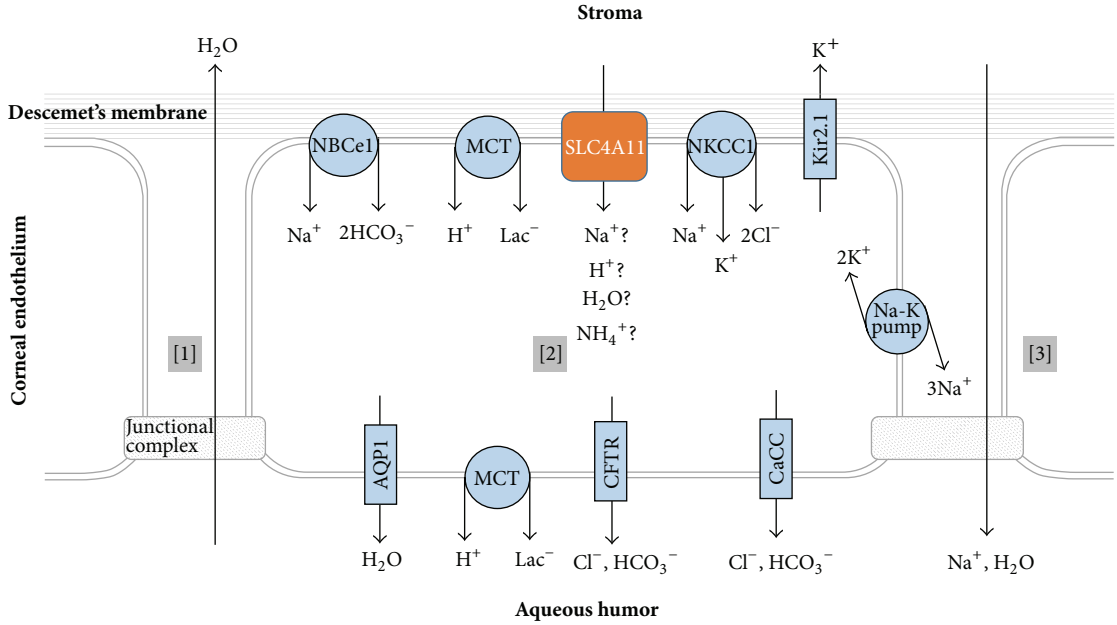

Figure 4: Transporters and channels that support corneal pump function. [1] Water is drawn from the aqueous humor into the stroma. [2] Endothelial cells secrete ions into the aqueous humor creating osmotic gradient that [3] draws fluid back into the aqueous humor.

phenotypes which may help advance our understanding of SLC4A11 dysfunction in CHED. In humans, the corneal endothelial monolayer is present and dystrophic or is absent with only rare fibrotic-appearing, atrophic endothelial cells present [6, 40–42]. In contrast, *slc4a11*-null mice have a corneal endothelial monolayer of cells present [30, 31]. However, disease is evidenced in these cells by the presence of vacuolization, gradual decrease in endothelial cell density, and loss of hexagonal cell morphology. Despite the presence of a corneal endothelial monolayer, these mice have corneal edema with corneal thickness increasing with age, thus arguing that the deficit in CHED lies with the efficacy of endothelial cell function.

The embryology of corneal endothelium and Descemet's membrane formation also supports the argument of disruption of endothelial cell function. The dysfunction occurs postnatally. The first wave of migration of neural crest cells from the rim of the invaginating optic cup forms the corneal endothelial monolayer and trabecular meshwork [43]. Two factors indicate that the defect in CHED is not with this initial migration. First, if the defect in CHED were in this initial migration, one might expect a higher incidence of glaucoma (due to comigration of cells forming trabecular meshwork) than is currently observed in individuals with CHED. Secondly, corneal endothelial cells secrete the anterior banded zone of Descemet's membrane beginning around the 3rd month and continuing through the 8th month of gestation [44]. The anterior banded zone is absent in conditions such as Peter's anomaly with defects in neural crest cell migration to form anterior segment structures [45, 46]. In contrast, the anterior banded zone is present in CHED with either normal or thickened morphology, thus indicating that the endothelial cells were present and functional during that period of development [40, 41]. During early postnatal development, the corneal endothelium begins formation of the posterior nonbanded zone (PNBZ) of Descemet's membrane. The PNBZ continues to thicken throughout life [47]. In diseases with dysfunctional endothelium, the PNBZ can merge with an abnormal posterior collagenous layer that is secreted by the endothelial cells. In humans with CHED, the PNBZ has variable thickness (thin or thick) with or without the presence of a posterior collagenous layer [40–42]. This variability in

thickness of the posterior portion of Descemet's membrane may reflect the variability in timing of demise of the corneal endothelial cells.

There are numerous ways a defective membrane transport protein could contribute to a disease state, the most obvious being loss of transport function. Others include loss of protein *per se* (and thence loss of docking sites for dependent interacting-proteins), cell stress due to the accumulation of misfolded transport protein, and maladaptive compensatory changes in the expression of other gene products. Many of the CHED mutations recapitulated in heterologous systems are predicted to generate a misfolded SLC4A11 protein and have been shown to accumulate in intracellular compartments when expressed in cultured cells [5]. As mentioned above, endothelial cells do exhibit signs of stress with vacuolization and deposition of the posterior layer of Descemet's membrane. However it does not seem that the anticipated stress from accumulation of misfolded protein is the sole driving force behind the manifestation of CHED. One CHED-linked mutation exhibits loss of H_2O and H^+/OH^- transport in model systems without any deleterious effect upon SLC4A11 protein expression [20, 21]. Moreover, corneal edema is recapitulated in a strain of *slc4a11*-null mouse that is predicted to express no misfolded slc4a11 protein product [31]. Thus, whatever its molecular action may be, the pathology behind CHED seems to involve a loss of SLC4A11-mediated support of "pump" function.

7. Conclusion

Current advances in genetic testing for individuals with CHED have narrowed the spectrum of disease to mutations in a single gene, *SLC4A11*. Mouse models of CHED with deficits in slc4a11 expression recapitulate many of the features of the disease and will allow us to better understand its development and pathophysiology. The functional role of SLC4A11 in the corneal endothelium is unsettled yet it likely impacts corneal endothelial ion transport as part of the "pump" mechanism to maintain corneal clarity.

Disclaimer

The opinions expressed herein do not necessarily represent those of the Veterans Administration or the U.S. Government.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Cultivated Oral Mucosa Epithelium in Ocular Surface Reconstruction in Aniridia Patients

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Purpose. Efficacy of cultivated oral mucosa epithelial transplantation (COMET) procedure in corneal epithelium restoration of aniridia patients. *Methods*. Study subjects were aniridia patients (13 patients; 17 eyes) with irregular, vascular conjunctival pannus involving visual axis who underwent autologous transplantation of cultivated epithelium. For the procedure oral mucosa epithelial cells were obtained from buccal mucosa with further enzymatic treatment. Suspension of single cells was seeded on previously prepared denuded amniotic membrane. Cultures were carried on culture dishes inserts in the presence of the inactivated with Mitomycin C monolayer of 3T3 fibroblasts. Cultures were carried for seven days. Stratified oral mucosa epithelium with its amniotic membrane carrier was transplanted on the surgically denuded corneal surface of aniridia patients with total or subtotal limbal stem cell deficiency. *Outcome Measures*. Corneal surface, epithelial regularity, and visual acuity improvement were evaluated. *Results*. At the end of the observation period, 76.4% of the eyes had regular transparent epithelium and 23.5% had developed epithelial defects or central corneal haze; in 88.2% of cases visual acuity had increased. VA range was from HM 0.05 before the surgery to HM up to 0.1 after surgery. *Conclusion*. Application of cultivated oral mucosa epithelium restores regular epithelium on the corneal surface with moderate improvement in quality of vision.

1. Introduction

During second decade of life, aniridic patients begin developing pathologic cellular exchange. Epithelial cells covering the cornea are removed by the surrounding conjunctival epithelium with its vascularized matrix [1]. This leads to a local disorder called limbal stem-cell deficiency (LSCD) [2]. Its stages lead to superficial conjunctival ingrowth, corneal haze, discomfort, and deep loss of visual acuity. Since the 1980s, the concept of limbal disease management has focused on limbal transplantation and removal of pathologic tissue followed by transplantation of autologous or allogeneic limbal tissue carried on the donor's sclera [3, 4]. Many studies have confirmed that delivery of healthy limbal tissue improves vision and decreases ocular discomfort [5–7].

Development of new culture techniques has changed the treatment of LSCD [8]. Transplantation became limited to cultured autologous cells, without additional tissues of

the donor's sclera or conjunctiva [9]. Application of oral mucosa epithelium was proposed by Shigeru Kinoshita and was turned into clinical practice by Nakamura et al. [10]. Another new concept was use of oral mucosa epithelium in reconstruction of the ocular surface [11]. With a phenotype similar to the cornea, it allowed the possibility of autologous treatment in patients with bilateral ocular involvement [12].

Congenital aniridia is a rare disorder of autosomal-dominant or sporadic occurrence. It occurs with an incidence of slightly less than 1 : 100,000 to 1 : 60,000 in the general population. It manifests with low visual acuity and amblyopia early. Later it usually is accompanied by cataract and glaucoma. From the second decade of life, an additional factor influences visual quality: corneal haze caused by the conjunctivalization of the cornea surface [13].

In aniridia patients, signs of LSCD are ocular discomfort, superficial vascular conjunctival pannus, and decreased visual acuity. Severity is mild; therefore, for several years those patients were disqualified from any surgery. Cultivated oral mucosa epithelium transplantation (COMET) offers minimal and effective surgical approach for this group of patients.

2. Materials and Methods

The experimental procedure was performed under the tenets of the Declaration of Helsinki. Signed written informed consent was obtained from all patients before the procedure began.

Oral mucosa epithelium specimens (3–5 mm^2) were collected under local anesthesia and decontaminated with 5% povidone-iodine solution. Biopsies were taken from the inferior buccal mucosa; no sutures were applied. The tissue was transferred immediately to corneal storage medium at 4°C and then taken to the laboratory.

The tissue specimen was treated with Dispase II for 1 hour, after which it was trypsinized with 1% trypsin/0.01% EDTA mixture for 10 min in 37°C to obtain a single cell suspension. Epithelial cells were prepared for seeding with a density of 4×10^4 cells per 1 mL (Cell counter, Coulter Z1, Miami, USA).

Culture media and chemicals were purchased from Sigma (Germany). The reagents for immunostaining were obtained from Santa Cruz Biotechnology Inc. (USA). The epithelial culture was carried in the presence of 3T3 fibroblasts, a source of growth factors. One week prior to specimen collection, six-well culture dishes (Becton Dickinson, USA) were prepared. Bottoms of the plates were covered with a monolayer of 3T3 fibroblasts (ATCC, USA). The 3T3 cells were cultivated in Dulbecco Modified Eagle's Medium (DMEM) with 10% bovine serum and a 100 μg/mL penicillin and streptomycin mixture. From the fifth to seventh day of culture, when the monolayer was reached, fibroblasts were inactivated by incubation in DMEM containing 2 μg/mL of Mitomycin C for 2 h.

The carrier for the oral mucosa epithelial sheet was the amniotic membrane located on the dish insert over the monolayer of fibroblasts. Amniotic membrane carriers were obtained from the Homograft Tissue Bank in Zabrze, Poland. Amniotic membrane samples were washed from the cryoprotective medium with Phosphate Buffered Saline (PBS), and the amniotic epithelium was removed gently with a culture scraper. Denuded amniotic membrane slides (15 × 15 mm) on nitrocellulose paper were put over the layer of fibroblasts in the culture plates.

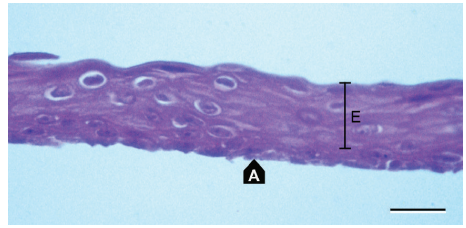


Figure 1: Cultivated oral epithelium (E) on the amniotic membrane (A) prior to transplantation. H&E stain. Scale bar: 20 μm.

Single oral mucosa cells were seeded on denuded amniotic membrane spread on nitrocellulose paper lying on the inserts over the fibroblast monolayer. Cultures were carried out in standard conditions of 37°C and a humidified atmosphere of 5% CO_2 and 95% air. The medium was a supplemented Dulbecco Modified Eagle's Medium (DMEM) with HAM F12 mixture with 10% of serum (bovine serum in first 12 experiments, autologous serum in the next 5 cultures), 0.5% dimethyl sulfoxide (DMSO), 10 ng/mL mouse Epidermal Growth Factor (EGF), 5 μg/mL bovine insulin, 0.1 nM cholera toxin, 0.18 mM adenine, 2 nM triiodothyronine, 4 mM L-glutamine, 0.4 mg/mL hydrocortisone, and 100 μg/mL penicillin and streptomycin mixture. The culture medium was changed every 48 h. Cultures were carried out on at least 2 amniotic membranes for each patient. On the seventh day of culture, the epithelial growth stage was evaluated under light microscope and small specimens of each membrane (Figure 1) were stained with hematoxylin-eosin (H-E). Amniotic membranes (A in Figure 1) covered completely with multilayer epithelium (E in Figure 1) were suitable for a graft. Cultivation over 7 days caused loss of superficial cells of the proliferating epithelium to the medium. At the time of microscopic qualification, airlifting was done. Cells were airlifted with a minimal amount of culture medium for 1 h.

One day before the transplantation, immunostaining for cytokeratin 4, cytokeratin 13, protein p63, and connexin 43 was performed to confirm the origin of the epithelium and identify the presence of low differentiated cells. Both cytokeratins 4 and 13 were described by Ang et al. as characteristic for oral mucosa epithelial cells, their presence confirming expected cellular structure of the graft just before application [14]. Protein p63 (transcription factor) plays an important role in epithelial cells proliferation and differentiation, in cultured cells it shows cellular potential for epithelial renewal [15]. Gap-junction protein connexin 43 is present in the basal epithelial layer, and positive staining confirms proper architecture of cultured epithelial layers [16]. Hematoxylin-eosin (H-E) staining was used in histological analysis of epithelial layer regularity and thickness. Regular structure

of intact epithelium on amniotic membrane presented on Figure 1 was expected by researchers. Only such grafts were qualified in surgery.

Study subjects were 13 aniridia patients (17 eyes) suffering from LSCD with central corneal involvement. Vascular conjunctival pannus involved the entire limbal area (total LSCD: 14 eyes) or at least 9 clock-hours of the limbus with central involvement (subtotal LSCD: 3 eyes). The main indication for the study was presence of semitransparent conjunctival pannus covering the cornea over the pupil. It caused decreased quality of vision even in cases of subtotal limbal involvement. The patients were both oral mucosa epithelium donors and recipients of the cultured epithelium. There were 3 men and 10 women. Ages ranged from 16 to 54 with an average of 31.1 ± 11.5 years. In 4 cases, surgery was bilateral and sequential (3–6 months between each surgery). Visual acuity before intervention ranged from HM to 0.05. Inclusion criteria were congenital aniridia, conjunctivalization of the corneal surface with neovascularization (with or without corneal scarring) and at least 6 months from last eye surgery. In addition, the study required healthy oral mucosa, no smoking for at least 7 days before taking a sample of epithelium, and regular teeth brushing (twice a day). Exclusion criteria were acute systemic infections, acute eye infection during the last 6 months, neoplasm history, pregnancy, and mental disorders. In addition patients with dense cataract coexisting corneal disorders were excluded.

Surgery was performed under topical anesthesia (Alcaine, Alcon, USA). A 360-degree peritomy was done, followed by conjunctival suturing at 1-2 mm behind the limbal area with single 10-0 nylon sutures. The conjunctival pannus was removed gently with a spatula or crescent knife. If necessary, fibrotic tissue spread under the conjunctival pannus was removed with gentle keratectomy to prepare a smooth corneal surface. Then, round, 14 mm diameter trephined amniotic carriers with oral mucosa epithelial cells were transplanted onto the denuded corneas. Peripheral, continuous 10-0 nylon suture was used to stabilize the graft. The entire cornea was covered, and suture was placed in the corneolimbal interface. Then, the graft was covered by a bandage contact lens.

After the procedure, levofloxacin (Santen, Finland) and preservative-free dexamethasone eye drops (Thea Laboratories, Paris, France) were administered 5 times a day for 2 weeks. Then, dexamethasone alone was applied 3 times a day for 6 weeks. All subjects received preservative-free lubricants every 2 hours, and in cases of epithelial defects or erosions, autologous serum drops were administered. On each control visit, stabilization of the corneal surface was evaluated with topical 10% fluorescein staining. Lack of epithelial defects (negative fluorescein staining) with regular, stratified epithelium or no more than 3 clock-hours of peripheral conjunctival neovascularization was classified as a successful case (coded as +++); partial success consisted of punctate epithelial defects or peripheral conjunctival neovascularization up to 9 clock-hours without pupil area involvement (coded as ++); and failure consisted of vascularization of the pupil area, persistent nonhealing epithelial defects or deep stromal vascularization (coded as +). Whether LSCD occurrence was total or subtotal made no differences in surgical management or postoperative treatment regarding 360-degree peritomy and removal of all fibrotic tissue or epithelia from the corneal surface.

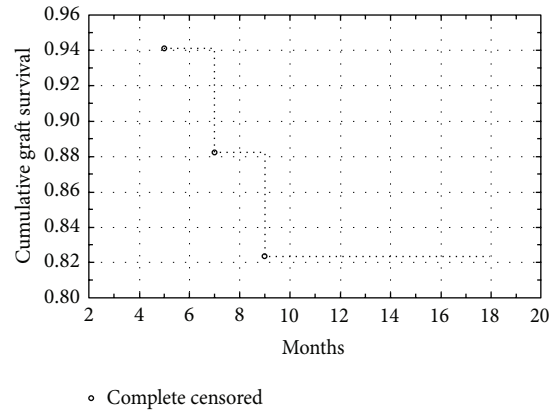


Figure 2: Cumulative graft survival: Kaplan-Meier curve for COMET in aniridia.

3. Results

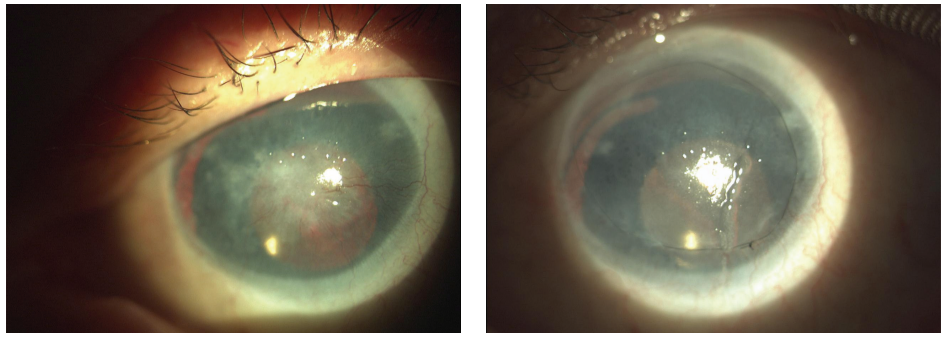
Local complications were not seen after oral mucosa donation. Transplanted specimens showed regular, stratified epithelium of 4–9 layers of cells. Epithelium origin was confirmed by previous immunostaining, as described in the culture procedure section.

Over the observation period of 12–18 months, 13 operated eyes showed stable epithelium; however, peripheral vascular irregularities were present in all of them. Table 1 shows detailed patient data. These corneas were not stained by fluorescein solution (qualified as +++); 4 eyes suffered various epithelial defects, from single local defects (++) to large areas of denuded cornea (+) with peripheral conjunctival ingrowth. At the end of the observation period, 3 eyes developed stromal scarring, conjunctival vascularization, or stromal vascularization. All 3 of these eyes were qualified as graft failure. In successful cases, the epithelium was smooth and regular (qualified as +++ and ++). Figure 2 presents the graft survival rate on a Kaplan-Meier curve. The first 9 postoperative months were critical to the final results. Occurrence of epithelial defect was the indication for autologous serum eye drops. Despite careful treatment, 3 failed cases developed after 6 months of progressive invasion of the central cornea by vascular conjunctival tissue. In other cases, the peripheral area was characterized by irregularities in the vascular network, with peripheral ingrowth of new vessels without central area involvement. Comparison of surfaces before and after surgery (Figures 3(a) and 3(b)) shows improvement of corneal transparency in the central area and irregular distribution of vessels in the limbal area. Inflammatory response, defined as hyperemia and neovascularization at the donor-host interface, was moderate and easily controlled with a steroid agent. Limited vascular ingrowth in eyes with stable autologous oral epithelium confirms that COMET with its autologous origin limits inflammatory response. There is also

Table 1: Overview of aniridia patients data.

Case	Age	Gender	Eye	BCVA pre	BCVA post	Epithelial regularity	Follow-up (m.)	Treatment
1	28	F	R	CF	0.05	+++	18	Routine
2	28	F	L	0.05	0.1	+++	12	Routine
3	27	F	R	0.02	0.1	+++	14	Routine
4	54	F	R	HM	CF	+	13	Routine, PSED, DEX
5	26	F	L	0.02	0.05	+++	18	Routine
6	29	F	R	0.05	0.05	+++	18	Routine
7	27	F	L	0.01	0.03	+	17	Routine, PSED, DEX
8	18	F	L	HM	0.1	+++	18	Routine
9	18	F	R	HM	0.05	+++	14	Routine
10	49	M	L	CF	0.1	+++	18	Routine
11	40	F	R	CF	0.05	+++	15	Routine, TSED
12	23	M	L	HM	CF	+++	18	Routine, TSED
13	38	F	L	0.01	0.02	+++	18	Routine
14	38	F	R	HM	0.05	++	15	Routine, PSED
15	49	M	L	CF	0.1	+++	12	Routine
16	16	F	L	HM	HM	+++	18	Routine, TSED
17	22	F	R	HM	0.05	+	16	Routine, PSED, DEX

Fluorescein staining: absent (+++), local defects (++), and present (+).
CF: counting fingers and HM: hand movements.
Routine treatment: Levofloxacin and dexamethasone eye drops 5 times a day for a 2-week period and then only dexamethasone 3 times a day for the next 6 weeks.
Additional treatment: PSED: permanent serum eye drops, TSED: temporary serum eye drops, and DEX: additional dexamethasone administration over 2nd month from surgery.



(a) (b)

Figure 3: (a) and (b) Aniridia patient, 49-year-old man, who underwent cataract surgery 5 years before COMET. Preoperative view with central fibrovascular scar on the left; on the right, 6 months after surgery, VA improvement from CF to 0.1 with central cornea covered by regular and smooth epithelium.

no immune reaction characteristic for allotransplantation in, for example, keratolimbal allografts (KLAL). Excluding the failed grafts, patients noted improvement in their quality of vision, as well as decreased ocular discomfort. Postoperative occurrence of epithelial defects did not interfere with visual acuity and were treated with autologous serum drops 7 times a day. Four eyes with epithelial defects received persistent therapy; in three others (graded as +++) therapy was temporary for up to four weeks. Visual acuity improvement was noted in 88.2% of cases. It ranged from HM up to 0.1. As shown in Figure 3 main benefit of COMET was restoring of transparent epithelium without pathologic vessels on the corneal surface. This 49-year-old, single-eyed man developed conjunctival pannus after cataract surgery performed 5 years ago. Improvement to 0.1 restored his ability to read; he pointed it as a main benefit of surgery. Despite moderate improvement of VA the majority of patients were satisfied after surgery.

4. Discussion

LSCD is a difficult therapeutic challenge in patients with congenital aniridia. This condition is mild in severity and is usually accompanied by amblyopia, cataracts, and corneal haze caused by conjunctival pannus. It is believed that progressive conjunctivalization with age is connected to congenital impairment of pluripotent cells located in the hem.

In 2003, Nakamura and Kinoshita proposed a cultivation method of oral epithelium to restore ocular epithelial surface based on experiments on rabbits [17]. Several benefits made the procedure very interesting. Damaged corneas covered by autologous epithelium have a lower risk of immunologic reaction. Oral epithelium transplanted onto the new locus can maintain its proliferative potential without developing keratinization. In addition, low differentiation and quick cellular turnover result in regular stratified epithelium.

An important factor of this method is the cellular phenotype of oral epithelium. The phenotype is similar to the corneal epithelium phenotype, making this procedure safe and easy to apply. Allografts often require systemic or local immunosuppressive agents administered for long periods postoperatively. Autografts do not have such requirements, as the induction of neovascularization is lower if the tissue comes from the same patient [18].

As described by Hata et al., oral epithelium culture is very effective for recipients, as these cells have a low stage of differentiation combined with fast cellular turnover [19]. To obtain a regular epithelial layer, only a few days of culturing are needed. In addition, these features are prolonged for the postoperative period. In long-term observation, it improves stability of the transplanted epithelium. In our patients, the surface epithelium in successful cases remained stable with temporary epithelial disorders. In local defects, autologous serum drops were administered to improve epithelial growth. For large defects autologous serum or bandage contact lenses were ineffective. Such patients in our study developed secondary conjunctival ingrowth leading to failure of the procedure.

COMET is widely proposed in Stevens-Johnson Syndrome (SJS), ocular cicatricial pemphigoid (OCP) and ocular burns. However, in those cases many patients require 2-step surgical approach to restore vision due to accompanied stromal damage. Sotozono et al. describes such cases showing that long-term visual improvement is possible even with high rate of postoperative epithelial defects (40%) [20]. In acute cases of SJS or OCP damage of the ocular surface and inflammation grade is high. Authors have only one case of aniridia and a few cases with slight ocular surface damage. In those cases visual improvement is significantly high. In aniridic patients ocular surface disease is limited to the epithelial surface and subepithelial fibrosis. There is no severe inflammation or destruction of surrounding tissues. In our group there was also no need of stromal procedures of lamellar or penetrating keratoplasty. Final vision remained low; however, main benefit was stable epithelial surface with only 3 failed grafts and 4 patients with epithelial defects (3 of them were temporary). If compared to severe cases of ocular surface disease in aniridia there was no indication to administration of systemic steroids.

Historically, therapeutic treatment has included Scheffer Tseng's approach of removing the corneal pannus combined with lamellar keratoplasty or penetrating keratoplasty combined with limbal allotransplantation. However, allotransplants require subsequent systemic immunosuppression, which can interfere with the patient's general health. Complicity and difficulty of the technique resulted in the need to transplant only those epithelial cells involved in the pathologic process. COMET offered the opportunity to focus on the affected area of the corneal surface.

Tsubota et al. describing keratolimbal allografts shows high risk of immune adverse reactions (graft rejection), infections, and systemic disorders associated with immunosuppressive agents administered for many postoperative months [21]. In his study stable ocular surface in achieved in 51% of the cases. If we compare improvements in vision, allografts limit the quality of vision [22]. Risk of rejection and revascularization result in loss of visual acuity in long-term observations. The Kaplan-Meier curves for the allograft approach show continuous reduction of success rate in the long term. Daya et al. reports results of allogeneic cultured corneal epithelium transplantation with success rate of 70% [23]. It indicates that limitation of surgical approach to epithelial surface only increases success rate. Nishida et al. report 100% success rate after COMET; however, in other studies authors achieve stabilization of corneal surface in 66.7% or even 57.5% [24–26]. The autologous approach with oral epithelium transplantation seems to cause less immunization, less inflammatory response, and less vascular ingrowth. These benefits should increase the success rate in bilateral limbal stem-cell insufficiency present in aniridia.

There were not many differences between culture procedures for the oral and corneal epithelium transplantation techniques. Amniotic membrane substrate as a cellular carrier and the source of basement membrane is widely applied [27, 28]. Its efficacy is confirmed in papers on cultured epithelial transplantation. Amniotic membrane makes the application of oral epithelial sheets safe and easy. In mild cases with partial limbal insufficiency in aniridia, transplantation limited to the application of amniotic membrane can support the corneal epithelium for a long period of time. Coculture with 3T3 fibroblasts results in fast proliferation and maintains low differentiation of the oral epithelium. This parameter is crucial for graft survival, despite fast cellular turnover. Another advantage is the almost unlimited source of tissue, which can be collected easily. The regenerative potential of oral epithelium is high. During cultivation, loss of superficial epithelial cells to the media was observed after seven days. In our opinion, a culture period of one week is sufficient to reach stable oral epithelial multilayers.

In 2003, Nakamura et al. described the first oral epithelium transplantations in humans with corneas severely damaged by Stevens-Johnson Syndrome (SJS) and severe ocular burns. Results were promising, and oral epithelium became a substitute for corneal epithelium with satisfactory corneal transparency and reduction of ocular discomfort. Nakamura et al. later described 17 patients treated with the COMET

procedure with a mean follow-up of 55 months [29]. All eyes manifested revascularization of the surface with various stages of severity. The most stable epithelia were observed within six months after surgery. Visual acuity improvement was observed in 95% of patients after surgery. After 36 months, only 53% of eyes noted improved visual acuity. The authors concluded that despite the decrease in vision quality, the reconjunctivalization was less severe than that before surgery. In failed cases presented in the paper, conjunctival invasion was not severe; however, involvement of visual axis caused failure of the entire treatment.

Another paper on COMET by Ang et al. described stabilization of the ocular surface without major complications in all investigated eyes for at least 12 months after surgery [14]. Graft survival was limited in COMET; however, scarification of the ocular surface was much less severe compared with untreated patients. In addition, combining COMET with penetrating keratoplasty led to improved ocular surface and decreased risk of PK failure [30].

Satake presented the results of 40 eyes after COMET. Procedure were performed on SJS, cicatricial pemphigoid, and ocular burn patients [26]. Results showed the fast decline of graft survival in the first six months after the procedures with subsequent stabilization. Outcomes depended on the severity of ocular involvement; patients with severe vascularization and damage to the cornea surface were at greater risk of early graft failure than those with less severe symptoms. Similarly, poor primary visual acuity resulted in a worse outcome for those in the aniridia group. However, analysis has not been performed on patients with congenital aniridia. According to our study, graft survival and epithelium regularity are much better in aniridia patients.

Visual acuity in patients with aniridia depends on the optical transparency of both the cornea and the lens. In 75% of patients, cataracts occurred next to the corneal pannus, which greatly affected visual acuity. Therefore, in our opinion, visual acuity is a poor success parameter of transplant-cultured oral mucosal epithelium. In the success group graded +++, there were eyes with no visual acuity improvement despite a stable surface, showing that other factors, such as foveal hypoplasia, optic nerve lesions, or cataracts, were disturbing vision. In our study, the criterion for success was restoration of the corneal epithelium, although it is known that the most important criterion for patients is visual acuity. Increased corneal vascularization with patient age is associated with epithelial cell dysfunction, but the exact cause has not been documented clearly yet. Some authors make effective use of anti-VEGF, but the literature does not describe the use of anti-VEGF in corneal neovascularization.

Application of COMET was successful in aniridia patients. The visual acuity improvement rate for aniridia cases was lower than that for SJS, ocular cicatricial pemphigoid, or ocular burn cases. While actual visual acuity improvement was lower, the main benefit was improvement of epithelial regularity. Chen et al. investigated cell survival in COMET patients [31]. The majority of recipients in the follow-up had oral mucosa-derived cells The present study has confirmed the usefulness of the procedure in cases of mild limbal insufficiencies, such as in aniridia patients. In conclusion, COMET is a very useful surgical procedure for patients with congenital aniridia, as it delivers benefits with minimal harm. Further studies are needed to compare epithelium restoring procedures including etiology of limbal stem deficiency.

Summary Statement

COMET procedure is a minimal invasive treatment, which enable exchanging irregular corneal epithelium for regular stratified oral mucosa epithelium. Aniridia causes huge discomfort; COMET not only restores stable ocular surface but also improves quality of life. Another advantage is simple surgical approach, autologous transplantation, and nonaggressive postoperative treatment. Visual acuity improvement is more efficient for near than for far distance; the majority of patients were able to read again.

Ethical Approval

Study design, patient's brochure, and consent form were approved by The Bioethics Committee at the Silesian Regional Medical Chamber, Agreement no. 24/2012, Katowice, Poland.

Disclosure

This paper was a poster presentation: American Academy of Ophthalmology Annual Meeting, November 2011, Best Poster Award.

Conflict of Interests

None of the authors has conflict of interests with the submission.

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