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Authors
Chauhan, Tulika
Encampira Luna, Erick
Le, Qihua
et al.

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Cell Morphology as an In Vivo Parameter for the Diagnosis of Limbal Stem Cell Deficiency

Clémence Bonnet, MD[*,†], **Tulika Chauhan, MD**[*,‡], **Erick Encampira Luna, MD**[*,§], **Qihua Le, MD, PhD**[*,¶], **Chi-Hong Tseng, PhD**[*], **Sophie X. Deng, MD, PhD**[*,‖]

[*]Stein Eye Institute, Department of Ophthalmology, David Geffen School of Medicine, University of California, Los Angeles, CA

[†]Cornea Department, Cochin Hospital, Paris University, AP-HP, Paris, France

[‡]Mahatma Gandhi Memorial Medical College, DAVV University, Indore, Madhya Pradesh, India

[§]Oftalmología Toluca, Col. Independencia, Toluca,Mexico

[¶]Department of Ophthalmology, Eye & ENT Hospital of Fudan University, Shanghai, China

[‖]Molecular Biology Institute, University of California, Los Angeles, CA.

Abstract

Purpose: To investigate basal epithelial cell morphology (CM) in the central cornea and limbal areas of eyes with limbal stem cell deficiency (LSCD).

Methods: Prospective, cross-sectional comparative study. We developed a CM scoring system based on basal epithelial cell phenotypes graded from 0 (normal) to 3 (severe morphologic alterations); this system was evaluated by 2 independent masked observers. The CM score was compared with the LSCD clinical score, the mean best corrected visual acuity (BCVA), and in vivo laser scanning confocal microscopy (IVCM) parameters used to stage the LSCD (ie., basal epithelial cell density [BCD], basal epithelial thickness [ET], and sub-basal corneal nerve fiber length density [CNFL]).

Results: 168 eyes with LSCD and 63 normal eyes were included. Compared with the control group, the LSCD group had significantly higher mean (± SD) CM scores in the central cornea (1.8 ± 0.7 vs 0.5 ± 0.4, respectively; $P = 0.01$) and limbal areas (1.6 ± 0.2 vs 1.3 ± 0.0, respectively; $P < 0.05$). The mean CM score in the central cornea was positively correlated with the clinical score ($P < 0.01$, r = 0.66) and negatively correlated with the BCVA ($P < 0.01$, r = 0.42). The CM scores were positively correlated with all other IVCM parameters in the central cornea and limbal areas (all $P < 0.001$).

Conclusion: Basal epithelial CM is altered in the central cornea and limbus of eyes with LSCD and thus can be used to stage the clinical severity of the disease.

Correspondence Sophie X. Deng, MD, PhD, Stein Eye Institute, UCLA, 200 Stein Plaza, Los Angeles, CA 90095, Tel: 310.206.7202; Fax: 310.794.7906, deng@jsei.ucla.edu.

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Keywords

Basal corneal epithelial cell density; cell morphology; corneal epithelial thickness; in vivo laser scanning confocal microscopy; limbal stem cell deficiency; limbal stem cells; diagnosis

INTRODUCTION

The corneal epithelium plays a major role in maintaining corneal transparency, a prerequisite to visual function.[1] It is widely accepted that the maintenance and renewal of the corneal epithelium rely on stem cells located in the limbus (limbal stem cells; LSCs), which acts as a barrier to prevent conjunctivalization of the cornea.[2] Complex interactions between cells of the extracellular matrix, vessels, nerves, melanocytes, and signaling molecules control the homeostasis of LSCs.[3] Their state of differentiation and proliferation is tightly regulated by their direct microenvironment, ie., the limbal niche.[4] In the human eye, the palisades of Vogt, the limbal crypts, and the limbal lacunae constitute the niche.[5–8] Disturbance of the limbal niche by any negative factor such as genetic mutation, inflammation, or trauma can lead to the reduction or destruction of the LSC pool.[2] Hence, the maintenance of corneal epithelium homeostasis and barrier function are altered and invasion of conjunctival epithelial cells on the corneal surface occurs, thereby defining limbal stem cell deficiency (LSCD).

Classic clinical signs of LSCD include stippling or granular fluorescein staining of the metaplastic/conjunctival epithelium, which can be difficult to detect in early stages.[2] Other clinical signs can be nonspecific, including neurotrophic keratopathy, persistent epithelial defects, corneal neovascularization, haze, and chronic inflammation.[2] To objectively define and stage the disease, a recent global consensus has been established.[9] Slit lamp examination and impression cytology have limitations as diagnostic methods; the diagnosis of LSCD may be confirmed by additional diagnostic tests such as in vivo laser scanning confocal microscopy (IVCM) or anterior segment optical coherence tomography.[9]

IVCM permits the visualization of central corneal parameters that correlate with disease severity and can be used to evaluate LSC function and stage LSCD. These IVCM parameters include the basal cell density (BCD), the epithelial thickness (ET), and the sub-basal corneal nerve fiber length density (CNFL) in the central cornea.[10–14] Changes in cell morphology (CM) have also been observed in eyes with LSCD.[15] In the mild stage of LSCD, basal epithelial cell borders become less distinct. During the moderate stage, the nuclei of these cells become more prominent, and in the severe stage the cells become enlarged and metaplastic.[15] This study aims to investigate whether basal epithelial CM can be another in vivo parameter for use in assessing LSCD severity.

MATERIALS AND METHODS

This prospective, cross-sectional study was conducted at the Stein Eye Institute after the approval of the Institutional Review Board at the University of California, Los Angeles (UCLA, IRB #10–001601). Appropriate consent was obtained from study subjects per

IRB protocol. The study was compliant with the HIPAA regulations and adhered to the Declaration of Helsinki.

Subjects with LSCD were consecutively recruited from the senior author's practice (S.X.D) between 2009 and 2017. The normal controls were recruited from the senior author's practice and the Comprehensive Division. The diagnosis of LSCD was based on clinical presentation, according to the criteria set by the International LSCD Working Group and confirmed by IVCM (HRT III, Heidelberg Engineering GmBH, Germany) and/or impression cytology.[9] Impression cytology was performed for the 56 subjects with LSCD (33.3%) who were willing to undergo the test.[16] All subjects with LSCD and 63 control subjects (63 eyes) underwent IVCM. Best corrected visual acuity (BCVA) using the Snellen chart was collected and converted to the logarithm of minimum angle of resolution (logMAR) for statistical analysis.

The stage of LSCD was classified as mild (2–4 points), moderate (5–7 points), or severe (8–10 points) based on the extent of corneal and limbal involvement defined by late stippling fluorescein staining, epithelial opacity with vortex pattern with or without epithelial defects, following a clinical scoring system previously described (Supplemental Figure).[14] The phenotype of the epithelial cells was further confirmed by in vivo imaging. The mild, moderate, and severe stages are correlated with stages I, II, and III, respectively, established by the LSCD International Working Group.[9, 17] The control group consisted of 10 eyes (15.9%) with, and 53 eyes (84.1%) without a history of contact lens wear. All control eyes were free of any ocular disease and any ocular surface abnormality that could have been detected by slit-lamp examination and had not undergone any ocular surgery other than cataract surgery.

In Vivo Laser Scanning Confocal Microscopy

IVCM was performed on the central cornea and the 4 limbal areas (superior, inferior, nasal, and temporal).[15] A minimum of 3 high-quality Z-scans were acquired in each area. Measurements were performed in the 5 areas of the basal epithelial layer, which was just above the sub-basal nerve plexus location. In vivo parameters of LSC function (BCD, ET, and CNFL) that were previously reported to correlate with the severity of the disease were collected for each area.[10–12] ET was defined as the scan depth difference between the most superficial layer of the epithelium and the basal layer.[11] BCD was measured as recommended by the manufacturer.[15] CNFL was measured as the fiber length density ($\mu m/mm^2$), which was evaluated by ACCMetrics as previously described (semiautomated software, University of Manchester, UK).[18]

CM findings of the basal epithelial cells previously described in eyes with LSCD of differing severity were used to develop a staging system consisting of 4 grades.[15] Morphologic criteria were the epithelial cell type (corneal or conjunctival), the intercellular cell border visibility, the cell body size and shape, the cytoplasm reflectivity, and the nucleus size and reflectivity (Table 1 and Figure 1).

Statistical Analysis

The average value of 3 measurements by IVCM in each area was obtained. These measurements in addition to the LSCD clinical grading were performed by 2 independent masked observers. The intraclass correlation coefficient between the 2 observers was 0.89, which confirmed their high level of agreement. Correlations between the CM score, the clinical score, the BCVA, and the IVCM parameters were characterized by box plots and Spearman correlation coefficients with all subjects. To compare the correlation coefficients, a bootstrap method was used. Statistical analysis was performed by a biostatistician (C.H.T) using R software (www.r-project.org). Any P value < 0.05 was considered statistically significant.

RESULTS

Subject Demographics

Demographics of the 231 eyes included in the study (LSCD, 168 eyes; control, 63 eyes) are presented in Table 2. The LSCD and control groups were comparable in terms of mean age and sex (all $P > 0.05$). The mean BCVA was significantly lower in the LSCD group ($P < 0.01$). The most frequent etiologies of LSCD were multiple ocular surgeries (85 eyes, 50.6%) and contact lens wear (37 eyes; 22.0%). LSCD stages, based on clinical scores, were mild in 63 eyes (37.5%), moderate in 55 eyes (32.7%), and severe in 50 eyes (29.8%).

Cell Morphology

According to the CM scoring presented in Figure 1, CM in the central cornea had a score of 0 (normal) in 5 eyes (3.0%), of 1 (mild) in 76 eyes (45.2%), 2 (moderate) in 42 eyes (25.0%), and 3 (severe) in 45 eyes (26.8%). In the control group, the central cornea CM score was 0 (normal) in 41 eyes (65.1%) and 1 (mild) in 22 eyes (34.9%; Table 2). There was no difference in the central cornea CM score between control eyes with and without history of contact lens wear ($P = 0.16$). The mean CM scores were significantly higher in the LSCD group than the control group in the central cornea (1.8 ± 0.7 in the LSCD group vs 0.5 ± 0.4 in the control group; $P = 0.01$) and limbal areas (1.6 ± 0.2 in the LSCD group vs 1.3 ± 0.0 in the control group; $P < 0.05$). The sensitivity and specificity of the scoring system using threshold values are presented in Table 3.

Significant correlations were found between the CM score, clinical score, BCVA, and IVCM parameters (BCD, ET, and CNFL). A positive correlation was observed between the CM score and the clinical severity score in the central cornea (Figure 2A; $P < 0.01$, r = 0.79) and limbal areas (Figure 3A; $P < 0.01$, r = 0.72). The CM scores of both the central cornea (Figure 2B; $P < 0.01$, r = 0.61) and limbal areas (Figure 3B; $P < 0.01$, r = 0.64) were correlated positively with the BCVA. When the CM score of the central cornea was compared with the IVCM parameters, we also found strong negative correlations with the central cornea BCD (Figure 2C; $P < 0.01$, r = −0.80), the central ET (Figure 2D; $P < 0.01$, r = −0.61), and the central CNFL (Figure 2E; $P < 0.01$, r = −0.71). The CM scores of all limbal areas were negatively correlated with BCD of all limbal areas (Figure 3C; $P < 0.01$, r = −0.80) and ET of all limbal areas (Figure 3D; $P < 0.01$, r = −0.73). Comparison of the central cornea correlations coefficients with the clinical scores revealed that BCD, CNFL,

and CM had higher correlations than ET (Supplemental Table 1; $P < 0.05$). No significant differences were found between the BCD, CNFL, and CM correlation coefficients. In the limbus, comparison of the correlation coefficients with the clinical score revealed that BCD and ET had higher correlations than CM (Supplemental Table 2; $P < 0.05$). No significant differences were found between BCD and CM correlation coefficients. Clinical examples of the CM scores in eyes with different stage of LSCD severity are presented in Figure 4.

DISCUSSION

A diagnosis of LSCD may be confirmed and the severity staged by using several biomarkers including BCD, ET, and CNFL.[10–13] The current study shows that CM changes in the central cornea and the limbus of eyes with LSCD are positively correlated with other in vivo parameters, specifically BCD, ET, and CNFL.[10–12, 14] Thus, CM is an additional biomarker that can be used to confirm the diagnosis and classify the severity of LSCD. Changes in epithelial CM observed using IVCM included the number of cell layers, cell size, and degree of reflectivity of the nucleus and the cell-cell junction.

CM changes and decreased cellular density are observed in other ocular surface diseases, such as dry eye diseases, keratoconus, vernal keratoconjunctivitis, and abnormalities after refractive surgeries.[19–23] However, in these diseases, the CM changes affect mostly the superficial corneal epithelial cells, nerves and anterior and/or posterior stromal keratocytes, whereas the basal epithelial cells remain largely unaffected. The CM changes of the basal epithelial cells described in this study are observed in LSCD.

Different morphologic features have been previously described to assess the epithelial phenotypes such as corneal, conjunctival, or mixed on the cornea surface.[15, 24–27]. The phenotype of the epithelial cells identified by IVCM has also been confirmed by impression cytology.[24, 26, 27] Lagali et al. reported that progression of LSCD in aniridia correlated with gradual loss of palisades structures, corneal epithelial cell phenotype, and corneal nerve.[25] Miri et al. reported that cell size and density was decreased in eyes with LSCD.[26] Shortt et al. developed a CM scoring system using 3 criteria: absence of epithelial cells visible; non-stratified epithelium 1 or 2 layers thick, with hyperreflective nuclei but loss of intercellular junctions; and stratified epithelium with clear intercellular boundaries indicating normal epithelial function. The study was able to correlate the morphologic presentation with phenotypic marker of conjunctival (cytokeratin 19) or corneal (cytokeratin 3) markers up to 3 years after transplantation of cultivated allogeneic limbal epithelial cells.[24] However, these studies remained descriptive, without providing correlations between the CM changes, the clinical stage and other in vivo parameters (BCD, ET, and CNFL). By providing such correlations, the current study further confirms CM as a biomarker of LSCD severity.

These in vivo biomarkers can also be used to evaluate the success of LSC transplantation. For example, Borderie et al. used the BCD to evaluate the success (i.e. the absence of recurrence of clinical signs of LSCD) of different type of LSC transplantation in eyes with stage III LSCD.[28] Three years after transplantation, a higher BCD (6558 cells/mm^2 in average) was observed in success cases. In an ongoing phase I clinical trial (NCT03957954) that investigates the safety and feasibility of cultivated autologous LSC for LSCD, all 4

biomarkers, clinical scores, ET, BCD, CNFL, and CM are being used to assess the LSC function.

Significant BCD and ET reduction are early signs of LSC dysfunction and are correlated with the severity of LSCD.[10, 11, 15] The correlation found between CM scoring and mild LSCD suggests that CM changes are also early findings of corneal epithelial dysfunction, which could be more objective than the subtle early clinical signs.[14] An inverse relationship between the reduction in BCD and the basal cell size diameter has been previously described.[10] Using our CM scoring system, we found that the central CM score had the strongest correlation with the central BCD than with ET or CNFL. The most relevant IVCM parameter to characterize LSC function remains to be determined. Each parameter has advantages and limitations. ET is a relatively objective measure. ET measured by anterior segment optical coherence tomography is a widely accessible, non-contact test that correlates with the severity of LSCD, making it a good screening tool for general ophthalmologists.[13] Compared with ET or CNFL, preliminary results show that BCD is better correlated with disease severity in both the central cornea and limbus.[10, 12] Current BCD analysis remains manual, time-consuming, and requires experienced observers, thus limiting its use to eye care centers with expertise in this type of analysis.

CNFL is another major criterion correlated with LSCD severity.[12, 29, 30] Close interactions between basal corneal epithelial cells and nerves are necessary to support the physiologic secretion of nerve growth factors.[31, 32] The loss of these interactions affect the maintenance of healthy nerves, corneal epithelial cells, and LSCs.[4, 12, 31, 32] Similar to BCD, CNFL analysis requires more sophisticated software and experienced observers.[12] Other limitations include compression artifacts that can occur during the scan acquisition and the presence of hyperreflective corneal scarring often seen in the severe stage of LSCD. CM is a more subjective analysis than the analyses of other IVCM parameters as evaluation of CM requires the knowledge of recognizing the cell morphologic phenotypes (corneal, metaplastic, or conjunctival). Machine deep-learning is a promising approach that enables automated and more objective quantification of these in vivo parameters.[33–35] Further studies are necessary to evaluate the weight of each biomarker to determine which one has a more accurate diagnostic value. It is likely that evaluation of a combination of all the IVCM parameters will be needed to obtain a comprehensive evaluation of LSC function.[9]

In summary, the CM score correlates with the severity of the LSCD and is an IVCM parameter that can aid in the diagnosis and staging of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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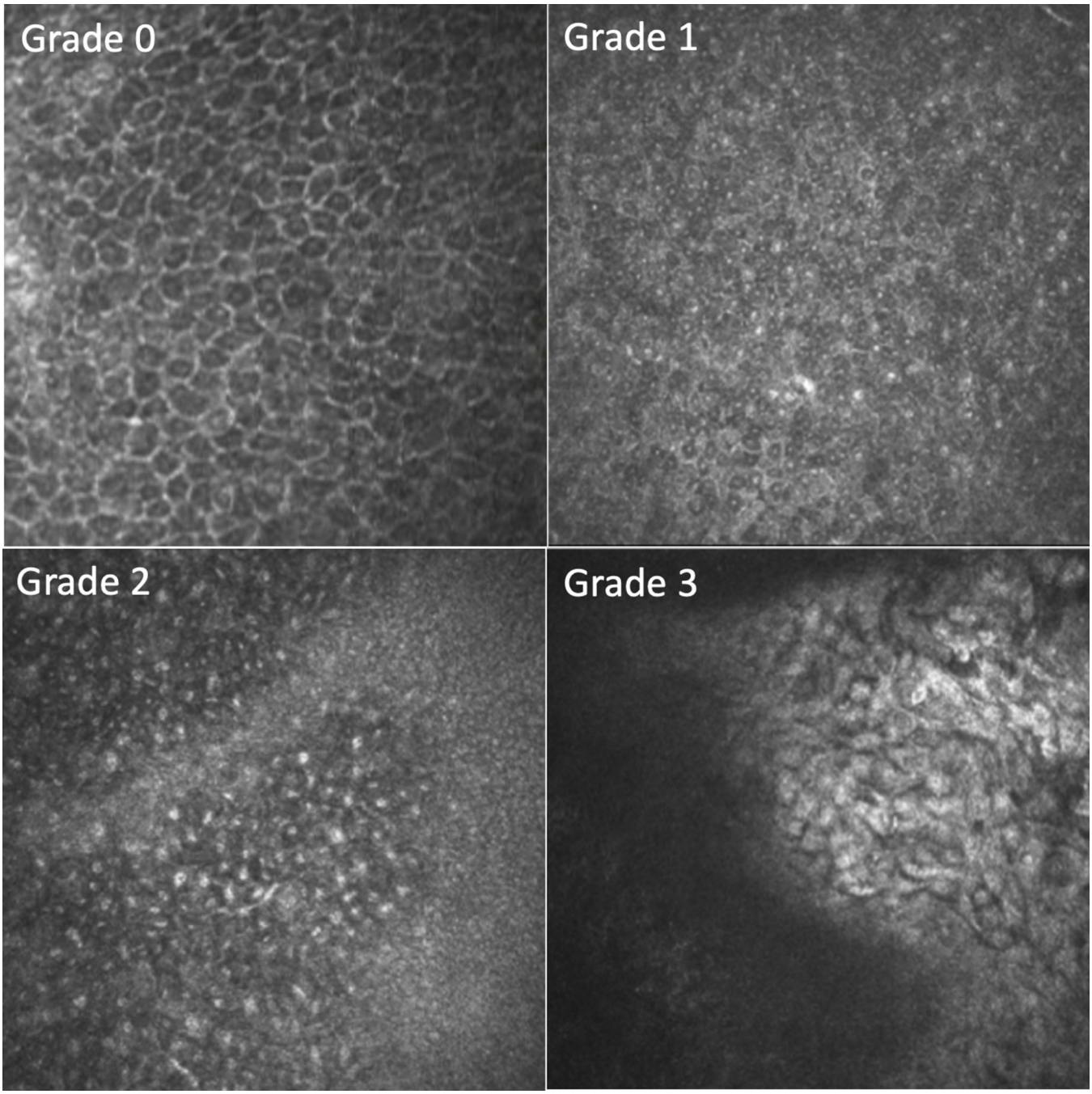

Figure 1.
Cell morphology evaluated by in vivo laser scanning confocal microscopy.

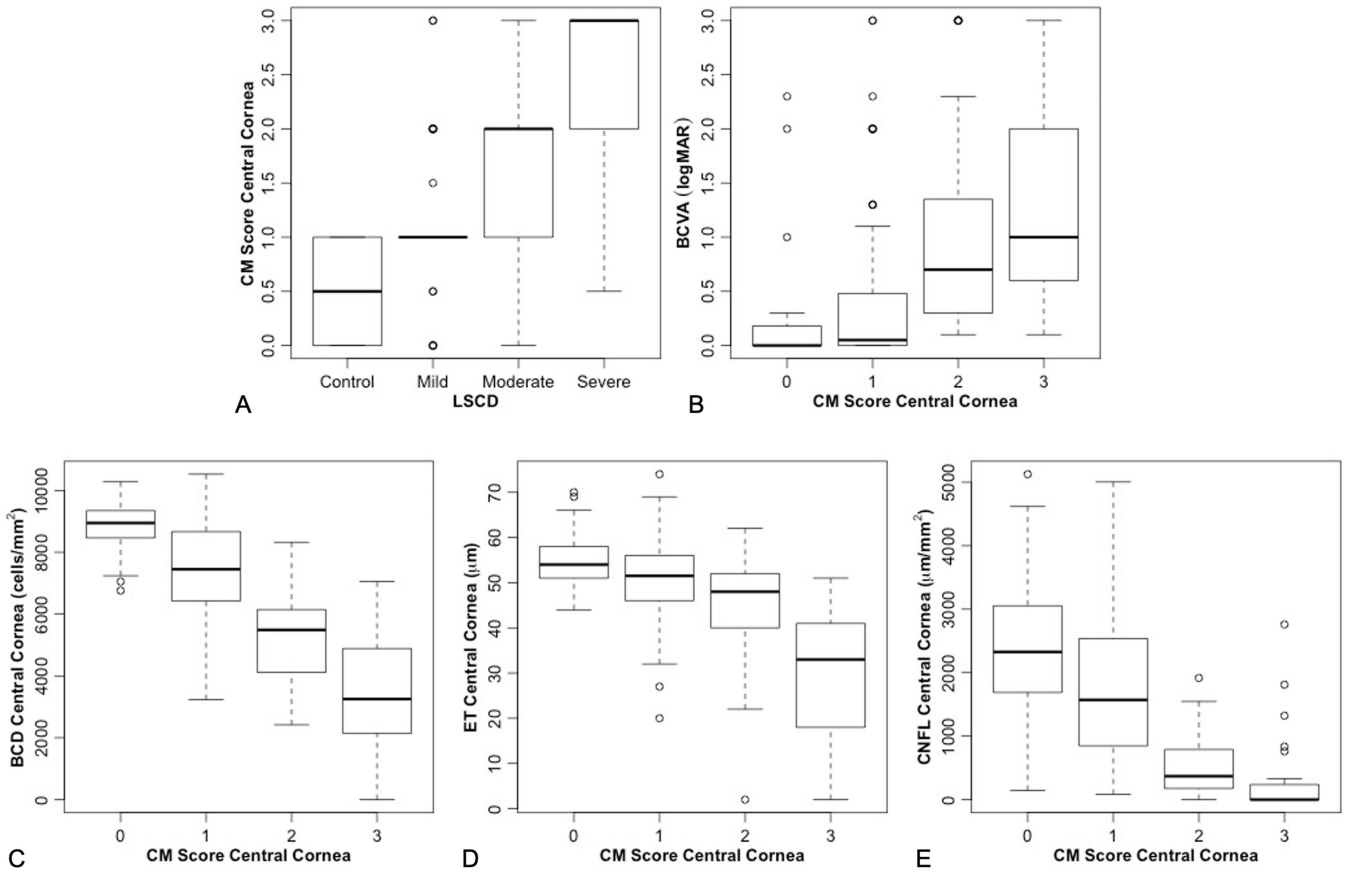

Figure 2.

Correlations of cell morphology stages in the central cornea.

A) Box and whiskers plots of CM scores in the central cornea and different LSCD stages ($P < 0.01$; r = 0.79). B) Box and whiskers plots of the BCVA and CM scores in the central cornea ($P < 0.01$; r = 0.61). C) Box and whiskers plots of the BCD and CM scores in the central cornea ($P < 0.01$; r = −0.80). D) Box and whiskers plots of the ET and CM scores in the central cornea ($P < 0.01$; r = −0.61). E) Box and whiskers plots of the CNFL and CM scores in the central cornea ($P < 0.01$; r = −0.71).

BCD = basal cell density; BCVA = best corrected visual acuity; CM = cell morphology; ET = epithelial thickness; LSCD = limbal stem cell deficiency. CNFL = central nerve fiber length density.

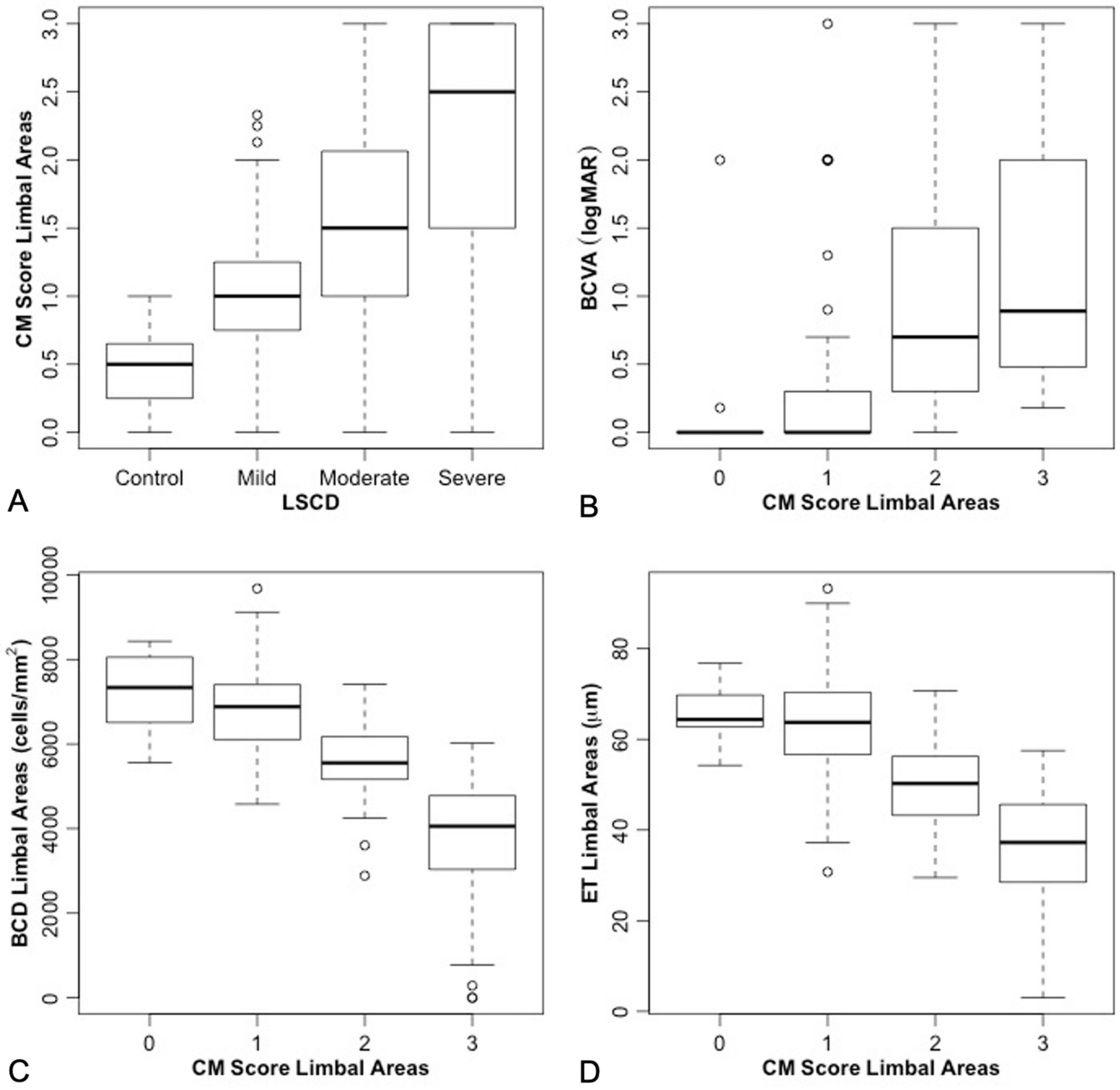

Figure 3.

Correlations of CM stages in the limbal areas.

A) Box and whiskers plots of the CM scores in the limbal areas and different LSCD stages ($P < 0.01$; r = 0.72). B) Box and whiskers plots of the BCVA and CM scores in the limbal areas ($P < 0.01$; r = 0.64). C) Box and whiskers plots of the BCD and CM scores in the limbal areas ($P < 0.01$; r = −0.80). D) Box and whiskers plots of the ET and CM scores in the limbal areas ($P < 0.01$; r = −0.73).

BCD = basal cell density; BCVA = best corrected visual acuity; CM = cell morphology; LSCD = limbal stem cell deficiency.

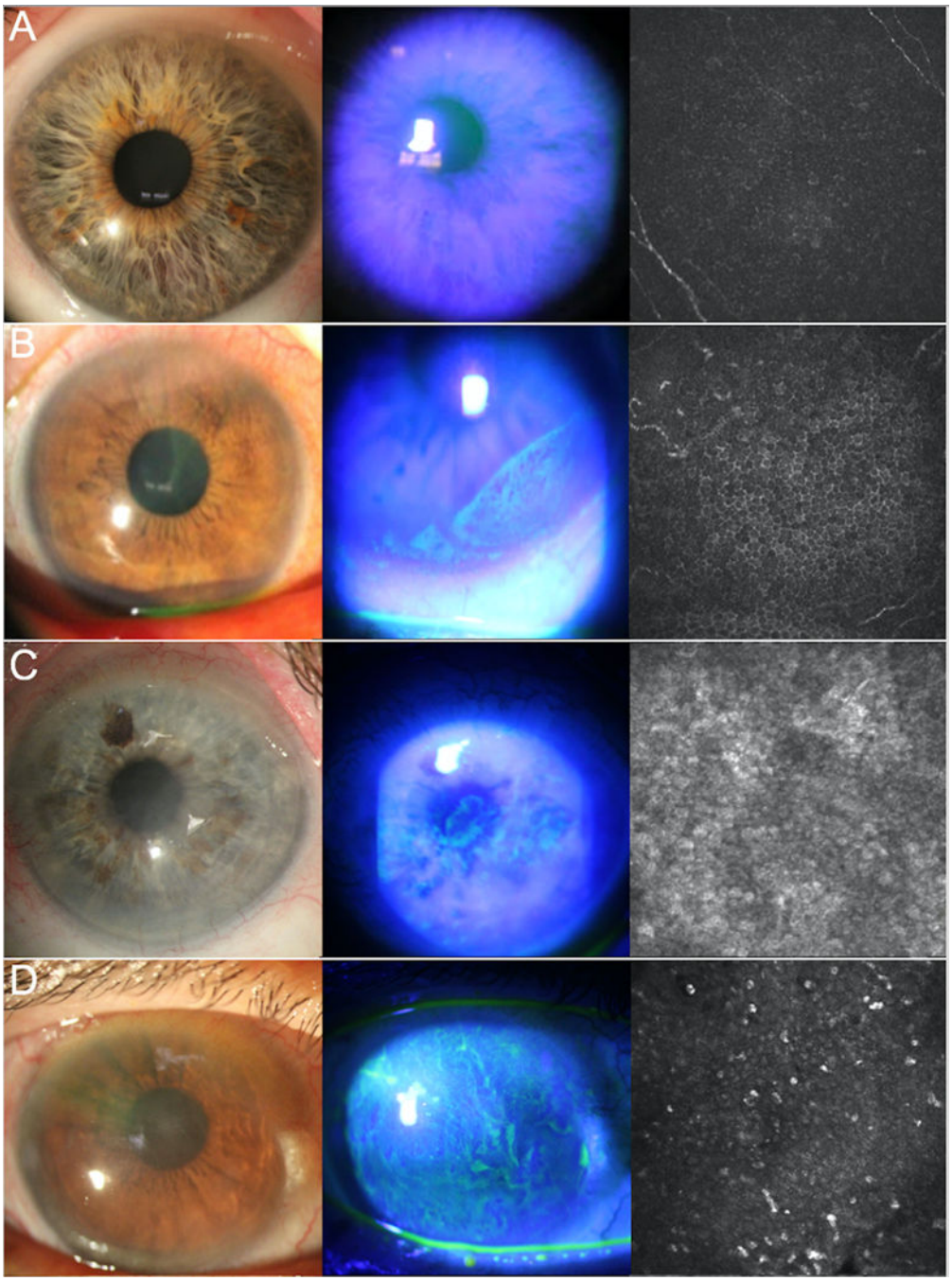

Figure 4.

Clinical examples of the CM scores in eyes with different stage of LSCD severity. Left panel: slit-lamp photography under bright light; central panel: fluorescein staining under blue cobalt light; right panel: IVCM of the basal central corneal epithelial layer. A) Control eye, stage 0. B) Mild LSCD, CM stage 1. C) Moderate LSCD, CM stage 2. D) Severe LSCD, CM stage 3.

CM = cell morphology; IVCM = in vivo laser scanning confocal microscopy; LSCD = limbal stem cell deficiency.

Table 1

Cell morphology score based on IVCM findings

IVCM criteria	Normal - grade 0	Mild - grade 1	Moderate - grade 2	Severe - grade 3*
Intercellular cell border	Distinct	Blurry	Not visible	Not visible
Cell body size shape	Small - Regular	Small - Regular	Enlarged - Irregular	Large - Loose
Cytoplasm reflectivity	Hyporeflective	Hypo/hyperreflective	Hypo/hyperreflective	Hyperreflective
Nucleus size - reflectivity	Not visible	Small - Hyperreflective (low N/C ratio)	Large - Hyperreflective (high N/C ratio)	Not visible

IVCM: in vivo laser scanning confocal microscopy; N/C ratio: nucleus/cytoplasm ratio.

*When cells were not visible, score was considered severe, grade 3.

Table 2.

Demographics of patients included

	All n (%)	Control	LSCD	*P* value
Eyes, n (%)	231 / 231	63 (27.3)	168 (72.7)	-
Sex female, **n** (%)	130 (56.2)	31(49.2)	99 (58.9)	0.23
Age, mean ± SD, years	56.9 ± 26.2	58.9 ± 20.4	56.1 ± 28.6	0.40
Etiology of LSCD				< **0.01**
Multiple ocular surgeries	85 (36.8)	-	85 (50.6)	
Contact lens use	37 (16.0)	-	37 (22.0)	
Cicatrizing conjunctivitis †	22 (9.5)	-	22 (13.1)	
Chronic ocular Inflammation	8 (3.5)	-	8 (4.8)	
Chemical injury	7 (3.0)	-	7 (4.2)	
Idiopathic	7 (3.0)	-	7 (4.2)	
Congenital aniridia	2 (0.9)	-	2 (1.2)	
LSCD clinical score, n (%)				
Mild	63 (27.3)	-	63 (37.5)	
Moderate	55 (23.8)	-	55 (32.7)	
Severe	50 (21.6)	-	50 (29.8)	
BCVA, mean ± SD, logMAR	0.65 ± 0.85	0.04 ± 0.09	0.89 ± 0.89	< **0.01**
BCVA in LSCD group, mean ± SD, logMAR				
Mild	-	-	0.43 ± 0.77	< **0.01** *
Moderate	-	-	1.02 ± 1.06	
Severe	-	-	1.67 ±1.08	
Cell morphology score				< **0.05** *
Central cornea				
0	46 (19.9)	41 (65.1)	5 (3.0)	
1	98 (42.4)	22 (34.9)	76 (45.2)	
2	42 (18.2)	0	42 (25.0)	
3	45 (19.5)	0	45 (26.8)	
Limbal areas				< **0.05** *
0	42 (18.2)	31 (49.2)	11 (6.5)	
1	105 (45.5)	32 (50.8)	73 (43.5)	
2	51 (22.1)	0	51 (30.4)	
3	33 (14.3)	0	33 (19.6)	

BCVA = best corrected visual acuity; logMAR = logarithm of the minimum angle of resolution; LSCD = limbal stem cell deficiency; n = number; SD = standard deviation.

† Mucous membrane pemphigoid and Stevens-Johnson syndrome

* Pairwise comparison

Table 3

Sensitivity and specificity of the cell morphology grading system with cutoffs

CM score	Controls, n eyes	LSCD, n eyes	Sensitivity (CI95%)	Specificity (CI95%)
< 1	41	9	94.6% (91.2–98.0)	65.1% (53.3–76.9)
≥ 1	22	159		
< 2	63	85	49.4% (41.8–57.0)	100%
≥ 2	0	83		

CM: cell morphology; LSCD: limbal stem cell deficiency.