In vivo confocal microscopy of the sclerocorneal limbus after limbal stem cell transplantation: Looking for limbal architecture modifications and cytological phenotype correlations

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Purpose: To correlate a biomicroscopic evaluation, an in vivo confocal microscopy examination, and impression cytologic findings of the corneal center and sclerocorneal limbus after cultured limbal stem cell transplantation and to test the effectiveness of in vivo confocal microscopy as a diagnostic procedure in ocular surface cell therapy reconstructive surgery.

Methods: Six eyes of six patients affected by limbal stem cell deficiency after chemical burns underwent ex vivo expanded limbal stem cell transplantation (two eyes) and ex vivo expanded limbal stem cell transplantation with subsequent penetrating keratoplasty (four eyes) to restore corneal transparency. One year after surgery, all patients underwent a biomicroscopic evaluation, central cornea impression cytology to detect cytokeratin 12 (CK12) positivity, and in vivo confocal microscopy of the central cornea and the sclerocorneal limbus to investigate the epithelial cellular morphology, limbal architecture, and corneal inflammation level.

Results: Impression cytology analysis showed CK12 positivity in five of six cases, in concordance with the biomicroscopic evaluation. Confocal microscopy pointed out irregular limbal architecture with the absence of the palisades of Vogt in all cases; the central epithelial morphology presented clear corneal characteristics in three cases and irregular morphology in the remaining three.

Conclusions: After successful ex vivo expanded limbal stem cell transplantation, in the presence of a complete anatomic architecture subversion, documented by support of in vivo confocal microscopy, the sclerocorneal limbus seemed to maintain its primary function. In vivo confocal microscopy confirmed the procedure was a non-invasive, efficacious diagnostic ocular surface procedure in the case of cell therapy reconstructive surgery.

Integrity and transparency of the cornea are fundamental for human vision whereas corneal disease or injuries represent leading causes of blindness worldwide. An intact corneal epithelium serves several important functions, including preservation of tissue clarity and protection from pathogens [1].

Differentiated cells are continuously replenished throughout an individual's lifespan by stem cell populations, limbal stem cells (LSCs), residing at the cornea's outer edge termed the limbus [2].

When the limbus is affected by an injury or a disease, it is unable to regenerate the corneal epithelium, defining a limbal stem cell deficiency (LSCD), characterized by corneal neovascularization, chronic inflammation, and ultimately blindness due to irreversible corneal opacity related to conjunctival epithelium migration on the corneal stroma [1]. The possibility of restoring the human corneal surface with autologous corneal epithelial sheets generated by serial cultivation of limbal cells has been considered; in this context, transplantation of in vitro culture-expanded limbal cell populations is a recent therapeutic approach to LSCD that successfully regenerated the functional corneal epithelium, with or without a pannectomy or an eventual keratoplasty to improve visual acuity [3].

The outcome of limbal stem cell transplantation (LSCT) surgery is usually defined based on subjective grading [4] of the interpretation of clinical signs, such as corneal vascularization, conjunctivalization, or ocular surface inflammation [5]. To overcome limitations related to the personal interpretation of clinical data in LSCD and to obtain objective evidence of this pathology, various diagnostic technological advances were introduced; the application of impression cytology (IC) provides objective analysis of LSCD, although invasiveness, evaluation limited to the epithelium, and poor repeatability remain the most obvious limitations [6].

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Laser scanning in vivo confocal microscopy (IVCM) provides reliable minimally invasive in vivo analysis of the ocular surface cell morphology and, specifically, of all corneal layers with a high degree of concordance with the IC analysis [7]; investigating microscopic limbal anatomy in healthy human subjects, the presence of regular mosaics of dark cell bodies and bright cell groups were identified whereas hyperreflective linear acellular structures, alternating with columns of epithelial cells, were considered to correspond to the limbal palisades of Vogt [8-10]. In various studies, IVCM and IC have been applied after ex vivo expanded limbal stem cell transplantation to determine surgical success and epithelial characteristics and to detect the limbal anatomic architecture [4,10,11]. The aim of this study was to determine, based on IC and IVCM analysis and concordance, real correspondence between the clinical and anatomic/structural outcome of cell therapy reconstructive surgery and to determine renewed staminal function in correlation with postoperative anatomic findings.

METHODS

The study adhered to the tenets of the Declaration of Helsinki and to the tenets of the ARVO Statement on Human Subjects and was approved by our Istitutional Review Board. Six eyes of six patients affected by total unilateral limbal stem cell deficiency (age 37–64) after chemical burns were enrolled. Informed consent was obtained from all patients.

All enrolled patients presented LSCD symptoms, including chronic ocular inflammation with conjunctivalization, pain, photophobia and vision loss, superficial and/or deep corneal neovascularization with corneal scarring, and general ocular discomfort. An initial diagnosis of total LSCD was obtained after slit-lamp biomicroscopy (corneal conjunctivalization) and confirmed by IC with cytokeratin 19 (CK19) >80% and CK3 <20% positivity as previously described by Rama et al. [12]. Two patients underwent ex vivo expanded LSCT for LSCD, and four patients underwent ex vivo expanded LSCT for LSCD and subsequent penetrating keratoplasty (PK) to restore corneal transparency after 6 months.

All surgical procedures, LSCT and PK, were performed at S. Giovanni Hospital of Rome (Italy) in 2013; the surgical technique performed for ex vivo expanded LSCT was performed as described in the literature [3,13]. Briefly, a limbal biopsy was performed by taking a 1×2-mm piece of limbal epithelium from the clear cornea of the contralateral healthy eye. The procedure was performed under aseptic conditions with topical anesthesia (Novesina, oxybuprocaine 0.4%, Novartis Farma S.p.A. Origgio (VA), Italy). Patients

were treated with topical 0.4% netilmicin for 7 days after the biopsy. Limbal keratinocytes were isolated and cultured on a feeder layer of lethally irradiated 3T3-J2 cells, approved for clinical use, and plated on fibrin glue. The fibrin-cultured epithelial sheets were placed in sterile contact lens containers and sent to the hospital. Growth of the primary cultures and preparation of a fibrin-cultured graft required 14–16 days. After retrobulbar anesthetic (2% carbocaine) was applied, conjunctival peritomy and pannectomy were performed in the damaged eyes before the fibrin-cultured epithelial sheets were placed on the corneal surface, under the dissected conjunctiva. The conjunctiva was sutured with 2 8/0 Vicryl sutures. Two 4/0 silk sutures were used to close the eyelids. The sutures were removed 3 days later, and prophylactic antibiotic eyedrops were given 3 times daily for 3 weeks. Systemic 1 mg/kg prednisone was given for 7 days after surgery and then reduced to 0.5 mg/kg for an additional 7 days. Subsequently, only preservative-free 0.15% dexamethasone was given topically for 1 month or more if necessary. The procedure was determined to be successful if the corneal epithelium was restored, the ocular surface was stable, and the symptoms improved after a 6-week follow-up. Four patients with a stable corneal epithelium but showing deep corneal stromal opacity were treated with subsequent PK to improve vision. All patients had been referred to the Ophthalmic Clinic of Chieti-Pescara University (Italy) for diagnostic evaluation after surgery (12 months after only LSCT and 18 months after LSCT/PK). In particular, all patients underwent IC of the central cornea and IVCM of the central cornea and the sclerocorneal limbus.

For IC, samples with more than 50% cellularity were considered suitable for diagnostic procedures, and immunofluorescence was stained using antibodies against CK12 (corneal-specific) to determine the phenotype of the cells populating the corneal surface [13]. Corneal impression cytology samples were collected using Millicell-CM 0.4 μm (Millipore, Bedford, MA), and the cells were fixed with cytology fixative (Bio-fix, Bio Optica, Milan, Italy).

For CK12 immunofluorescence staining, the Millicell membranes were hydrated with distilled water, and 80% alcohol was added for 2 min. The membranes were washed in distilled water, and then PBS (1X; 0,14 M NaCl ,0,0027 KCl, 0,01M NaPO$_4$, pH 7.4 at 25° C) was added for 2 min, followed by two washes with wash buffer (Dako, Glostrup, Denmark) of 2 min each. Then RNase A (Sigma-Aldrich, St. Louis, MO) diluted 1:290 in PBS was incubated for 20 min at room temperature. The specimens were washed, and PBS-bovine serum albumin (BSA) 1% was added for 1 h at room temperature. Finally, CK12 antibody (Santa Cruz
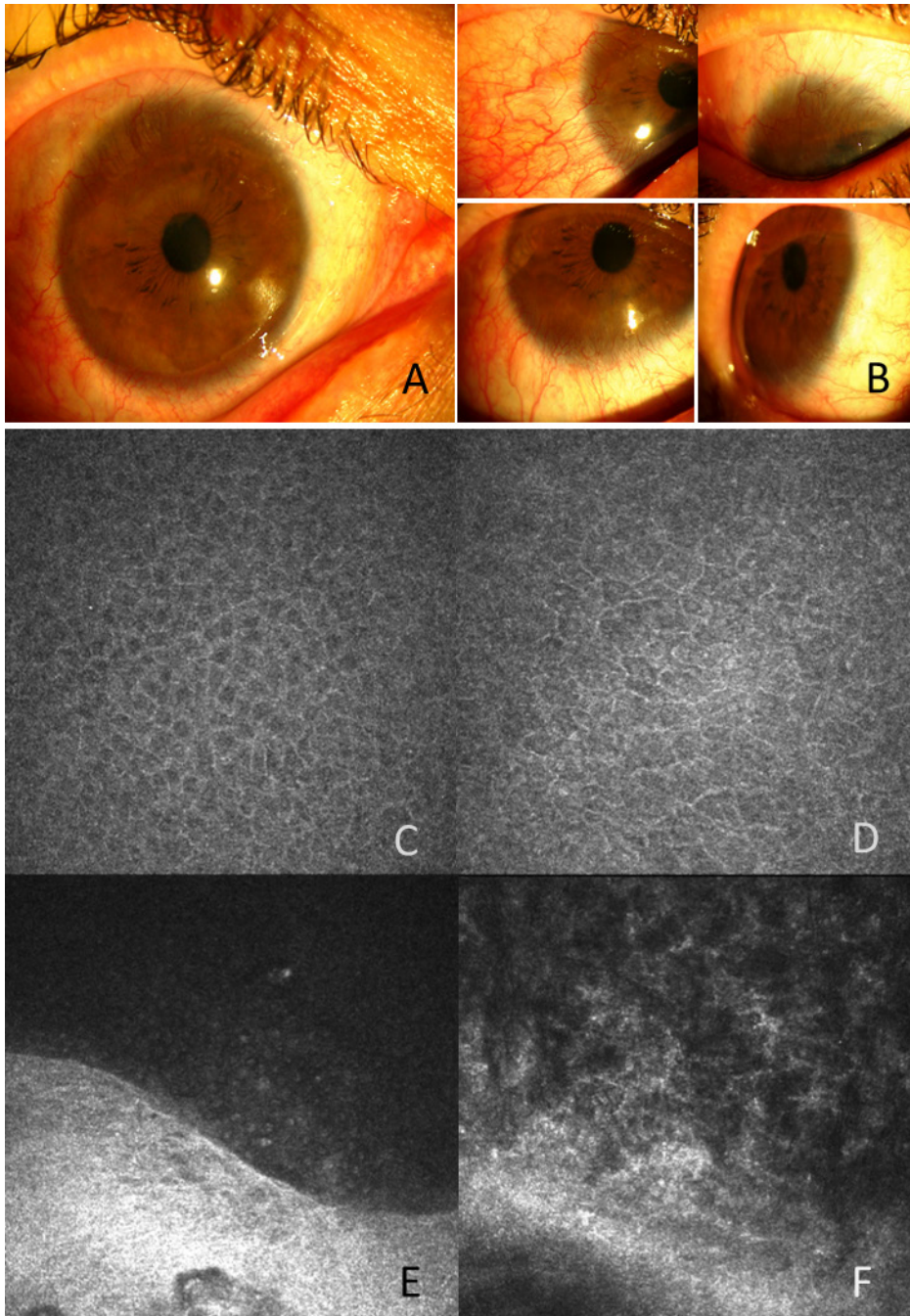
Figure 1. Biomicroscopic findings of central cornea and different limbal quadrants. **A-B**: Biomicroscopic findings of central cornea (**A**) and different limbal quadrants (**B**).

Biotechnology, Santa Cruz, CA) diluted 1:50 in antibody diluent (Dako) was incubated overnight at 4 °C. Samples were washed, and anti-goat Alexa Fluor 488 (Invitrogen, San Giuliano Milanese, Italy) diluted 1:200 and propidium iodide at 1:150 both in antibody diluent (Dako) were added and incubated for 1 h at room temperature. Membranes were mounted with a drop of fluorescent mounting medium (Dako), and Zeiss Confocal LSM 510 (Carl Zeiss MicroImaging GmbH, Vertrieb, Germany) was used to visualize the cells.

CK12 positivity was considered to indicate the corneal epithelium, while CK12 negativity was considered to indicate the presence of the non-corneal epithelium and a consequent diagnosis of persistent LSCD. All evaluations of the impression cytology specimens were performed by two independent observers masked to the details of the staining technique used (R.C., M.L.).
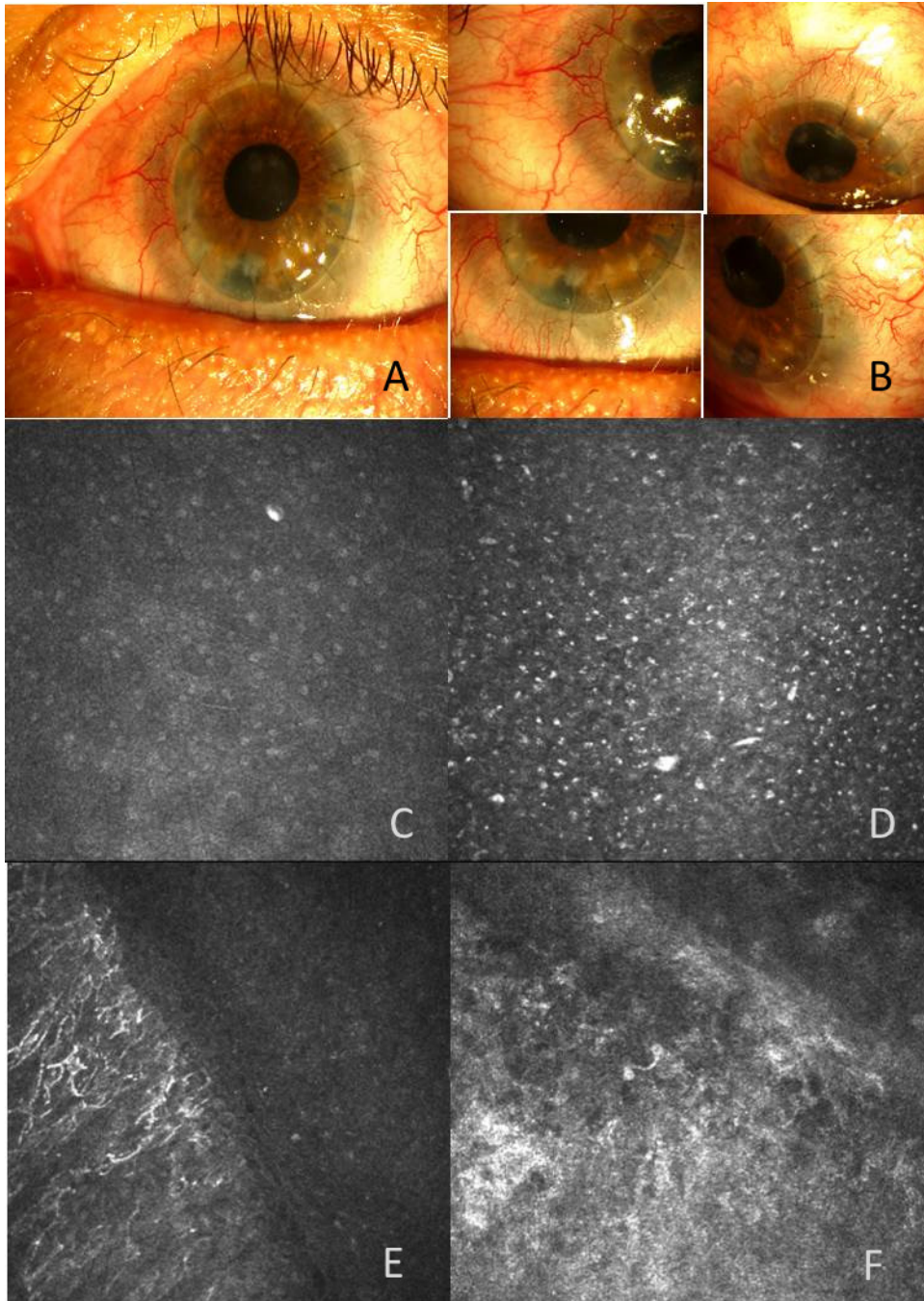
Figure 2. IVCM morphological epithelial characteristics of central cornea: presence in the central cornea of a distinct epithelium with specific morphological characteristics of the stratified corneal epithelium was identified in Figure 1, Figure 2, and Figure 3, and more polymorphic in Figure 3. In Figure 4 and Figure 5, the alternation of different areas of the normal stratified corneal epithelium and irregular morphology and stratification of central epithelial cells was identified. **C-D**: IVCM morphological epithelial characteristics of central cornea: presence in the central cornea of a distinct epithelium with specific morphological characteristics of the stratified corneal epithelium was identified in Figure 1, Figure 2, and Figure 3, and more polymorphic in Figure 3. In Figure 4 and Figure 5, the alternation of different areas of the normal stratified corneal epithelium and irregular morphology and stratification of central epithelial cells was identified.

All patients underwent laser scanning IVCM examination of the limbus and of the corneal surface by an experienced examiner (M.N.). In vivo confocal microscopy scans were performed using a digital corneal confocal laser-scanning microscope (HRT II Rostock Cornea Module, diode-laser 670 nm; Heidelberg Engineering GmbH, Heidelberg, Germany). The confocal laser scanning device was equipped with a water immersion objective (Zeiss, Jena, Germany; 633/N.A. 0.95 W) and provided automatic z-scan determination of the depth of focus within the cornea. Thus, high-contrast digital images with a field of view of 300×300 μm were acquired of all corneal layers. The theoretical confocal section thickness is approximately 10 μm. This is the slice thickness (voxel), which is imaged by the confocal microscope to form a two-dimensional pixel-based digital image. The lateral and transverse resolution was 4 μm.
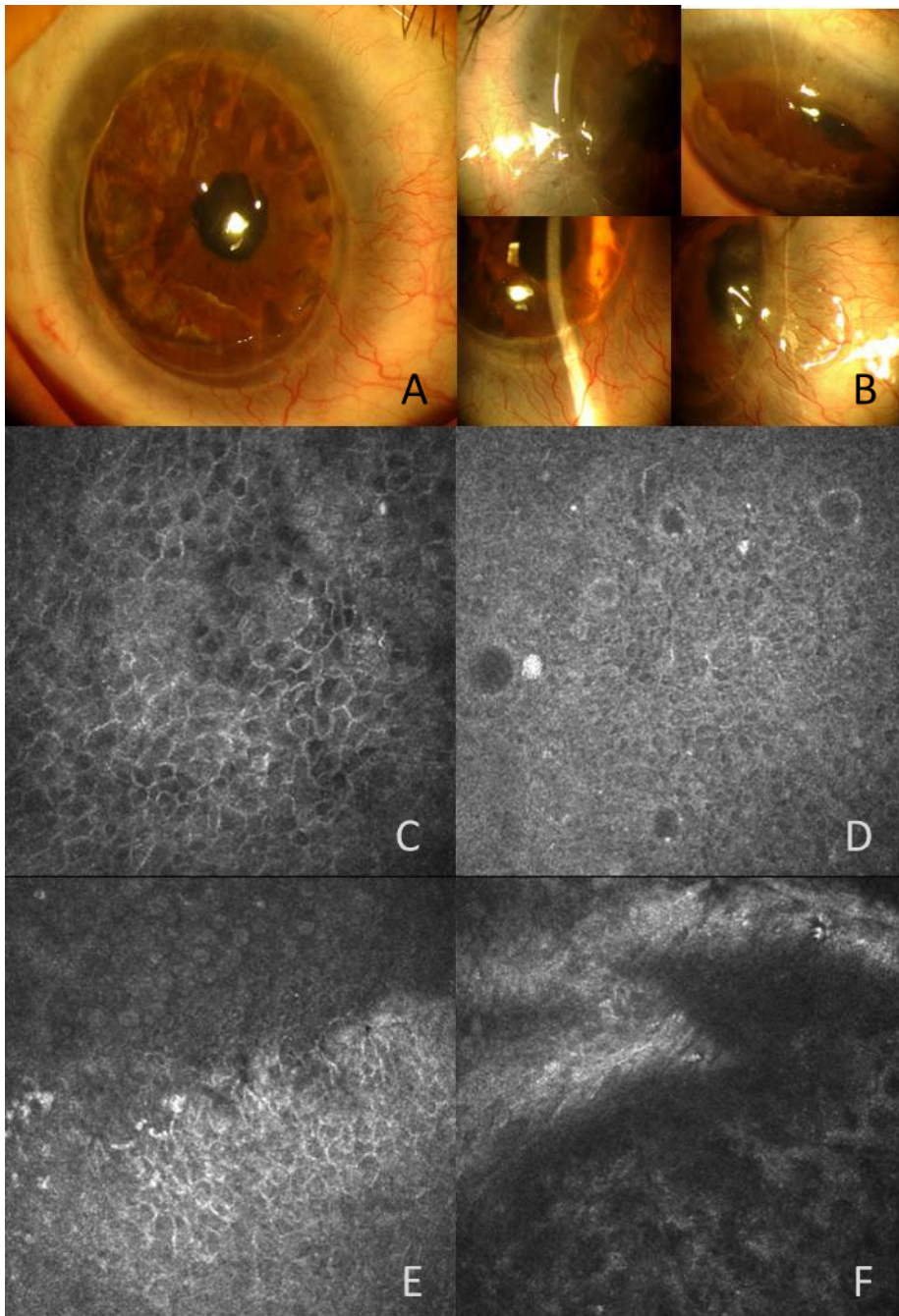
Figure 3. The last case presented a discontinuous epithelium in the central cornea, also characterized by cellular irregular morphology, not clearly corneal (Figure 6). **E–F**: In vivo confocal microscopy (IVCM) limbal analysis of the superficial and deep limbal transition in IVCM.

IVCM was performed under topical anesthesia with 0.4% oxybuprocaine. Proper alignment and positioning of the head were maintained with the help of a dedicated movable-target red fixation light for the contralateral eye. A digital camera mounted on a side arm provided a lateral view of the eye and objective lens to monitor the position of the objective lens on the surface of the eye. A drop of 0.2% polyacrylic gel served as the coupling medium between the poly(methyl methacrylate) contact cap of the objective lens and the cornea (contact lens).

In vivo confocal microscopy examination was performed in the central and paracentral cornea, and in four clock hour positions of the limbal area (12, 6, 3, 9) corresponding to the superior, inferior, nasal, and temporal limbus using a previously described method [7,14]. Sequential images derived from automatic scans and manual frame acquisition throughout the area of interest were obtained with emphasis on visualizing the pathologic and nontransparent superficial tissue of the corneal surface.
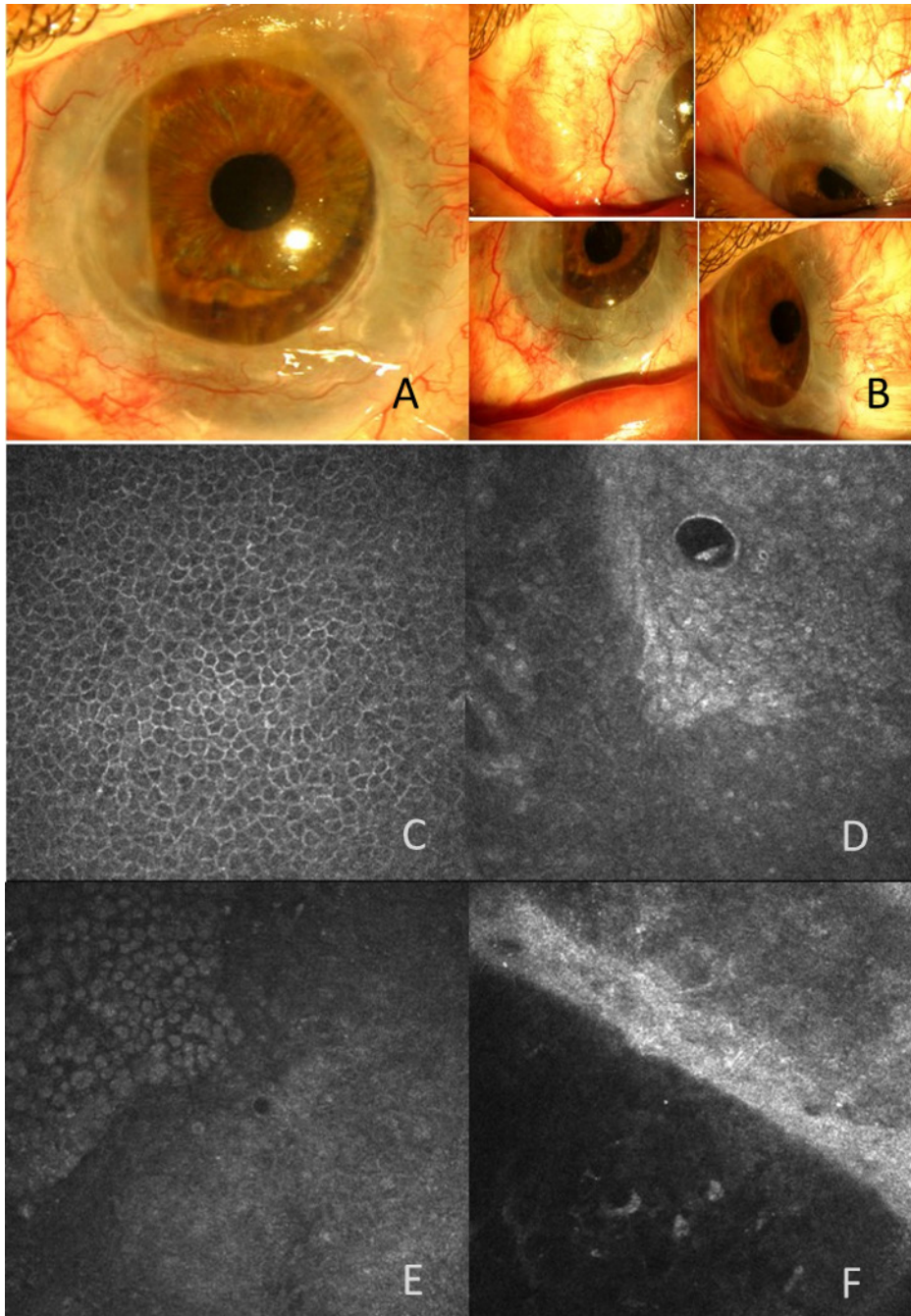
Figure 4. The palisades of Vogt were absent in all quadrants of all patients. The transition of superficial epithelial morphology in all cases (100%) was abrupt with an immediate transition from a conjunctival-shaped epithelium to a corneal-shaped epithelium in five of the six patients (Figures 1E–5E) and to a discontinuous and irregular central epithelium in the last patient (Figure 6E).

For corneal scans, the epithelial stratification and cellular morphology were evaluated in the central and paracentral cornea. Particular emphasis was used to analyze the epithelial cell morphology and morphometry, the presence of irregular cells or epithelial discontinuity, subepithelial vascularization, or fibrous tissue. The normal corneal epithelium was defined as a multilayered epithelium with specific morphologic characteristics: polygonal and flat cells with hyperreflective nuclei in the superficial layer that progressively decreased in size in the intermediate layers and small cells without detectable nuclei with reflective borders in the basal layer [15]. In the limbal scans, the presence of the palisades of Vogt and the progressive morphologic superficial and deep transition of epithelial cells from the conjunctival to the corneal phenotype in the peripheral cornea adjacent to the limbus were considered an indicator of normal anatomy [16].
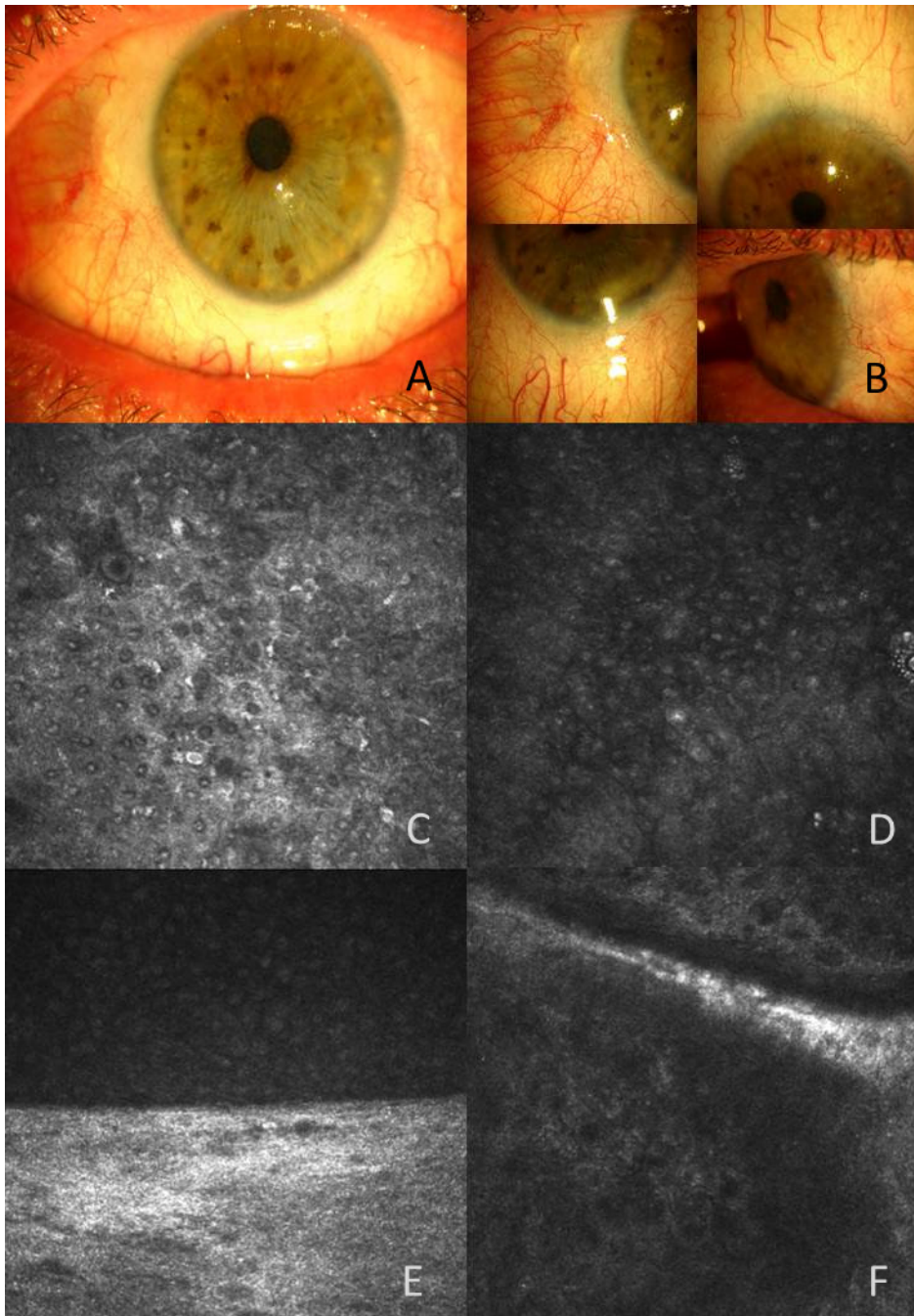
Figure 5. Similarly, deeper in the anterior stroma, an immediate transition between the hyperreflective limbal stroma, without detectable keratocytes and a corneal peripheral stroma with a hyporeflective extracellular matrix and keratocytes was observed in all cases (100%; Figures 1F–6F).

The palisades of Vogt and the limbal epithelial transition were considered present when they were revealed in at least three limbal quadrants examined, partially present if revealed in only one or two quadrants, and absent if not detected at all. The transition between the cornea and the conjunctiva was evaluated in superficial layers and between the cornea and the sclera was investigated in deeper layers together with the presence of the palisades of Vogt.

Epithelial morphology and stratification were evaluated with microscopic signs of inflammation. We considered markers of inflammation the presence of inflammatory cell infiltration in the subepithelial and anterior stroma, tissue edema, and activated keratocytes. Grading of inflammation severity was reported on an absolute scale (+=mild inflammation, ++=moderate inflammation, +++=severe inflammation) as reported in the literature [8,17].
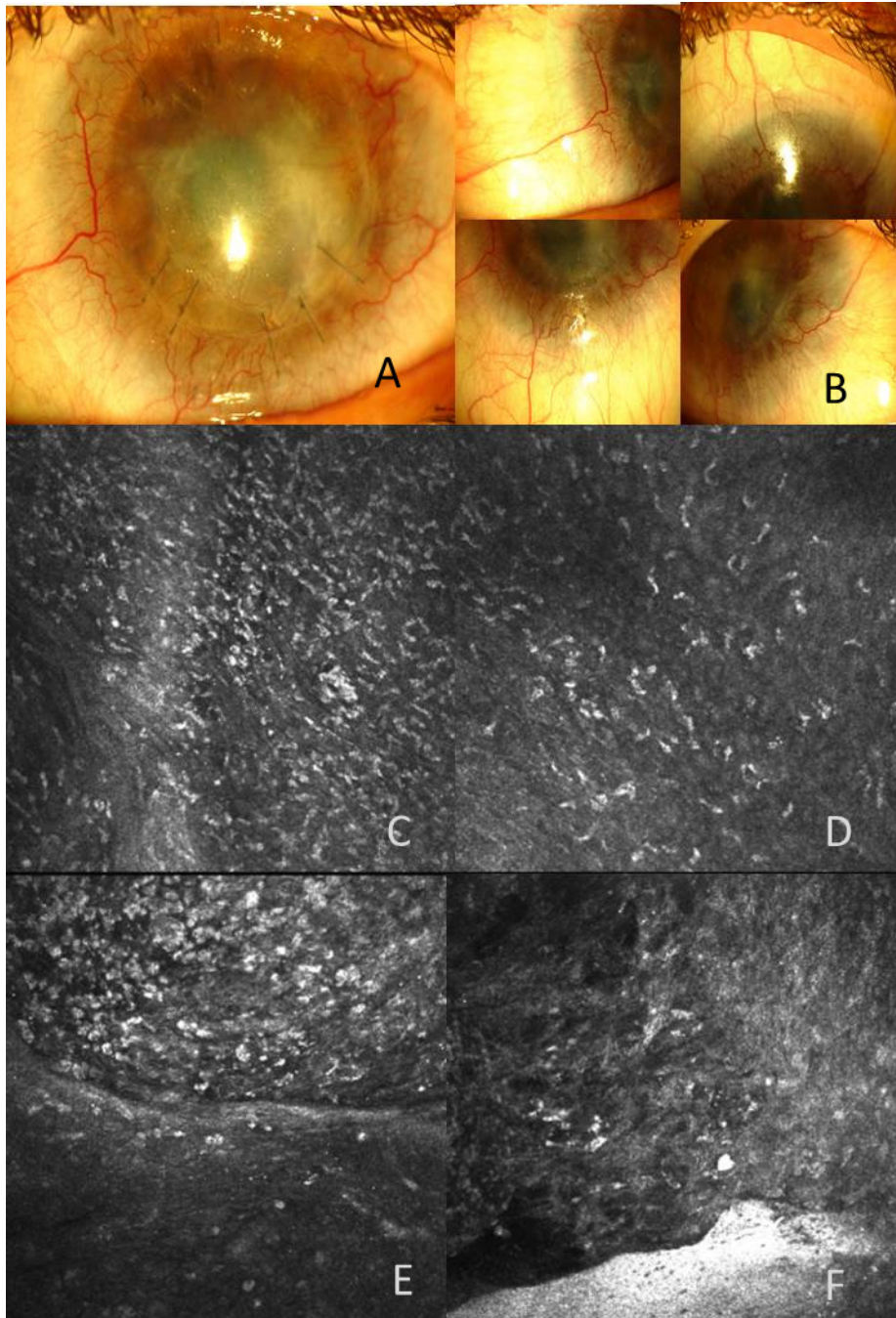
Figure 6. The last case presented a discontinuous epithelium in the central cornea, also characterized by cellular irregular morphology, not clearly corneal, in concordance with CK12 staining negativity.

RESULTS

The biomicroscopic evaluations are reported in the figures (Figure 1A, Figure 2, Figure 3, Figure 4, Figure 5, and Figure 6A) with focus on the superior, inferior, nasal, and temporal limbus (Figure 1B, Figure 2, Figure 3, Figure 4, Figure 5, and Figure 6B) and, for impression citology examination, K12 positivity, indicating the corneal epithelium phenotype, was detected in five of six cases (83.3%), in concordance with the biomicroscopic examination reported in Table 1 and Figure 7A,B.

IVCM: In three cases (50%), the presence in the central cornea of a distinct epithelium with the specific morphological characteristics of the stratified corneal epithelium was identified, in concordance with immunofluorescence staining for CK12, confirming the normal corneal epithelial phenotype (Figure 1C,D, Figure 2C,D, and Figure 3C,D). In

TABLE 1. IVCM EVALUATION OF CENTRAL CORNEA AND LIMBAL AREA.

| Pt.	Surgery	Biomicroscopic examination	cK 12	Superficial transition	Deep transition	Vogt's palisades	Inflammation	IVCM analisys
1	LSCT/PK	Transparent cornea, fibrovascular pannus that reach graft margin	+	sudden	sudden	absent	+	Different areas of normal/ corneal and irregular cells
2	LSCT	Transparent cornea, superficial corneal neovascularization in all quadrants	+	sudden	sudden	absent	++	corneal
3	LSCT/PK	Subepithelial fibrosis, fibrovascular pannus that reach graft margin	+	sudden	sudden	absent	++	corneal
4	LSCT/PK	Corneal Haze, Diffuse corneal neovascularization in superficial and basal layers	-	sudden	sudden	absent	+++	Irregular cells,
5	LSCT/PK	Transparent cornea, fibrovascular pannus that reach graft margin.	+	sudden	sudden	absent	++	corneal
6	LSCT	Transparent cornea, Diffuse epithelial irregularity, superficial neovascularization in one quadrant.	+	sudden	sudden	absent	+	Different areas of normal/ corneal and irregular cells
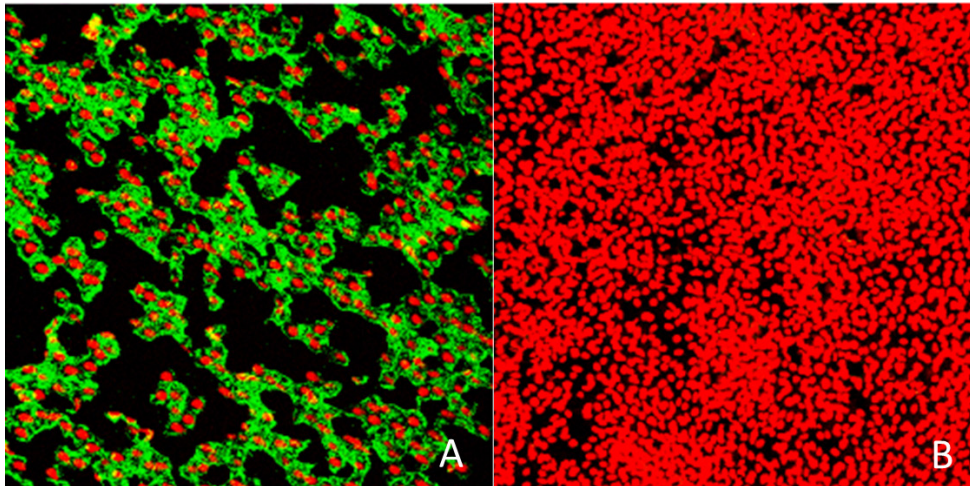
Figure 7. Immunofluorescence: representative images of CK12 positivity and negativity in the central cornea after limbal stem cell transplantation (LSCT) and LSCT/penetrating keratoplasty (PK). Immunofluorescence: representative images of (**A**) CK12 positivity and (**B**) negativity in the central cornea after limbal stem cell transplantation (LSCT) and LSCT/ penetrating keratoplasty (PK).

two cases (33.3%), alternation of different areas of the normal stratified corneal epithelium and irregular morphology and stratification of central epithelial cells were identified even in the presence of CK12 positivity (Figure 4C,D and Figure 5C,D). The last case presented a discontinuous epithelium in the central cornea, also characterized by cellular irregular morphology, not clearly corneal, in concordance with CK12 staining negativity (Figure 6C,D).

All eyes (100%) examined showed irregular morphology of the limbal architecture. The palisades of Vogt were absent in all quadrants of all patients.

The transition of the superficial epithelial morphology in all cases (100%) was abrupt with an immediate transition from a conjunctival-shaped epithelium to a corneal-shaped epithelium in five of the six patients (Figure 1E, Figure 2, Figure 3, Figure 4, and Figure 5E) and to a discontinuous and irregular central epithelium in the last case (Figure 6E). Similarly, deeper in the anterior stroma, an immediate transition between a hyperreflective limbal stroma, without detectable keratocytes and corneal peripheral stroma, and a hyporeflective extracellular matrix and keratocytes was observed in all cases (100%; Figure 1F, Figure 2, Figure 3, Figure 4, Figure 5, and Figure 6F). Different levels of inflammation expressed by the presence of the infiltration of inflammatory cells in the subepithelial and anterior stroma, edema, and activated keratocytes were detected and reported in absolute scale in (Table 1; Figure 8).

DISCUSSION

Ocular burns may destroy the limbus, causing LSCD. Corneal invasion of conjunctival epithelial cells leads to restoration of the ocular surface but also leads to inflammation,

neovascularization, and corneal opacity with the consequent inevitable loss of vision [3,18].

Sclerocorneal limbal restoration represents the only possibility to avoid this process and reconstitute normal corneal transparency; LSCT offers an opportunity to treat patients who have severe uni- or bilateral loss of the corneal epithelium and is applicable when at least a minimum part of the limbus is spared in one of the two eyes [3,18]. Elucidating and quantifying the microstructural changes in the corneal epithelium and in the sclerocorneal limbus, before and after this kind of procedure, is fundamental to understand the surgical prognosis and eventual therapeutic possibilities. Various studies in the literature have described diagnostic alternatives in LSCD [19,20]. IC has been the gold standard diagnostic test for LSCD. Conventional histological stainings, such as hematoxylin, or periodic acid–Schiff, or the Papanicolaou test, are useful methods for detecting epithelial morphology and distinguishing corneal and conjunctival cells; nevertheless, false-negative results are common when the evaluation is based only on cellular morphology and subjective interpretation. Therefore, the application of immunocytochemistry or immunofluorescence on impression cytology specimens is often decisive for identifying the epithelium phenotype. Although several conflicting reports are present in the literature regarding the specificity of cytokeratin expression, cytokeratins 3–12 are regarded as markers of corneal epithelial differentiation while cytokeratin 19 has been reported to be a conjunctiva-specific cytokeratin [19-22].

IVCM provides high-resolution images of the ocular surface at the cellular level with increased application over the last decade that includes the study of the affected limbal microstructures in LSCD [20,23]. In our opinion, both
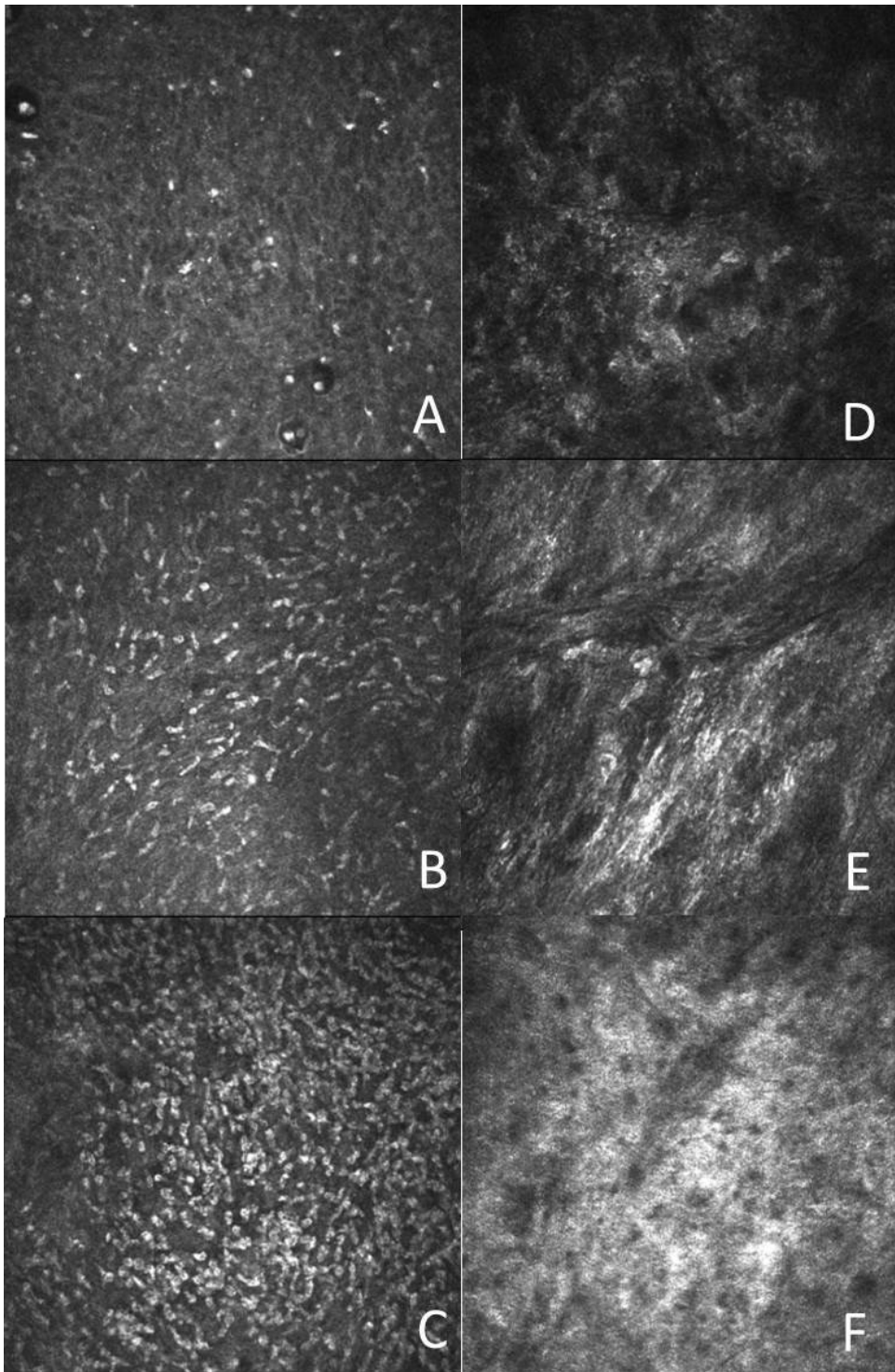
Figure 8. In vivo confocal microscopy inflammation grading. Gradual presence of inflammatory cell infiltration in the subepithelium ranging from (**A**) mild/+ to (**C**) severe/+++ and in the anterior stroma edema and activated keratocytes ranging from (**D**) mild/+ to (**F**) severe/+++.

diagnostic techniques are determinants and not mutually exclusive in evaluating the ocular surface, when accompanied by a proper biomicroscopic examination. The aim of this study was to define, based on IC and IVCM analysis and concordance, real correspondence between clinical and anatomic outcomes of LSCT to correlate staminal function and postoperative anatomic findings. Four of the six patients underwent PK after LSCT to reconstitute corneal transparency, providing a measure of the stromal scar severity after ocular burns. Biomicroscopic characteristics, reported in

Table 1, underlined in four patients (two LSCT and two LSCT/PK) a transparent cornea with fibrovascular pannus or neovascularization that reaches the corneal (and graft) margin without central cornea invasiveness, and in one patient (LSCT/PK) only an associated unperceivable subepithelial fibrosis; these data correlated well in all cases with a diagnosis of CK12 positive corneal phenotype (IC; Figures 1C,D, 2C,D, 3C,D, 4C,D, and 5C,D).

In contrast, one patient presented biomicroscopically a corneal haze with diffuse neovascularization in the superficial and basal layers to support the surgical failure of the limbal stem cell and (subsequent) corneal transplantation, confirmed with IC analysis (CK12 negative staining; Figure 6C,D). The substantial described concordance between the biomicroscopic evaluation and IC was enriched and completed by central corneal IVCM analysis.

IVCM showed evident corneal morphological characteristics in three patients with a certified CK12 positive corneal phenotype and multiple areas of corneal and irregular cells in two patients with CK12 positivity. This disagreement is likely a consequence of the fact that IVCM evaluates corneal surface areas larger than impression cytology immediate examination, which also identifies the presence of morphologically different cellular types (mixed epithelium), despite a positive corneal phenotype presence. Further investigations and studies (i.e., goblet cell presence evaluation in IVCM and/or positivity for other cytokines in IC) would obtain specific cellular distinction.

In the only case of CK12 negative staining, irregular epithelial cells, not clearly corneal, and diffuse hyperreflective cells (likely inflammatory cells suggestive of altered immune status of ocular surface) were identified in the IVCM analysis. Regarding the sclerocorneal limbus IVCM evaluations, the palisades of Vogt were absent in all cases, despite CK12 staining or IVCM analysis results with a total loss of physiological conjunctival/corneal transition.

As already known in the literature, degradation of the palisades structures is correlated with progressive limbal stem cell deficiency in patients not yet submitted to cell therapy reconstructive surgery [10]. Our data emphasized the absence of these structures after LSCT and indifferently in the presence or absence of CK12 positivity.

In 2009, Dua et al. pointed out the existence and survival of a healthy sheet of corneal epithelial cells in the presence of clinically apparent total limbal stem cell (SC) deficiency, suggesting a limited role of limbal epithelial SC in physiological homeostasis of the corneal epithelium. The authors reported, from eight examined eyes, that probably some limbal stem cells or stem cell niches continue to survive though not clinically visible, and contribute to the suggestion, therefore, that the limbus may not have a critical role in physiological corneal epithelial homeostasis [24]. In particular, the authors postulated that stem cells from cell therapy reconstructive surgery are repositioned in the basal layers of the corneal epithelium to sustain the corneal surface cell mass [24].

Similarly, our data showed that physiological corneal epithelial homeostasis can be maintained in the absence of clinically detectable limbal epithelial stem cells and that after LSCT the normal limbal anatomic structure is not reconstructed after successful LSCT as well as not. Therefore, we conclude that IVCM, as a non-invasive and repeatable diagnostic technique, together and in concordance with a biomicroscopic evaluation and IC, provides an adequate evaluation of corneal epithelium characteristics after LSCT and to improve our knowledge about ocular surface conditions in terms of inflammation and renewed stemness.

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