A Proof-of-Concept Clinical Trial Using Mesenchymal Stem Cells for the Treatment of Corneal Epithelial Stem Cell Deficiency

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List of abbreviation

AEMPS: Spanish Agency of Medicines and Sanitary Products

CLET: cultivated limbal epithelial transplantation

hAM: human amniotic membrane

LSCD: limbal stem cell deficiency

MSC: mesenchymal stem cells

MSCT: mesenchymal stem cell transplantation

ABSTRACT

Ocular stem cell transplantation derived from either autologous or allogeneic donor corneoscleral junction is a functional cell therapy to manage extensive and/or severe limbal stem cell deficiencies that lead to corneal epithelial failure. Mesenchymal stem cells have been properly tested in animal models of this ophthalmic pathology, but never in human eyes despite their potential advantages. We conducted a 6- to 12-month proof-of-concept, randomized, double-masked pilot trial to test whether allogeneic bone marrow-derived mesenchymal stem cell transplantation (MSCT, n=17) was as safe and as equally efficient as allogeneic cultivated limbal epithelial transplantation (CLET, n=11) to improve corneal epithelial damage due to limbal stem cell deficiency. Primary endpoints demanded combination of symptoms, signs, and the objective improvement of the epithelial phenotype in central cornea by *in-vivo* confocal microscopy. This proof-of-concept trial showed that MSCT was as safe and efficacious as CLET. Global success at 6-12 months was 72.7%-77.8% for CLET cases and 76.5%-85.7% for MSCT cases (not significant differences). Central corneal epithelial phenotype improved in 71.4% and 66.7% of MSCT and CLET cases, respectively at 12 months (p=1.000). There were no adverse events related to cell products. This trial suggests first evidence that MSCT facilitated improvement of a diseased corneal epithelium due to lack of its stem cells as efficiently as CLET. Consequently, not only CLET but also MSCT deserves more preclinical investigational resources before the favorable results of this proof-of-concept trial could be transformed into the larger numbers of the multicenter trials that would provide stronger evidence. (ClinicalTrials.gov number, NCT01562002.)

Key words: clinical trial, corneal blindness, corneal epithelial stem cells, human proof-of-concept, *in vivo* confocal microscopy, limbal stem cell deficiency, mesenchymal stem cell, stem cell transplantation

INTRODUCTION

Corneal epithelial failure due to extensive or severe limbal stem cell deficiency (LSCD) is an end-stage pathology resulting from multiple diseases that destroy the corneal epithelium stem cell niche, located at the sclerocorneal limbus. LSCD results in recurrent corneal epithelial ulceration, neovascularization, and opacification because of the inability of the limbal niche to renew the corneal epithelium.[1–3] Corneal transplantation is not a viable primary solution as the donor tissue cannot replace the damaged corneal epithelial stem cells.[4] The first attempts to replace native limbal epithelial cells were to transplant whole limbal tissue from donor eyes.[5] More recently, single limbal epithelial transplantation places small pieces of limbal tissue (not isolated cells) from the healthy fellow eye.[6] In 1997, stem cell-based therapies based on cultivated limbal epithelial cells commenced a significant breakthrough in regenerative medicine[7] and are currently an established therapy, both from autologous and allogeneic sources, the latter used when there is no possibility of a healthy donor contralateral eye.[2,4,8–12] Human amniotic membrane (hAM) transplantation, useful in sectoral and mild LSCD and an excellent cell carrier for stem cell growth and transplantation, has not been shown to help LSCD cases that are total and/or severe.[13,14] In this study, we explored, for the first time in human eyes in which medical therapy had failed, the potential capacity of mesenchymal stem cells (MSC) to treat corneal epithelial pathology due to LSCD.[15–17] MSC could have potential advantages over limbal epithelial stem cells for this purpose because they can be easily obtained from many tissue types without dependence of deceased donors.[18] Additionally, they can be cultured *in vitro* to clinical scales in a short period of time, overcoming the dependence on and the limitations of limbal epithelial cells, which are difficult to obtain, isolate, and culture and have limited availability.[19,20]

Moreover, cryopreserved MSC can be transplanted without loss of potency,[21] whereas cryopreserved limbal epithelial stem cells have not been transplanted in humans yet.[22,23] Finally, allogeneic MSC can be transplanted without the need of host immunosuppression,[20,24,25] while allogeneic transplantation of limbal epithelial stem cells requires one year of systemic immunosuppression to avoid immune rejection.[9,11]

We report herein a proof-of-concept clinical trial aimed to evaluate the initial safety and clinical efficacy of MSC *versus* the established therapy with limbal epithelial cells for corneal epithelial pathology due to LSCD. An initial clinical success would warrant the economic expenditure necessary to carry out a more thorough investigation of the mechanism of action of not only limbal stem cells but also MSC before proceeding with larger clinical trials.

MATERIAL AND METHODS

Study design and patients

This was a Phase I-II randomized, controlled, double-masked, unicenter clinical trial based on the hypothesis that allogeneic bone marrow-derived mesenchymal stem cell transplantation (MSCT) is as safe and effective as allogeneic cultivated limbal epithelial transplantation (CLET) to treat patients with total and/or severe LSCD.

It was designed as a proof-of-concept clinical trial to include only the minimum required number of transplants necessary to prove the hypothesis.

The protocol (EudraCT 2010-023535-42) was approved by the local Ethics Committee of our institution and the Spanish Agency of Medicines and Sanitary

Products (AEMPS, www.aemps.gob.es). The Clinical Trials.gov Identifier is NCT01562002.

All procedures were conducted in accordance with the principles of the Declaration of Helsinki, good manufacturing and clinical practice guidelines, and the European Union Tissues and Cells Directive. All patients gave written informed consent. The trial was sponsored by our institution, Advanced Therapies Unit, University of Valladolid, Spain, under the guidance of the Advanced Therapies National Program (Ministry of Heath, Government of Spain).

We enrolled adult patients with bilateral and severe disease, as it is ethically indicated in an exploratory proof-of-concept clinical trial. The following inclusion/exclusion criteria were met: (1) diagnosis of target disease characterized by (a) corneal epithelial failure because of LSCD graded as total and/or severe, meaning that at least three quadrants of the limbus were damaged (as visualized by slit-lamp) and/or that the central cornea was involved[9]; (b) invariably accompanied by blindness or low vision due to opacified central cornea; and (c) in which all available medical therapies (i.e., topical medications and hAM transplantations mainly in acute phases of chemical injuries) had failed (Tables 1 and 2 for detailed previous treatment in each patient); (2) no ocular surgeries in the previous 6 months other than another cell transplant within this trial; (3) the affected eye had to have undergone medical therapies to quiet and reverse as much as possible any treatable limbal dysfunction; and (4) no contraindications for immunosuppressive therapies.

As the final outcome of cell transplantation strongly depends on the etiology of limbal damage, the following three etiological categories were considered[11] at the initial visit: Chemical injuries; immune-based inflammatory diseases (e.g. Stevens-Johnson

syndrome, mucous membrane pemphygoid, atopic keratoconjunctivitis); and other less inflammatory conditions (e.g., sequelae from multiple surgeries, chronic sequelae from infectious keratitis, congenital aniridia). The allocation to the two treatment groups (see below) was balanced according to these etiologies.

Randomization and masking

After screening for inclusion/exclusion criteria and balancing the allocation of the three prognostic etiologic categories, all scheduled transplants were randomly allocated in a 1:1 ratio to CLET or MSCT. Randomization was balanced by the use of permuted blocks of varying block size with a maximum size of 6. The randomization schedule was computer-generated (R Statistical Software). When both eyes of a patient were to receive transplants, the use of CLET or MSCT was randomly assigned so that both eyes did not necessarily receive the same type of transplant. Some eyes, due to the failure of the transplant, received more than one transplantation. For each repeated surgery, the assignment of CLET or MSCT was random. Thus the new transplants, CLET or MSCT, were not necessarily the same as the preceding one that failed.

The Cell Processing Unit staff was aware of group assignment to prepare either cell product. The only difference in the final package that arrived at the Medical Institution the day of surgery was the type of cell cultivated, which was impossible to discern by the naked human eye. Everything else, including the packaging, was identical and followed the good manufacturing procedures. The products were identified by the randomization number, and only the Statistical Unit and the Cell Processing Unit

knew the identity of each product. All attending sanitary personnel and the patients themselves were completely masked as to the type of cells being transplanted.

Procedures

Cells destined for CLET or MSCT (investigational products 09-137 and 10-134, respectively) were cultured on top of hAM at the University of Valladolid Cell Processing Unit, operating under good manufacturing practices and licensed and accredited by the AEMPS. Donor hAM and cadaveric limbal rings (≤60 years of age) came from a registered and accredited tissue bank (Blood-Tissue Community Center, Oviedo, Spain). Bone marrow from iliac crest was collected from allogeneic donors ≤60 years of age who gave written consent and were under other approved trials.[26–28]

De-epithelialized hAM (size 2.5x2.5 cm) were prepared using our published standard protocol[11] and served as the substratum for both cell types. For CLET, two 2x2 mm pieces of allogeneic limbal rings were processed and cultured, as described.[11] For MSCT, allogeneic MSC were obtained and characterized as reported,[26–28] analyzing CD90, CD73, CD166, and CD105 as positive markers and CD14, CD34, CD45, and HAD-DR as negative markers in accordance with the International Society for Cellular Therapy (ISCT) position statement.[29] The cell products were harvested when the cultured cells were ~90% confluent (~250,000 cells). For the limbal epithelial cells, this took 3-4 weeks, and for the MSC it took 3-5 days. The quality criteria for the cell products were (1) sterility, (2) hAM integrity, (3) adherence of the hAM to the plate, (4) 80-90% of cell confluence (monolayer) observed under inverted phase contrast microscopy, and (5) cell morphology (polygonal shape for CLET and spindle shape for MSCT) observed under inverted phase contrast microscopy. After

determining that the cultures were negative for aerobes, anaerobes, fungi, and mycobacteria, they were delivered within 4 h of surgery. Some of the cultures that were assigned to patients who cancelled their surgeries (see below) were allowed to finish growing and later processed for immunostaining to test for limbal, mesenchymal, and differentiated corneal epithelial cell markers, as previously published[30,31] (see Supplementary Appendix for more details). To prevent any possible immune allograft rejection, patients receiving CLET underwent a mild immunosuppressive therapy.[11] While patients receiving MSCT would not normally need such therapy due to the absence of immune rejection by allogenic MSC,[15–17,24,25] oral immunosuppression was instituted to eliminate immune suppression as a variable and to maintain the double masked nature of the study; otherwise, the trial could not have been masked. Thus, all patients were started at the initial visit on 1.5-2.0 g/day of mycophenolate mofetil; 3-5 mg/kg/day of cyclosporine A, or 1-2 mg/kg/day of azathioprine were also permitted if, for any reason, mycophenolate mofetil was not available or the patient was already using one of the other two immunomodulating agents. This treatment was maintained for 12 months after transplantation and discontinued in the next 3 months. We closely monitored potential side effects clinically and by blood/urine work-ups every 1-2 months. No other systemic medications were added.

Surgery took place 3-4 weeks after the initial visit, and all were performed using the identical technique by the same experienced surgeon.[11] Briefly, after preparing the recipient corneal-limbal bed (i.e. scraping off the corneal-limbal pannus), hAM with cells for either CLET or MSCT were placed with the membrane facing up and the cells facing down, to facilitate their fast as possible access to the damaged corneal and limbal bed. In this way, the cells were in direct physical and functional contact

with the tissues to be repaired and protected from the external environment by the hAM. The transplant was sutured to the bared sclera and covered with a bandage contact lens for 4 weeks.

Twenty-four hours after surgery, each patient was evaluated and topical treatment with the fixed combination of 1% prednisolone acetate and 0.3% tobramycin (Tobradex®, Alcon Laboratories, FT. Worth, TX, USA) was prescribed 4 times per day until the hAM dissolved. The stitches and the contact lens were also removed between 4 and 6 weeks. Then, 1 mg/ml dexamethasone (Maxidex®, Alcon Laboratories) was instilled 4 times a day and slowly tapered in the next 3 months. Anti-glaucoma medications were the only other topical medication allowed (other than lubricants) and only in those patients who were previously using them, as they had already been diagnosed with glaucoma. The patients were evaluated 24 h, 1 week, and 4 weeks after surgery. Evaluations were then performed every month for the first 6 months, and every two months until the first year. All personnel related to patient care, and the patients themselves, were masked as to the type of cells transplanted.

Outcomes

Evaluation endpoints were collected at the initial baseline visit and at 6 months and 12 months. Three self-administered questionnaires evaluated symptoms and quality of life: the Single Item Dry Eye Questionnaire (SIDEQ), the Ocular Surface Disease Index (OSDI), and the National Eye Institute 25-item Visual Function Questionnaire (NEI-VFQ25).[32,33] The following clinical signs were evaluated by slit-lamp biomicroscopy: conjunctival redness, central corneal epithelial opacity, corneal

epithelial integrity manifesting as superficial keratitis and persistent epithelial defects, and corneal superficial neovascularization (area/length) (Table 3).

Visual acuity was determined as mandatory in clinical trials, although improved acuity is never the aim of these kind of trials. Procedures such as stem cell transplantation are intended to promote recovery of the corneal epithelium. Even with successful repair of the corneal epithelium, visual deficiency may continue due to deeper corneal damage (e.g., full thickness corneal destruction in severe chemical burns) or involvement of other parts of the eye (e.g., pre-existing cataract or various anomalies such as glaucoma, retinal pathology, etc.). In fact, we determined the presence of these factors before surgery to inform patients of what to expect in terms of visual recovery (Table 3).

We used *in vivo* confocal microscopy (IVCM) (Heidelberg Retinal Tomograph HRT-3 and Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) to image the basal epithelium phenotype in the central cornea, as described by us[11,34] and others.[35–37] The confocal images are used to determine the presence of the normal homogenous corneal epithelial cell phenotype as well as conjunctival-like or mixed epithelial cell phenotypes typically present in the damaged central cornea. This provided an objective assessment of the presence of LSCD in central cornea and of the efficacy of restoration therapies (Table 3). See Supplementary Appendix for details on all end-points.

The primary outcomes were as follows: (1) improvement in any of the three questionnaires; (2) improvement by at least one step in at least two of the three following clinical parameters: conjunctival redness, central corneal epithelial opacity, or superficial punctate keratitis; (3) complete absence of persistent epithelial defects;

and (4) presence of a more corneal epithelial-like phenotype in the central cornea. The change in epithelial phenotype could be either a change from a conjunctival-like epithelium to either a corneal-like or a mixed epithelial phenotype or from a mixed epithelial phenotype to a corneal-like epithelium phenotype.[11] Secondary outcomes included (1) amelioration of at least a one-step in superficial corneal peripheral neovessels (area/length) and (2) vision improvement of two lines or more in those cases that had the potential of vision gain with this cell transplantation alone and no additional surgeries (Table 3, column: Visual Prognosis and Potential for Visual Recovery, Grade 1 patients).

The outcome was considered successful when either a complete or a partial success was accomplished. A complete success meant that all four primary outcomes were achieved, and a partial success meant that at least two of the four primary outcomes or one primary and one secondary outcome were achieved. Failure meant that only one or none of the primary outcomes was met.[11]

Statistical analysis

Statistical analyses were performed by a PhD licensed statistician, who estimated that a sample of 10 transplants per group would give 80% statistical power to detect the non-inferiority of the experimental group. The calculations assumed a success rate of 80% in each group and a non-inferiority margin of 25% at an alpha level of 0.05. Consequently it was considered that a minimum of 10 transplants per group was sufficient for the exploratory nature of this proof-of-concept trial.

Quantitative characteristics were expressed as means ± standard deviations (SD), and qualitative variables were described as percentages. The median and interquartile range (IQR) were used to summarize distributions of ordinal variables. Normality assumptions were checked by the Shapiro-Wilk test. Differences between the means of two independent groups were tested by Student's t-test or the non-parametric alternative, Mann-Whitney U test, if the normality assumption was not valid. Levene's test was used to check homogeneity of variance. Relationships between two qualitative variables were evaluated by chi-square test or Fisher's exact test with small expected cell counts. Analysis of variance (ANOVA) with repeated measures on one factor was utilized to test for mean differences over time. The sphericity assumption was checked by Mauchly's test and, in case of violation of sphericity, the Greenhouse-Geisser correction was used. If there were differences in the repeated ANOVA measures, Bonferroni post hoc testing was used to determine where differences lay in a pairwise analysis. When data had marked deviations from the normality assumption Friedman's test was used, followed by the post hoc analysis based on Wilcoxon-Nemenyi-McDonald-Thompson test. Kaplan-Meier survival analysis was applied to estimate transplant survival. The log-rank test was used to compare the survival curves of each transplant type. The R Statistical Software version 3.1.3 was used (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical trial sample

We initially recruited 27 Caucasian patients (36 eyes, 42 potential transplants) (Fig 1). Five patients (10 eyes) cancelled surgery due to different personal/logistical reasons. Therefore, the final number of transplant surgeries was 37 (26 eyes, 22 patients). Among them, 16 cases were randomized to CLET and 21 to MSCT. Nine cases (24.3% of the total 37; 95% confidence interval [CI], 12.4 to 41.6), 5 CLET and 4 MSCT, lost their transplants within the first week due to loss of the bandage contact lens. Based on our preclinical data (unpublished), we considered that stem cells may have not completely reached their tissue target in less than 7 days, thus these transplants were excluded.

We consequently included in this trial 28 transplant surgeries from 23 eyes (20 patients), 11 cases were randomized to CLET and 17 to MSCT that were fully assessable at the minimum established period of 6 months (Fig 1). Of those, 23 reached 12 month follow-up and only 5 transplants did not: one (MSCT-2) was withdrawn due to a violent relapse of concomitant atopic dermatitis and its ocular component, atopic keratoconjunctivitis, that ruined his transplant and also worsened considerably his fellow non-transplanted eye. The other 4 cases were successfully re-grafted for the benefit of the patient (2 failed MSCTs; 1 failed CLET, 1 partially successful CLET). See Tables 1 and 2 for more details.

Table 4 shows the summary characteristics of the 28 fully assessable cell transplants; the detailed characteristics at baseline, 6 months, and 12 month of each case are shown in Tables 1 and 2.

Patients with assessable transplants had a mean age of 49.3±10.8 years (range, 28-77 years). Females comprised 42.9% (95% CI, 25 to 62.6) of the transplant recipients and males 57.1% (95% CI, 37.4 to 75) (p=0.253). The assignment of

patients to the CLET or MSCT groups was statistically independent of age or gender (Table 4). Time from LSCD to cell transplant was not significantly different between CLET and MSCT (Table 4).

The etiology groups leading to the target disease and the severity and extension of the disease were equally distributed between CLET and MSCT patients (Table 4). Consequently the different nature and severity of the background disease had no influence in the results. As this was an initial pilot trial, we decided not to restrict access regardless of the etiology.

Although we intended to transplant only one eye in this proof-of-concept trial, 4 cases had transplants in both eyes to attend patient demands, because both eyes were highly symptomatic and had not responded to medical therapy or to previous hAM transplantations (see inclusion criteria).

There were no intra- or post-operative complications. No episodes of immune rejection were recorded. Oral immunosuppression was used in all 28 transplant cases. Mycophenolate mofetil was prescribed in 12 cases (3 CLET, 9 MSCT), cyclosporine A in 6 cases (2 CLET, 4 MSCT); and azathioprine in 7 cases (4 CLET, 3 MSCT). Three patients had two immunosuppressants concomitantly due to their systemic disease. The drugs were well tolerated in all cases, and no discontinuations were necessary. Mycophenolate mofetil had to be lowered from 2 g/day to 1.5 or 1 g/day in 3 cases due to asthenia. Cyclosporine A was also lowered from 5 to 3 mg/kg/day in two cases due to mild elevation in blood pressure.

There were 3 serious adverse events and 21 non-serious adverse events, including 10 that were mild and 11 that were moderate to severe (Table 5). All were unrelated to the type of cell transplantation. Most were due to activation or recurrence of

baseline disease, and some were attributable to the concomitant immunosuppression.

Final outcome and survival analysis

The overall success for all cell transplants after 6 months was 75% (21 of 28 cases: 13 complete successes, 8 partial successes). After 12 months, the success rate was 82.6% (19 of 23: 15 complete successes, 4 partial successes). Five transplants were evaluated until month 6 (4 regrafts, one withdrawal), accounting for the different percentages. Except for CLET-2 and MSCT-15 that went from partial successes at 6 months to complete successes at 12 months, the final fate of all transplants was already established at 6 months.

The percentage of successful cases at 6 and 12 months was slightly higher for MSCT (76.5% and 85.7% respectively) than for CLET (72.7% and 77.8% respectively), but the differences were not statistically significant (Table 4). Thus, the final results were statistically independent of the type of cell transplant.

All failures were in eyes with either chemical injuries (4 of 7; 57.1%) or immune-mediated inflammatory diseases (3 of 7; 42.9%). None of the five non-inflammatory disease cases failed. Among chemical burns transplants, 75% were successful. Two of the failed cases were CLET, and two were MSCT. For transplants performed in inflammatory immune-based diseases, 57.1% were successful. One CLET transplant failed and two MSCT transplants failed in the same patient. Similarly, 25% of all cell transplants were immune-mediated diseases and of those, 57.1% were successful (2 MSCT in the same patient and 1 CLET failed).

For primary and secondary evaluation endpoints, there were no significant differences between the two groups except for central corneal opacity, which was slightly more improved in MSCT cases (Table 4). Within each of the two groups, one of the symptom questionnaires showed improvement. Conjunctival redness and central corneal opacity also decreased and superficial corneal integrity, both keratitis and ulceration, improved for both groups (Fig 2).

Evaluation of the epithelial phenotype in the central cornea by laser IVCM was the most objective primary endpoint. Based on the established criteria, complete success required that the corneal epithelial phenotype of the transplanted eyes must have improved by at least one step towards the normal corneal epithelial phenotype. At baseline, there was no significant difference in the distribution of the conjunctival-like or the mixed epithelial phenotype in the central cornea (Fisher's exact test, baseline p=0.226). Consequently, the outcome of each type of cell transplant could not have been influenced by a more frequent presence of a more favorable phenotype before cell therapy in any of the three etiologic groups (Table 4). The change in epithelial phenotype at the central cornea (Fig 2) was not significantly different between CLET and MSCT at 6 months (p=0.524) or at 12 months (p=0.5562). After 6 months, 50% of CLET cases (95% CI, 23.7 to 6.3) and 62.5% of MSCT cases (95% CI, 35.9 to 83.7) had improved the epithelial phenotype in the central cornea. At 12 months after surgery, 66.7% of CLET (95% CI, 30.9 to 91) and 71.4% of MSCT (95% CI, 42 to 90.4) had improved the epithelial phenotype in the central cornea. The differences between CLET and MSCT were not significant (p=0.6891 and p=1.000 at 6 and 12 months, respectively).

For CLET, a corneal epithelial phenotype was present in 20.0% (95% CI, 3.5 to 55.8) and 33.3% (95% CI, 9 to 69.1) of the transplants at 6 and 12 months, respectively

(differences were not significant). For MSCT, the corneal epithelial phenotype was present in 43.8% (7 cases, 95% CI, 20.8 to 69.5) at 6 months and 57.1% (8 cases, 95% CI, 29.7-81.2) at 12 months (p=0.0469, and p=0.0234, respectively). The percentage of cases reaching a corneal-like epithelial phenotype was not significantly different between CLET and MSCT at 6 (p=0.4152) or 12 months (p=0.854). Survival curve analysis showed that the differences between CLET and MSCT survival was not significant at either 6 or 12 months (log-rank test, p = 0.817, Fig 2). See Fig 3 as example.

In summary, our hypothesis of non-inferiority for MSCT versus CLET was confirmed at 6 and 12 months (p=0.0446 and p=0.0244, respectively).

DISCUSSION

This is the first clinical trial showing that allogeneic bone marrow-derived MSC can be safely transplanted to the human ocular surface, as far as we know. By doing that in the context of a controlled double-masked randomized trial, we demonstrated that non-epithelial stem cells are safe and as efficient (MSCT 85.7% success) as corneal epithelial stem cells derived from the limbus (CLET 77.8% success) in restoring the corneal epithelial phenotype damaged due to LSCD. Due to its proof-of-concept design, the number of transplants was small but statistically sufficient to prove our hypothesis. Additionally, through the balanced allocation of different etiologies to either group, we guaranteed no bias in this sense. Finally, as in any initial exploratory trial, only severe LSCD of diverse etiology were included.

hAM was used as a substrate to culture both limbal epithelial cells and MSC in the present clinical trial. hAM itself has re-epithelizing, anti-fibrotic, anti-inflammatory,

441 anti-angiogenic, and anti-microbial features.[38] In patients with partial and/or mild
442 LSCD, which maintain residual stem cell function, hAM transplantation can improve
443 their clinical situation by supporting regeneration of residual limbal stem cells.[39]
444 However, cell-free hAM transplantation is insufficient for regeneration of the ocular
445 surface in patients with severe and/or total LSCD.[13,14]

446 Because this is the first study of its kind, our results cannot be compared with similar
447 studies in humans; nevertheless, they can be compared to other CLET series,
448 including our own in which Ramirez et al. compared the CLET success rates among
449 previous studies and found that ours was similar to or better than the others.[11]

450 At present, the only techniques accepted as established therapies to recuperate from
451 corneal epithelial failure due to limbal niche destruction is either full tissue (limbal)
452 transplant[5,6,40] or cell-based therapy.[2,4,8–12,41] While other cell therapies have been
453 proposed and tested in animals, the only established cell therapy approved so far for
454 humans is CLET, which is based on *in vitro* cultivation of limbal niche cells. At
455 present, a cell product (Holoclar®) has just been approved by the European Agency
456 of Medicines based on a previous large study.[9] The authorization includes the
457 performance of a post-authorization multicenter trial. The approved indication for this
458 product is moderate to severe limbal deficiency due to chemical/thermal burns, and
459 the cells must be autologous, thus restricting all other etiologies and all bilateral
460 cases unless a 1-2 mm^2 limbal biopsy can be safely removed from one of the eyes.
461 For bilateral cases or other etiologies, allogeneic CLET has proved to be effective in
462 our hands,[11] confirming previous studies.[8,10,41,42]

463 However, CLET has several limitations. The main one is that epithelial stem cells
464 must be extracted from their niches where they are thought to represent less than

10% of all cells.[1,3] This means that the procedure is dependent upon healthy donor eyes or cadaveric donations, which may be limited. In practice, the small limbal biopsies can be lost because of contamination and/or lack of adequate growth. Typically, it takes 3-5 weeks to cultivate a sufficient quantity for transplantation. These factors make both autologous and allogenic CLET expensive and time-consuming. The natural step forward is to turn to the most commonly used adult stem cell in regenerative medicine, MSC. Currently these cells are being used in clinical trials to treat multiple diseases including osteo-articular, liver, kidney, cardiac, hematological (graft-versus-host disease), lung problems, keratoconus, and preclinically in retinal repair.[43–46] There is also abundant literature on the successful use of these cells in animal models of limbal deficiency or corneal burns.[17,47–53]

Although it remains unclear if MSC can transdifferentiate into corneal epithelial cells,[51–56] strong evidence suggests that other multiple mechanisms contribute simultaneously to the therapeutic action. These cells have the capacity to migrate into injured tissues[53] and exert anti-inflammatory and immunomodulatory properties. They have paracrine activity via the production of multiple trophic and growth factors that reduce tissue injury and protect tissue from further adverse effects while enhancing tissue repair. Finally, they are able to stimulate development of resident stem cells.[16,17,24,25,53–60] However, all of these effects have not been demonstrated in humans yet. In the case of CLET, there are preclinical data indicating that transplanted limbal cells migrate to the damaged limbal and corneal areas and that they repopulate and regenerate them to some extent.[61–63] However, in humans this has never been demonstrated, mainly due to the technical difficulty of tracking *in vivo* the transplanted cells.

Most of our failures (57.1%) were chemical injuries, confirming other authors' reports[9] and our own previous results.[11] The second most frequent failures (42.9%) were immune-mediated inflammatory diseases. None of the cases with non-inflammatory diseases failed. The overall effectiveness of the both CLET and MSCT was high considering that 57% of the cases were chemical burns, of which 75% were successful regardless of which transplantation protocol was used. Similarly, 25% of the cases were immune-mediated inflammatory cases, of which 57% were successful.

Although within each transplant type, one of the symptom questionnaires showed improvement, our experience during this trial and the previously published one[11] is that patients had a lot of difficulty in expressing in writing what they were feeling. For instance, many complained about the numerous questions they needed to answer, and at the end they were not sure what to answer. Plus it was very confusing for them to answer when they had a useful remaining eye, especially in the vision-related quality of life questionnaire.

This trial included only severe end-stage LSCD syndromes, as is typical for initial clinical trials. Vision improvement is usually dependent on multiple factors beyond the LSCD, and consequently this was not the goal of either transplant procedure in this trial. Although some patients proceeded with further surgical measures to improve vision after the 12-month mandatory follow-up period, these procedures were beyond the scope of this study. Nevertheless, our encouraging results indicate the possibility of restoring to health less severe limbal disease with these transplants, thus preventing further corneal damage and visual deterioration. Finally, these cell transplant procedures may ultimately improve the health of the limbal niche where stem cells and other important cells normally reside. This will result in greater

success if corneal transplantation from either cadaveric donors or by artificial corneas becomes necessary, making the cell transplantation procedures complementary to the tissue transplantation procedures.

CONCLUSIONS

In summary, we have shown in this proof-of-concept clinical trial that MSC used in MSCT can safely and effectively help treating corneal pathology due to LSCD. Further progress in treating severe and blinding pathology due to LSCD will depend on more research that explores the mechanism by which the transplanted stem cells improve the corneal surface cell phenotype. One of the next steps is to organize multicenter clinical trials of both MSCT and CLET. However before that can occur, it is essential to develop a commonly agreed upon set of diagnostic criteria for LSCD so that the prevalence of this pathology can be determined and that data developed among the participating centers can be effectively compared.

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FIGURE LEGENDS

Figure 1. Consort flow diagram. Twenty-eight transplants (23 eyes of 20 patients) were included and fully assessable at the minimum established period of 6 months. Eleven (9 eyes, 9 patients) were CLET and 17 (16 eyes, 14 patients) were MSCT.

Figure 2. Evaluation Endpoints and Final Outcome for Cultured Limbal Epithelial Transplantation (CLET, N=11) and Mesenchymal Stem Cell Transplantation (MSCT, N=17).

Panel A shows all clinical signs evaluated with anterior segment slit-lamp biomicroscopy: ocular (conjunctival) redness, corneal epithelial integrity, and central corneal epithelial opacity. Boxes extend from the 25th to the 75th percentile, horizontal bars represent the median, and whiskers extend 1.5 times the length of the interquartile range (IQR) above and below the 75th and 25th percentiles, respectively. The mean of each group is shown by black diamonds. Individual values for each subject are indicated by filled circles. Conjunctival redness and central corneal epithelial opacity improved significantly from baseline to final evaluation at 12 months. Superficial keratitis improved significantly by 6 months. Both CLET and MSCT groups improved similarly, except for corneal opacity, which was significantly better for MSCT at 6 and 12 months (see Table 4 for mean numerical values and Tables 1 and 2 for individual values). Panel B shows mosaic plots. The area of each rectangle is proportional to the observed frequency in that cell. Labels show the conditional relative percentages of each possible epithelial phenotype for each transplant type at baseline, 6, and 12 months. The number of cases is indicated in brackets. Mosaic plots represent contingency tables as a matrix of rectangles, the

762 dimensions of which are proportional to the observed frequencies of each cross-
763 classification. Cases with conjunctival-like (Conj), corneal-like (Corn), or mixed
764 phenotypes were divided into the relative proportions across transplant type. CLET
765 and MSCT performed equally well (no significant differences) regarding this main
766 objective evaluation outcome. The conjunctival-like phenotype decreased while the
767 corneal-like phenotype increased over time. Panel C shows the successful outcome
768 for each type of transplant at each visit (left). Partial success rates are represented
769 by the lighter-colored area. There were no significant differences between the
770 percentage of successful cases with CLET or MSCT at 6 months (72.7% vs 76.5%)
771 or 12 months (77.8% vs 85.7%) months. Kaplan-Meier survival curve analysis (right)
772 shows a probability of success after CLET of 0.818 (95% CI, 0.6191 to 1.00) at 6
773 months and of 0.716 (95% CI, 0.488 to 1.00) at 12 months. MSCT survival probability
774 was 0.812 at 6 months (95% CI, 0.642 to 1.00) and 0.75 at 12 months (95% CI,
775 0.565 to 0.995). The difference in survival between the two types of cell transplants
776 was not significant at either of the two periods (log-rank test, p = 0.817).

777

778 **Figure 3.** This 44-year-old woman (patient No. 10, see Tables 1 and 2) had bilateral
779 corneal epithelial failure due to 360º limbal stem cell deficiency caused by a 20-year
780 duration of Stevens-Johnson's syndrome. Opacity was restricted to the anterior
781 cornea (epithelium and anterior stroma) and the lens (cataract; not seen in this
782 photograph). Before entering this trial, she was treated aggressively for her extremely
783 severe secondary dry eye (a spot of squamous metaplasia can be seen at the lower
784 inner periphery of her left limbal area) during the course of 6 months. She lost her
785 first bilateral CLET transplants prematurely (48 h after surgery) due to inadequate
786 contact lens fitting. After her second bilateral transplants (right eye, CLET; left eye,

MSCT), her eyelids were maintained closed for 10 days except to quickly deliver eyedrops, thus keeping the transplants in place. Panels A and B show her right eye and left eye, respectively, 5 weeks before (upper), 6 months (middle) and 12 months (lower) after a successful CLET and after a successful MSCT. *In vivo* confocal microscopy images of the basal central corneal epithelium showed a mixed epithelial phenotype before surgery (upper) and corneal-like phenotypes at 6 months (middle) and 12 months (lower) after both CLET (Panel A) and MSCT (Panel B). Baseline visual acuity in her right eye was 0.25 (upper) and improved to 0.32 at 6 months (middle) and 12 months (lower). Baseline visual acuity in her left eye was 0.01 (upper) and improved to 0.25 at 6 months (middle) and to 0.32 after 12 months (lower). The patient did not want to undergo any other surgery as she was able to carry on with her life, and we did not encourage further surgery as any trauma in these patients carries the risk of triggering violent inflammatory relapse.

TABLES

Table 1. Baseline data (0) and outcomes at 6 months (6) and 12 months (12) after cultivated limbal epithelial transplantation (CLET) in the 11 assessable cases (9 eyes, 9 patients) suffering from ocular surface failure due to limbal stem cell deficiency syndrome (LSCD).

CLET No./Eye	Patient No. Gender/ Age	LSCD etiology (months elapsed till transplant): Etiology*/Grade† 2nd diagnoses	SIDEQ 0/6/12	OSDI 0/6/12	VFQ25 0/6/12	Visual Potential‡	BCVA (ETDRS) 0/6/12	Conj redness (0-4) 0/6/12	Central corneal epithelial opacity (0-4) 0/6/12	Corneal neovessel area (0-4) 0/6/12	Corneal neovessel length (0-4) 0/6/12	Corneal staining (0-4) 0/6/12	Corneal PED (0-4) 0/6/12	Central corneal epithelial phenotype (IVCM) 0/6/12	Days to AM re-absorption from surgery	Final outcome 6 and 12 months	*Comments*
1/OD	1/F/49	Post-infectious keratitis (240)+2 previous PKP: 3/T Glaucoma	24/9/16	89.6/ 50.0/65.9	45.4/ 57.3/55.0	4	0.025/ 0.001/0.001	1/1/1	3/3/3	2/2/1	2/1/1	4/2/1	0/0/0	Conj/ Conj/Conj	24	Partial success/ Partial success	PKP performed at month 18. Although remaining clear, vision deteriorated due to advanced glaucoma
2/OS	2/F/77	Persistent corneal ulcer (20) 2nd to recurrent ocular surface carcinoma (120): 3/T	17/17/13	87.5/ 50.0/60.0	46.8/ 49.0/35.3	1	0.04/ 0.2/0.25	2/2/1	1/1/0	2/1/1	2/1/1	3/1/0	3/0/0	Conj/ NP/Corn	30	Partial success/ Success	Patient chronically immunosuppressed due to heart transplant
3/OD	3/M/47	Chemical+mechanical injury (24): 1/T Irreversible retinal pathology	8/6/8	57.5/ 25.0/32.5	47.1/ 32.5/34.5	0	0.001/ 0.001/0.001	2/2/1	4/3/2	3/1/1	3/2/1	3/2/1	0/0/0	Conj/ Mix/Mix	30	Success/ Success	-
4/OS	7/M/48	Chemical injury (46)+2 previous AMT: 1/T	14/13/-	62.5/ 83.3/-	50.9/ 43.8/-	4	0.001/ 0.001/-	3/2/-	3/3/-	4/3/-	3/3/-	4/3/-	2/1/-	Conj/ Conj/-	90	Partial Success/-	Regrafted CLET-5, for further potential improvement
5/OS	7/M/48	Chemical injury (54)+2 previous AMT+previous CLET-4: 1/T	9/8/14	83.3/ 62.5/80.6	52.9/ 50.9/55.0	4	0.001/ 0.001/0.001	2/1/1	3/2/2	3/3/3	3/2/2	3/1/0	1/0/0	Conj/ Mix/Mix	90	Success/ Success	-
6/OS	9/M/62	Chemical injury (600)+2 previous AMT+ PKP: 1/T Glaucoma, exotropia	13/18/-	61.1/ 85.4/-	53.8/ 57.0/-	4	0.001/ 0.001/-	3/3/-	2/3/-	4/4/-	3/3/-	2/2/-	3/2/-	Conj/ Conj/-	21	Failure/-	Regrafted CLET-7
7/OS	9/M/62	Chemical injury (608)+2 previous AMT+previous PKP+previous CLET-6: 1/T Glaucoma, exotropia	17/22/16	93.8/ 72.7/85.4	53.6/ 53.4/56.0	4	0.001/ 0.01/0.01	3/2/2	3/3/2	4/4/4	3/3/3	2/0/0	2/0/0	Conj/ Conj/Conj	30	Partial success/ Partial Success	-
8/OD	10/F/44	Stevens Johnson+multiple AMT (120): 2/T Cataract	28/20/21	100.0/ 93.8/97.5	47.4/ 49.8/47.3	2	0.25/ 0.32/0.32	3/3/1	3/1/1	3/1/1	3/2/1	2/1/1	0/0/0	Mix/ Corn/Corn	8	Success/ Success	-
9/OD	13/M/48	Chemical injury (84)+3 previous AMT: 1/T	16/11/15	81.8/ 72.9/79.2	30.6/ 35.8/39.8	4	0.0001/ 0.0001/0.0001	2/2/1	4/4/4	4/4/4	4/4/4	0/0/0	0/0/0	Conj/ Mix/Mix	30	Failure/ Failure	-
10/OD	16/M/50	Graft vs host disease (168)+5 previous PKP+conj flap+sclera patch: 2/T	17/22/18	87.5/ 90.0/89.6	56.3/ 59.1/52.5	0	0.001/ 0.001/0.001	4/3/2	4/4/4	4/4/4	4/4/4	0/0/0	0/0/0	Conj/ Conj/Conj	10	Failure/ Failure	Extremely thin cornea under pannus prevented its removal at surgery. Thickness increased so as to proceed with a keratoprothesis
11/OS	21/M/41	Chemical injury (36)+5 AMT: 1/T Cataract, glaucoma	10/11/9	62.5/ 64.6/87.5	53.0/ 55.1/48.4	4	0.32/ 0.5/0.4	2/1/1	2/2/2	3/2/1	2/1/1	2/0/0	0/0/0	Mix/ Corn/Corn	34	Success/ Success	PKP at month 14 +cataract surgery +hard contact lens recovered full vision
Mean (SD)		181.8 (219.2)	15.7 (6.1)/ 14.3 (5.8)/ 14.4 (4.1)-	78.8 (15.1)/ 68.2 (20.7)/ 75.4 (19.8)	48..9 (7.0)/ 49.4 (8.8)/ 47.1 (8.6)			-	-	-	-	-	-	-	36.1 (27.9)		-
Median (IQR)		-	-	-	-			2 (1)/ 2 (1)/ 1 (0)	3 (1)/ 3 (1)/ 2 (1)	3 (1)/ 3 (2.5)/ 1 (3)	3 (0.5)/ 2 (1.5)/ 1 (2)			-			-

Assessable cases were those reaching at least 6 postoperative months; *1: chemical injuries, 2: immune-based inflammatory diseases, 3: non-inflammatory diseases; †T: total, S: severe; ‡Visual potential: 1, improvement with CLET only (corneal opacity was only superficial); 2, improvement with one surgery different form corneal transplant after CLET (i.e. cataract removal); 3, improvement with subsequent corneal transplant after CLET (corneal opacity was full thickness); 4, improvement with subsequent corneal transplant plus another surgery (cataract removal unless otherwise specified) after CLET, and 0: No possibility of improvement (i.e., due to irreversible retinal pathology); BCVA, Best corrected visual acuity; BCVA values 0.01, 0.001, 0.0001, and 0.00001 equivalent to counting fingers, hand motion, light perception, and no light perception respectively; ; ETDRS, Early Treatment Diabetic Retinopathy Study; Conj, conjunctival; Corn, corneal; IQR, interquartile range; IVCM, in vivo confocal microscopy; SIDEQ, Single Item Dry Eye Questionnaire; VFQ25, National Eye Institute 25-item Visual Function Questionnaire (0-100); OSDI, Ocular Surface Disease Index (0-100); PED, persistent epithelial defect; AMT, amniotic membrane transplantation; PKP, penetrating keratoplasty; SD, standard deviation; M, male; F, female; NP: not performed; OS, left eye; OD, right eye.

Table 2. Baseline data (0) and outcomes at 6 months (6) and 12 months (12) after bone marrow-derived mesenchymal stem cell transplantation (MSCT) in the 17 assessable cases (16 eyes, 14 patients) suffering from ocular surface failure due to limbal stem cell deficiency syndrome (LSCD).

MSCT No. /Eye	Patient Nº Gender /Age	LSCD etiology (months elapsed till transplant): Etiology*/Grade†/ 2nd diagnoses	SIDEQ 0/6/12	OSDI 0/6/12	VFQ25 0/6/12	Visual Potential‡	BCVA (ETDRS) 0/6/12	Conj redness (0-4) 0/6/12	Central corneal epithelial opacity (0-4) 0/6/12	Corneal neovessel: area (0-4) 0/6/12	Corneal neovessel length (0-4) 0/6/12	Corneal staining (0-4) 0/6/12	Corneal PED (0-4) 0/6/12	Central corneal epithelial phenotype (IVCM) 0/6/12	Days to AM reabsorption from surgery	Final outcome 6/12	*Comments*
1/OS	4/F/31	Chemical injury (24)+ 2 previous conj resection+AMT: 1/T Cataract	18/15/8	39.6/ 47.9/64.6	22.6/ 43.0/29.4	2	0.125/ 0.158/ 0.125	1/1/1	1/2/3	3/3/3	2/2/3	2/0/2	1/0/0	Mix/ Conj/Conj	10	Failure/ Failure	Poor compliance
2/OS	6/M/53	Atopic kerato conjunctivitis (170): 2/T Post-infectious keratitis Unsuccessful cataract surgery	24/23/-	95.8/ 93.8/-	43.6/ 41.0-	2	0.04/ 0.025/-	3/0/-	2/0/-	3/0/-	2/0/-	1/0/-	0/0/-	Mix/ NP/-	17	Partial success/ -	Withdrawn at month 7: intense systemic flare-up worsened both eyes, perforating OS cornea; PKP required
3/OD	7/M/48	Chemical injury (46)+multiple AMT: 1/T Glaucoma	12/15/-	77.1/ 85.4/-	55.0/ 56.8/-	4	0.001/ 0.001/-	3/3/-	3/3/-	4/4/-	4/4/-	3/2/-	0/0/-	Conj/ Conj/-	25	Failure/ -	Regrafted MSCT-4
4/OD	7/M/48	Chemical injury (54)+multiple AMT+previous MSCT-3: 1/T Glaucoma, cataract	9/8/11	83.3/ 62.5/77.8	54.2/ 50.9/57.8	4	0.001/ 0.001/ 0.001	3/2/1	3/2/1	4/4/4	4/3/2	2/2/1	0/0/0	Conj/ Mix/Corn	60	Success/ Success	PKP at month 15
5/OD	8/M/70	Multiple surgeries for proliferative vitreoretinopathy (48): 3/S Irreversible macular pathology	6/3/7	12.5/ 15.6/6.3	54.6/ 63.8/62.4	4	0.001/ 0.001/ 0.001	1/1/0	2/0/1	2/1/1	1/1/1	2/1/1	1/0/0	Mix/ Corn/Corn	10	Success/ Success	PKP at month 30
6/O	10/F/44	Stevens Johnson (120)+multiple AMT: 2/T Cataract	28/23/16	100..0/ 93.8/97.5	46.1/ 49.3/41.8	2	0.01/ 0.25/ 0.32	3/3/1	4/3/1	4/3/2	3/2/1	3/2/2	0/0/0	Mix/ Corn/Corn	8	Success/ Success	-
7/OS	11/F/37	Congenital aniridia (132): 3/S Cataract, nystagmus	16/14/12	38.9/ 45.5/63.6	39.3/ 41.6/43.3	4	0.062/ 0.1/0.1	1/0/0	4/3/2	3/2/1	2/2/2	0/0/0	0/0/0	Conj/ Mix/Mix	22	Success/ Success	-
8/OD	12/F/42	Atopic kerato conjunctivitis (132)+2 previous AMT: 2/T Cataract	21/20/-	79.2/ 77.1/-	39.2/ 40.2/-	2	0.031/ 0.025/-	1/2/-	1/1/-	2/2/-	2/2/-	1/1/-	0/0/-	Conj/ Conj/-	15	Failure/ -	Regrafted MSCT-9 (Table 2)
9/OD	12/F/42	Atopic kerato conjunctivitis (140)+2 previous AMT+previous MSCT-8: 2/T Cataract.	17/21/23	66.7/ 66.7/75.0	33.9/ 39.7/27.8	2	0.05/ 0.05/ 0.05	2/2/1	1/1/1	2/2/2	2/2/2	1/1/1	0/0/0	Conj/ Conj/Conj	15	Failure/ Failure	Poor compliance Systemic disease poorly controlled
10/O	14/F/53	Congenital aniridia (160): 3/S Cataract, nystagmus, glaucoma	21/20/21	83.3/ 75.0/85.4	44.7/ 43.8/47.8	2	0.1/ 0.08/ 0.08	1/1/0	2/1/1	2/1/1	2/1/1	3/1/0	0/0/0	Mix/ Corn/Corn	28	Success/ Success	Had a previous CLET (lost transplant No. 7) Cataract surgery at month 20 did not recover vision
11/OD	17/F/28	Chemical injury (72)+previous cadaveric limbal transplant +AMT+PKP: 1/T Severe perennial allergic conjunctivitis, cataract.	19/13/13	64.6/ 33.3/33.3	40.3/ 35.8/46.8	4	0.01/ 0.01/ 0.01	3/2/1	1/1/1	4/4/4	3/2/2	1/1/0	0/1/0	Conj/ Mix/Conj	20	Partial success/ Partial success	PKP at month 13
12/OD	18/M/65	Chemical injury (360)+previous AMT+PKP+intracorneal rings: 1/T Cataract	24/16/16	81.3/ 70.8/79.2	44.1/ 33.6/37.2	4	0.12 0.5/0.4	2/1/1	1/1/1	3/3/3/	2/2/2	2/0/0	0/0/0	Mix/ Corn/Corn	29	Success/ Success	PKP at month 20
13/OS	19/F/49	Chemical injury (80)+oral mucosal transplant+2 AMT: 1/S	23/19/18	91.7/ 97.9/83.3	37.5/ 48.8/38.6	3	0.01/ 0.2/0.2	1/1/1	2/1/0	2/1/1	2/1/1	1/0/0	0/0/0	Conj/ Corn/Corn	20	Success/ Success	PKP at month 24
14/OS	20/F/44	Stevens Johnson (90)+3 previous AMT: 2/T Cataract	23/16/21	83.3/ 62.5/81.3	46.1/ 47.8/42.5	4	0.158/ 0.5/0.32	3/2/2	2/2/1	2/2/2	1/1/1	4/3/1	0/0/0	Mix/ Corn/Corn	15	Success/ Success	Cataract progression
15/OD	21/M/41	Chemical injury (45)+5 previous AMT+PKP: 1/T Cataract, glaucoma	9/12/7	64.6/ 64.6/70.8	44.0/ 33.6/48.0	4	0.01/ 0.01/ 0.001	4/3/2	4/2/1	4/3/3	3/2/1	4/NP/1	4/2/0	Conj/ Conj/Mix	85	Partial success/ Success	Initially performed for impending perforation
16/OD	22/M/54	Chemical injury (84)+2 previous AMT: 1/T Cataract	20/18/7	70.8/ 66.7/89.6	40.6/ 21.7/36.3	3	0.25/ 0.25/ 0.25	2/1/1	1/0/0	1/1/1	2/1/1	0/0/0	0/0/0	Mix/ Corn/Corn	30	Success/ Success	-
17/OS	22/M/54	Chemical injury (95)+3 previous AMT: 1/T Cataract	28/22/19	77.1/ 70.8/56.3	37.0/ 34.4/33.6	4	0.001/ 0.001/ 0.001	4/2/1	4/4/3	4/4/4	3/3/2	3/1/0	3/0/0	Conj/ Conj/Conj	60	Partial success/ Partial success	-
Mean (SD)		108.9 (77.8)	18.7 (6.5)/ 16.4 (5.4)/ 14.2 (5.7)	71.2 (22.5)/ 66.5 [21.8)/ 68.9 (24.0)	42.5 (8.0)/ 42.7 (9.9)/ 42.4 (9.8)			-	-	-	-				27.6 (21.1)		
Median (IQR)			-	-	-	-	-	2 (2)/ 2 (1)/ 1 (0)	2 (2)/ 1 (1)/ 1 (0)	3 (2)/ 2 (2)/ 2 (2)	2 (1)/ 2 (1)/ 1.5 (1)			-		-	

Assessable cases were those reaching at least 6 postoperative months; *1: chemical injuries, 2: immune-based inflammatory diseases, 3: non-inflammatory diseases; †T: total, S: severe; ‡Visual potential: 1, improvement with CLET only (corneal opacity was only superficial); 2, improvement with one surgery different form corneal transplant after CLET (i.e. cataract removal); 3, improvement with subsequent corneal transplant after CLET (corneal opacity was full thickness); 4, improvement with subsequent corneal transplant plus another surgery (cataract removal unless otherwise specified) after CLET, and 0: No possibility of improvement (i.e. due to irreversible retinal pathology); BCVA, Best corrected visual acuity; BCVA values 0.01, 0.001, 0.0001, and 0.00001 equivalent to counting fingers, hand motion, light perception, and no light perception respectively; Conj, conjunctival; Corn, corneal; IQR, interquartile range; IVCM, in vivo confocal microscopy; VFQ25, National Eye Institute 25 Visual Function Questionnaire (0-100); OSDI, Ocular Surface Disease Index (0-100); SIDEQ, Single Item Score Dry Eye questionnaire (0-28); AMT, amniotic membrane transplantation; PED, persistent epithelial defect; PKP, penetrating keratoplasty; SD, standard deviation; M, male; F, female; NP, not performed; OS, left eye; OD, right eye.

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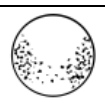
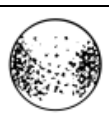
806 Table 3. Ocular Surface Clinical Signs Evaluated to Define and Grade Limbal Stem Cell Deficiency and Score the Evaluation End-
807 Points.

	Conjunctival Redness*+	Central Corneal Epithelial Opacity*	Corneal Epithelial Integrity		Corneal Superficial Neo Vascularization Area/Length*	Visual Prognosis and Potential for Visual Recovery		Central Corneal Phenotype (In *Vivo* Confocal Microscopy)†
			Superficial Punctate Keratitis*	Epithelial Ulceration Area*		Previous Ocular Media Opacity	Surgeries Judged Necessary to Recover Full Potential Vision	
Grade 0	White conjunctiva	None		None	None /None	Any grade of corneal opacity plus non-corneal irreversible visual loss (e.g., irreversible retinal pathology, advanced glaucoma)	No potential for gain: Stem cell transplant performed to deal with pain and avoid globe removal	CORNEAL: Regular, hexagonal cells with a cell diameter <20 μm; dark cytoplasm, dark nucleus; hyper-reflective, bright well-defined cell margins.
Grade 1	Widening of the vessels	Mild: clearly visible pupil		≤¼	≤¼ / 1mm	Corneal opacity restricted to anterior cornea (and anterior stroma)	One surgical procedure: stem cell transplant only	CONJUNCTIVAL: Closely packed round or irregularly shaped cells; cell diameter of >20 μm (irregular size); large nucleus/cytoplasm ratios; dark cytoplasm and bright, hyperreflective nucleus with ill-defined cell margins. Occasional goblet cells
Grade 2	Mild redness	Moderate: hazily visible pupil		>¼ and ≤½	>¼ and ≤½ / 2-3 mm	Corneal opacity restricted to anterior cornea (as Grade 1) plus another non-corneal reason for visual loss (e.g., cataract)	Two surgical procedures: stem cell transplant + non-corneal surgery (e.g., cataract removal)	MIXED: Both corneal and conjunctival phenotypes are present
Grade 3	Moderate redness	Severe: faintly visible pupil		>½ and ≤¾	>½ and ≤¾ / 4-5 mm	Full thickness corneal opacity	Two surgical procedures: stem cell transplant + corneal transplant	
Grade 4	Intense redness	Severe: no visible pupil		>¾	>¾ / ≥6 mm	Full thickness corneal opacity plus another non-corneal reason for visual loss (e.g., cataract)	Three surgical procedures: stem cell transplant + corneal transplant + non-corneal surgery (e.g. cataract removal)	

*Evaluated by slit-lamp biomicroscopy.

+ Evaluated following the Efron Scale for conjunctival redness.[64]

†Evaluated with the Heidelberg Retinal Tomograph HRT-3 and Rostock Cornea Module (HRT3, Heidelberg Engineering GmbH, Heidelberg, Germany).

Table 4. Characteristics, Endpoint Values, and Outcome of the 28 Assessable Cases (23 eyes from 20 patients) of Corneal Epithelial Failure Due to Limbal Epithelial Stem Cell Deficiency Randomized to Cultivated Limbal Epithelial Transplantation (CLET) or Mesenchymal Stem Cell Transplantation (MSCT).

CHARACTERISTIC / ENDPOINT	CLET (N = 11)	MSCT (N = 17)	p-values*
Females/Males — no. (%); 95% confidence interval (CI)	3 (27.3); 7.3 to 60.7/ 8 (72.7); 39.1 to 92.7	9 (52.9); 28.5 to 76.1/ 8 (47.1); 23.9 to 71.5	0.2530
Age — years (mean±SD)	52.4±10.5	47.2±10.8	0.3448
Limbal Stem Cell Deficiency			
Grade: Total/ Severe — no. (%), 95% CI	11 (100) /0 (0)	13 (76.5); 49.8 to 92.2/ 4 (23.5); 7.8 to 50.2	0.1324
Etiology — no. (%); 95% CI			
Chemical Burns —16 (57.1)	7 (63.6); 31.6-87.6	9 (52.9); 28.5 to 76.1	0.8669
Immune-based Inflammatory Diseases — 7 (25.0)	2 (18.2); 3.2 to 52.3	5 (29.4); 11.4 to 56	0.8232
Non-inflammatory Diseases, Other — 5 (17.9)	2 (18.2); 3.2 to 52.3	3 (17.7); 4.7 to 44.2	1
Months from Disease Onset to Cell Transplant — mean±SD	181.8±219.2	108.9±77.8	0.9437
Days from Cell Transplant to Supporting Amniotic Membrane Reabsorption — mean±SD	36.1±27.9	27.6±21	0.1634
Primary Evaluation End-Points Baseline/ 6 months/ 12 months			
Symptoms/Quality of Life* Questionnaires (range) — mean±SD			
Single Item Dry Eye Questionnaire SIDEQ (0-28)	15.7±6.1/ 14.3±5.8/ 14.4±4.1	18.7±6.5/ 16.4±5.4/ 14.2±5.7 p=0.0237and p=0.0336 between baseline and 6 or 12 months	0.2379/ 0.3408/ 0.9178
Ocular Surface Disease Index OSDI (0-100; severe>32)	78.8±15.1/ 68.2±20.7/ 75.4±19.8 p=0.0318 and p=0.0072 between baseline and 6 or 12 months	71.2±22.5/ 66.5±21.8/ 68.9±24	0.4795/ 0.8352/ 0.4306
National Eye Institute 25-item Visual Function Questionnaire NEI-VFQ25 (0-100)	48.9±7/ 49.4±8.8/ 47.1±8.6	42.5±8/ 42.7±9.9/ 42.4±9.8	0.0239/ 0.0774/ 0.2524
Clinical signs (range) — median (interquartile range [IQR])			
Conjunctival redness (0-4)	2 (1)/ 2(1)/ 1 (0) p=0.0012 between baseline and 12 months	2 (2)/ 2(1)/ 1 (0) p<0.0001 between baseline and 12 months	0.6057/ 0.2638/ 0.2438
Central corneal epithelial opacity (0-4)	3 (1)/ 3 (1)/ 2 (1) p=0.0129 between baseline and 12 months	2 (2)/ 1 (1)/ 1 (0) p=0.0023 between baseline and 12 months	0.1323/ 0.0275/ 0.04
Corneal epithelial integrity: superficial punctate keratitis (0-4)	2 (1)/ 1 (2)/ 0 (1) p=0.0428 and p=0.0012 between baseline and 6 or 12 months	2 (2)/ 1 (1.25)/ 0.5 (1) p=0.0263 and p=0.0006 between baseline and 6 or 12 months	0.4693/ 0.7356/ 0.3532
Corneal epithelial integrity: persistent epithelial defect or ulceration (0-4)	0 (2)/ 0 (0)/ 0 (0)	0 (0)/ 0 (0)/ 0 (0)	0.2558/ 0.3422/ 1
Epithelial phenotype in central cornea (*in vivo* confocal microscopy) — no. (%); 95%			

CI			
Conjunctival phenotype 18 (64.3)/ 11 (42.3)/ 7 (30.4)	9 (81.8); 47.8 to 96.8/ 5 (50); 23.7 to 76.3/ 3 (33.3); 9 to 69.1	9 (52.9); 28.5 to 76.1/ 6 (37.5); 16.3 to 64.1/ 4 (28.6); 9.6 to 58	0.2486/ 1/ 0.2486
Mixed phenotype 10 (35.7)/ 6 (23.1)/ 5 (21.7)	2 (18.2); 3.2 to 52.3 / 3 (30); 8.1 to 64.6/ 3 (33.3); 9 to 69.1	8 (47.1); 23.9 to 71.5/ 3 (18.8); 5 to 46.3/ 2 (14.3); 2.5 to 43.9	1/ 0.4915/ 0.5735
Corneal phenotype 0 (0)/ 9 (34.6)/ 11 (47.8)	0 (0); 0 to 32.2/ 2 (20); 3.5 to 55.8/ 3 (33.3); 9 to 69.1	0 (0); 0 to 22.9/ 7 (43.8); 20.8 to 69.5%)/ 8 (57.1); 29.7 to 81.2	0.8261/ 0.4152/ 0.854
Secondary Evaluation End-Points — baseline/ 6 months/ 12 months			
Corneal neovessels: area (0-4) — median (IQR)	3 (1)/ 3 (2.5)/ 1 (3) **p=0.0129** between baseline and 12 months	3 (2)/ 2 (2)/ 2 (2) **p=0.0307** and **p=0.0045** between baseline and 6 or 12 months	0.3197/ 0.594/ 0.8416
Corneal neovessels: length (0-4) — median (IQR)	3 (0.5)/ 2 (1.5)/ 1 (2) **p=0.0307** and **p=0.0044** between baseline and 6 or 12 months	2 (1)/ 2 (1)/ 1.5 (1) **p=0.0055** between baseline and 12 months	0.074/ 0.2271/ 0.7037
Best-corrected visual acuity: all cases† — mean±SD	0.06±0.11/ 0.09±0.17/ 0.11±0.16	0.06±0.07/ 0.13±0.16/ 0.13±0.14	0.148/ 0.1171/ 0.3668
Best-corrected visual acuity: successful cases† — mean±SD	0.08±0.13/ 0.13±0.19/ 0.14±0.18	0.06±0.08/ 0.15±0.18/ 0.14±0.15	0.6837/ 0.4592/ 0.9307
SUCCESSFUL OUTCOME 6 months/ 12 months — no. (%); 95% CI	8 (72.7); 39.3 to 92.7/ 7 (77.8); 40.2 to 96.1	13 (76.5); 49.8 to 92.2/ 12 (85.7); 56.2 to 97.5	0.8232/ 0.6241

* Between CLET and MSCT groups. Significant p-values are highlighted in bold characters. Only significant P-values are shown in the 2nd (CLET) and the 3rd (MSCT) columns.

†Only one case, CLET nº2 (Table 1), had a grade 1 potential to recover visual acuity, meaning that it was previously considered that her damage was restricted to the corneal epithelium. Her visual acuities were 0.04, 0.2 and 0.25 at baseline, 6, and 12 months, respectively.

827 Table 5. Serious and Non-serious Adverse Events Encountered in All Cell Transplants
828 Performed (N = 37), Including 9 Transplants that Did Not Reach the Minimum
829 Established 6 Months of Follow Up (5 CLET and 4 MSCT) Plus the 28 That Did (11
830 CLET and 17 MSCT)*.

EVENT	CLET (N = 16)			MSCT (N = 21)		
	No. of Events (%)/ Transplant-Patient No./ Baseline Disease/ Immuno-suppressants	Relation to Study medication (Cell Transplant)/ Severity/ Attributable Relation	Final Outcome/ Comments	No. of Events (%)*/ Transplant-Patient No./ Baseline Disease/ Immuno-suppressants	Relation to Study Medication (Cell Transplant)/ Severity/ Attributable Relation	Final Outcome/ Comments
Serious adverse events						
Herpes simplex keratitis				1 (2.7)/ 8-15/ Persistent corneal ulcer due to herpes simplex keratitis/ Mycophenolate mofetyl	Unrelated/ Moderate/ Recurrence of baseline disease, facilitated by surgical trauma and/or immuno suppression used	Solved with sequelae/ Penetrating corneal transplant performed due to risk of perforation
Corneal perforation				1 (2.7)/ 2-6/ Atopic keratoconjunctivitis secondary to severe atopic dermatitis/ Cyclosporine+ azathioprine	Unrelated/ Severe/ Intense relapse of baseline disease, already poorly controlled systemically	Solved with sequelae/ Tectonic corneal transplant
Ocular surface neoplasia	1 (2.7)/ 2-2/ Moderate/ Persistent corneal ulcer due to ocular surface neoplasia and its required treatments/ Cyclosporine+ azathioprine	Unrelated/ Moderate/ Recurrence of baseline disease at same rate as before cell transplant (patient long-term immuno suppressed due to previous heart transplant)	Solved (excisional surgery) with no sequelae/ Cell transplant action was not interrupted as persistent corneal wound healed			
Non-serious adverse events†						
Loss of transplant within 24 hr after surgery (Not assessable at first evaluation)	1 (2.7)/ 4-5/ Severe/ Chemical injury/ mycophenolate mofetil	Unrelated/ Baseline disease? (same fate for all previous ammiotic membranes grafts)	Unsolved with no sequelae/ No further actions taken; patient was withdrawn	3 (8.1)/ 1,2,3-5/ Severe/ Chemical injury/ mycophenolate mofetil	Unrelated/ Severe/ Baseline disease (same fate for all previous amniotic membrane grafts)	Unsolved with no sequelae/ No further actions taken; patient was withdrawn
Loss of transplant within 48 hr after	4 (10.8)/4, 6-14; 7-14; 9-19/ Severe/ Stevens-Johnson (No. 5, 6);	Unrelated/ Severe/ Bandage contact lens displacement?	Solved with o sequelae (all regrafted successfully)			

surgery	congenital aniridia (No. 7); chemical injury (No. 9)/ Cyclosporine (No.5, 6); azathioprine (No. 7); mycophenolate mofetil (No. 9)					
Corneal erosion				2 (5.4)/ 14-20/ Moderate/ Stevens-Johnson's syndrome/ Micophenolate mofetil	Unrelated/ Mild/ Misdirected lashes rubbing cornea due to underlying disease	Solved with no sequelae/ Lashes were removed permanently
Flu episode				1 (2.7)/ 10-14/ Moderate/ Congenital aniridia/ azathioprine	Unrelated/ Mild/ Patient was not vaccinated	Solved with no sequelae/ no effect in transplant

* CLET, cultivated limbal epithelial transplantation; MSCT, mesenchymal stem cell transplantation.

† Non-serious adverse events shown are those that were moderate or severe. The remaining non-serious adverse events were mild, easily solved and all were considered to be unrelated with cell transplant: nausea, vomiting, pharyngitis, twisted ankle, and reconstruction of anophthalmic socket to improve existent cosmetic prosthesis in the contralateral eye. Three patients complained of mild asthenia and their mycophenolate mofetil was lowered to 1.5 d/day in one patient and to 1 mg/kg in 2 more patients. Two patients had transient blood pressure mild elevation that was brought under control by lowering their cyclosporine dose from 5 to 3 mg/kg/day.

Figure 1

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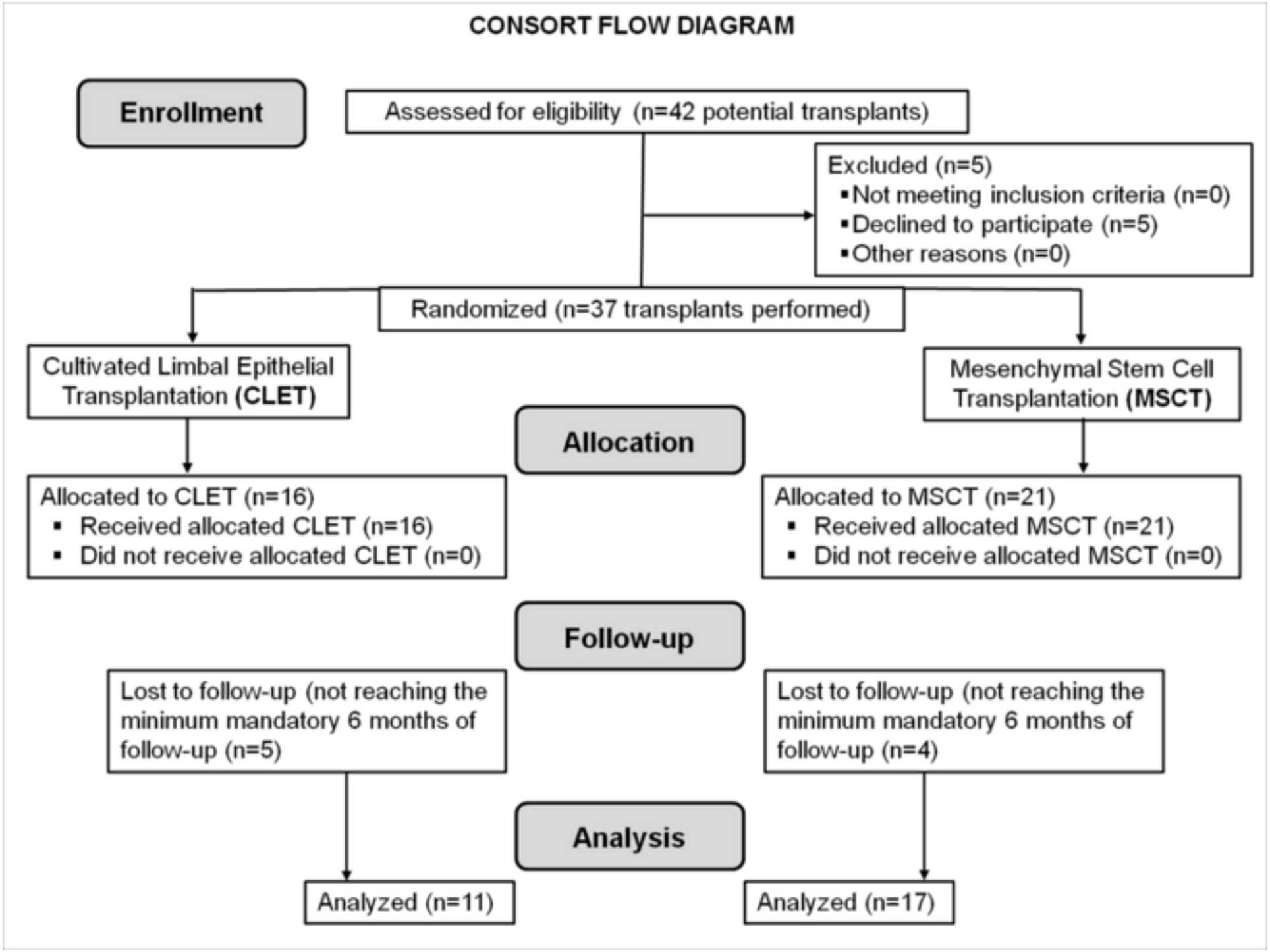

Figure 2

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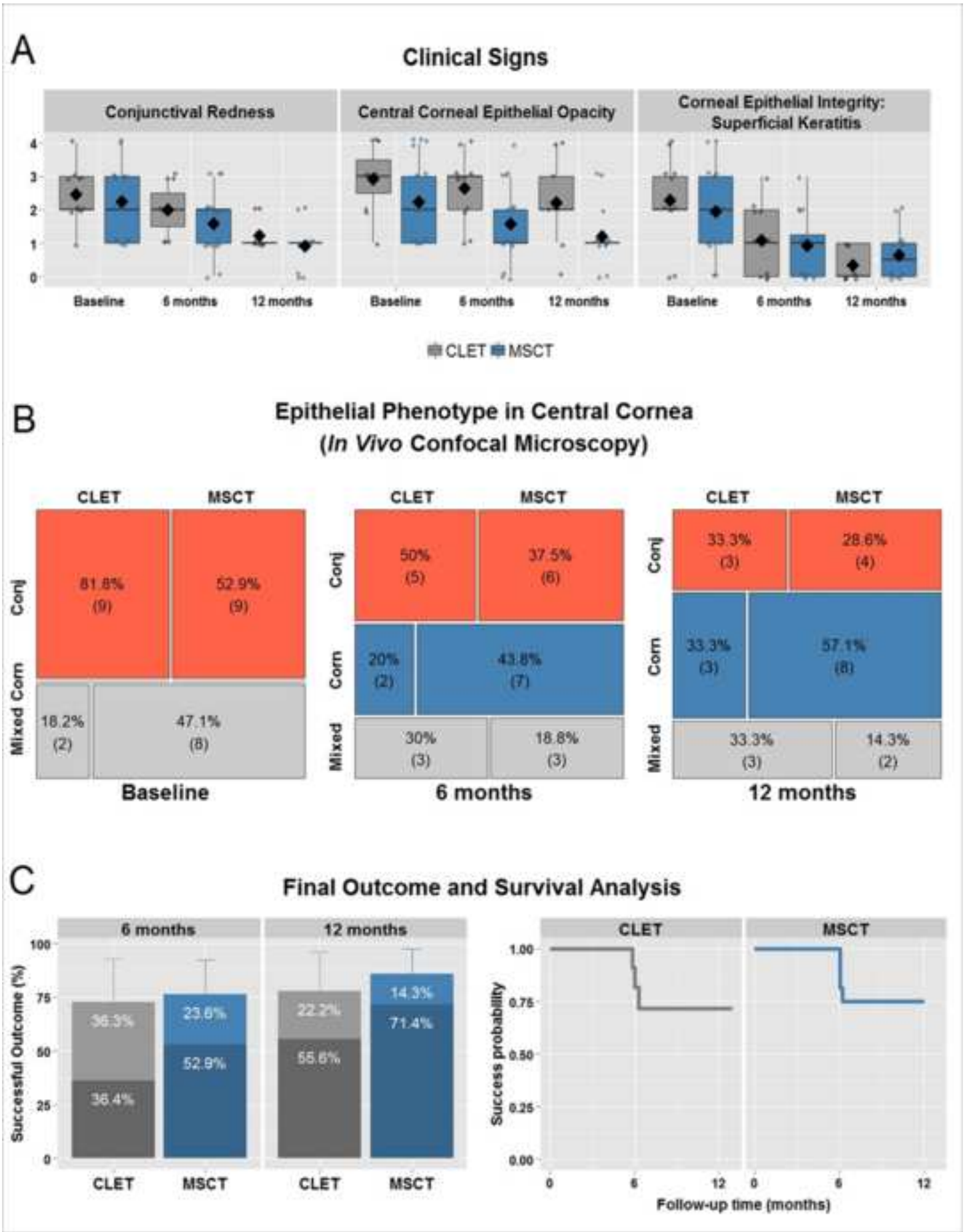

Figure 3

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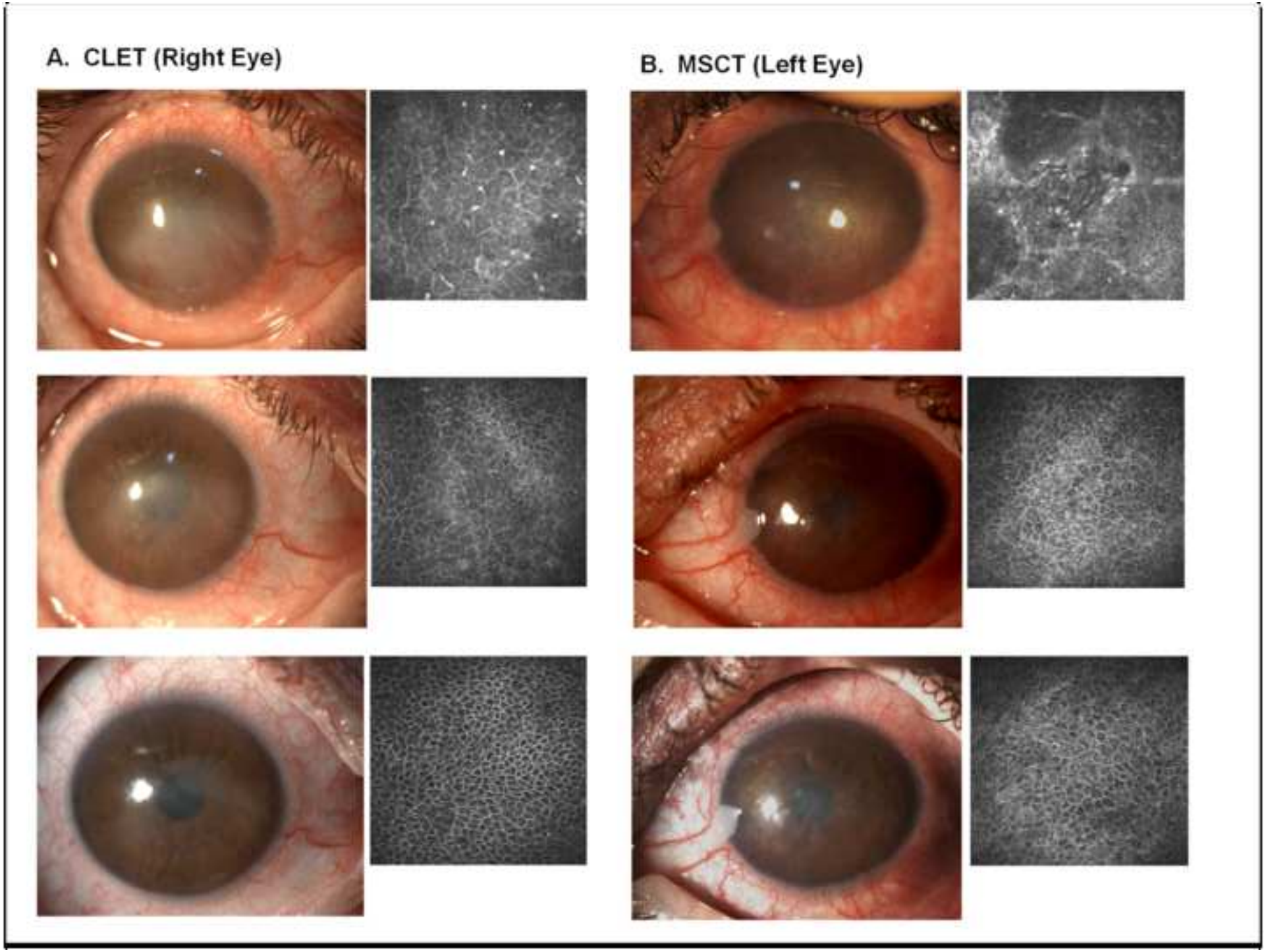

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about this study.

Supplement to: Calonge M, Pérez I, Galindo S, Nieto-Miguel T, López-Paniagua M, Fernández I, Alberca M, García-Sancho J, Sánchez A, Herreras JM. **A Proof-of-Concept Clinical Trial Using Mesenchymal Stem Cells for the Treatment of Corneal Epithelial Stem Cell Deficiency**

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Supplemental Methods

Preparation of amniotic membrane

Human amniotic membranes (2.5x2.5 cm) were used as carriers for cells, and they were prepared using our previously described method.[1] Briefly, the membranes were stored at -80ºC upon arrival. Immediately before use, they were thawed, washed with phosphate buffered saline (PBS, Life Technologies-Gibco, Carlsbad, CA, USA), treated with trypsin for 15 min at room temperature, and gently scraped to remove the epithelial cells from the underlying basement membrane. Afterwards, the samples were washed twice in PBS to remove cellular debris to obtain the de-epithelialized amniotic membranes. Each amniotic membrane was attached to the bottom of a 35-mm cell culture dish with the basement membrane side up. The quality criteria for the amniotic membrane were as follows: (1) minimum size to almost cover a 35-mm diameter culture plate, (2) tissue sterility, (3) tissue integrity, and (4) adherence to the plate.

Preparation of cultivated limbal epithelial cells for transplantation (CLET)

Cadaveric limbal rings were preserved within 7 days from donor death, and they were processed during the 4 h after arrival following a modification of our previously reported protocols.[1,2] Briefly, two 2x2 mm pieces of limbal tissue (limbal explants) were extracted from limbal ring, and both were plated onto the de-epithelialized amniotic membranes (2.5x2.5 cm). The limbal explants were maintained initially under a drop of fetal bovine serum (FBS) (Life Technologies-Gibco) in standard conditions of 37ºC, 95% humidified air, and 5% CO_2 gas mixture. After 24 h, 3 ml of the following culture medium were added: DMEM/F12 media (1:1 mixture) (Life Technologies-Gibco), 5% FBS (Life Technologies-Gibco), 50 µg/ml hydrocortisone (Sigma Aldrich, St. Louis, MO, USA), 0.5 ng/ml

cholera toxin (Gentaur, Kampenhout, Belgium), 5 ng/ml insulin-transferrin-selenium (ITS) (Sigma Aldrich), 0.5% dimethylsulfoxide (Sigma Aldrich), 2.5 ng/ml human epidermal growth factor (Life Technologies-Gibco), and 0.5 mg/ml gentamicin (Life Technologies-Gibco). The limbal explants were kept in culture until a cellular outgrowth front of approximately 2 mm was present, and then they were removed to allow further cell proliferation until cells reached 90% confluence (3-4 weeks). The mean time for explant removal was 12.18 ± 1.17 days (mean ± standard error of the mean, SEM). The culture medium was changed every 3 days. The quality criteria for the limbal explants were as follows: (1) 2x2 mm size, (2) tissue sterility, (3) adherence to the amniotic membrane, and (4) appearance of cell outgrowth in less than 15 days.

Preparation of bone marrow-derived mesenchymal stem cells for transplantation (MSCT)

Bone marrow was processed as we previously reported,[3,4] obtaining 20-200x10^6 of mesenchymal stem cells from every donor after 2 weeks of culture. Subsequently, the mesenchymal stem cells were characterized following the International Society for Cellular Therapy (ISCT) position statement.[3,5,6] Expression of the positive markers CD73, CD90, CD105, and CD166 and the negative markers CD14, CD34, CD45, and HAD-DR was analyzed by flow cytometry. In addition, cell viability was studied by trypan blue staining. Data were reported as means ± SEMs. For MSCT use, 100,000 fresh cells (passage 2) were seeded in a drop of FBS onto a 2.5x2.5 cm piece of de-epithelized amniotic membrane. After 2 h, 2 ml of DMEM medium (High glucose; Life Technologies-Gibco) containing 20% FBS (Life Technologies-Gibco), and 0.5 mg/ml gentamicin (Life Technologies-Gibco) were added, and incubation continued at 37ºC, 95% humidified air, and 5% CO_2 gas mixture until cells achieved 90% confluence (3-5 days) The culture medium was changed every 3 days.

Immunofluorescence microscopy

Amniotic membrane-cell grafts, with either cultivated epithelial limbal cells or mesenchymal stem cells, were monitored under a phase contrast microscope (Eclipse TS100, Nikon, Tokyo, Japan) and fixed with 4% formaldehyde (Panreac, Barcelone, Spain). Before immunofluorescence assays, each amniotic membrane-cell graft was cut into 5 pieces of about 1 cm^2 each. Immunofluorescence assays were performed following a previously reported protocol.[2] The samples were permeabilized for 10 min with 0.3% Triton X-100 (Sigma-Aldrich), blocked with 5% donkey serum (Sigma-Aldrich) for 1 h at room temperature, and incubated overnight at 4ºC with specific primary antibodies (Table S1). Subsequently, samples were incubated 1 h at room temperature with the corresponding secondary antibody (Alexa Fluor® 488 donkey anti-mouse 1:200 or donkey anti-rabbit 1:300; Life Technologies). Cell nuclei were counterstained with propidium iodide (1:6,000; Life Technologies). Each piece obtained from a single amniotic membrane-cell graft was incubated with one primary antibody. The marker analyzed in each piece was randomly assigned. Regarding the total number of experiments performed, the same marker was analyzed in different areas of the total amniotic membrane-cell graft surface (near the explant or at the graft edge). Images were acquired with an inverted fluorescence microscope (DM4000B, Leica, Wetzlar, Germany). The percentage of positive cells was estimated for each marker. Negative controls included the omission of primary antibodies. All antibodies were previously validated in different positive controls by our research group.[2,7] At least four samples from different cell donor were analyzed for each condition (n=4).

Patients

Prior to the initiation of these procedures, all patients and all allogeneic tissue donors underwent mandatory screening for the following transmittable diseases: human immunodeficiency virus, human T-cell leukemia-lymphoma virus, syphilis, and hepatitis B-C.

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

Twenty-four hours after surgery, each patient was evaluated and topical treatment with the fixed combination of 1% prednisolone acetate and 0.3% tobramycin (Tobradex®, Alcon Laboratories, FT. Worth, TX, USA) was prescribed 4 times per day until the amniotic membrane dissolved. The stitches and the contact lens were also removed between 4 and 6 weeks. Then, 1 mg/ml dexamethasone (Maxidex®, Alcon Laboratories) was instilled 4 times a day and slowly tapered in the next 3 months.

Evaluation end-points

Limbal stem cell deficiency-related symptoms and their impact on daily life activities were evaluated with three self-administered questionnaires. The Single Item Dry Eye Questionnaire (SIDEQ) gives a 0-4 score to each of 5 different questions about the presence of dryness, foreign body sensation, burning/stinging, pain, itching, sensitivity to light, and blurred vision (maximum score: 28); the Ocular Surface Disease Index (OSDI) also evaluates ocular surface symptoms with 12 questions, and scores >12 indicate abnormal symptomatology, and >32 means severe symptoms (maximum score 100).[8] The visual function-related aspects of the quality of life were evaluated with The National Eye Institute 25-item Visual Function Questionnaire (NEI-VFQ25), where higher scores on a 0 to 100 scale indicate better function.[9]

Right after the questionnaires were administered, best corrected visual acuity was measured using the standard Early Treatment Diabetic Retinopathy Study (ETDRS), as is mandatory in clinical trials. It is crucial to note that vision improvement is never the primary goal of this kind of cell transplantation because this technique intends only to reconstruct the corneal epithelium. It will not affect deeper corneal opacification, cataract, glaucoma that often accompanies these pathologies, or other potential causes of diminished vision such as concomitant retinal pathology in post-multiple surgery cases, nystagmus in congenital aniridia, and others. For these conditions, if they are not irreversible, other visual rehabilitation techniques might be needed after CLET or MSCT. To avoid misinterpretation by the patient, the potential dependence of the visual prognosis on the surgical procedures judged to be necessary to restore vision after cell transplantation was explained at the initial visit and given a grade as shown in Table 1.

After determination of the best corrected visual acuity, the ocular surface clinical status was evaluated as routinely done by anterior segment biomicroscopy using a slit lamp and taking photographs

(IMAGENet program Fuji Fujifilm Finepix S1 Pro. Fuji Photo Film Co., LTD., Tokyo, Japan; Slit lamp Topcon SL-8Z, Topcon Corp., Hasunuma-cho, Habasi-ku, Tokyo, Japan) at each visit. All evaluated parameters at the initial visit and at 6 and 12 months after transplantation and associated scales are shown in Table 1. Ocular redness was evaluated in the bulbar conjunctiva, proximal to the cornea. Nasal and temporal areas were assessed independently based on the Efron scale (score 0-4),[10] and the final score was obtained after averaging both values. Corneal epithelial integrity was evaluated with the vital stain sodium fluorescein using a commercial strip previously wetted and applied to the inferior fornix. After 2 min, the degree of staining was recorded using a cobalt blue filter (Topcon Corp., Tokyo, Japan) over the light source of the slit-lamp biomicroscope and a yellow Wratten #12 filter (Eastman Kodak, Rochester, NY, USA). Both superficial punctuate keratitis (Oxford scheme, 0-5 score)[11] as well as the potential presence of persistent epithelial defect were recorded.

In vivo laser confocal microscopy was the last-performed evaluation end-point (always by same coauthor IP). We used the Heidelberg Retinal Tomograph HRT-3 and Rostock Cornea Module (HRT3, Heidelberg Engineering GmbH, Heidelberg, Germany) and followed the protocol as previously described.[1] Topical anesthesia was achieved with 0.1% tetracaine chlorhydrate and 0.4% oxibuprocaine chlorhydrate solution (Colircusí Anestésico Doble®, Alcon Laboratories) Optical sections from the central cornea were taken at all layers of the epithelium, and the basal layers were then evaluated for the defined phenotypes, as explained in Table 1.

Several other tests were performed at the initial visit and at 6 and 12 months that were not related to outcomes but are part of any routine ophthalmic evaluation. Schirmer test without topical anesthesia evaluated tear production. One Schirmer sterile strip (Tearflo; HUB Pharmaceuticals LLC, Rancho Cucamonga, CA, USA), was placed in the lateral canthus of the inferior lid margin. The length of wetting was measured after 5 min, with eyes closed. Intraocular pressure was evaluated using a Perkins tonometer (Perkins MK 2; HS Clemens Clarke International, Essex, United Kingdom). Fundus

evaluation was by indirect ophthalmoscopy under pharmacologic pupil dilation. When media opacity prevented visualization of intraocular structures by slit-lamp examination or funduscopy, anterior segment optical coherence tomography (OCT) and posterior segment echography (ultrasound) were routinely performed.

During the course of all visits, patients were carefully questioned for potential medication side-effects or any other possible adverse event by two clinicians (co-authors MC and JMH) who also evaluated clinical parameters independently. In case of disagreement, the average score was recorded.

Supplemental Results

Characterization of bone marrow-derived mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells had the phenotype defined by the ISCT. The positive markers CD73, CD90, CD105 and CD166 were expressed by 99.7 ± 0.1%, 99.9 ± 0.02%, 97.8 ± 0.5%, and 98.98 ± 0.31% of the cells, respectively. Negative markers CD14, CD34, CD45 and HLA-DR were expressed by 0.1 ± 0.04%, 0.03 ± 0.02%, 0.09 ± 0.03%, and 0.05 ± 0.02% of the cells, respectively. The viability of the bone marrow-derived mesenchymal stem cells was 98.6 ± 0.002%.

Immunofluorescence microscopy

Protein markers K15 and p63alpha for limbal epithelial cells and K3 for differentiated corneal epithelial cells were analyzed in both types of cells cultured on amniotic membrane. The percentage of limbal epithelial cells positive for limbal stem cell markers K15 and p63alpha was 90% and 70% respectively (Figure S1 and Table S2). The corneal differentiated epithelial protein K3 was expressed

by 80% of limbal cells (Figure S1 and Table S2). These markers K15, p63alpha, and K3 were expressed by 90% of mesenchymal stem cells cultured on amniotic membrane (Figure S1 and Table S2).

Supplemental Tables

Table S1. Primary antibodies used for immunodetection assays.

Antibody	Specificity	Category	Clone	Source	Working dilution
Keratin 3 (K3)*	Differentiated corneal epithelial cells	Mouse monoclonal	AE5	Mp Biomedical (Illkirch, France)	1:50
Keratin 15 (K15)	Limbal epithelial stem cells	Mouse monoclonal	LHK15	Millipore (Billerica, MA, USA)	1:50
Alpha isoform of nuclear protein 63 (p63alpha)	Limbal epithelial stem cells	Rabbit polyclonal	-	Cell Signaling (Danvers, MA, USA)	1:50

*K3 and K12 are the most specific markers for the corneal epithelium, and they are not expressed in limbal epithelial stem cells. Either of these markers can be used to determine the corneal epithelial phenotype.

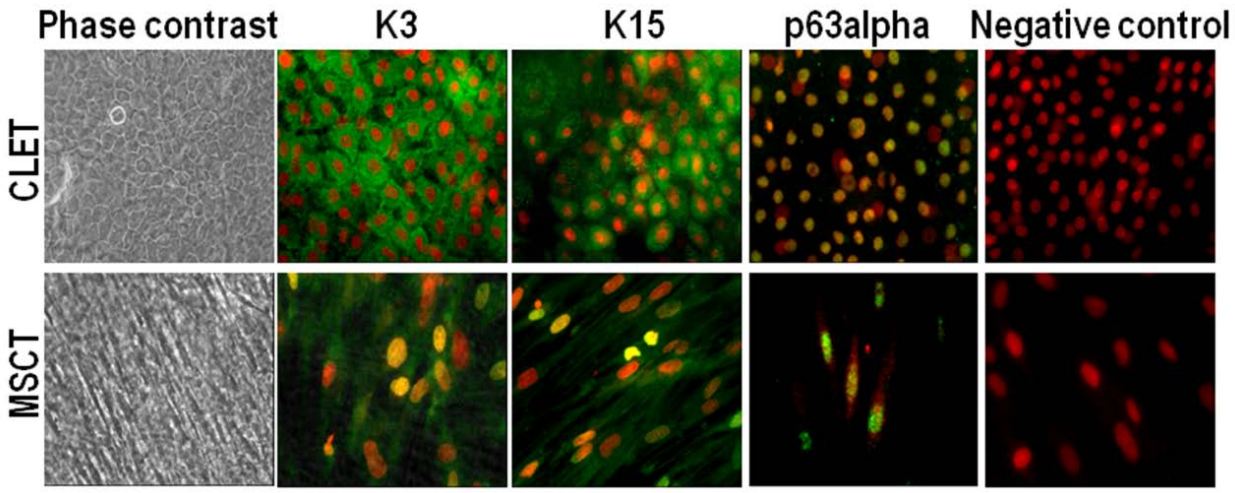
Table S2. Characterization of cultivated limbal epithelial cells and bone marrow-derived mesenchymal stem cells for transplantation (CLET and MSCT, respectively).

Sample	Mean time ofculture on amniotic membrane	Mean % of K3	Mean % of K15	Mean % of p63alpha	Cell morphology	Cell stratification
CLET	3 – 4 weeks	80	90	70	Cuboidal	No
MSCT	3 – 5 days	90	90	90	Elongated	No

K: Keratin; p63alpha: Alpha isoform of nuclear protein 63.

Supplemental Figure

Figure S1. Characterization of cultivated limbal epithelial cells and bone marrow-derived mesenchymal stem cells for transplantation (CLET and MSCT, respectively). Representative images captured by phase contrast (20X magnification) and immunofluorescence microscopes (40X magnification), n=4. Corneal epithelial cell (K3) and limbal epithelial stem cell (K15 and p63alpha) markers were analyzed. Green, K3, K15, and p63alpha marker expression; red, nuclei counterstained with propidium iodide.



Supplemental Discussion of Immunofluorescence Results

The quality of the transplanted amniotic membrane-containing cell grafts (CLET or MSCT) were characterized in parallel. We observed that around 80% of limbal epithelial cells cultured on amniotic membranes expressed the limbal epithelial stem cell markers K15 and p63alpha. This was consistent with the results previously reported by Zakaria et al.,[12] although the data are not directly comparable due to different culture media and scaffolds that were used by these authors. In their amniotic membrane-limbal epithelial cell grafts, the predominant phenotype (>50%) consisted of cells that expressed ABCG2, ΔNp63, and K14 markers. Moreover, these authors reported negative expression for the corneal proteins K3/12 and desmoglein.[12] In contrast, we found that protein K3 was also expressed by about 80% of limbal cells on grafts, showing that at least some of these cells expressed limbal epithelial stem cell markers and corneal markers at the same time. These results could suggest the presence of a high percentage of transient amplifying cells ($K15^+$, $p63alpha^+$, and $K3^+$) in amniotic membrane-limbal epithelial cell grafts. These data agree with the fact that human limbal epithelium contains mainly transient amplifying cells and that limbal epithelial stem cells represent less than 10% of the total limbal basal cell population.[13–15] Therefore, our grafts would be suitable for ocular surface treatment. This is consistent with the finding of Rama et al.,[16] who reported that cultures containing more than 3% p63 positive cells have a high probability of leading to successful corneal epithelial regeneration. In contrast, cultures with 3% or less p63 positive cells have a lower probability for successful corneal regeneration. Their data are not directly comparable to ours because of the different culture conditions. On the other hand, it is not possible to rule out the potential migration of limbal MSC from the limbal explant stroma to the amniotic membrane under culture conditions. In fact, this could be an explanation for the high expression of K3 and p63alpha observed in CLET cultures, as different authors have reported K3 and p63alpha expression in MSC

cultured on amniotic membrane.[17–22] However, the morphology observed by both phase contrast and immunofluorescence microscopy in the LESC cultures was polygonal, more similar to epithelial-like cells, suggesting that it is very unlikely that MSC from limbal stroma were contaminating the CLET cultures. Mesenchymal stem cells cultivated on amniotic membranes were positive for K3 and K15 proteins. These data agree with previous studies in which different cytokeratins (K3, K12, K18) were expressed by cells obtained from bone marrow or adipose tissues.[17–21] In addition, we detected the p63alpha marker in cells cultivated on the amniotic membranes, in accordance with results reported by other investigators.[22–24] However, the expression of this marker by mesenchymal stem cells is currently controversial because several groups showed that MSC did not express p63 protein.[18,20]

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