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## Global consensus on the definition, classification, diagnosis and staging of limbal stem cell deficiency

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### Abstract

**Purpose:** Despite extensive knowledge gained over the last three decades regarding limbal stem cell deficiency (LSCD), the disease is not clearly defined, and there is lack of agreement on the diagnostic criteria, staging and classification system among treating physicians and research scientists working on this field. There is therefore an unmet need to obtain global consensus on the definition, classification, diagnosis and staging of LSCD.

**Methods:** A Limbal Stem Cell Working Group was first established by the Cornea Society in 2012. The Working Group was divided into subcommittees. Four face-to-face meetings, frequent email discussions and teleconferences were conducted since then to obtain agreement on a strategic plan and methodology from all the participants after a comprehensive literature search,

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and reached final agreement on the definition, classification, diagnosis and staging of LSCD. A writing group was formed to draft the current manuscript, which has been extensively revised to reflect the consensus of the Working Group.

**Results:** A consensus was reached on the definition, classification, diagnosis and staging of LSCD. The clinical presentation and diagnostic criteria of LSCD were clarified, and a staging system of LSCD based on clinical presentation was established.

**Conclusions:** This global consensus provides a comprehensive framework for the definition, classification, diagnosis and staging of LSCD. The newly established criteria will aid in the correct diagnosis and formulation of an appropriate treatment for different stages of LSCD that will facilitate a better understanding of the condition, help with clinical management, research and clinical trials in this area.

## Summary

Limbal stem cell deficiency can be caused by genetic defect(s) or secondary insult to LSCs and their niche, which can result in abnormal corneal epithelial wound healing and/or conjunctivalization of the corneal surface. Late corneal fluorescein staining, recurrent or persistent epithelial defect, and epithelial thinning with or without vascularization while frequently seen in LSCD, are not unique to LSCD. The presence of one or more of these signs might not be sufficient to make the diagnosis. The clinical presentation of LSCD varies based on the severity. The severity of LSCD is classified into three defined stages based on clinical presentation. Diagnostic tests include detection of conjunctival cells using impression cytology, IVCN, and AS-OCT of the cornea and limbus. Because of the limitations of clinical examination, a diagnostic test is recommended, if available, to confirm the diagnosis of LSCD before any surgical invention. This global consensus statement provides a comprehensive framework for the definition, classification, staging and diagnosis of LSCD.

## Keywords

limbal stem cell deficiency; limbal stem cells; conjunctiva; cornea; definition; classification; diagnosis; markers; impression cytology; anterior segment optical coherence tomography; in vivo confocal microscopy

## Introduction

Normal function of the epithelial stem cells at the corneoscleral limbus is a prerequisite for the maintenance and regeneration of the corneal epithelium. Although there is disagreement on which marker(s) is specific for corneal epithelial stem cells, it is generally believed that they reside in the basal epithelium of the limbus, which is the transition zone between the transparent cornea and the opaque sclera.<sup>1</sup> For this reason, these stem cells are referred to as limbal epithelial stem cells (LSCs). The normal limbus and LSCs act as a barrier against the invasion of conjunctival epithelium onto the corneal surface.<sup>2</sup>

Evidence for the location of stem cells at the limbus originates mainly from studies of their proliferation and differentiation. Within the limbal basal epithelium exists a population of cells that are normally quiescent (slow-cycling).<sup>3</sup> These cells lack the differentiation features

of the corneal epithelium and are the most undifferentiated cells within the corneolimbal epithelium.<sup>1</sup> Furthermore, these cells express numerous markers that are characteristic of stem and progenitor cells in other tissues.<sup>4,5</sup>

One key characteristic of stem cells is the tight regulation of their state of differentiation and proliferation by the microenvironment in their immediate neighborhood, which is commonly referred to as the stem cell niche.<sup>6,7</sup> In the human eye the palisades of Vogt (POV) with the underlying limbal vasculature function as a niche for LSCs.<sup>8</sup> In addition to the stem and progenitor cells organized in clusters, the limbal niche contains several types of supporting cells (niche cells), a specific extracellular matrix, and soluble factors mediating biochemical and biophysical signals.<sup>9,10</sup> An orchestrated interaction of the various factors within the niche is a prerequisite for the undisturbed function of LSCs to keep a stable ocular surface.<sup>11-13</sup> Therefore, the disturbance of the niche in the context of disease, injuries, and uncontrolled inflammation will lead to aplasia and depletion of LSCs; subsequently the ability to sustain the regeneration of the corneal epithelium is lost. Therapeutic strategies to repair LSC function not only must replenish the stem cell population but also attempt to restore the stem cell niche.

The important role of LSCs in the maintenance of the normal homeostasis of corneal epithelium and wound healing has been confirmed in a variety of experimental animal models.<sup>14-16</sup> In vivo evidence has demonstrated that a certain number of LSCs is necessary to maintain a healthy corneal epithelium.<sup>15,17,18</sup> Based on the concept of LSCs, Kenyon and Tseng demonstrated in 1989 that the corneal epithelial phenotype can be reconstituted in patients with unilateral limbal stem cell deficiency (LSCD) by transplantation of autologous corneal epithelial stem cells from the contralateral healthy eye.<sup>19</sup> For bilateral disease, allograft transplantation was later introduced with much lower success rates, primarily because of immunologic rejection.<sup>17,20</sup> Cultivated autologous LSCs were subsequently developed to treat patients with LSCD.<sup>21,22</sup>

The establishment and application of the concept of LSCs have led to extraordinary progress in the development of procedures and cell-based therapies for ocular surface rehabilitation, benefiting patients tremendously over the last 3 decades. The multitude of surgical approaches of transplanting LSCs onto diseased eyes has led to a classification system for ocular surface rehabilitation procedures developed by the Cornea Society. The purposes of this classification system are to improve the clarity of communication and to enable the comparison of outcomes of these innovative procedures.<sup>23</sup>

In contrast to the surgical treatment of LSCD, there is no agreement on the disease entity of LSCD. Since the introduction of the LSC concept in 1980s', numerous reports have used a wide variety of terms to describe LSCD and have provided various definitions of this disease. Currently, there is neither a generally accepted classification nor a staging system for this disease entity. Especially in the context of therapeutic strategies, numerous diagnostic approaches have been described to identify LSCD, and the variety in these approaches makes it impossible to compare the results of clinical trials. The Cornea Society realized that there was a need for an international agreement about the definition, classification, diagnosis and staging of LSCD.

## Methods

The Cornea Society formed the Limbal Stem Cell Working Group in 2012 to obtain a consensus from a group of experts in the field of LSCD. The working group consisted of members of the Cornea Society, the Asian Cornea Society, PanCornea, and EU-Cornea; these members were experts on cornea and external diseases with extensive experience in the field of LSCD, had been authors of high-impact scientific publications, were recognized internationally by the cornea community, and were willing to attend face-to-face meetings and comply with project timelines. A subcommittee was established to devise a proposed definition, classification system, and staging and diagnostic criteria for LSCD. Literature searches were performed by members who were assigned topics; their aim was to identify criteria reported for which inclusion in the new consensus would be appropriate. The working group met face to face on 4 occasions between 2012 to 2013 to formulate a strategic plan and methodology from all the participants and discuss the definition, classification system, and diagnostic and staging criteria. Subsequently, frequent email discussions and multiple teleconferences occurred to further refine the criteria. Members who attended 2 or more of the meetings are listed in Acknowledgments. Once there was a broad consensus on all topics, a writing committee was formed. The results were summarized by the writing committee at the final meeting in 2017. The draft of the manuscript was circulated for further comments. The final draft was then approved by all members of the writing committee of this manuscript.

## Results

### Definition of limbal stem cell deficiency

Limbal stem cell deficiency is an ocular surface disease caused by a decrease in the population and/or function of corneal epithelial stem/progenitor cells; this decrease leads to the inability to sustain the normal homeostasis of the corneal epithelium. The disease is characterized by conjunctivalization (i.e., replacement of the normal corneal epithelium by conjunctival epithelium) and/or other signs of epithelial dysfunction such as persistent or recurrent epithelial defects with or without neovascularization, ocular surface inflammation, and scarring. Frequent consequences are decreased vision and discomfort leading to reduced health-related quality of life (HRQOL). Limbal stem cell deficiency may present alone as a single entity or associated with abnormalities of other components of the ocular surface such as the conjunctiva, meibomian glands, lacrimal glands, tears, corneal nerves and immune system.

Partial LSCD is characterized by incomplete conjunctivalization of the corneal surface and the presence of residual limbal and consequent corneal epithelial cells (Figure 1C, 1D). Total LSCD is characterized by conjunctivalization of the entire corneal surface due to a complete loss of corneal epithelial stem/progenitor cells (Figure 1E, 1F).

### Classification of limbal stem cell deficiency

Limbal stem cell deficiency is due to a variety of causes (Table 1). The cause of LSCD in some cases are unknown (idiopathic LSCD).

## 1 Acquired limbal stem cell deficiency

### 1.1 Acquired nonimmune-mediated limbal stem cell deficiency

**1.1.1 Chemical and thermal injury:** Whereas data regarding the epidemiology of LSCD are not available, a reported series of patients with LSCD showed that the leading cause of LSCD is ocular burn: at least two-thirds of cases of LSCD that had undergone LSC transplantation are due to ocular surface burn (Suppl Table 1). Chemical ocular injuries in general appear to be more common in males than females.<sup>24</sup> Chemical and thermal injuries can result in the destruction of LSCs and their niche. At the acute stage of ocular burns, evidence of more than 50% of limbal ischemia is a strong risk factor for the development of LSCD. Among chemical injuries, alkali burns appear to be more common and result in more severe tissue damage than acid burns as alkali chemicals induce saponification of cellular membranes with deeper penetration in the limbal tissue, potentially destroying the limbal niche and LSCs.<sup>25,26</sup> Sulfur mustard, which is a potent chemical warfare vesicating agent can result in delayed-onset keratopathy and LSCD.<sup>27</sup> Prolonged ocular surface inflammation and denervation after chemical injury may cause further damage to the residual LSCs. Thermal burns appear to be much less frequent than chemical injuries (Suppl Table 1).

**1.1.2 Contact lens wear:** The term *contact lens–induced keratopathy* has been used to describe the clinical presentation of LSCD associated with contact lens wear. The exact pathophysiology of contact lens–induced LSCD<sup>28</sup> is unclear, but it is probably multifactorial as a result of poor contact lens fitting, low oxygen permeability of the contact lens material, prolonged/extended wear, and sensitivity of some contact lens wearers to the toxicity of contact lens cleaning and storage solutions. Most cases of contact lens–induced LSCD are reversible after the discontinuation of contact lens wear and medical treatment.

**1.1.3 Surgery:** Eye surgery with limbal involvement, including the excision of limbal and conjunctival tumors, repeated and extensive pterygium surgery,<sup>29,30</sup> and trabeculectomy with the use of anti-metabolites<sup>31–33</sup> can induce LSCD as a result of the direct destruction of LSCs and the limbal niche. The extent of LSCD is often sectoral. The term *iatrogenic LSCD* has been used to categorize LSCD caused by ocular surgeries and medications.<sup>30</sup>

**1.1.4 Toxicity from medications:** Both topical drugs, in particular, antimetabolites mitomycin C<sup>33–35</sup> and 5-fluorouracil;<sup>31</sup> preservatives in eye medications;<sup>36</sup> and systemic chemotherapies (hydroxyurea,<sup>37</sup> S-1,<sup>38</sup> hydroxycarbamide<sup>39</sup>) have been reported as causes of LSCD.

**1.1.5 Bullous keratopathy:** Long-standing, advanced bullous keratopathy could cause LSCD, and squamous metaplasia is associated with LSCD in eyes with this type of keratopathy.<sup>40,41</sup> After successful corneal transplantation, LSCD may improve in affected eyes.

**1.1.6 Other:** Limbal stem cell deficiency is considered as a part of the pathophysiology of pterygium, and excision of extensive pterygium may also cause LSCD. Severe microbial keratitis including trachoma could lead to LSCD.<sup>42,43</sup> Potential mechanisms for LSC destruction by microbial keratitis include severe inflammation of the ocular surface,

infectious agent-induced LSC necrosis, toxicity of the anti-microbial medications, and progressive fibrosis of the ocular surface following the acute stage of infection. In the case of trachoma, chronic microtrauma to the corneal surface by the eyelid abnormality contributes to the pathogenesis of LSCD.

Other possible causes of LSCD include severe chronic rosacea blepharoconjunctivitis often in the setting of other ocular surface diseases, advanced ocular surface squamous cell carcinoma,<sup>44</sup> and radiation.<sup>45</sup> These etiologies of LSCD each represent less than 5% of all etiologies.

## 1.2 Acquired primary immune-mediated LSCD

**1.2.1 Stevens-Johnson syndrome/toxic epidermal necrolysis spectrum disease:** Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) spectrum disease is a rare hypersensitivity reaction that typically involves the skin and various mucous membranes including the ocular surface. The most severe ocular outcome in SJS/TEN spectrum disease is the destruction and resultant aplasia of the LSCs.<sup>17,42,46</sup> Primary corneal involvement in SJS is not common; LSCD more commonly results from secondary corneal involvement due to chronic microtrauma and persistent ocular surface inflammation.<sup>47</sup> This microtrauma may occur in the context of pre-existing structural anomalies of eyelid position, trichiasis, or symblepharon. Moreover, scarring of lacrimal duct orifices and the destruction of conjunctival goblet cells and meibomian gland orifices may lead to a combination of severe aqueous, mucin, and lipid tear deficiencies, exacerbating LSCD.<sup>46,48,49</sup>

**1.2.2 Mucous membrane pemphigoid (MMP):** This group of chronic inflammatory, subepithelial, blistering diseases predominantly affects the mucous membranes through the deposition of IgG, IgA, and/or C3. Ocular involvement in mucous membrane pemphigoid (ocular MMP, formerly referred to as *ocular cicatricial pemphigoid*) is characterized by chronic or relapsing inflammation and subconjunctival fibrosis, followed by fornix foreshortening and obliteration, symblepharon formation, and, in severe cases, ankyloblepharon.<sup>50,51</sup> Scarring of lacrimal duct orifices may lead to severe dry eye, which exacerbates LSC dysfunction. The alterations of the adnexa and the blink-related microtrauma exacerbate inflammation in the ocular surface unit, including limbus, weakening stromal support and leading to the gradual loss of LSCs.<sup>52</sup>

## 1.2.3 Allergic ocular surface diseases

**1.2.3.1 Vernal keratoconjunctivitis:** The palpebral and limbal forms of vernal keratoconjunctivitis (VKC) is sometimes associated with LSCD.<sup>53,54</sup> In the palpebral form, fibroblast activation and collagen hyperproduction lead to giant papillary hypertrophy with associated lid abnormalities and poor ocular surface conditions. In the limbal form, alterations of the extracellular matrix molecules caused by inflammatory insults erode the stromal support of the limbal niche. Eosinophils and other inflammatory cells also produce toxins that can directly damage and destroy LSCs.<sup>55</sup>

**1.2.3.2 Atopic keratoconjunctivitis:** Similarly to VKC, chronic ocular surface inflammation in atopic keratoconjunctivitis is associated with mechanical trauma and

palpebral scarring that leads to LSCD.<sup>56</sup> Neovascularization and lipid deposition typically affect the superior one-third of the cornea but can also become circumferential in severe cases.<sup>57</sup> In addition, inflammatory cells produce toxins that can directly damage and destroy LSCs as described for VKC.

**1.2.3.3 Graft-vs-host disease:** Ocular complications are common in chronic graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell transplantation.<sup>58</sup> Inflammation stimulates fibrosis in the limbal area, which is detrimental to LSCs, and associated dry eye further exacerbates the chronic inflammatory process with more damage. Chronic severe chronic GVHD could lead to LSCD.<sup>59</sup>

**2 Hereditary Limbal Stem Cell Deficiency**—A primary genetic defect is an uncommon etiology of LSCD (Table 2); however, it is important to be able to diagnose the LSCD early and manage it accordingly.

**2.1 Congenital Aniridia:** Congenital aniridia occurs in 1 in 50,000 to 100,000 newborns around the world, and this condition is usually inherited as an autosomal dominant pattern or is due to sporadic mutations in the *PAX6* gene. Patients with aniridia can have other associated eye problems, e.g. glaucoma, cataract, and foveal and optic nerve hypoplasia. The corneal surface and limbal stem cells function are normal at birth but LSCD or aniridia-associated keratopathy usually develops at teenage years and progress to total LSCD accompanied by stromal opacity over time. Autosomal dominant keratitis could be a variant of aniridia.<sup>60</sup> This type of keratitis is characterized by a partial or complete absence of the iris, typically in both eyes, but a variant with minimally affected irides has been reported.<sup>61</sup>

**2.2 Autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy/dysplasia:** Autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy/dysplasia, also known as multiple endocrine deficiency and autoimmune polyendocrine syndrome type 1, is inherited in an autosomal recessive fashion, and the autoimmune component of the syndrome affects multiple organs including parathyroid, adrenal glands and ectodermal tissues. Keratitis secondary to LSCD can occur in a subpopulation of affected individuals.<sup>42,62,63</sup>

**2.3 Other:** Uncommon genetic disorders that can cause LSCD include xeroderma pigmentosum,<sup>64</sup> dyskeratosis congenital,<sup>65</sup> ectrodactyly-ectodermal dysplasia-clefting syndrome,<sup>66,67</sup> keratitis–ichthyosis–deafness syndrome,<sup>68,69</sup> lacrimo-auriculo-dento-digital syndrome (Levy-Hollister) syndrome,<sup>70</sup> and epidermolysis bullosa,<sup>71,72</sup> Other genetic disorders such as autosomal dominant keratitis and dominantly inherited keratitis which have been reported only once are not included in Table 2. Additional reports are needed to confirm the genetic etiology of LSCD in these diseases.

## Diagnosis of limbal stem cell deficiency

### 1 Clinical Presentation

**1.1 Symptoms of limbal stem cell deficiency:** Patients with LSCD may be asymptomatic initially. When symptomatic, patients may describe any or all of the following: ocular



discomfort, irritation, foreign body sensation, conjunctival redness, tearing, dryness, blepharospasm, pain, photophobia, decreased vision, and blindness.<sup>73</sup> These symptoms can be debilitating and lead to a decreased HRQOL.

**1.2 Signs of limbal stem cell deficiency:** The absence of the corneal epithelium phenotype or conjunctivalization of the cornea produces clinical signs of LSCD. Slit lamp examination, particularly of the superior cornea in early disease, and fluorescein staining pattern could reveal signs of LSCD. Fluorescein staining of the ocular surface is a test that can differentiate between normal healthy corneal epithelium and abnormal pathologic epithelium. Under normal conditions, corneal epithelial cells on the surface are interconnected by tight junctions, which are impermeable to larger molecules. In contrast, the conjunctival epithelium is characterized by relatively loose cell-cell contacts, which result in a permeability that is up to 40 times greater than that of the corneal epithelium.<sup>74</sup> In LSCD the epithelium on the corneal surface may be either conjunctival with neovascularization, a mixture of metaplastic corneal epithelial cells and conjunctival epithelial cells, or only conjunctival epithelial cells without neovascularization (Figure 1C, 1D).<sup>75,76</sup> The conjunctival epithelium, which is thinner and hazy has a different protein profile that differs from those of the healthy cornea.<sup>77-79</sup>

The diagnosis of LSCD is aided significantly by the fluorescein staining pattern of the conjunctival epithelium. Under cobalt blue light, fluorescein allows to detect both the presence of abnormal epithelial cells in LSCD as well as their pattern of distribution. In contrast to the epithelial defects that are immediately stained by fluorescein, the dye diffuses into the paracellular space of the conjunctivalized surface and abnormal delayed staining is observed 10 or more minutes after fluorescein instillation.<sup>17,56,80</sup> This abnormal staining pattern can be visualized even after rinsing with balanced salt solution or eye wash. In general, there is a marked difference in the thickness between the corneal epithelium, which tends to be regular and thick, and the conjunctival epithelium, which tends to be irregular and thin on the cornea (Figure 1D).<sup>79</sup> This difference in thickness often sharply demarcates the area of conjunctivalization from the adjacent healthy corneal epithelium and is rendered more obvious by the pooling of fluorescein (Figure 1D).<sup>80</sup>

The pattern and intensity of fluorescein staining vary according to the severity of the disease. In mild or early disease, which often is sectoral, only punctate fluorescein staining in a curve-like path may be visible in the affected area (Figure 1D).<sup>32</sup> As LSCD progresses and conjunctival-type epithelium spreads across the cornea, areas may have a dulled light reflex, an irregular and hazy opaque epithelium (easier seen with fluorescein staining under cobalt blue lighting), superficial vascularization of the cornea, and peripheral pannus (Figure 1E).<sup>76,81</sup> In addition, absence or flattening of the limbal palisades of Vogt may be noted, although this finding can also be age-related.<sup>82</sup> In the more severe stage, the abnormal epithelium spreads in a spiral pattern from the limbus and has been described as vortex keratopathy or whorl-like epitheliopathy.<sup>83</sup> Characteristics of LSCD in its more advanced stages (Figure 1E, 1F) include subepithelial fibrosis, fibrovascular pannus, corneal stromal scarring and neovascularization (since conjunctival epithelium lacks anti-angiogenic properties compared to corneal epithelium), and persistent and/or recurrent epithelial defects.<sup>84,85</sup>

**2 Diagnostic tests**—There are limitations of slit lamp clinical examination and fluorescein staining test in the diagnosis of LSCD.<sup>86,87</sup> The signs of abnormal epithelium can be subtle and often not specific to LSCD. Adjunct diagnostic tests are of great value in confirming LSCD whether or not associated with other ocular surface abnormalities such as tear deficiency, corneal neovascularization, keratinization, or if goblet cells are absent as determined by impression cytology and in vivo laser scanning confocal microscope (IVCM). Phenotypic analysis of cells sampled from the corneal surface by impression cytology or in vivo imaging can confirm the diagnosis of LSCD.

### 2.1 Cell sampling from the cornea to detect conjunctival cells

**2.1.1 Cell sampling methods:** Several methods can be used to obtain cells from the ocular surface. Impression cytology can collect cells only from the superficial layers of the epithelium.<sup>88,89</sup> Cell scraping can collect cells from the deeper layers of the epithelium, which may reflect more accurately the phenotype of the epithelium under examination.<sup>90</sup> Tissue biopsies can accurately provide the anatomic features of the ocular surface but is more invasive than other cell sampling methods; however, pathologic examination of surgically excised tissue may aid in definitely characterizing the pathophysiology of disease.

The use of impression cytology for the diagnosis of LSCD was established in 1989 by Tseng and coworkers.<sup>2,42</sup> In the context of therapeutic strategies, impression cytology has been used to demonstrate preoperative conjunctivalization and postoperative restoration of the normal corneal phenotype after autologous limbal transplantation.<sup>19</sup> Impression cytology is considered as the “gold standard” in diagnostic testing for LSCD. The method is simple, minimally invasive, and able to detect goblet cells or conjunctival epithelial cells on the corneal surface, which is the hallmark of LSCD. A filter paper made of nitrocellulose acetate, cellulose acetate, or hydrophilic Polytetrafluoroethylene (Biopore membrane) is placed on the cornea after instillation of topical anesthetics.

The sensitivity of impression cytology in the diagnosis of LSCD is affected by many factors: (I) The material used for sampling affects the outcome;<sup>91, 92</sup> (II) The pressure of the application of the filter onto the ocular surface also affects the results of the sampling; (III) The location and size of sampling are important, especially in cases of sectoral LSCD; (V) Retrieval of conjunctival cells is more efficient than for corneal epithelial cells. Therefore, it is important to highlight that the absence of corneal epithelial cells on the filter paper does not necessarily indicate an absence of corneal epithelial cells on the ocular surface as is often the case in patients with normal corneas. IVCM, if available, could be used to detect residual limbal and corneal epithelial cells.<sup>86,93</sup> Due to the above sampling bias, impression cytology is not a quantitative test to stage LSCD.

### 2.1.2 Evaluation of cell biomarkers

**Conventional histochemistry:** Traditionally, histologic stains such as hematoxylin and eosin (H&E) or periodic acid-Schiff stain (PAS) are used to detect goblet cells collected by impression cytology or cell scraping (Figure 2E, F). The presence of goblet cells is indicative of conjunctivalization of the cornea. However, conjunctival goblet cells may be completely absent in patients with other ocular conditions such as keratinization.<sup>94,95</sup>

Therefore, a positive corneal impression cytology result demonstrating the presence of goblet cells is a confirmatory of a diagnosis of LSCD, whereas a negative result cannot rule out LSCD. In these cases, additional tests to detect conjunctival epithelial cells are required for a definitive diagnosis of LSCD.

**Immunohistochemistry:** Because of the low sensitivity of goblet cells in the diagnosis of LSCD, specific markers of conjunctival epithelial cells have been sought to indicate the presence of these cells on corneal impression cytology specimens. Immunohistochemistry allows detection of intracellular proteins (e.g., cytokeratins, CK) and secreted proteins that are typically expressed in conjunctival epithelial and goblet cells (e.g., mucins)<sup>96</sup>. CK19 has been used in the past, but there are conflicting data regarding its specificity.<sup>97–101</sup> Newer markers, (e.g., CK7,<sup>102</sup> CK13,<sup>100,102,103</sup> MUC1,<sup>97</sup> and MUC5AC<sup>104</sup>) can also be used to support the diagnosis of LSCD (Figure 2 A–C).

**Reverse transcription polymerase chain reaction:** Reverse transcriptase polymerase chain reaction (RT-PCR) has been investigated as a method of detecting specific mRNA that are expressed in conjunctival cells collected by impression cytology. Because of its high sensitivity, RT-PCR can detect conjunctival markers such as CK19, CK7, CK13, MUC1, and MUC5AC. Using RT-PCR to detect MUC 5AC seems to be more sensitive than using immunocytology to detect goblet cells,<sup>104</sup> if any.

## 2.2 In vivo imaging techniques in the diagnosis of LSCD

**2.2.1 In vivo confocal microscopy:** In vivo laser scanning confocal microscopy has emerged as a diagnostic tool for LSCD, in part, because this method does not require the removal of corneal epithelial cells for analyses. IVCM provides information about disease severity and can be used in both the diagnosis and monitoring of LSCD. In addition, IVCM may be useful in evaluating the outcomes of cultured LSC grafts<sup>105</sup> and recovery from trauma such as chemical injury.<sup>106</sup>

Corneal, conjunctival, and limbal epithelial cells can be distinguished on the basis of their different cell morphology.<sup>32,107</sup> The absence of the corneal epithelium and/or the presence of conjunctival cells on the cornea is diagnostic of LSCD (Figure 3). Goblet cells can be detected by IVCM (Figure 3E).<sup>86</sup> Because of the limitation in using goblet cells in the diagnosis of LSCD, the differences in cell morphology between the corneal and conjunctival epithelium detected by IVCM appears to be a more sensitive approach in diagnosing LSCD. Other features of IVCM findings suggestive of LSCD include epithelial thinning and reduction of subbasal nerve plexus and basal cell density.<sup>107–110</sup>

**2.2.2 Anterior segment optical coherence tomography:** Anterior segment optical coherence tomography (AS-OCT) has emerged as an alternative imaging technique for LSCD, allowing for both noninvasive imaging of the ocular surface, including the limbus, and a larger field of view at the expense of resolution.<sup>111–115</sup> Although AS-OCT does not offer the same degree of resolution at the cellular level as IVCM does, AS-OCT may prove useful in measuring epithelial thickness, pannus depth,<sup>116</sup> assessing POV, limbal crypts, and the clear transition between the hyporeflexive corneal epithelium and the hyperreflective conjunctival epithelium in the limbal region (i.e. cross-sections parallel and perpendicular to

the limbus and en face sections).<sup>117,118</sup> Compared with normal eyes, eyes with LSCD feature an absence of the POV and limbal crypts (limbal niche characteristics), and have greater variability in their central corneal epithelial thickness and, in general, a significant reduction in the thickness of limbal epithelium, as measured by AS-OCT (Figure 4).<sup>115,118</sup> AS-OCT is also helpful in the diagnosis of LSCD with complex concomitant ocular surface diseases, such as ocular surface squamous neoplasia.<sup>119</sup> Moreover, the results of AS-OCT are usually consistent with the findings of IVCN regarding the reduction of epithelial thickness or the morphologic changes in the POV.<sup>115,118</sup> However, absence of POV is not pathomognic for LSCD because the presence of POV decreases with age even in normal eyes.<sup>82</sup>

**2.2.3 Correlations between AS-OCT and IVCN, and impression cytology:** In vivo confocal microscopy has a high degree of concordance with impression cytology in the diagnosis of LSCD.<sup>86</sup> Because of cell sampling bias in impression cytology as metioned above, IVCN may prove to be more sensitive than impression cytology in detecting the presence of the corneal epithelium in sectoral or partial LSCD.<sup>86</sup> This sensitivity is particularly important in staging LSCD accurately. In eyes with the clinical features of total LSCD as seen under slit-lamp biomicroscopy and confirmed by impression cytology, IVCN sometimes is able to detect well-demarcated, lacunae-like structures that are occasionally found in certain deep limbal stroma, which contain clusters of highly packed, normal limbal epithelial cells.<sup>93</sup> However, the correlation between AS-OCT and impression cytology has not been investigated because the resolution of AS-OCT is not at the cellular level.

### Staging of limbal stem cell deficiency based on clinical presentation

Limbal stem cell deficiency can be categorized into 3 stages based on the extent of corneal and limbal involvement detected by clinical examination as described in Table 2 and graphically illustrated in Figure 5. Staging of LSCD important to guide therapeutic recommendations and surgical planning. The most important factor is whether or not the visual axis or central 5 mm of cornea is affected (Stages II and III), and whether more than 50% of the limbal stem cells are intact should be considered. The final stage (Stage III) involves total LSCD where the whole corneal surface is affected. Abnormalities of other components of the ocular surface such as the conjunctiva, meibomian glands, lacrimal glands, tears, corneal nerves and immune system are important in the management of LSCD<sup>120</sup> and will be addressed in a separate document on LSCD therapies.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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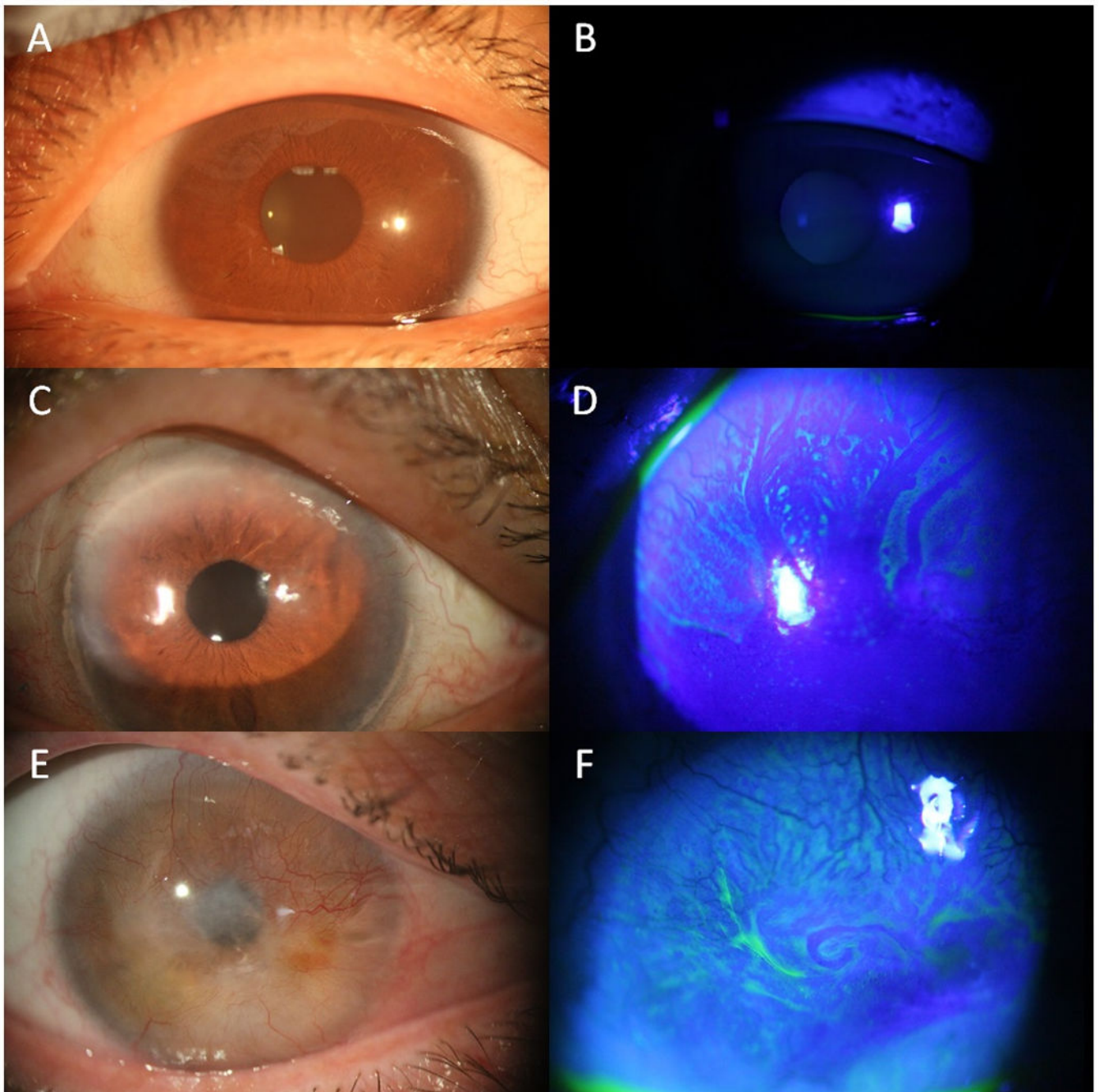
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**Figure 1.**

Slit lamp photos (bright light and fluorescein staining). Slit lamp photos of normal eyes (A,B), sectoral limbal stem cell deficiency (LSCD, C,D) and total LSCD eyes (E, F). The epithelium in the normal eye is transparent (A), which cannot be stained by fluorescein (B). In the eye with sectoral LSCD, the epithelium of affected area superiorly is greyish (C) under bright light. It can be better visualized with fluorescein staining under cobalt blue light, which often have a whorl-like pattern and fluorescein pooling in area where there is epithelial thinning (D). In eyes with total LSCD, corneal opacity and superficial

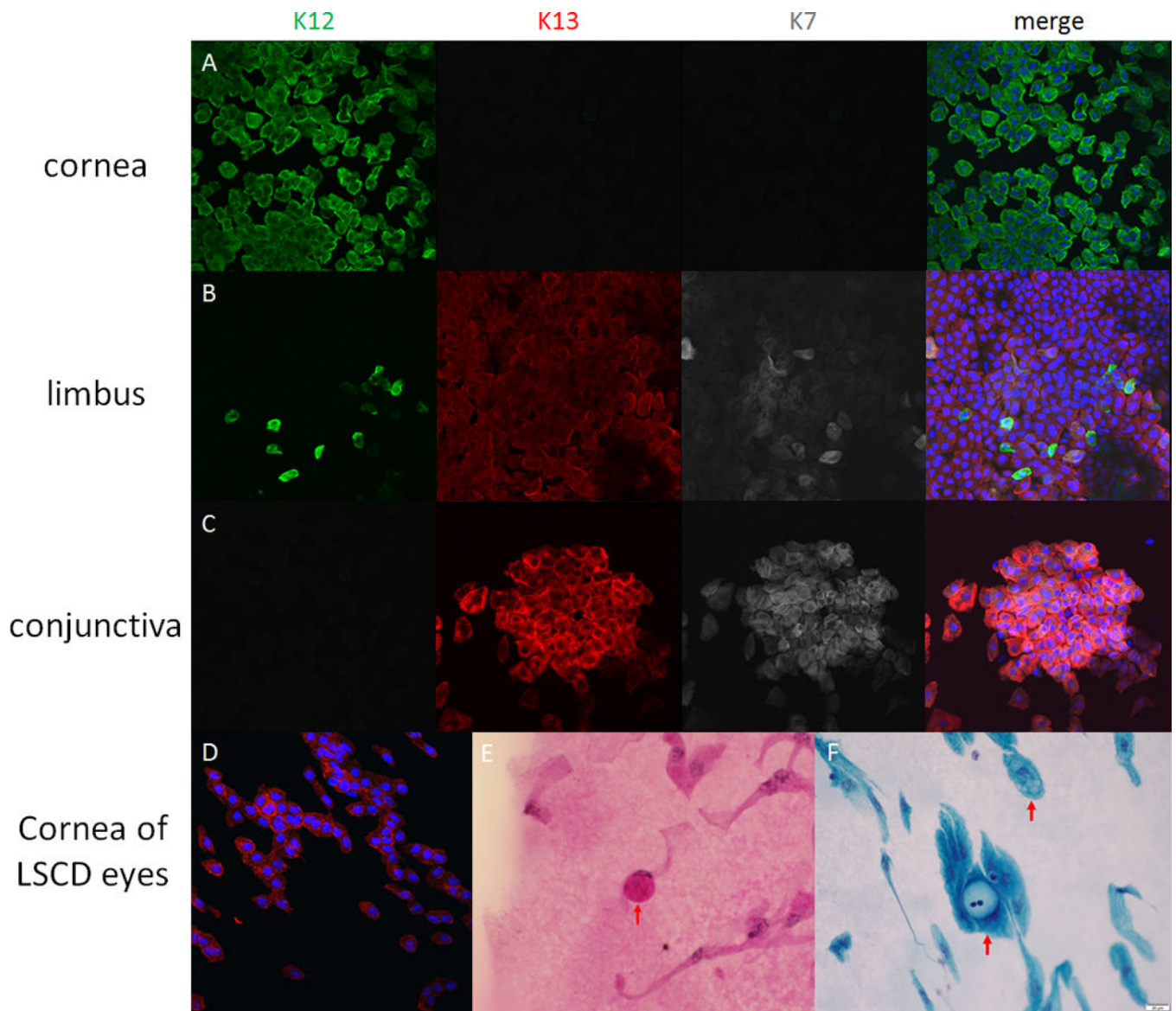
neovascularization affecting the entire cornea surface are visible under bright light (E).  
Fluorescein staining shows epithelial irregularity (F).

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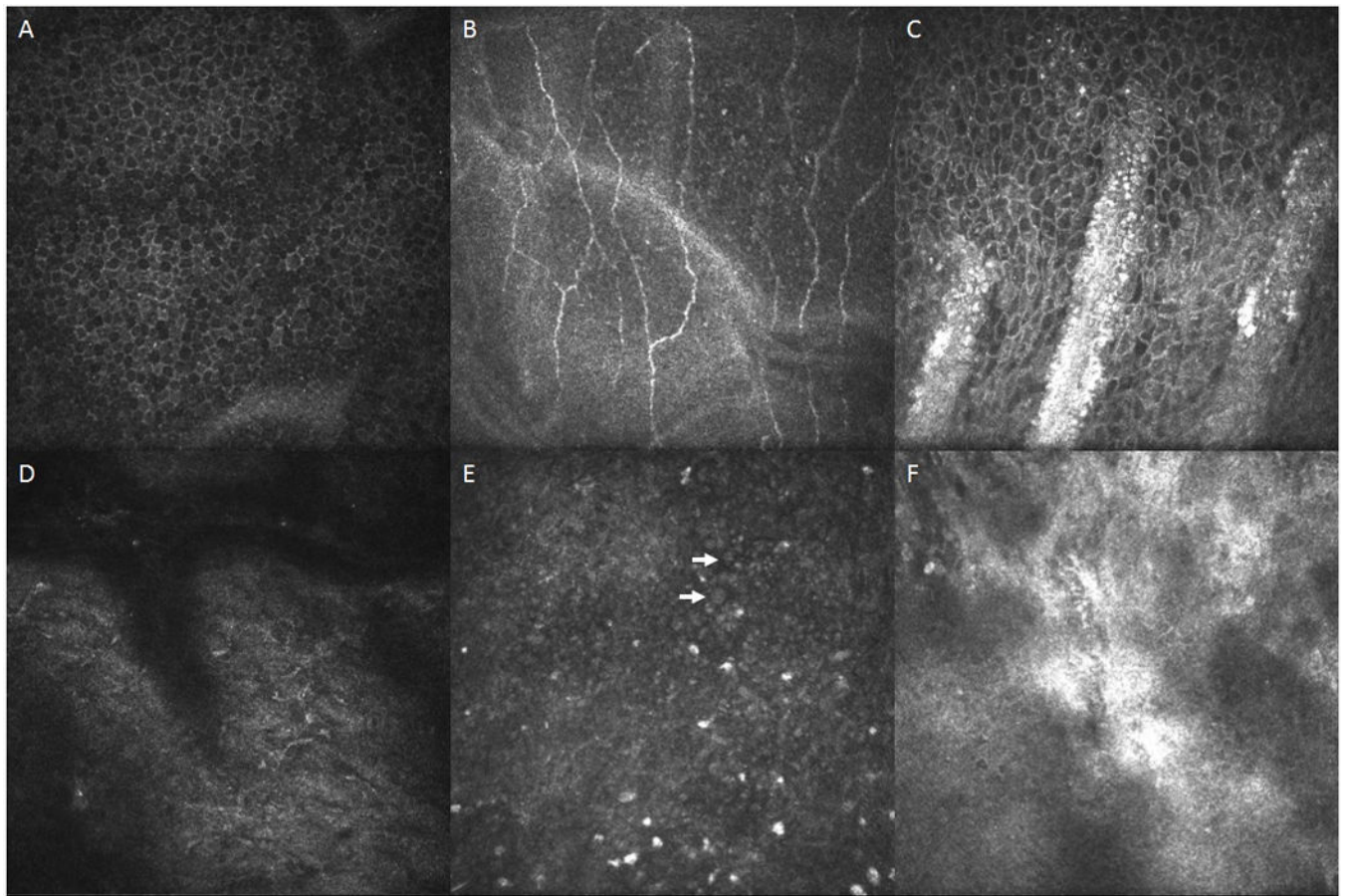
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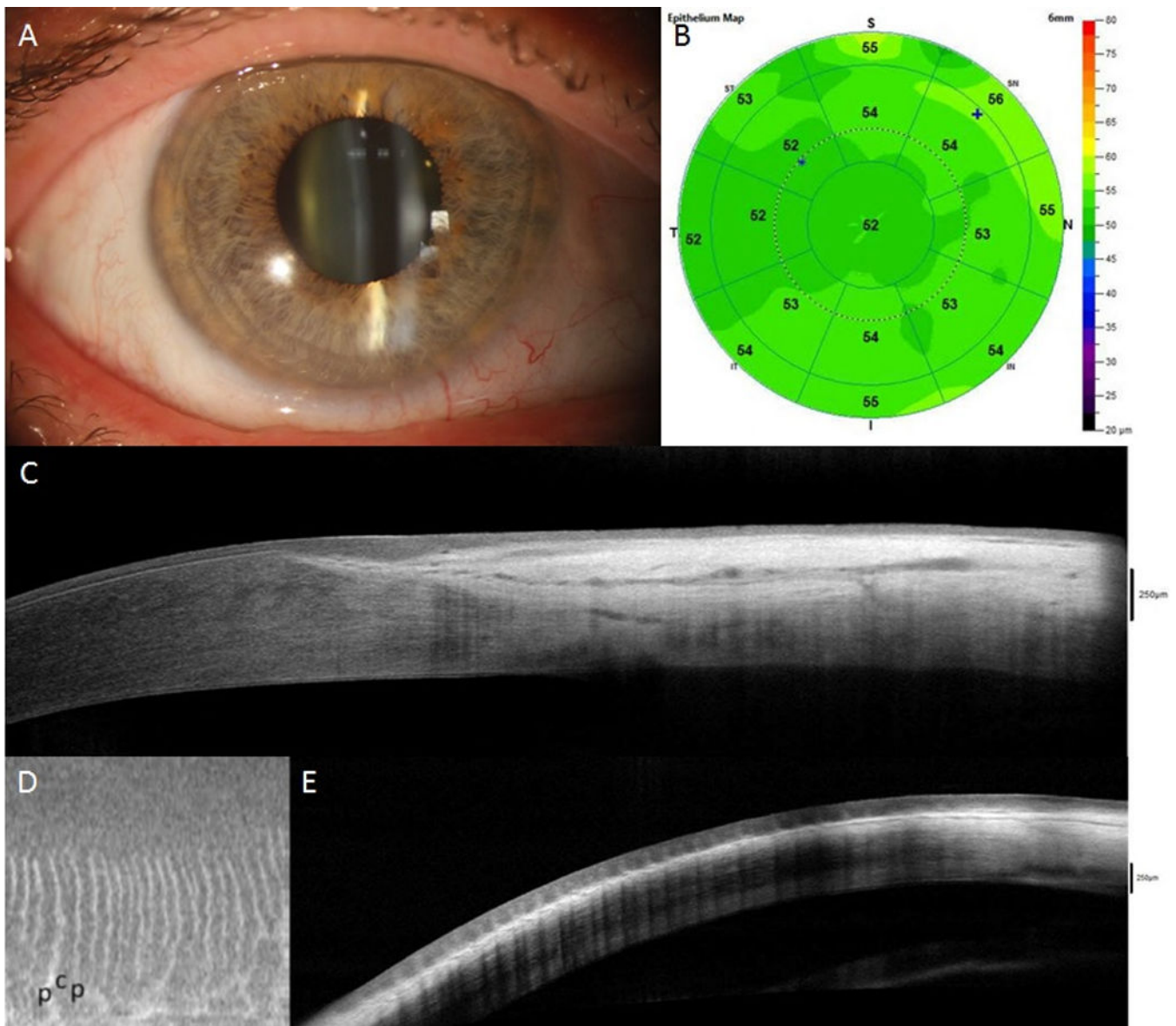
**Figure 2.**

Immunofluorescein and pathological staining of corneal impression cytology specimens. Figure 2A, 2B and 2C are specimens taken from central cornea, limbus and conjunctiva of a normal cadaveric eye, respectively. The expression of cytokeratin (CK) 12 is only found in corneal epithelial cells, and cannot be found in conjunctival epithelial cells. CK13 and CK7 are only expressed in conjunctival cells. At the limbal area, both CK12-positive cells and CK13-CK7-positive cells are visible. Figure 2 D-F are the samples taken from the eyes with limbal stem cell deficiency. CK13 positive cells are visible on the cornea (2D). The goblet cells can be found through periodic acid-Schiff staining (2E) or Papanicolaou staining (2F) in the samples taken in limbal stem cell deficiency eyes after immunostaining. The goblet cells have 2–3 times larger cell size compared with epithelial cells, and mucus blobs are visible within the cells. The mucus blobs are usually visualized as pink by PAS staining.



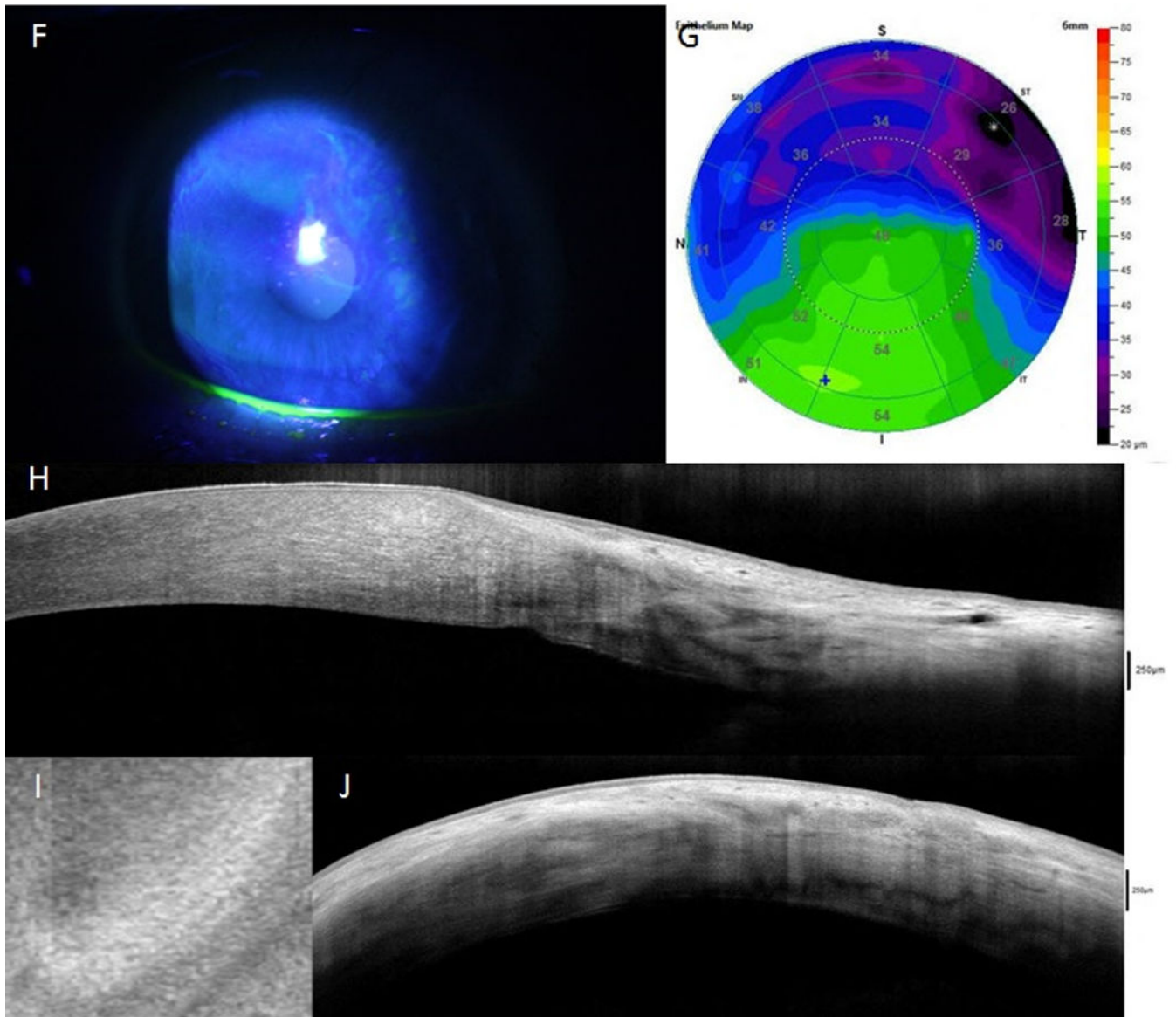
**Figure 3.**

In vivo confocal microscopy imaging. Confocal microscopic images of normal eyes and eyes with limbal stem cell deficiency (LSCD). Panels A-C are images of normal eyes. Panels D-F are images of eyes with LSCD. Normal corneal basal epithelial cells (A) have well-defined, bright cell borders and hypo-reflective cytoplasm, with a regular and uniform arrangement. The nuclei are not visible or are very faint. Sub-basal nerve plexus (B) and the palisades of Vogt (C) are clearly visible in normal eyes. In eyes with severe LSCD, corneal basal epithelial cells are absent and sub-basal nerves were fragmented and the nerve density decreases significantly (D). Goblet cells (arrow) are occasionally visible (E). The palisades of Vogt (F) are not present in eyes with LSCD, but inflammatory cell infiltrates are sometimes visible.



**Figure 4.**

AS-OCT assessment of normal eyes (A-E) and eyes with limbal stem cell deficiency (LSCD, F-J). In eyes with sectoral LSCD, the corneal epithelial thickness in the affected area is decreased (G) compared to normal eye (B) and unaffected area (G); this reduction is consistent with the slit-lamp finding (F). Cross-sections perpendicular to the limbus show a clear transition between the hyporeflexive corneal epithelium and the hyperreflective conjunctival epithelium with limbal epithelial thickening in normal eyes (C) but not in eyes with LSCD (H). The limbal epithelium in the affected area becomes thinner (H). The palisades of Vogt (p in figure) and limbal crypts (c in figure) are clearly visualized in normal eyes on en face mode (D) and parallel section (E), whereas they are absent in LSCD eyes (I and J).



**Figure 5.** Staging system of limbal stem cell deficiency (LSCD). The severity of LSCD is classified into 3 stages. Stage I disease only involves the periphery of the cornea and the degree of involvement is staged into A, B and C (top panel). Stage II disease involves both the periphery and the central 5 mm of the cornea (second panel). Stage III disease involves the entire corneal surface (bottom panel).



**Table 1.**

## Etiology of limbal stem cell deficiency.

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<b>Acquired limbal stem cell deficiency</b>
Acquired nonimmune-mediated
Chemical injury <sup>27,42</sup>
Thermal injury <sup>42</sup>
Radiation injury <sup>45</sup>
Contact lens wear <sup>28,42</sup>
Multiple surgeries involving the limbus <sup>30,42</sup>
Bullous keratopathy <sup>40,41</sup>
Infectious ocular disease <sup>28,43,71</sup>
Chronic lid disease
Severe blepharitis-rosacea <sup>43</sup>
Trachoma <sup>42</sup>
Tumors of the ocular surface <sup>44</sup>
Severe pterygium <sup>29,30</sup>
Drug induced LSCD
Mitomycin C <sup>33-35</sup>
5-fluorouracil <sup>31</sup>
Preservative <sup>36</sup>
Systemic chemotherapy and immunotherapy <sup>37-39</sup>
Acquired primary immune-mediated
Stevens-Johnson syndrome/toxic necrolysis <sup>17,42,46,47</sup>
mucous membrane pemphigoid <sup>50,51</sup>
Allergic ocular surface disease
Vernal keratoconjunctivitis <sup>53,54</sup>
Atopic keratoconjunctivitis <sup>56,57</sup>
Graft-vs-host disease <sup>58,59</sup>
Idiopathic
<b>Hereditary limbal stem cell deficiency</b>
Congenital aniridia <sup>43,71</sup>
Dyskeratosis congenita <sup>65</sup>
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy/dysplasia (Multiple endocrine deficiency, APS1) <sup>62,63</sup>
Xeroderma pigmentosum <sup>64</sup>
Keratitis ichthyosis deafness syndrome <sup>68,69</sup>
Ectrodactyly-ectodermal dysplasia-clefting syndrome <sup>66,67</sup>
Lacrimo-auriculo-dental-digital syndrome <sup>70</sup>
Epidermolysis bullosa <sup>71,72</sup>

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**Table 2.**

Staging of limbal stem cell deficiency based on clinical presentation.

		<b>A</b>	<b>B</b>	<b>C</b>
Stage I	Normal corneal epithelium within the central 5 mm zone of the cornea	<50% of limbal involvement	50% but <100% limbal involvement	100% of limbal involvement
Stage II	The central 5 mm zone of the cornea is affected	< 50% of limbal involvement	50% but <100% limbal involvement	
Stage III	The entire corneal surface is affected			

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