

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

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44 **Running head:** Mesenchymal stem cells for corneal failure

45

46 **List of abbreviation**

47 AEMPS: Spanish Agency of Medicines and Sanitary Products

48 CLET: cultivated limbal epithelial transplantation

49 hAM: human amniotic membrane

50 LSCD: limbal stem cell deficiency

51 MSC: mesenchymal stem cells

52 MSCT: mesenchymal stem cell transplantation

53

54

55 **ABSTRACT**

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

78

79 **Key words:** clinical trial, corneal blindness, corneal epithelial stem cells, human

80 proof-of-concept, *in vivo* confocal microscopy, limbal stem cell deficiency,

81 mesenchymal stem cell, stem cell transplantation

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83

84 **INTRODUCTION**

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

109 Moreover, cryopreserved MSC can be transplanted without loss of potency,<sup>21</sup>  
110 whereas cryopreserved limbal epithelial stem cells have not been transplanted in  
111 humans yet.<sup>22,23</sup> Finally, allogeneic MSC can be transplanted without the need of  
112 host immunosuppression,<sup>20,24,25</sup> while allogeneic transplantation of limbal epithelial  
113 stem cells requires one year of systemic immunosuppression to avoid immune  
114 rejection.<sup>9,11</sup>

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

121

## 122 **MATERIAL AND METHODS**

### 123 ***Study design and patients***

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

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

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

132 Products (AEMPS, [www.aemps.gob.es](http://www.aemps.gob.es)). The Clinical Trials.gov Identifier is  
133 NCT01562002.

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

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

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



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

160

### 161 ***Randomization and masking***

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

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

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

181

## 182 ***Procedures***

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

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

203 determining that the cultures were negative for aerobes, anaerobes, fungi, and  
204 mycobacteria, they were delivered within 4 h of surgery. Some of the cultures that  
205 were assigned to patients who cancelled their surgeries (see below) were allowed to  
206 finish growing and later processed for immunostaining to test for limbal,  
207 mesenchymal, and differentiated corneal epithelial cell markers, as previously  
208 published<sup>30,31</sup> (see Supplementary Appendix for more details). To prevent any  
209 possible immune allograft rejection, patients receiving CLET underwent a mild  
210 immunosuppressive therapy.<sup>11</sup> While patients receiving MSCT would not normally  
211 need such therapy due to the absence of immune rejection by allogenic MSC,<sup>15-  
212 17,24,25</sup> oral immunosuppression was instituted to eliminate immune suppression as a  
213 variable and to maintain the double masked nature of the study; otherwise, the trial  
214 could not have been masked. Thus, all patients were started at the initial visit on 1.5-  
215 2.0 g/day of mycophenolate mofetil; 3-5 mg/kg/day of cyclosporine A, or 1-2  
216 mg/kg/day of azathioprine were also permitted if, for any reason, mycophenolate  
217 mofetil was not available or the patient was already using one of the other two  
218 immunomodulating agents. This treatment was maintained for 12 months after  
219 transplantation and discontinued in the next 3 months. We closely monitored  
220 potential side effects clinically and by blood/urine work-ups every 1-2 months. No  
221 other systemic medications were added.

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

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

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

244

## 245 **Outcomes**

246 Evaluation endpoints were collected at the initial baseline visit and at 6 months and  
247 12 months. Three self-administered questionnaires evaluated symptoms and quality  
248 of life: the Single Item Dry Eye Questionnaire (SIDEQ), the Ocular Surface Disease  
249 Index (OSDI), and the National Eye Institute 25-item Visual Function Questionnaire  
250 (NEI-VFQ25).<sup>32,33</sup> The following clinical signs were evaluated by slit-lamp  
251 biomicroscopy: conjunctival redness, central corneal epithelial opacity, corneal

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

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

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

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

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

285 The outcome was considered successful when either a complete or a partial success  
286 was accomplished. A complete success meant that all four primary outcomes were  
287 achieved, and a partial success meant that at least two of the four primary outcomes  
288 or one primary and one secondary outcome were achieved. Failure meant that only  
289 one or none of the primary outcomes was met.<sup>11</sup>

290

### 291 ***Statistical analysis***

292 Statistical analyses were performed by a PhD licensed statistician, who estimated  
293 that a sample of 10 transplants per group would give 80% statistical power to detect  
294 the non-inferiority of the experimental group. The calculations assumed a success  
295 rate of 80% in each group and a non-inferiority margin of 25% at an alpha level of  
296 0.05. Consequently it was considered that a minimum of 10 transplants per group  
297 was sufficient for the exploratory nature of this proof-of-concept trial.

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

318

## 319 **RESULTS**

### 320 ***Clinical trial sample***

321 We initially recruited 27 Caucasian patients (36 eyes, 42 potential transplants) (Fig  
322 1). Five patients (10 eyes) cancelled surgery due to different personal/logistical  
323 reasons. Therefore, the final number of transplant surgeries was 37 (26 eyes, 22  
324 patients). Among them, 16 cases were randomized to CLET and 21 to MSCT. Nine  
325 cases (24.3% of the total 37; 95% confidence interval [CI], 12.4 to 41.6), 5 CLET and  
326 4 MSCT, lost their transplants within the first week due to loss of the bandage contact  
327 lens. Based on our preclinical data (unpublished), we considered that stem cells may  
328 have not completely reached their tissue target in less than 7 days, thus these  
329 transplants were excluded.

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

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

342 Patients with assessable transplants had a mean age of  $49.3 \pm 10.8$  years (range, 28-  
343 77 years). Females comprised 42.9% (95% CI, 25 to 62.6) of the transplant  
344 recipients and males 57.1% (95% CI, 37.4 to 75) ( $p=0.253$ ). The assignment of



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

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

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

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

366 There were 3 serious adverse events and 21 non-serious adverse events, including  
367 10 that were mild and 11 that were moderate to severe (Table 5). All were unrelated  
368 to the type of cell transplantation. Most were due to activation or recurrence of

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

371

### 372 ***Final outcome and survival analysis***

373 The overall success for all cell transplants after 6 months was 75% (21 of 28 cases:  
374 13 complete successes, 8 partial successes). After 12 months, the success rate was  
375 82.6% (19 of 23: 15 complete successes, 4 partial successes). Five transplants were  
376 evaluated until month 6 (4 regrafts, one withdrawal), accounting for the different  
377 percentages. Except for CLET-2 and MSCT-15 that went from partial successes at 6  
378 months to complete successes at 12 months, the final fate of all transplants was  
379 already established at 6 months.

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

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

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

398 Evaluation of the epithelial phenotype in the central cornea by laser IVCM was the  
399 most objective primary endpoint. Based on the established criteria, complete success  
400 required that the corneal epithelial phenotype of the transplanted eyes must have  
401 improved by at least one step towards the normal corneal epithelial phenotype. At  
402 baseline, there was no significant difference in the distribution of the conjunctival-like  
403 or the mixed epithelial phenotype in the central cornea (Fisher's exact test, baseline  
404  $p=0.226$ ). Consequently, the outcome of each type of cell transplant could not have  
405 been influenced by a more frequent presence of a more favorable phenotype before  
406 cell therapy in any of the three etiologic groups (Table 4). The change in epithelial  
407 phenotype at the central cornea (Fig 2) was not significantly different between CLET  
408 and MSCT at 6 months ( $p=0.524$ ) or at 12 months ( $p=0.5562$ ). After 6 months, 50%  
409 of CLET cases (95% CI, 23.7 to 6.3) and 62.5% of MSCT cases (95% CI, 35.9 to  
410 83.7) had improved the epithelial phenotype in the central cornea. At 12 months after  
411 surgery, 66.7% of CLET (95% CI, 30.9 to 91) and 71.4% of MSCT (95% CI, 42 to  
412 90.4) had improved the epithelial phenotype in the central cornea. The differences  
413 between CLET and MSCT were not significant ( $p=0.6891$  and  $p=1.000$  at 6 and 12  
414 months, respectively).

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

417 (differences were not significant). For MSCT, the corneal epithelial phenotype was  
418 present in 43.8% (7 cases, 95% CI, 20.8 to 69.5) at 6 months and 57.1% (8 cases,  
419 95% CI, 29.7-81.2) at 12 months ( $p=0.0469$ , and  $p=0.0234$ , respectively). The  
420 percentage of cases reaching a corneal-like epithelial phenotype was not significantly  
421 different between CLET and MSCT at 6 ( $p=0.4152$ ) or 12 months ( $p=0.854$ ). Survival  
422 curve analysis showed that the differences between CLET and MSCT survival was  
423 not significant at either 6 or 12 months (log-rank test,  $p = 0.817$ , Fig 2). See Fig 3 as  
424 example.

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

427

## 428 **DISCUSSION**

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

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

441 anti-angiogenic, and anti-microbial features.<sup>38</sup> 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.<sup>39</sup>  
444 However, cell-free hAM transplantation is insufficient for regeneration of the ocular  
445 surface in patients with severe and/or total LSCD.<sup>13,14</sup>

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.<sup>11</sup>

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<sup>5,6,40</sup> or cell-based therapy.<sup>2,4,8-12,41</sup> 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.<sup>9</sup> 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<sup>2</sup> 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,<sup>11</sup> confirming previous studies.<sup>8,10,41,42</sup>

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

465 10% of all cells.<sup>1,3</sup> This means that the procedure is dependent upon healthy donor  
466 eyes or cadaveric donations, which may be limited. In practice, the small limbal  
467 biopsies can be lost because of contamination and/or lack of adequate growth.  
468 Typically, it takes 3-5 weeks to cultivate a sufficient quantity for transplantation.  
469 These factors make both autologous and allogenic CLET expensive and time-  
470 consuming. The natural step forward is to turn to the most commonly used adult stem  
471 cell in regenerative medicine, MSC. Currently these cells are being used in clinical  
472 trials to treat multiple diseases including osteo-articular, liver, kidney, cardiac,  
473 hematological (graft-versus-host disease), lung problems, keratoconus, and  
474 preclinically in retinal repair.<sup>43-46</sup> There is also abundant literature on the successful  
475 use of these cells in animal models of limbal deficiency or corneal burns.<sup>17,47-53</sup>

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

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

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

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

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

517

## 518 **CONCLUSIONS**

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

528

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- 737

738 **FIGURE LEGENDS**

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

742

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

746 Panel A shows all clinical signs evaluated with anterior segment slit-lamp  
747 biomicroscopy: ocular (conjunctival) redness, corneal epithelial integrity, and central  
748 corneal epithelial opacity. Boxes extend from the 25th to the 75th percentile,  
749 horizontal bars represent the median, and whiskers extend 1.5 times the length of the  
750 interquartile range (IQR) above and below the 75th and 25th percentiles,  
751 respectively. The mean of each group is shown by black diamonds. Individual values  
752 for each subject are indicated by filled circles. Conjunctival redness and central  
753 corneal epithelial opacity improved significantly from baseline to final evaluation at 12  
754 months. Superficial keratitis improved significantly by 6 months. Both CLET and  
755 MSCT groups improved similarly, except for corneal opacity, which was significantly  
756 better for MSCT at 6 and 12 months (see Table 4 for mean numerical values and  
757 Tables 1 and 2 for individual values). Panel B shows mosaic plots. The area of each  
758 rectangle is proportional to the observed frequency in that cell. Labels show the  
759 conditional relative percentages of each possible epithelial phenotype for each  
760 transplant type at baseline, 6, and 12 months. The number of cases is indicated in  
761 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 360° 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,

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

800 TABLES

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

CLET No./Eye	Patient No. Gender/ Age	LSCD etiology (months elapsed till transplant): Etiology*/Grade† 2 <sup>nd</sup> 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 keratoprosthesis PKP at month 14
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	+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 from 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+/ 2 <sup>nd</sup> 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, performing 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	0.25/ 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 <sup>+</sup>	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) <sup>†</sup>
			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.<sup>64</sup>

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



808 Table 4. Characteristics, Endpoint Values, and Outcome of the 28 Assessable Cases  
809 (23 eyes from 20 patients) of Corneal Epithelial Failure Due to Limbal Epithelial Stem  
810 Cell Deficiency Randomized to Cultivated Limbal Epithelial Transplantation (CLET) or  
811 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
<b>Limbal Stem Cell Deficiency</b>			
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
<b>Primary Evaluation End-Points</b>			
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 <b>p=0.0237</b> and <b>p=0.0336</b> 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 <b>p=0.0318</b> and <b>p=0.0072</b> 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	<b>0.0239</b> / 0.0774/ 0.2524
Clinical signs (range) — median (interquartile range [IQR])			
Conjunctival redness (0-4)	2 (1)/ 2(1)/ 1 (0) <b>p=0.0012</b> between baseline and 12 months	2 (2)/ 2(1)/ 1 (0) <b>p&lt;0.0001</b> between baseline and 12 months	0.6057/ 0.2638/ 0.2438
Central corneal epithelial opacity (0-4)	3 (1)/ 3 (1)/ 2 (1) <b>p=0.0129</b> between baseline and 12 months	2 (2)/ 1 (1)/ 1 (0) <b>p=0.0023</b> between baseline and 12 months	0.1323/ <b>0.0275</b> / <b>0.04</b>
Corneal epithelial integrity: superficial punctate keratitis (0-4)	2 (1)/ 1 (2)/ 0 (1) <b>p=0.0428</b> and <b>p=0.0012</b> between baseline and 6 or 12 months	2 (2)/ 1 (1.25)/ 0.5 (1) <b>p=0.0263</b> and <b>p=0.0006</b> 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 ( <i>in vivo</i> 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) <b>p=0.0129</b> between baseline and 12 months	3 (2)/ 2 (2)/ 2 (2) <b>p=0.0307</b> and <b>p=0.0045</b> 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) <b>p=0.0307</b> and <b>p=0.0044</b> between baseline and 6 or 12 months	2 (1)/ 2 (1)/ 1.5 (1) <b>p=0.0055</b> 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

812  
813 \* Between CLET and MSCT groups. Significant p-values are highlighted in bold characters.  
814 Only significant P-values are shown in the 2nd (CLET) and the 3rd (MSCT) columns.

815 †Only one case, CLET n<sup>o</sup>2 (Table 1), had a grade 1 potential to recover visual acuity,  
816 meaning that it was previously considered that her damage was restricted to the corneal  
817 epithelium. Her visual acuities were 0.04, 0.2 and 0.25 at baseline, 6, and 12 months,  
818 respectively.

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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 amniotic 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

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

833 † Non-serious adverse events shown are those that were moderate or severe. The  
834 remaining non-serious adverse events were mild, easily solved and all were  
835 considered to be unrelated with cell transplant: nausea, vomiting, pharyngitis, twisted  
836 ankle, and reconstruction of anophthalmic socket to improve existent cosmetic  
837 prosthesis in the contralateral eye. Three patients complained of mild asthenia and  
838 their mycophenolate mofetil was lowered to 1.5 d/day in one patient and to 1 mg/kg in  
839 2 more patients. Two patients had transient blood pressure mild elevation that was  
840 brought under control by lowering their cyclosporine dose from 5 to 3 mg/kg/day.

---

Figure 1  
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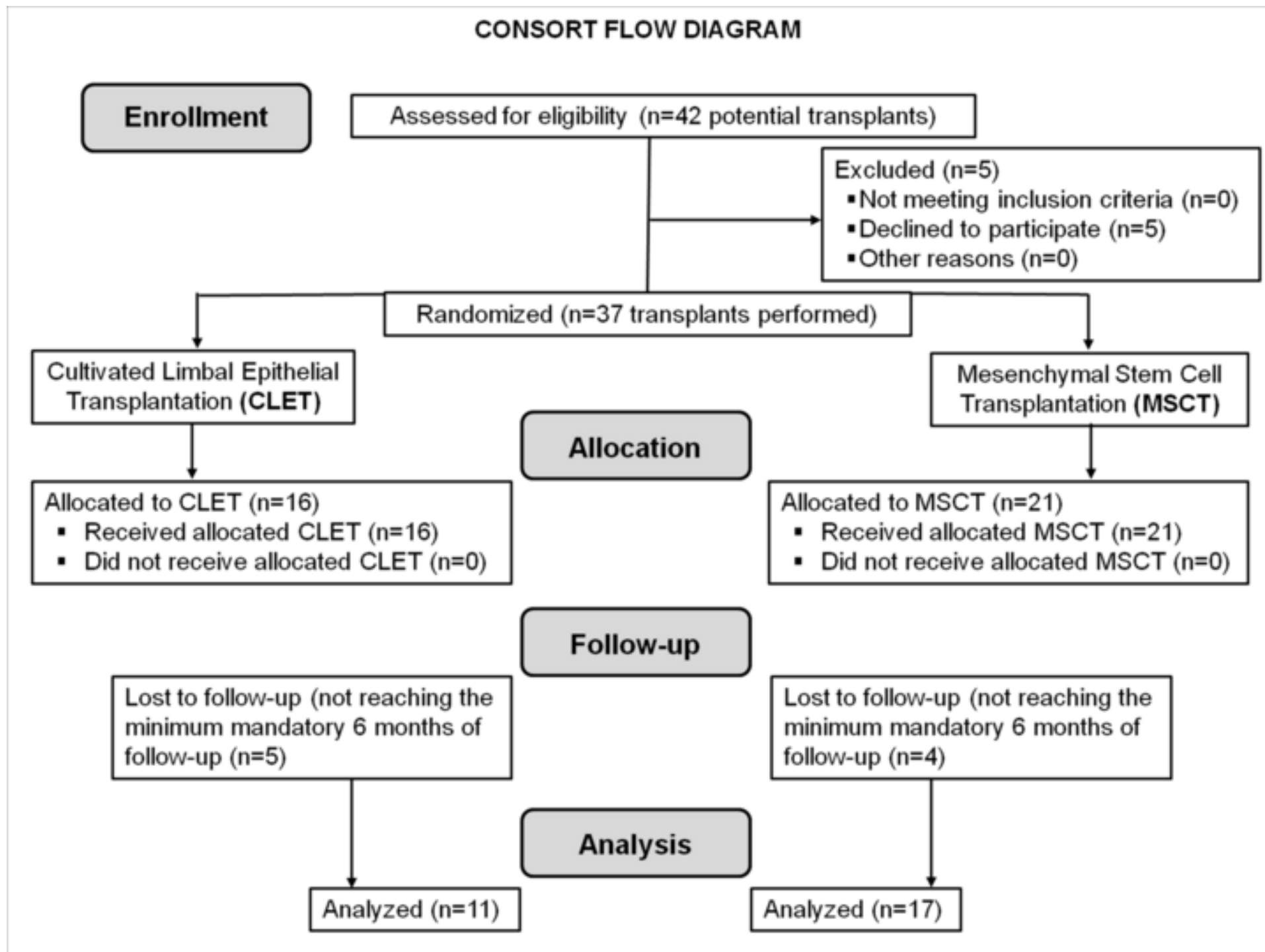


Figure 2  
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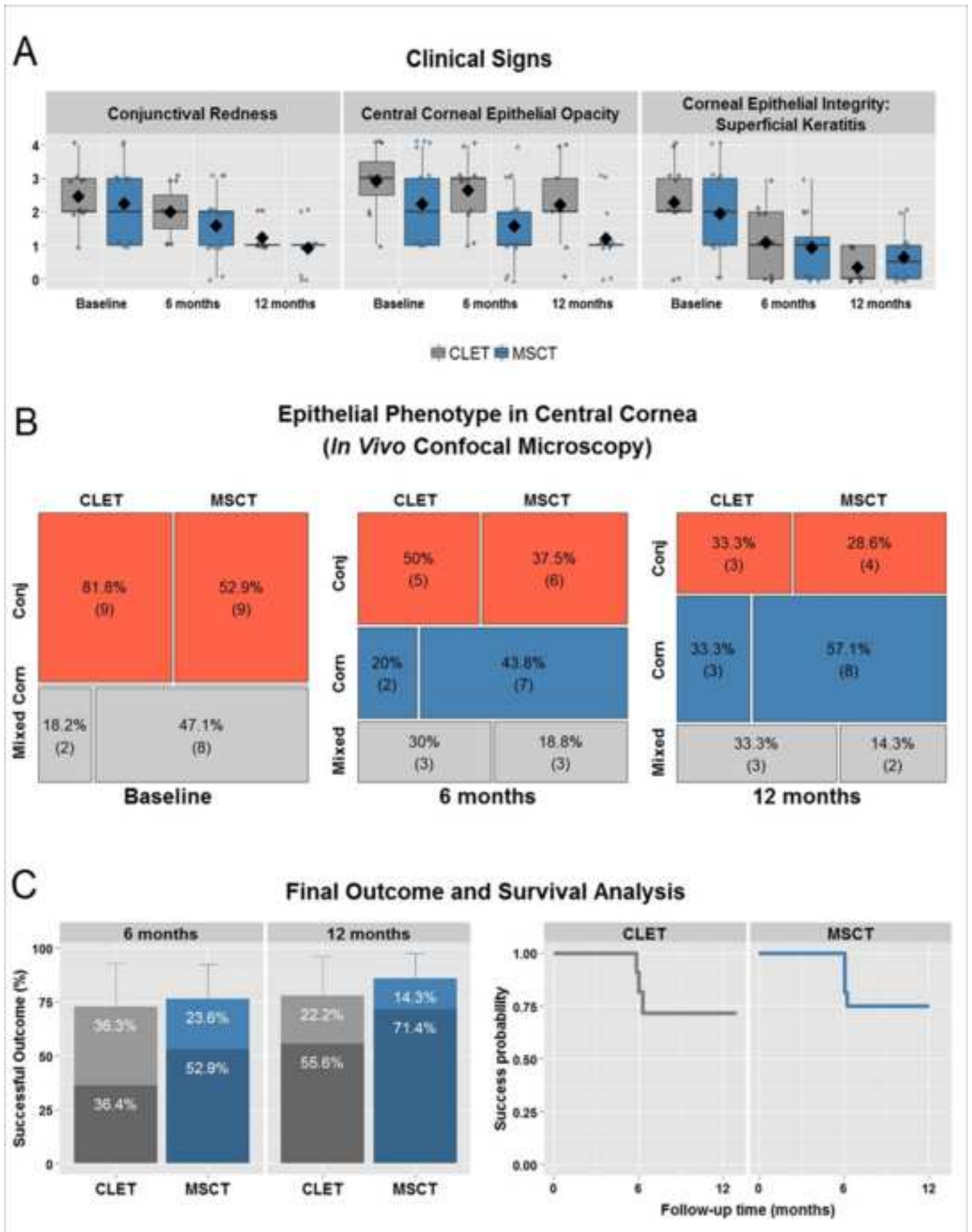
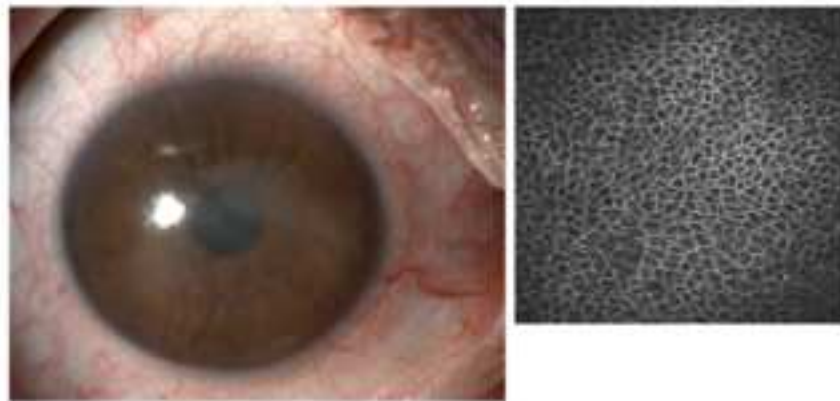
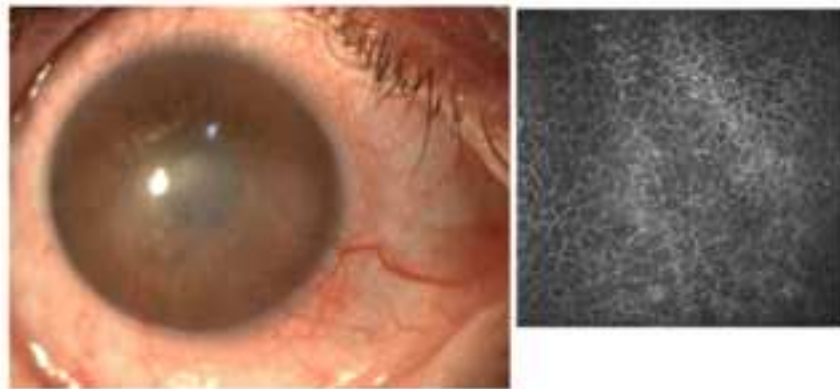
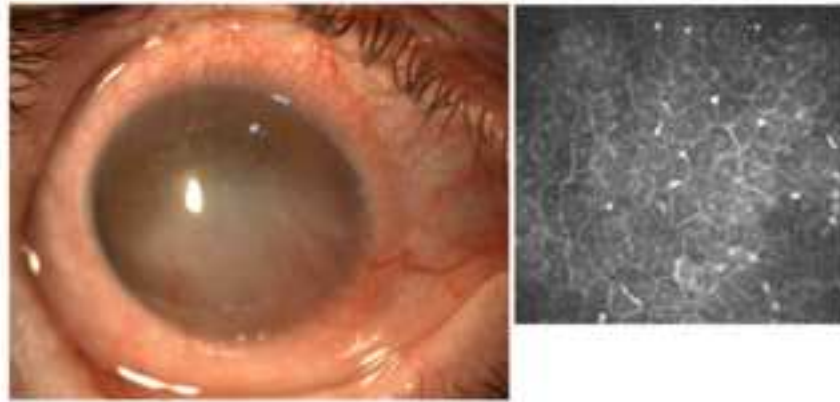
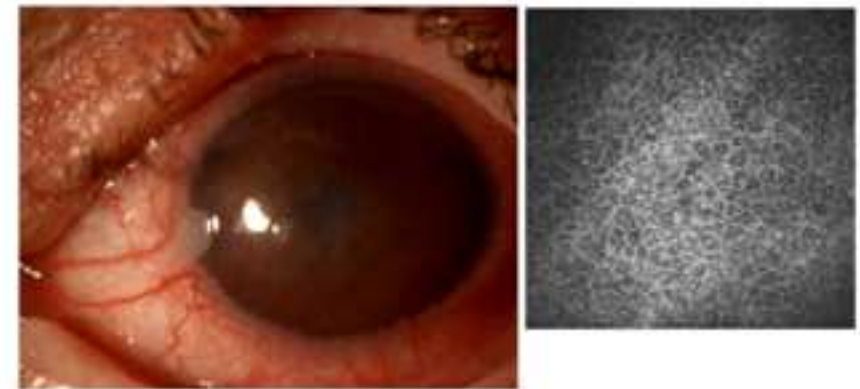
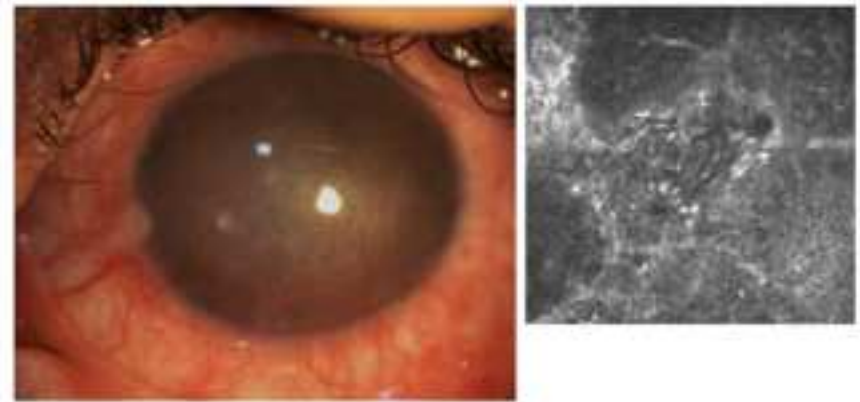


Figure 3  
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A. CLET (Right Eye)



B. MSCT (Left Eye)



## 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**

### Table of Contents

Supplemental Methods .....	2
Preparation of amniotic membrane .....	2
Preparation of cultivated limbal epithelial cells for transplantation (CLET) .....	2
Preparation of bone marrow-derived mesenchymal stem cells for transplantation (MSCT) .....	3
Immunofluorescence microscopy .....	4
Patients .....	5
Evaluation end-points .....	6
Supplemental Results .....	8
Characterization of bone marrow-derived mesenchymal stem cells .....	8
Immunofluorescence microscopy .....	8
Supplementary Tables .....	10
Supplementary Table S1 .....	10
Supplementary Table S2 .....	10
Supplementary Figure.....	11
Supplementary Figure S1 .....	11
Supplemental Discussion of Immunofluorescence Results .....	12
Supplemental References .....	14



## **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.<sup>1</sup> Briefly, the membranes were stored at -80°C 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.<sup>1,2</sup> 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 37°C, 95% humidified air, and 5% CO<sub>2</sub> 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  $12.18 \pm 1.17$  days (mean  $\pm$  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,<sup>3,4</sup> obtaining  $20\text{-}200 \times 10^6$  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.<sup>3,5,6</sup> 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  $\pm$  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 37°C, 95% humidified air, and 5% CO<sub>2</sub> 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, Barcelona, Spain). Before immunofluorescence assays, each amniotic membrane-cell graft was cut into 5 pieces of about 1 cm<sup>2</sup> each. Immunofluorescence assays were performed following a previously reported protocol.<sup>2</sup> 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 4°C with specific primary antibodies (Table S1). Subsequently, samples were incubated 1 h at room temperature with the corresponding secondary antibody (Alexa Fluor<sup>®</sup> 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.<sup>2,7</sup> 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).<sup>8</sup>

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.<sup>9</sup>

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),<sup>10</sup> 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 punctate keratitis (Oxford scheme, 0-5 score)<sup>11</sup> 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.<sup>1</sup> 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  $99.7 \pm 0.1\%$ ,  $99.9 \pm 0.02\%$ ,  $97.8 \pm 0.5\%$ , and  $98.98 \pm 0.31\%$  of the cells, respectively. Negative markers CD14, CD34, CD45 and HLA-DR were expressed by  $0.1 \pm 0.04\%$ ,  $0.03 \pm 0.02\%$ ,  $0.09 \pm 0.03\%$ , and  $0.05 \pm 0.02\%$  of the cells, respectively. The viability of the bone marrow-derived mesenchymal stem cells was  $98.6 \pm 0.002\%$ .

### **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.

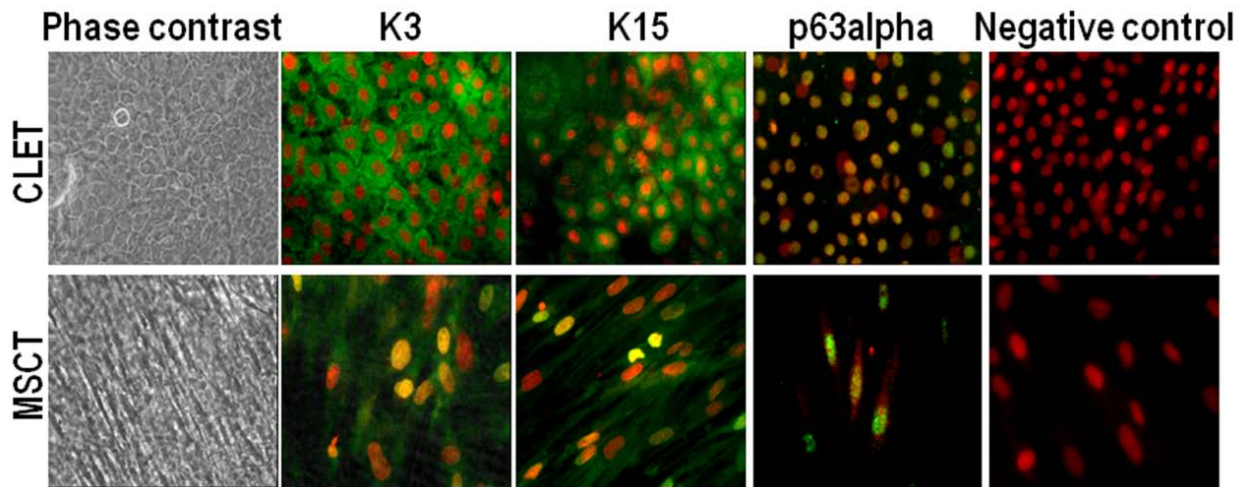
**Table S2. Characterization of cultivated limbal epithelial cells and bone marrow-derived mesenchymal stem cells for transplantation (CLET and MSCT, respectively).**

Sample	Mean time of culture 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.,<sup>12</sup> 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.<sup>12</sup> 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<sup>+</sup>, p63alpha<sup>+</sup>, and K3<sup>+</sup>) 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.<sup>13–15</sup> Therefore, our grafts would be suitable for ocular surface treatment. This is consistent with the finding of Rama et al.,<sup>16</sup> 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.<sup>17-22</sup> 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.<sup>17-21</sup> In addition, we detected the p63alpha marker in cells cultivated on the amniotic membranes, in accordance with results reported by other investigators.<sup>22-24</sup> However, the expression of this marker by mesenchymal stem cells is currently controversial because several groups showed that MSC did not express p63 protein.<sup>18,20</sup>

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