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In vivo confocal microscopy assessment of the corneoscleral limbal stem cell niche before and after biopsy for cultivated limbal epithelial transplantation to restore corneal epithelium

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Summary. Autologous cultivated limbal epithelial transplantation (CLET) is a successful therapy to restore corneal epithelium when limbal epithelial stem cells are damaged unilaterally, which can result in corneal blindness. We used *in vivo* confocal microscopy (IVCM) to identify the best location in the corneoscleral limbal niche and to harvest autologous epithelial stem cells for CLET. We also ascertained the completeness of limbal structure removal after biopsy and followed the healing process for any evidence of limbal structure reappearance. The 4 meridians of the corneoescleral limbus of 5 healthy donor eyes were scanned clinically and by IVCM before biopsy and 1 week, 1, 3, and 6 months after.

IVCM detected palisades of Vogt, the limbal niche hallmark, more efficiently (100%) than clinically (60%), and were consistently better defined at the 12 o'clock meridian, and so this was the site selected for biopsy. The depth of palisades was 80.4±19.8 μm, and of the limbal biopsies was 136.8±19.1 μm, thus assuring that the limbal niche was completely harvested in all cases. Re-epithelialization of the donor site was complete at 1 week. The limbal wound was refilled with fibrovascular tissue, and no limbal-like structures reappeared.

The study shows that clinical absence of palisades of Vogt is not necessarily an exclusionary criterion for autologous CLET. IVCM was useful to select the best place for limbal biopsy and identified features not visible clinically. IVCM also confirmed complete removal of limbal tissue by the biopsy. Limbal niche structures did not reappear by 6 months after surgery.

Key words: Palisades of Vogt, Limbal biopsy, *In vivo* confocal microscopy

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Introduction

The corneoscleral limbus is a transition zone where the transparent cornea meets with the opaque sclera and conjunctiva. It contains the limbal niche composed of the palisades of Vogt that harbor the limbal epithelial stem cells (LESC) (Goldberg and Bron, 1982; Li et al., 2007). These cells play a crucial role in the maintenance of corneal epithelial integrity, renewal, and regeneration (Thoft and Friend, 1983). In fact, LESC depletion, damage, or loss of function results in the so-called limbal stem cell deficiency syndrome (LSCD), which is the hallmark of conjunctivalization of the cornea. This results in an unstable ocular surface that is prone to recurrent epithelial breakdown and chronic non-healing ulceration, causing pain and susceptibility to infection, neovascularization, corneal opacity, and thus resulting in eventual blindness (Dua and Azuara-Blanco, 2000; Dua et al., 2000).

Limbal transplantation, first performed in 1989 (Kenyon and Tseng, 1989), is indicated for corneal epithelial restoration in patients with total LSCD (Tsubota et al., 1999; Miri et al., 2010). More recently, small (2-4 mm^2) limbal explants have been expanded *in*

vitro to circumvent potential complications related to the transplantation of large portions of limbal tissue. Cultivated limbal epithelial transplantation (CLET), first reported by Pellegrini et al. (1997) is currently considered a successful alternative for corneal epithelial reconstruction in cases of LSCD (O'Callaghan and Daniels, 2011). When the source of limbal cells is autologous, a 2-4 mm^2 limbal biopsy is removed from the healthy fellow eye and further cultivated in special clean rooms or cell processing units (Shortt et al., 2008).

It has been suggested that the superior and inferior limbal areas are where the palisades of Vogt are better developed (Shortt et al., 2007a; Miri et al., 2010). However, there are only a few reports describing the method to assure that the biopsy is taken from the limbal location with the best chance to result in a successful transplant (Shortt et al., 2007a). Additionally, there are no descriptions of how to assure that the biopsy is deep enough to remove the entire limbal niche. Lastly, there is little information on the healing process of the limbus as evaluated by *in vivo* confocal microscopy (IVCM) after removal of the donor tissue (Shortt et al., 2007a; Miri et al., 2011).

IVCM is able to generate images of the ocular surface quite close to histologic sections (Baudouin et al., 2008), and it has been used for research on normal ocular surface tissues (Lemp et al., 1985; Cavanagh et al., 1990; Zhivov et al., 2005; Eckard et al., 2006) and to describe various pathological and post-surgical processes (Guthoff and Stave, 2066; Guthoff et al., 2009) there are limited studies that characterize the corneoscleral limbus *in vivo*. However, the following structures have been observed by IVCM in the healthy limbus: (1) palisades of Vogt, characterized by the presence of palisade ridges (Shortt et al., 2007a; Patel et al., 2006; Takahashi et al., 2009) rete pegs or limbal crypts (Patel et al., 2006) and focal stromal projections (Shortt et al., 2007a); (2) limbal-cornea epithelium (Zheng and Xu, 2008); (3) melanocytes in the basal layers of limbal palisades (Patel et al., 2006; Zheng and Xu, 2008) and (4) Langerhans cells in the basal layer of limbal palisades (Zheng and Xu, 2008). IVCM allows direct observation of these features in the limbal niche (Romano et al., 2003; Shortt et al., 2007a)

CLET is being carried out at the Institute of Applied Ophthalmobiology (IOBA), University of Valladolid (Ramírez, 2013) in compliance with the so-called good manufacturing practice (GMP) standards and the European Union Tissues and Cells Directive. Therefore we undertook this study using IVCM with a three-fold aim. The first aim was to analyze the preoperative features of the corneoscleral limbus and identify the regions richer in palisade ridges and rete pegs, and based on this analysis, select the best area for harvesting limbal tissue for therapeutic CLET. Secondly, we wanted to ascertain whether or not the depth of the biopsy was adequate, therefore showing that the structures traditionally described as the limbal niche were contained in the limbal biopsy. Finally, we followed the healing process after limbal biopsy to evaluate whether or not new limbal-like structures would appear.

Materials and methods

Design: Prospective, noncomparative, interventional case series.

Patients participating in this study were already enrolled in a clinical protocol in which the efficacy of CLET was evaluated at IOBA (Ramírez et al., 2013). Inclusion criteria were the diagnosis of unilateral LSCD and the subsequent scheduling of autologous CLET in which a limbal biopsy was used to harvest the LESC. If the potential donor eye had participated in the causative etiology of the LSCD, this event had to have occurred at least 5 years before. In all cases, the donor eye had to be totally asymptomatic and within normal limits under slit-lamp examination (SLE). Exclusion criteria for the donor eye were clinical signs of mild or sectorial LSCD, presence of any concomitant pathology (i.e., allergy, dry eye, and glaucoma), previous ocular surgeries, contact lens wear, or the current use of any topical medication. Only patients who could adequately be followed and subjected to IVCM were included.

Ethical approval was granted by the University of Valladolid Ethics Committee and informed consent was obtained from all patients. All procedures were conducted in accordance with the Declaration of Helsinki regarding human subject research and the provisions of Spanish Organic Law 15/1999 on the Protection of Personal Data, in terms of patient identification, data storage, and statistical analysis.

As part of the CLET protocol, all patients were screened for human immunodeficiency virus 1 and 2, human T-cell leukemia-lymphoma virus, hepatitis B and C, and syphilis prior to the procedure. After clinical history and ophthalmic examination were completed, the donor eye of included patients was again examined by SLE and IVCM 15 days prior to limbal biopsy to select the most suitable site to harvest the LESC. Once the 2x2 mm biopsy was removed, patients were examined the next day by SLE. They were examined again one week later by SLE and IVCM to determine the depth of biopsy and to determine if all limbal niche structures had been removed. Finally, to follow the healing process, the limbal wound site was assessed by SLE for 3 years and by IVCM 6 months after the biopsy.

SLE and limbal area

SLE and anterior segment photographs of the donor limbus were performed 15 days before surgery from all 360° of the limbus (16x magnification) and from the superior, inferior, nasal, and temporal quadrants (25x magnification). The same evaluations were performed after 24 h, 1 week, 1 month, 3 months, 6 months, 12 months, 2 years, and 3 years after biopsy, but only in the quadrant where the limbal biopsy was removed.

Fluorescein staining under SLE was used to evaluate the epithelial defect left after surgical biopsy and epithelial stability in the central cornea. Variables assessed by this method were the presence of palisades of Vogt, fibrosis, vascularization, fluorescein staining in the biopsy site or at any other place on the cornea, and grade of hyperemia according to Efron scale (Efron et al., 2001). Photographs were taken with slit-lamp TOPCON SL-8Z (Topcon Corp., Tokyo, Japan) and IMAGENet software (Fuji Fujifilm Finepix S1 Pro, Tokyo, Japan).

In vivo confocal microscopy (IVCM) of the limbal area

The Rostock Cornea Module and Heidelberg Retinal Tomograph HRT-3 (HRT3. Heidelberg engineering GmbH, Heidelberg, Germany) were used for IVCM examination of the limbal niche. Topical anesthesia was achieved with 0.1% tetracaine chlorhydrate and 0.4% oxibuprocaine chlorhydrate solution (Colircusí Anestésico Doble®, Alcon Laboratories, Ft. Worth, TX, USA). For depth measurement, IVCM optical sections from each limbal quadrant were collected by scanning from the epithelium towards the limbal palisades and anterior stroma at a single point. The emergence of the anterior corneal stroma was taken as the bottom of the wounded area. Then a lateral scan was performed to observe the following limbal features: (1) palisades of Vogt consisting of (a) palisade ridges, (b) limbal crypts or rete pegs, and (c) focal stromal projections, (2) limbal-corneal epithelium present as light and dark cell areas, (3) melanocytes appearing as hyperreflective cells, (4) Langerhans cells appearing as bright corpuscular cells, (5) neovascularization, and (6) fibrosis. Before biopsy, each of the 4 quadrants was evaluated to choose the most suitable one for biopsy. After surgery, IVCM evaluation was done only at the biopsied site at 1 week, 1 month, 3 months, and 6 months postoperatively.

Biopsy surgical procedure

Autologous biopsies were obtained from the region of the corneoscleral limbus where the palisades of Vogt were better defined by IVCM, i.e., where more palisade ridges, rete pegs and focal stromal projections were visualized. All surgical operations were performed by the same surgeon (coauthor JMH) and were carried out in a standard manner, under topical anesthesia, as described for IVCM, and following the standards for aseptic procedures. A lamellar biopsy of the limbus measuring 1-2 mm in tangential diameter was made. Beginning in the sclera, 0.5-1.0 mm posterior to the limbus, the biopsy continued through the limbus and extended 0.5-1.0 mm into the clear cornea. The incision deepened up to the anterior corneal stroma. The biopsied tissue was immediately placed in sterile transport medium and sent to the Cell Processing Clean Room (Institute of Molecular Biology and Genetics, University of Valladolid) for expansion on amniotic membrane under the explant culture method previously described (Shortt 2007b). After 3-4 weeks, the expanded tissue was returned to our institution for CLET. The donor eye was patched until the next day. A mixture of 0.3% tobramycin and 0.1% dexamethasone eye drops (Tobradex®, Alcon Laboratories) was applied 3 times daily for 2 weeks after surgery.

Statistical analysis

Descriptive statistics such as means and standard deviations were determined with the Statistical Package for the Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, USA).

Results

Five out of the eleven autologous CLET cases enrolled in our CLET study (Ramírez et al., 2013) were recruited. The first four eyes undergoing autologous CLET were done before the current clinical protocol was approved, and therefore images of the limbus were not obtained. Therefore, 5 healthy eyes of 5 Caucasian patients (3 males, 2 females; 44.0±17.3 years old, range 26-69 years) were recruited and followed for 6 months. The etiologies of LSCD in the diseased eyes, as well as

Table 1. Patient and palisades of Vogt characteristics before and after limbal biopsy.

Patient No./ Gender age	Cause of LSCD	Palisades of Vogt by SLE*	Palisades of Vogt structures in each quadrant by IVCM†				Depth of palisades of Vogt†	Biopsy site depth‡
			Superior	Inferior	Nasal	Temporal	Starting/ending	
1/Male 26	Acanthamoeba keratitis. failed PKP	M6, M12	PR, RP, FSP	PR,RP	PR, RP	PR, RP	25/95 μm	146 μm
2/Female 69	Infectious keratitis (unknown origin) in childhood	None	PR, RP	PR,RP	PR, RP	None	32/46 μm	111 μm
3/Male 36	Chemical burn	M6, M12	PR, RP	PR,RP, FSP	PR,RP	None	25/91 μm	161 μm
4/Female 55	Multiple surgeries due to retinal detachment, failed PKP	M6, M12	PR, RP, FSP	PR,RP	PR, RP	PR, RP	30/88 μm	126 μm
5/Male 53	Chemical burn	None	PR, RP, FSP	PR,RP	None	None	24/82 μm	140 μm

LSCD, limbal stem cell deficiency syndrome; PKP, penetrating keratoplasty; SLE, slit-lamp examination; IVCM, *in vivo* confocal microscopy; M6, 6 o'clock limbal meridian; M12, 12 o'clock limbal meridian; PR, palisade ridges; RP, rete pegs; FSP, focal stromal projections. Biopsy depth= 136.8±19.1 μm. *, determined by SLE of donor eye 15 days before biopsy; †, determined by IVCM of donor eye 15 days before biopsy; ‡, determined by IVCM of donor eye one week after biopsy.

the clinical SLE and IVCM characteristics of the donor eyes are shown in Table 1. Three donor eyes had no prior history of disease. Two patients thought that their healthy eye had been somewhat damaged by the same cause of LSCD as the affected eye. In both cases, the causes, infectious keratitis for Patient 2 and chemical injury for Patient 5, were more than 5 years in the past. These two donor eyes were completely asymptomatic and the ocular surface of all 5 eyes looked totally normal at SLE evaluation, therefore meeting the inclusion criteria.

The expansion and culture on amniotic membrane of the biopsy-derived limbal explants were successful and presented similar outgrowth rates in all cases. CLET surgeries were uneventful, and the clinical results were defined as successful after one year of follow-up in all 5 patients (Ramírez et al., 2013). The donor eye best corrected visual acuity was 20/20 for all 5 eyes before the biopsy, and this was unaffected by the procedure. There were no postoperative infections or any other complication. The healing process for the donor eye was limited to the area of the biopsy, without side effects on the central cornea.

Preoperative selection of biopsy site

At the preoperative assessment of the donor eye by SLE, palisades of Vogt were found in 60% of patients (Table 2). Patients 1, 3, and 4 presented palisades of Vogt only in the vertical meridians (Table 1). These structures were not seen in the horizontal meridians in any patient. In Patients 2 and 5, palisades were not observed in any limbal meridian, in spite of showing no signs of LSCD. In contrast, palisades of Vogt were detected by IVCM in all four meridians in Patients 1 and 4, in the superior, inferior, and nasal meridians in Patients 2 and 3, and in the 12 and 6 o'clock meridians in Patient 5. Figure 1 shows findings for Patient 5 by SLE and IVCM.

Palisade ridges and rete pegs became visible at a

Table 2. Slit-lamp examination findings during wound healing of the limbal biopsy donor site.

Elapsed-time from limbal biopsy	Palisades of Vogt %	Fluorescein staining %	Fibrosis %	Vascularization %
-15 days	60	0	0	0
1 day	0	100	0	0
1 week	0	0	100	20
1 month	0	0	100	60
2 months	0	0	100	100
3 months	0	0	100	100
6 months	0	0	100	100
1 year	0	0	100	100
2 years	0	0	100	100
3 years	0	0	100	100

N= 5 eyes

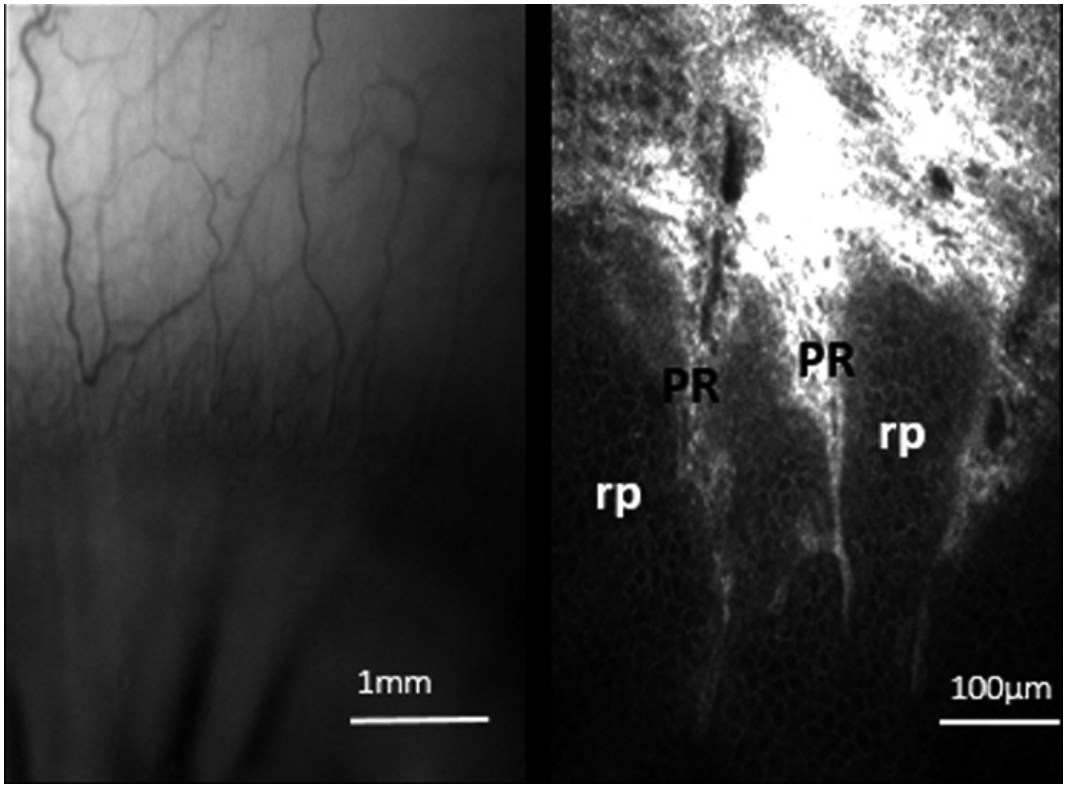

Fig. 1. Comparison of slit-lamp examination and *in vivo* confocal microscopy (IVCM) ability to resolve the palisades of Vogt (Patient 5). The slit-lamp image (left) showed no clinical evidence of palisades of Vogt in the donor eye. In contrast, the palisades were evident in the same site by IVCM (right). PR, palisade ridges; rp, rete pegs.

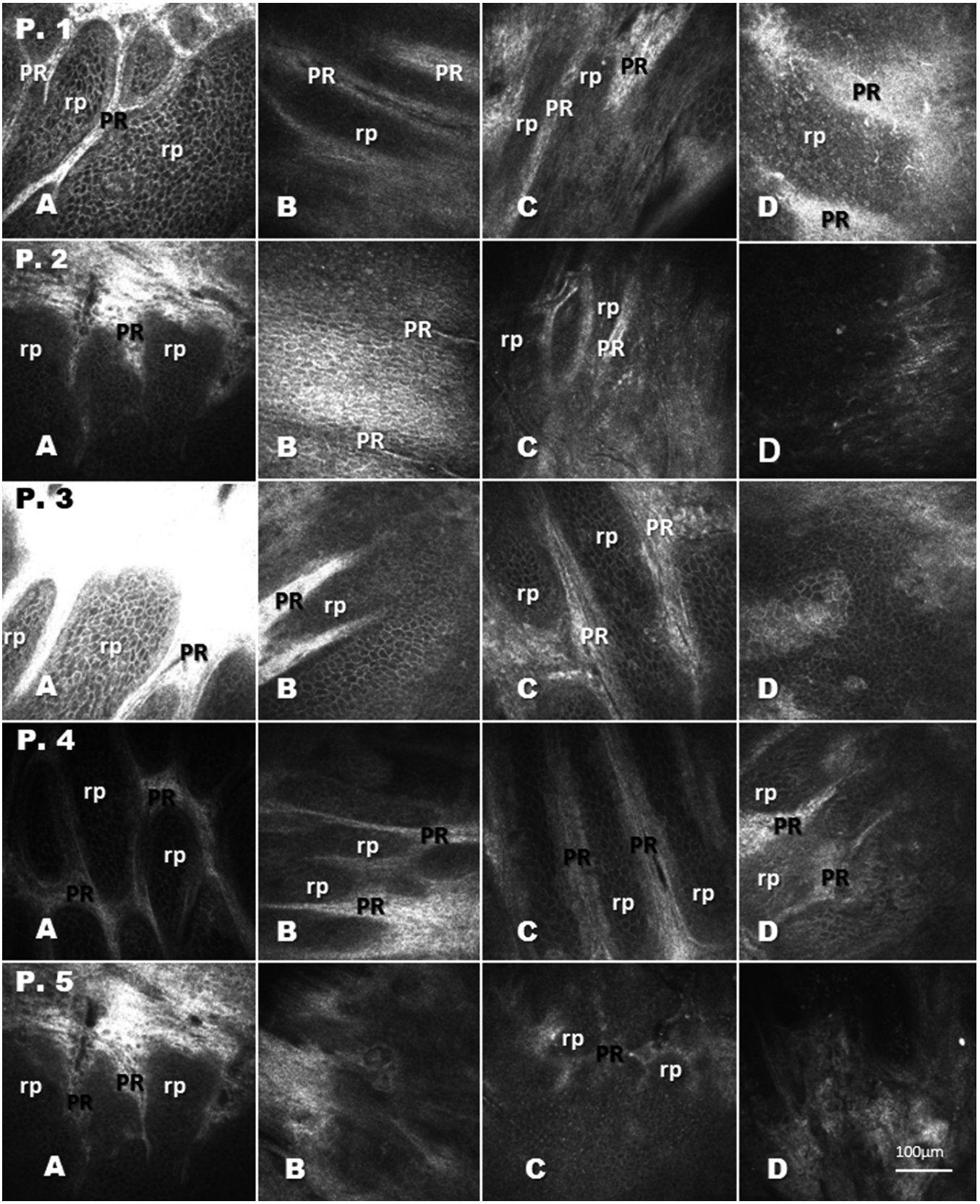

Fig. 2. *In vivo* confocal microscopy images of the palisades of Vogt in the four meridians of Patients 1 to 5 (P. 1 - P.5). **A.** Superior meridian. **B.** Nasal meridian. **C.** Inferior meridian. **D.** Temporal meridian. PR, palisade ridges; rp, rete pegs, more frequently observed in the superior meridian.

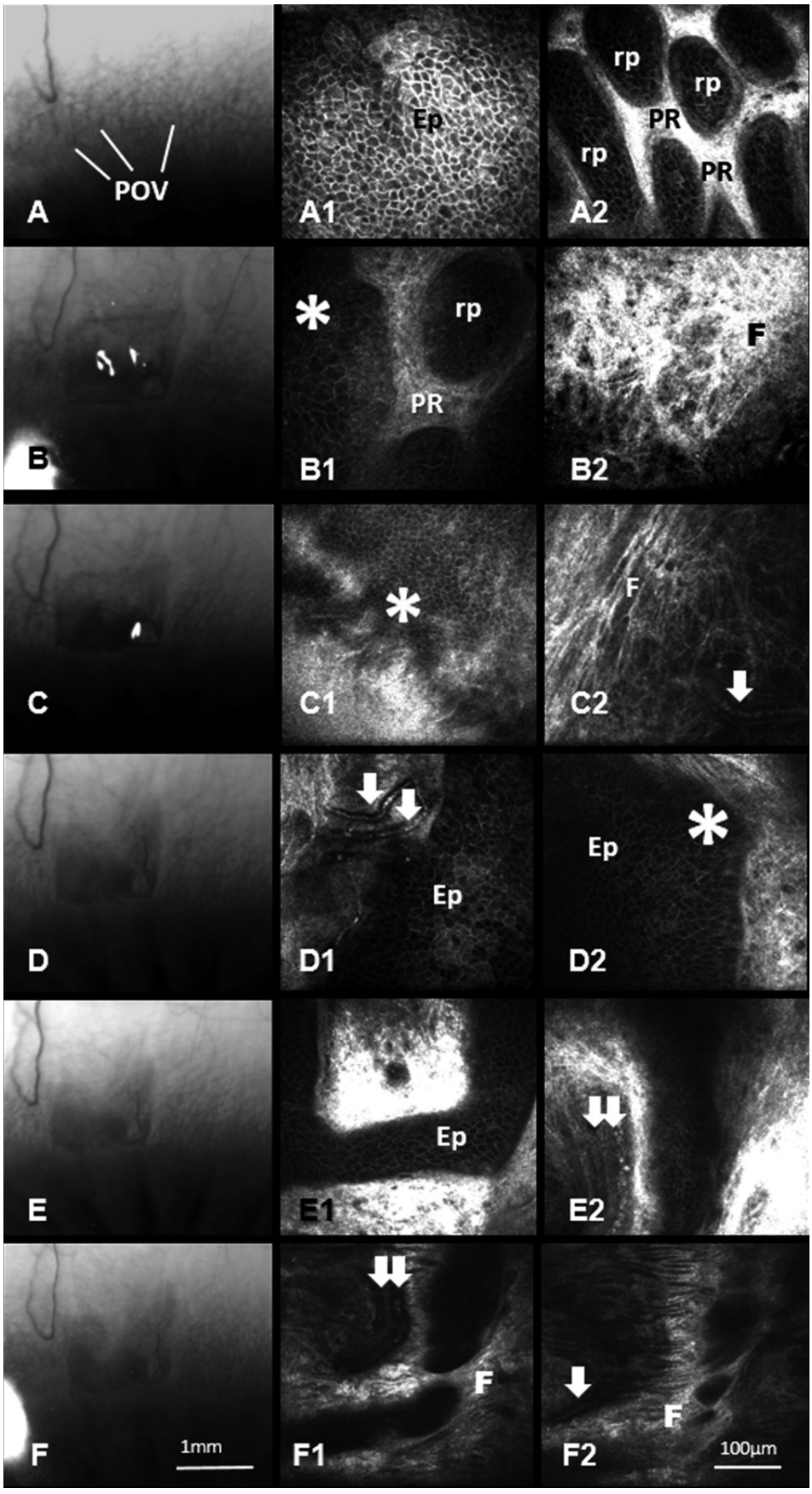

Fig. 3. Slit-lamp examination (SLE) and *in vivo* confocal microscopy (IVCM) images of the healing process in Patient 4. SLE (left panel) and IVCM photographs (middle and right panels, showing two different areas of 400 µm^2 each), of the biopsied site at the following time points: **A-A2,** initial evaluation 15 days before biopsy; **B-B2,** 1 week post-biopsy; **C-C2,** 1 month; **D-D2,** 2 months; **E-E2,** 3 months, and **F-F2,** 6 months. POV, palisades of Vogt; Ep, normal epithelium; PR, palisade ridges; rp, rete pegs; F, Fibrosis; white and black arrows, blood vessels; asterisk, biopsy limit.

mean depth of 27.2±3.56 μm and were not visible below 80.4±19.8 μm. Thus the mean height was 53.2±22.5 μm. Palisades of Vogt structures were better defined in the 12 o'clock meridian of the superior limbal quadrant (Fig. 2). Additionally, the rete pegs and focal stromal projections were more frequently observed in the superior quadrant (Table 1). Consequently, the 12 o'clock meridian of the superior quadrant was the site selected as best to harvest limbal stem cells in all 5 eyes.

Assessment of biopsy depth and limbal remnants

By IVCM at 7 days post-biopsy, the mean wound depth was 136.8±19.1 μm (Table 1). The bottom of the biopsy was usually preceded by fibrosis in the center of the wound area. Blood vessels were usually present at the periphery. After the biopsy, normal structures of the palisades of Vogt were not observed and no remnants were found by IVCM in any eye.

Healing process of the biopsy wound site

At 1 day after biopsy and on all other follow-up days, the patients were asymptomatic or had only mild and transient irritation. Grade 3 hyperemia lasted one week in Patient 3 and persisted for one month in Patients 1, 2, and 4. In Patient 5, hyperemia remained until the third month, but only around the biopsy site.

For all patients on the first day after biopsy, there was fluorescein staining in the site but not beyond (Table 2). The absence of fluorescein staining at 1 week

Table 3. *In Vivo* confocal microscopy findings in the limbal biopsy wound healing process.

Elapsed-time from limbal biopsy	Palisades of Vogt structures			LCE %	Melanocytes %	Fibrosis %	Vasc %	Goblet Cells %	Langerhans cells %
	PR %	RP %	FSP %						
-15 days	100	100	40	60	0	0	20	0	0
1 week	0	0	0	60	0	100	20	0	40
1 month	0	0	0	60	0	100	100	20	60
2 months	0	0	0	100	0	100	100	0	60
3 months	0	0	0	100	0	100	100	0	40
6 months	0	0	0	100	0	100	100	20	40

Limbal normal findings: Palisades of Vogt structures: PR, palisade ridges;RP, rete pegs; FSP, focal stromal projections; LCE, limbal-corneal epithelium; Vasc, vascularization; N= 5 eyes. % indicates the percentage of patients in which a specific finding was encountered.

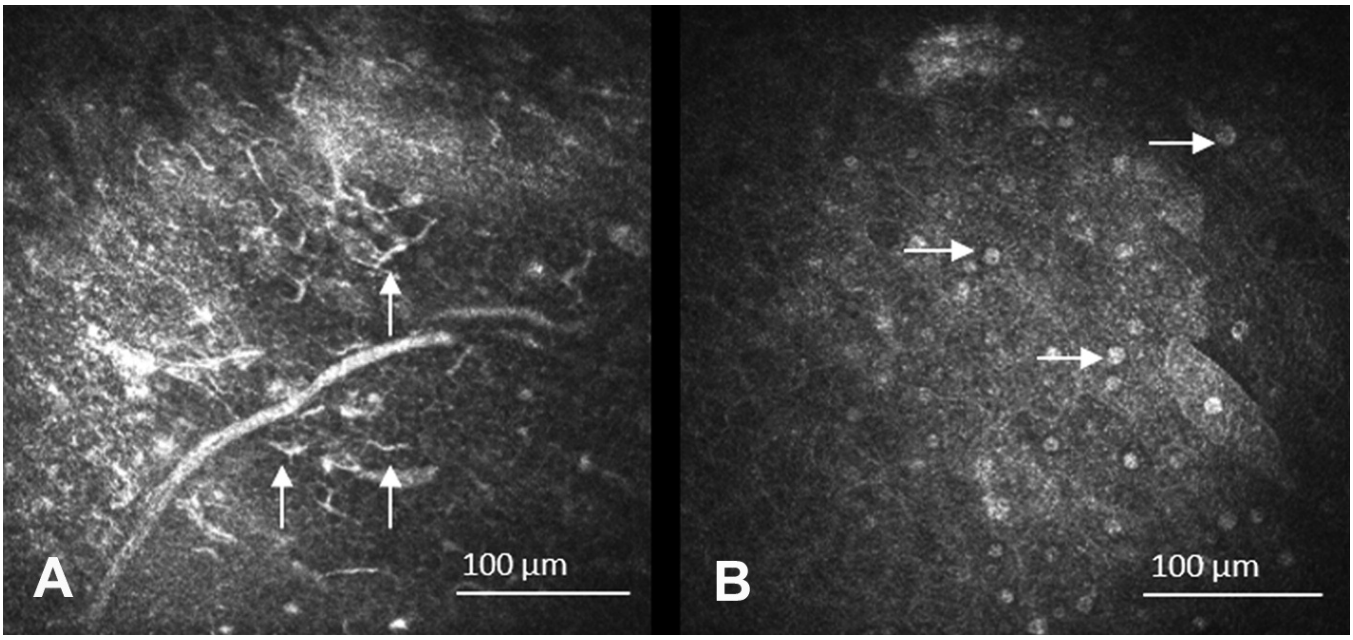

Fig. 4. *In vivo* confocal microscopy images of the healing limbal area one month after biopsy, showing (arrows): **A.** Langerhans cells (Patient 3) and **B.** goblet cells (Patient 2).

indicated that the superficial re-epithelialization was complete. At the first month after biopsy, fibrosis was observed in all cases, moving forward from the scleral edge of the wound towards the corneal edge. There was vascularization surrounding the biopsy edges in one eye at 1 week, in 3 eyes at 1 month, and in one eye at 3 months. From the third month and on, the fibrotic tissue remained stable.

IVCM allowed a more complete and detailed observation of the healing process (Table 3, Fig. 3). At 1 week post-biopsy, there was fibrosis in the periphery and at the bottom of the donor limbal site in all 5 patients. Instead of the normal palisades, the epithelium was deeper than in the adjacent zones. At the bottom of the donor site, the epithelium was surrounded by anterior stroma, and in more superficial zones it was surrounded by the normal palisades of Vogt located outside the wounded area. After 1 month, all patients had vascularization at the edges and bottom of the donor site. Vascularization and fibrosis persisted, moving upwards and forward to the center of the donor site until 6 months after the biopsy. Langerhans cells were frequently seen after the first week post-biopsy (Fig. 4). The limbal-corneal epithelium was identified in 60% of the patients at 1 week (Patients 2, 3, and 5), in 80% at 3 months (Patients 1, 3, 4, and 5), and in all patients at 6 months. Melanocytes were never observed.

IVCM also enabled the observation of changes in the epithelium overlying the biopsy site. For Patient 1 at one week after the procedure, the epithelium at the site of the biopsy had the corneal epithelial phenotype. However 6 months later, the epithelium had acquired a conjunctival phenotype, composed of large polymorphic cells, though no goblet cells were detected. In Patient 2, the epithelial phenotype was conjunctival both before and after the biopsy. For this patient, goblet cells were observed at 1 and at 6 months (Fig. 4). In Patients 3, 4, and 5, the epithelium presented the corneal phenotype in every evaluation. These changes were limited to the biopsy site, as the central cornea was never affected.

Discussion

While the length (0.031 mm) and width (0.04 mm) of palisades of Vogt have been described (Townsend, 1991), to the best of our knowledge, we are the first to quantify the height and depth of the palisades of Vogt, which contain the limbal stem cells. Several reports state that the palisades are concentrated in the upper and lower limbal meridians (Goldberg and Bron, 1982; Townsend, 1991; Shortt et al., 2007a; Zeng and Xu, 2008; Takahashi et al., 2009) and our results agree with those findings. Furthermore, we found, that in all our patients, the 12 o'clock meridian was the best location in the corneoscleral limbal niche, for harvesting limbal stem cells, for autologous CLET. We also determined that the removal of limbal structures after biopsy was complete in all cases. Additionally, we have shown that the limbal healing process at the biopsy site replaced niche structures with fibrovascular tissue in the absence of re-growth of any limbal structures.

In other reports, the method of evaluation and selection of the limbal biopsy donor site was not specified (Pellegrini et al., 1997; Nakamura et. a., 2006; Rama et al., 2010). Therapeutic CLET depends, among other factors, on the adequate harvesting of LESC. It is therefore of paramount importance to select the best possible site for the limbal biopsy to optimize the LESC culture and *in vitro* expansion. We found in this study that IVCM images can help target the best region of the limbus to collect the LESC. This was accomplished by directly observing and acquiring high quality images of the rete pegs and focal stromal projections that are the niche of the LESC (Chen and Tseng, 1990; Thoft and Friend, 1983; Shortt et al., 2007a). While all SLE findings were corroborated by IVCM, some structures could not be observed by routine SLE. Thus, the clinical absence of palisades of Vogt as observed by SLE is not necessarily an exclusionary criterion for autologous CLET. In two of our patients, IVCM identified limbal features that were not visible by SLE. Thus IVCM was useful to select the best place for the limbal biopsy.

Takahashi et al. (2009) found that by IVCM, the palisades of Vogt were more frequently detected in the superior and inferior meridian, which is consistent with our findings. Using SLE, they also found palisades of Vogt in more patients than by IVCM. These findings do not match ours, as we observed palisades of Vogt structures by IVCM in 2 patients in which they were not clinically evident by SLE. The reason for this disagreement could be due to the age of the patients, the oldest of which was 87 years in Takahashi's study, while our oldest patient was 69 years old.

Shortt et al. (2007a,b) reported that the focal stromal projections and the rete pegs were prominently located in the vertical meridians, similar to our observations of palisade ridges, rete pegs, and the focal stromal projections in the 12 and 6 o'clock meridians. We found that the rete pegs were more abundant in the 12 o'clock meridian, which suggested that this site was the best location to harvest the LESC.

Using IVCM, Miri et al. (2012) recently described solid cords of cells extending from the bottom of the conjunctival side of the palisade ridges, referring to these structures as the limbal epithelial crypts. We did not observe such structures, but we collected only a few IVCM images from the conjunctival side of the limbus. Similar to their findings, we also observed interconnections in the adjacent palisade ridges. Miri et al. also found that the appearance of the basal epithelial cells was better defined in moderately pigmented subjects. We observed the basal cells in all patients even though none of them presented pigmented palisades. This absence of pigment might be due to the Caucasian origin of the patients evaluated.

Initially, Patients 2 and 5 were going to have allogeneic CLET, but due to the IVCM findings where palisades of Vogt were clearly identified, we decided to

harvest and cultivate a limbal biopsy. The outgrowth rate of LESC on amniotic membrane culture for these two patients was similar to the other cases. Moreover the successful outcome of CLET in these patients was similar to the others (Ramirez et al., 2013). This suggests that IVCM is a useful technique to evaluate and select the site for limbal biopsy and can be especially useful in those patients for whom clinical evaluation by SLE is uncertain. Limbal palisades of Vogt cannot be clinically identified in up to 20% of healthy individuals (Townsend, 1991) Our results suggest that the absence palisades of Vogt detectable by SLE should not be an absolute exclusion criteria for autologous CLET provided that palisade ridges, rete pegs and focal stromal projections can be demonstrated by IVCM in an otherwise healthy ocular surface.

The presence of stem cells in the cultured cell population is usually evaluated by the colony forming ability of transplanted cells, the immunohistological analysis of the grafts, and the evidence of donor cell survival (Shortt et al., 2007b). We found that prior to all these evaluations, IVCM of the biopsy site can ensure that the structures harboring the LESC are appropriately harvested. We also showed that the depth of the biopsy site was consistently greater than the depth of the palisades.

As observed by IVCM after limbal biopsy, the healing process began with re-epithelialization of the donor site. Later growth of fibrovascular tissue, often not visible by SLE up to 2 months, developed in the bottom and edges of the donor site, under the epithelium. Vascularization was evident by IVCM in all cases from the first month, but by SLE it was detected in only 60% of patients at two months and in all patients thereafter. As seen by IVIVCM at six months after biopsy and by SLE after 3 years, there was no re-establishment of the corneoscleral limbus niche structures, i.e., palisade ridges, rete pegs, or focal stromal projections in the wound site.

Kinoshita et al. (1982) found goblet cells on the cornea after epithelial wound healing when the limbal epithelium was removed. In our study, goblet cells were found in Patient 2 only at one month, and it was limited to the biopsy area. The central cornea was always stable in this patient, which suggests that the presence of the goblet cells was a normal part of the healing process, as has been described when the limbus is partially removed.

Miri et al. (2011) evaluated the limbal donor site by SLE and by *in vivo* IVCM. They showed that removal of two limbal pieces, superior and inferior, was not associated with any residual long-term adverse effect in the donor eye. They also found that 10 eyes were re-epithelialized with conjunctival epithelium and 5 with normal corneal epithelium. Three of our patients showed normal corneal epithelium on the donor site while the other two cases had the conjunctival epithelial phenotype. In both studies these changes were limited to the donor site. The time to complete re-epithelialization in our patients was one week faster, probably due to the smaller size of our biopsies (1-2 mm^2 versus 2 o'clock hours).

In Patients 2, 3, and 5, Langerhans cells were present at 1 month. In Patients 1 and 4, Langerhans cells were observed only in the evaluations at 1 and 2 months respectively. It is possible that Langerhans cells were present at each evaluation period but not observed by IVCM because only a small portion of the biopsy site was imaged. For all 5 patients, both SLE and IVCM revealed the absence of pigment and the presence of fibrosis in every evaluation.

There are some drawbacks in the use of IVCM for the types of analyses described here. First, the area of observation is very small, 400 x 400 μm. Second, IVCM depends entirely on patient cooperation. In patients with photophobia, nystagmus, etc., it is a serious challenge to correctly capture the necessary images. Third, there is a learning curve for the IVCM operator, and it requires an experienced examiner to capture high quality images. Finally, IVCM incurs additional costs and from 10-20 minutes of extra examination time. Nevertheless, it is a useful technique, not only for the reasons presented above, but also because it is a reliable and objective way to judge the corneal phenotype, a necessary evaluation endpoint in clinical trials involving CLET (Shortt et al., 2007b). Though IVCM is not currently mandatory for patients undergoing CLET, it may have advantages over corneal impression cytology, which removes 3-4 layers of epithelium, in assessing the healing of the biopsy wound site.

In conclusion, we corroborated other authors' findings regarding the limbal vertical meridians, especially the superior one, as the best site for harvesting limbal biopsies for autologous CLET. We also showed that the clinical absence of palisades of Vogt as assessed by SLE does not always represent the absence of the limbal niche structures. Thus, when imaged by IVCM, cases that might be judged by SLE as unsuitable for autologous CLET could actually be suitable. We also measured the depth of the rete pegs and showed that regular limbal biopsies completely removed all of the necessary structures for harvesting limbal cells. Lastly, we described the healing process of a limbal biopsy up to 6 months by IVCM and up to 3 years by SLE, documenting the absence of side effects in healthy eyes and that the limbal niche structures do not reappear.

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References

Baudouin C., Labbé A. and Dupas B. (2008). Imaging the ocular surface with confocal microscopy: histology without samples [in French]. J. Fr. Ophtalmol. 31,308-316.

Cavanagh H.D., Jester J.V., Essepian J., Shields W. and Lemp M.A. (1990). Confocal microscopy of the living eye. CLAO J. 16, 65-73.

Chen J.J. and Tseng S.C. (1990) Corneal epithelial wound healing in partial limbal deficiency. Invest. Ophthalmol. Vis. Sci. 31, 1301-1314.

Dua H.S. and Azuara-Blanco A. (2000). Limbal stem cells of the corneal epithelium. Surv. Ophthalmol. 44, 415-425.

Dua H.S., Saini J.S., Azuara-Blanco A. and Gupta P. (2000). Limbal stem cell deficiency: concept, etiology, clinical presentation, diagnosis and management. Ind. J. Ophthalmol. 48, 83-92.

Eckard A., Stave J. and Guthoff R.F. (2006). *In vivo* investigations of the corneal epithelium with the confocal Rostock Laser Scanning Microscope (RLSM). Cornea 25, 127-131.

Efron N., Morgan P.B. and Katsara S.S. (2001). Validation of grading scales for contact lens complications. Ophthalmic. Physiol. Opt. 21,17-29.

Goldberg M.F. and Bron A.J. (1982). Limbal palisades of Vogt. Trans Am. Ophthalmol. Soc. 80, 155-171.

Guthoff R.F. and Stave J. (2006). *In vivo* micromorphology of the cornea: confocal microscopy principles and clinical applications. In: Cornea and external eye disease. Reinhard T. and Larkin D.F.P. (eds). In: Essentials in Ophthalmology. Krieglstein G.K. and Weinreb R.N. (series eds). Springer. Berlin. pp 173-208.

Guthoff R.F., Zhivov A. and Stachs O. (2009). *In vivo* confocal microscopy, an inner vision of the cornea - a major review. Clin. Exp. Ophthalmol. 37, 100-117

Kenyon K.R. and Tseng S.C. (1989). Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96, 709-723.

Kinoshita S., Kiorpes T.C., Friend J. and Thoft R.A. (1982). Limbal epithelium in ocular surface wound healing. Invest. Ophthalmol. Vis. Sci. 23, 73-80.

Lemp M.A., Dilly D.N. and Boyde A. (1985). Tandem-scanning (confocal) microscopy of the full thickness cornea. Cornea 4, 205-209.

Li W., Hayashida Y., Chen Y.T. and Tseng S.C. (2007). Niche regulation of corneal epithelial stem cells at the limbus. Cell Res. 17, 26-36.

Miri A., Al-Deiri B. and Dua H.S. (2010). Long-term outcomes of autolimbal and allolimbal transplants. Ophthalmology 117, 1207-1213.

Miri A., Said D.G. and Dua H.S. (2011). Donor site complications in autolimbal and living-related allolimbal transplantation. Ophthalmology 118, 1265-1271.

Miri A., Al-Aqaba M., Otri A.M., Fares U., Said D.G., Faraj L.A. and Dua, H.S. (2012). *In vivo* confocal microscopic features of normal limbus. Br. J. Ophthalmol. 96, 530-536.

Nakamura T., Inatomi T., Sotozono C., Ang L. P., Koizumi N., Yokoi N. and Kinoshita, S. (2006). Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 113, 1765-1772.

O'Callaghan A.R. and Daniels J.T. (2011). Concise review: limbal epithelial stem cell therapy: controversies and challenges. Stem Cells 29, 1923-1932.

Patel D.V., Sherwin T. and McGhee C.N. (2006). Laser scanning *in vivo* confocal microscopy of the normal human corneoscleral limbus. Invest. Ophthalmol. Vis. Sci. 47, 2823-2827.

Pellegrini G., Traverso C.E., Franzi A.T., Zingirian M., Cancedda R. and De Luca M. (1997). Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 349, 990-993.

Rama P., Matuska S., Paganoni G., Spinelli A., De Luca M. and Pellegrini, G. (2010). Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. 363, 147-155.

Ramírez B.E., Pastor M., Herreras J., Pérez I., Garcia-Sancho J., Sanchez A. and Calonge M. (2013). Treatment of ocular surface failure due to limbal stem cell deficiency by cultivated limbal epithelial cell transplantation (CLET). Invest. Opthalmol. Vis. Sci. [Suppl] 54, 5433

Romano A.C., Espana E.M., Yoo S.H., Budak M.T., Wolosin J.M. and Tseng S.C. (2003). Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. Invest. Ophthalmol. Vis. Sci. 44, 5125-5129.

Takahashi N., Chikama T., Yanai R. and Nishida T. (2009). Structures of the corneal limbus detected by laser-scanning confocal biomicroscopy as related to the palisades of Vogt detected by slit-lamp microscopy. Jpn. J. Ophthalmol. 53, 199-203.

Thoft R.A. and Friend J. (1983). The X, Y, Z hypothesis of corneal epithelial maintenance [letter]. Invest. Ophthalmol. Vis. Sci. 24, 1442-1443.

Townsend W.M. (1991). The limbal palisades of Vogt. Trans. Am. Ophthalmol. Soc. 89, 721-756.

Tsubota K., Satake Y., Kaido M., Shinozaki N., Shimmura S., Bissen-Miyajima H. and Shimazaki J. (1999). Treatment of severe ocular surface disorders with corneal epithelial stem-cell transplantation. N. Engl. J. Med. 340, 1697-1703.

Shortt A.J., Secker G.A., Munro P.M., Khaw P.T., Tuft S.J. and Daniels, J.T. (2007a). Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit *in vivo* observation and targeted biopsy of limbal epithelial stem cells. Stem Cells 25, 1402-1409.

Shortt A.J., Secker G.A., Notara M.D., Limb G.A., Khaw P.T., Tuft S.J. and Daniels J.T. (2007b). Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. Surv. Ophthalmol. 52, 483-502.

Shortt A.J., Secker G.A., Rajan M.S., Meligonis G., Dart J.K., Tuft S.J. and Daniels J.T. (2008). Ex vivo expansion and transplantation of limbal epithelial stem cells. Ophthalmology 115, 1989-1997.

Zheng T. and Xu J. (2008). Age-related changes of human limbus on *in vivo* confocal microscopy. Cornea 27, 782-786.

Zhivov A, Stave J, Vollmar B, Guthoff R. (2005). *In vivo* confocal microscopic evaluation of Langerhans cell density and distribution in the normal human corneal epithelium. Graefes Arch. Clin. Exp. Ophthalmol. 243, 1056-1061.

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