Corneal Stroma Enhancement With Decellularized Stromal Laminas With or Without Stem Cell Recellularization for Advanced Keratoconus

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• PURPOSE: This phase 1 study seeks to preliminarily evaluate the safety and efficacy of decellularized human corneal stromal lamina transplantation with or without autologous adipose-derived adult stem cell recellularization within the corneal stroma of patients with advanced keratoconus.
• DESIGN: Phase 1 clinical trial.
• METHODS: Femtosecond-assisted 120-μm thickness and 9-mm diameter laminas were obtained from the anterior stroma of human donor corneas and decellularized with a sodium dodecyl sulfate solution. Autologous adipose-derived adult stem cells were obtained by elective liposuction and cultured onto both sides of the lamina. Five patients received the decellularized lamina alone and 4 patients the recellularized lamina into a femtosecond-assisted 9.5-mm diameter lamellar pocket under topical anesthesia. The total duration of follow-up was 6 months.
• RESULTS: No case showed clinical haze or scarring by month 3. Six months after surgery, patients showed a general improvement of all visual parameters, with a mean unaided visual acuity from 0.109 to 0.232 ($P = .05$) and corrected distance visual acuity from 0.22 to 0.356 ($P = .068$). Refractive sphere improved in all patients (from −4.55 diopters [D] to −2.69 D; $P = .017$), but refractive cylinder remained stable (from −2.83 to −2.61; $P = .34$). An improvement tendency of all anterior keratometric values was observed. A mean improvement of 120 μm in all thickness parameters was confirmed ($P = .008$), as well as an improvement in the spherical aberration ($P = .018$), coma ($P = .23$) and total higher order aberrations ($P = .31$). No significant differences among groups were detected.
• CONCLUSIONS: Decellularized human corneal stromal laminas transplantation seems safe and moderately effective for advanced keratoconus. Potential benefits of its recellularization with autologous adipose-derived adult stem cells remains unclear. (Am J Ophthalmol 2018;186:47–58. © 2017 Elsevier Inc. All rights reserved.)

CORNEAL ECTASIAS, SUCH AS KERATOCONUS, ARE characterized by a progressive thinning, bulging, and distortion of the cornea, with secondary loss of vision caused by high irregular astigmatism.[1] Visual rehabilitation of advanced corneal ectasias requires penetrating or lamellar corneal transplantation techniques, which have several drawbacks, such as graft rejection, failure, and slow visual recovery because of high levels of induced postoperative astigmatism in relation with the suture.[1] It should also be considered that in many countries access to donor corneal tissue is limited: approximately 53% of the world's population has no access to corneal transplantation.[2]

Tissue engineering of the cornea aims to solve this problem, although the highly complex structure of the corneal stroma limits the usefulness of these corneal substitutes in real clinical practice generated, to date, in the laboratory, because of a lack of either transparency or strength properties.[3] Cellular therapy of corneal stroma is gaining interest because stem cells from either ocular or extraocular sources are capable of not only surviving and differentiating in vivo into adult human keratocytes, but also of producing new collagen within the host stroma.[4,5] They improve pre-existing scars or corneal transparency in animal models for corneal dystrophies by corneal stroma remodeling and host keratocyte modulation by paracrine secretion,[6–11] and also show immunomodulatory properties in syngeneic, allogeneic, and even xenogeneic scenarios.[11,12] Recently, our group reported for the first time in a pilot clinical trial the possible benefits of cellular therapy of the corneal stroma with extraocular stem cells in patients with advanced keratoconus.[13] Although additional

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research studies are still necessary, cellular therapy of the corneal stroma will unlikely be able to fully rehabilitate the thickness of an already severely thinned cornea, because the collagen production by the transplanted cells has been shown to be limited.[4,13]

Several corneal decellularization techniques have been described to supply the collagen component of the corneal stroma and provide an acellular corneal extracellular matrix.[14] These scaffolds have become more popular in the last few years because they provide a natural environment for the growth and differentiation of cells and are tolerated well even by xenogeneic recipients, as components of the extracellular matrix are generally preserved among species.[15]

The aim of the present study is to preliminary evaluate the safety and efficacy of the intrastromal implantation of decellularized human corneal stromal laminas for the thickness rehabilitation of advanced keratoconic eyes in a human phase 1 pilot study, and to define the possible advantages of the addition of autologous adipose-derived adult stem cells (ADASCs) to these implants. To the best of our knowledge, this is the first report regarding the use of implants of decellularized human corneal stroma tissue, with or without mesenchymal stem cells, for human corneal transplantation.

METHODS

• **STUDY APPROVAL, DESIGN, AND SUBJECTS:** This investigation is a prospective consecutive series of cases based on the cooperation between the Research, Development and Innovation Department of Vissum Instituto Oftalmologico de Alicante, Miguel Hernandez University, Alicante (Spain), Optica General (Saida, Lebanon), Laser Vision Center (Beirut, Lebanon) and the Reviva Research and Application Center (Middle East Hospital, Beirut, Lebanon). The Institutional Review Board Ethical Committee of the Reviva Research and Application Center (Lebanese University, Beirut) prospectively approved this study. All patients gave informed written consent for all procedures described in this study. The study was conducted in strict adherence to the tenets of the Declaration of Helsinki and it was registered at ClinicalTrials.gov (NCT02932852).

Nine consecutive patients were enrolled in the study and were randomly distributed into 2 study groups. Group 1 included decellularized human corneal stroma transplantation (5 patients); group 2 included autologous ADASC recellularized human corneal stroma transplantation (4 patients).

Inclusion criteria. Inclusion criteria were as follows: advanced keratoconus defined as stage ≥IV according to the RETICS keratoconus classification[16]; age ≥18 years; negative HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) serology; and no history of malignancy.

Exclusion criteria. Exclusion criteria were as follows: corrected distance visual acuity (CDVA) <0.1 in the contralateral eye; active concomitant inflammatory eye disease; other ophthalmic comorbidity, such as cataract, retinal diseases, or glaucoma; previous ocular surgical interventions other than cataract; previous corneal hydrops or central corneal scars; history of cognitive impairments or dementia that may impact the patient's ability to participate in the informed consent process and to appropriately complete evaluations; any immunodeficiency or immunosuppressive therapy; serologic evidence of infection with HBV, HCV, or HIV; and pregnancy or breastfeeding. Keratoconus progressive status was not considered as an exclusion or inclusion criteria.

• **AUTOLOGOUS ADASC ISOLATION, CHARACTERIZATION, AND CULTURE:** Patients were subjected to standard liposuction after informed consent. All procedures were performed in good medical practice conditions. Approximately 250 mL of fat mixed with local anesthesia was obtained from each patient. Adipose tissue was processed according to previous publications.[17,18] Briefly, adipose tissue was washed in phosphate-buffered saline (PBS) and digested in collagenase I for 40 minutes at 37°C in agitation. Then, collagenase was inhibited adding autologous human serum extracted from each patient. Erythrocytes were lysed in erythrocyte lysis buffer (Gibco-Life Technologies, Waltham, MA) and then the pelleted cells were cultured in Dulbecco's modified eagle medium with Glutamax and sodium pyruvate (Gibco), 10% autologous human serum, 1% penicillin-streptomycin (Gibco) and 0.2% amphotericin B (Gibco). Cell characterization was performed by $CD34^{+}CD45^{-}CD105^{+}$ labeling and flow cytometry analysis as requested by the International Federation of Adipose Therapeutics.[19] Sixty to 80 hours before surgery, quiescence was induced by reducing the amount of serum to 0.5% in order to transplant the ADASCs in a physiological status more closely resembling the natural nonproliferative stromal keratocytes, because proliferative stem cells within the corneal stroma could potentially induce stromal scarring or haze. Quiescence and the absence of apoptosis and aneuploidy were verified by propidium iodide labeling (Invitrogen, Waltham, MA), and cell cycle analysis by flow cytometry as described in previous articles.[4,13,15] Twenty-four hours before implantation, ADASCs were harvested by tripsinization (Sigma, St. Louis, MO) and 0.5×10^{6} cells were cultured on each side of the decellularized corneal stroma lamina for 24 and 12 hours, respectively (37°C in standard CO_2 incubator).

• **DECELLULARIZATION AND RECELLULARIZATION OF HUMAN CORNEAL STROMA LAMINAS:** Corneal stroma from donor human corneas with nonviable endothelium

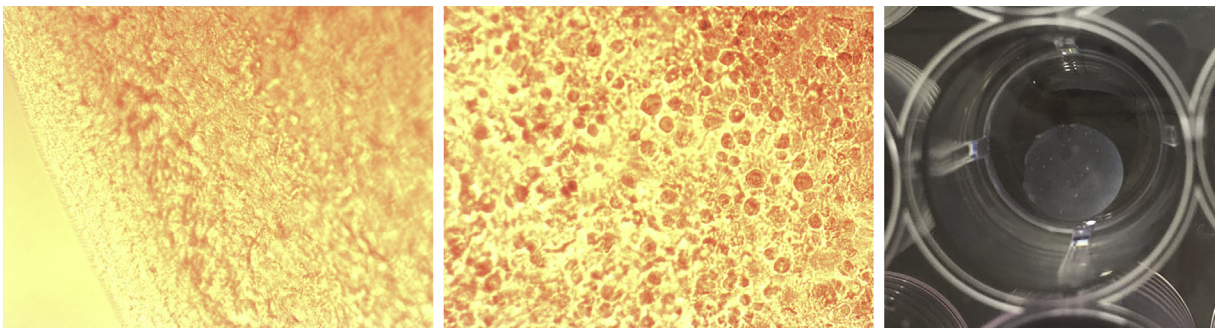

FIGURE 1. (Left) Phase-contrast photograph of a lamina after decellularization and just before recellularization (×10, photograph taken at the peripheral border). (Middle) Phase-contrast photograph of a lamina 11 hours after adding adipose-derived adult stem cells to the second side of the lamina (×10; total culture time 23 hours; photograph taken at the center of the lamina). (Right) Macroscopic appearance of the lamina under phosphate-buffered saline in a culture well.

but with negative viral serology were used. Corneas were provided by the eye bank Banco de Ojos para el tratamiento de la Ceguera of Centro de Oftalmología Barraquer (Barcelona, Spain). Directive 2004/23/EC on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells; and Directive 2006/17/EC of the Council regarding certain technical requirements for the donation, procurement, and testing of human tissues and cells were followed.

Donor corneas were mounted on an artificial anterior chamber (Barron; Katena Products, Denville, NJ). The epithelium was mechanically removed with surgical sponges, and the anterior corneal stroma cut with 60-kHz IntraLase iFS femtosecond laser (AMO, Santa Ana, CA) in 2 consecutive laminas of 120 μm thickness and 9.0 mm diameter. These were subsequently washed in PBS (Sigma) supplemented with 1% antibiotic-antimicotic (Gibco). Femtosecond laser parameter settings were equivalent to the ones used for a standard laser in situ keratomileusis flap dissection, except by an anterior side cut angle of 360°. With this procedure, 2 laminas were obtained, a superficial one containing the Bowman membrane and a deeper one without this layer. The remaining posterior cornea was discarded. The decellularization protocol was based on previous publications.[15,20] Briefly, the laminas were immersed in 1% (wt/vol) sodium dodecyl sulfate solution (Sigma) with a protease inhibitor cocktail (P8340; Sigma), and incubated in an orbital shaker (75 rpm) for 24 hours at room temperature. Then, the laminas were washed 8 times in PBS with 1% antibiotic-antimicotic in the same conditions for 15 minutes each at room temperature. To remove DNA, the laminas were incubated in DNAse (Benzonase Nuclease 6.5 U/mL; Merck, Darmstadt, Germany) in PBS with the same protease inhibitor cocktail in the same conditions at 37°C for 72 hours. Finally, the laminas were washed 8 times for 15 minutes each in PBS with 1% antibiotic-antimicotic (Figure 1, Left). Twenty-four hours before implantation, laminas for patients receiving recellularized tissue were then placed in tissue culture wells for recellularization with autologous ADASCs (0.5×10^6 cells were cultured on each side of the lamina) (Figure 1, Middle). After finishing the recellularization process, the laminas were transferred to the theatre in PBS at room temperature for their implantation (Figure 1, Right). Superficial or deep laminas were randomly used for the transplantation in both groups.

• **SURGICAL PROCEDURE: LENTICULE IMPLANTATION:** Topical anesthesia with oral sedation was used for all surgeries. The 60-kHz IntraLase iFS femtosecond laser was used in single pass mode for the recipient corneal lamellar dissection by creating an intrastromal lamellar cut 9.5 mm in diameter at half depth of the preoperative thinnest pachymetry point measured by the Visante anterior segment optical coherence tomography (OCT) (Carl Zeiss, Berlin, Germany). The femtosecond laser–assisted corneal dissection ended with a 50° anterior side cut as a corneal incision. Laser parameter settings were equivalent to those recently reported by our group for this type of dissection.[13] The corneal intrastromal pocket was then opened by blunt dissection with a Morlet lamellar dissector (Duckworth & Kent, Hertfordshire, United Kingdom), and subsequently the lamina inserted, centered, and unfolded through gentle taping and massaging from the host epithelial surface. A temporal limbal paracentesis was performed just before implantation to reduce intraocular pressure. In patients receiving a recellularized lamina, in order to compensate the expected cellular damage by the implantation process, the pocket was irrigated immediately before and after insertion with a solution containing an additional 1 million autologous ADASCs in 1 mL PBS through a 25 G cannula. The incision was closed with one interrupted 10-0 nylon suture that was removed 1 week after the operation. Topical antibiotic and steroids (TobraDex; Alcon, Fort Worth, TX) were applied at the end of the surgery. All surgeries were performed by the same surgeons (J.L.A. and J.L.A.B.) at Laser Vision (Beirut).

• **POSTOPERATIVE CARE AND FOLLOW-UP SCHEDULE:** Topical antibiotic and steroids (TobraDex) were applied every 6 hours for 1 week, followed by a descending dose of topical dexamethasone 0.1% (Maxidex, Alcon) for 3 more weeks.

Patients were followed-up at 1 day, 1 week, and 1, 3, and 6 months postoperatively. The following data were recorded throughout the preoperative assessment and postoperative months 1, 3, and 6: unaided visual acuity, CDVA, rigid contact lens visual acuity, manifest refraction, slit lamp biomicroscopy, fundoscopy, intraocular pressure, endothelial cell count by specular microscopy (Nidek, Osaka, Japan), corneal topography (Pentacam, Oculus Inc., Wetzlar, Germany), corneal aberrometry (Sirius; CSO, Firenze, Italy) with 6-mm pupils, anterior segment OCT-Visante (Carl Zeiss), and corneal confocal biomicroscopy with the Heidelberg Retinal Tomograph Rostock Cornea Module (Heidelberg Engineering Inc., Heidelberg, Germany). Intrastromal in vivo keratocyte cell count was performed as previously described[21]: anterior stroma defined as the stroma immediately after the Bowman membrane up to the anterior edge of the implanted lamina. Posterior stroma is defined as the stroma in between the posterior edge of the lamina and immediately anterior to the Descemet membrane. The transplanted mid-stroma is defined as the tissue in between the anterior and posterior edges of the implanted lamina. Three clear images without motion blur or compression lines were selected from each sector (anterior, lamina, and posterior). Therefore, 9 frames per subject were selected for analysis and reviewed by an experienced observer (M.E.Z.). For all images, a standard frame size of 100 × 100 μm was selected, and keratocytes with clear cell borders within this area (using a medium image brightness and contrast) were manually counted. Subsequent keratocyte density (cells/mm^2) was recorded.

• **STATISTICAL ANALYSIS:** The statistical analysis was performed with SPSS software version 20.0 for Windows (SPSS Inc., Chicago, IL). Normality of the study data was confirmed by the Kolmogorov–Smirnov test, which determined that all variables followed a normal distribution ($P = .05$). Because of the small size of the study sample ($n = 9$), the Wilcoxon sign test was performed to test for statistically significant differences ($P \leq .05$).

RESULTS

THE 9 CONSECUTIVE PATIENTS HAD A MEAN AGE OF 34 years (range 24–49 years). The study sample was composed of 7 females and 2 males as well as 6 right eyes and 3 left eyes (Table 1). None of these eyes had previously received corneal collagen crosslinking or any other ophthalmic intervention. All surgeries were performed without any intraoperative complications, except for a limited anterior stromal incision tear during the implantation of the graft in 1 patient. This was managed with bandage contact lens for a week and the patient made a full recovery without further complications. All patients completed the full follow-up (6 months), and the results are summarized in Table 2.

TABLE 1. Epidemiologic Data of the Study Groups Regarding Age and Sex

	Group 1	Group 2
Mean age, y (range)	32.2 (24–43)	36.25 (30–49)
Female:male ratio	4:1	3:1

• **VISUAL ACUITY:** A general improvement for all visual parameters was observed (Figure 2, Top left), with a mean unaided visual acuity from 0.109 (range 0.05–0.33) to 0.232 (range 0.1–0.475) 6 months after surgery ($P = .05$), a mean CDVA from 0.22 (range 0.1–0.4) to 0.356 (range 0.1–0.55) ($P = .068$), and a mean unaided visual acuity from 0.541 (range 0.2–0.875) to 0.565 (range 0.2–0.9) ($P = .73$). Visual acuity parameters showed, as expected, an initial worsening within the first postoperative month (in relation with a mild graft edema), with a subsequent progressive improvement over time, observing already a net improvement compared with preoperative values 3 months after surgery (for unaided visual acuity and CDVA) and 6 months postoperatively (for unaided visual acuity) (Figure 2, Left). No statistically significant differences in the improvement of visual parameters were observed between groups 1 and 2 (Table 2).

• **MANIFEST REFRACTION:** Refractive sphere improved in all patients (Figure 2, Top right), with a preoperative mean value of −4.55 diopters (D) (range −12 to −0.25) and −2.69 D (range −6.5 to −0.25) 6 months after surgery ($P = .017$). On the other hand, refractive cylinder remained stable, showing only a mild improvement tendency (Figure 2, Right), from a preoperative mean value of −2.83 (range −5.5 to −1.5) to a 6-month postoperative mean value of −2.61 (range −4 to −1.25) ($P = .34$). No statistically significant differences were observed between groups (Table 2).

• **SLIT LAMP BIOMICROSCOPY:** No postoperative complications including inflammation or rejection signs were recorded throughout the follow-up period in all patients. The implanted lamina showed a mild haziness in relation with a mild lenticule edema during the first postoperative month (what correlated well with the initial loss in the visual parameters) (Figure 3, Top middle and bottom left). Corneal transparency progressively improved throughout the follow-up period, showing complete restoration 3 months postsurgery (Figure 3, Top right and bottom

TABLE 2. Visual, Refractive, Keratometric, and Pachymetric Outcomes for Groups 1 and 2[a]

	Preoperatively, Mean (Range)		Postoperatively, Mean (Range)					
			1 Month		3 Months		6 Months	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
UVA (decimal)	0.07 (0.05–0.15)	0.158 (0.05–0.33)	0.11 (0.1–0.15)	0.137 (0.1–0.2)	0.21 (0.15–0.3)	0.17 (0.1–0.233)	0.25[b] (0.15–0.475)	0.212 (0.1–0.45)
CDVA (decimal)	0.169 (0.1–0.265)	0.287 (0.1–0.4)	0.173 (0.15–0.265)	0.191 (0.1–0.266)	0.291 (0.2–0.425)	0.310 (0.15–0.5)	0.361 (0.2–0.5)	0.35 (0.1–0.55)
CLVA (decimal)	0.525 (0.2–0.85)	0.562 (0.4–0.875)	0.338 (0.2–0.475)	0.412 (0.2–0.75)	0.443 (0.266–0.65)	0.575 (0.45–0.8)	0.483 (0.2–0.7)	0.668 (0.55–0.9)
Rx Sphr (D)	−5.15 (−12 to −1)	−3.812 (−6.75 to −0.25)	−5.25 (−12 to −1)	−3.38 (−5 to −0.25)	−3 (−5.5 to −1)	−3.187 (−5.5 to −0.25)	−2.5 (−4 to −1)	−2.937 (−6.5 to −0.25)
Rx Cyl (D)	−2.65 (−5.5 to −1.5)	−3.062 (−3.25 to −3)	−2.75 (−4 to −1.5)	−2.937 (−3.25 to −2.5)	−2.30 (−3.25 to −1.25)	−2.875 (−3.50 to −2.50)	−2.4 (−4 to −1.25)	−2.875 (−3.5 to −2.5)
Anterior Km (D)	60.02 (50.5–66.5)	56.83 (47.9–65.4)	59.34 (49.4–66.3)	55.8 (47.5–64.3)	59.26 (52.9–65.1)	55.825 (46.9–65.5)	58.62 (48.4–66.4)	55.525 (46.7–62.2)
Posterior Km (D)	−9.58 (−11.3 to −7.6)	−8.7 (−10.2 to −7.1)	−9.62 (−11.3 to −7.7)	−8.6 (−10.2 to −7)	−9.64 (−11.1 to −7.8)	−8.55 (−10.2 to −6.9)	−9.66 (−11.3 to −7.5)	−8.5 (−9.9 to −6.9)
Kmax (D)	69.2 (59.3–75.5)	66.25 (55.6–81.2)	68.06 (56.3–76.9)	65.725 (54.3–83.8)	66.62 (56.1–77.1)	65.575 (53.8–82.9)	67.14 (54.8–80.3)	63.6 (54.1–74.4)
Topo Cyl (D)	−4.72 (−6.3 to −2.7)	−3.78 (−7.4 to −1.5)	−3.3 (−4.7 to −0.8)	−4.2 (−7.6 to −2.6)	−4.4 (−8.7 to −0.9)	−3.925 (−7.6 to −1.1)	−4.92 (−11.4 to −0.9)	−3.85 (−8.1 to −0.9)
CCT (μm)	389.20 (306–502)	428.25 (330–464)	509 (385–599)	523 (420–572)	510.2 (422–617)	544.75 (428–593)	517[b] (427–617)	551 (471–594)
Thinnest point (μm)	360 (255–477)	383.25 (275–451)	468.4 (320–583)	487.75 (359–561)	472.4 (367–575)	496.25 (367–553)	483.6[b] (370–595)	495.75 (410–553)
Visante CCT (μm)	376.4 (280–482)	417.5 (300–459)	497.6 (390–591)	519 (393–578)	496.4 (400–591)	556.25 (428–641)	507.4[b] (426–607)	531.75 (440–568)

CCT = central corneal thickness; CDVA = corrected distance visual acuity; CLVA = rigid contact lens visual acuity; D = diopter; Kmax = maximum keratometry; Km = mean keratometry; Rx Cyl = refractive cylinder; Rx Sphr = refractive sphere; Topo Cyl = topographic cylinder; UVA = unaided visual acuity.

[a]Group 1 underwent decellularized human corneal stroma transplantation ($n = 5$) and group 2 underwent autologous adipose-derived adult stem cell recellularized human corneal stroma transplantation ($n = 4$).

[b]Statistically significant ($P \leq .05$) differences between the preoperative and 6-month postoperative values for each parameter and for each study group separately.

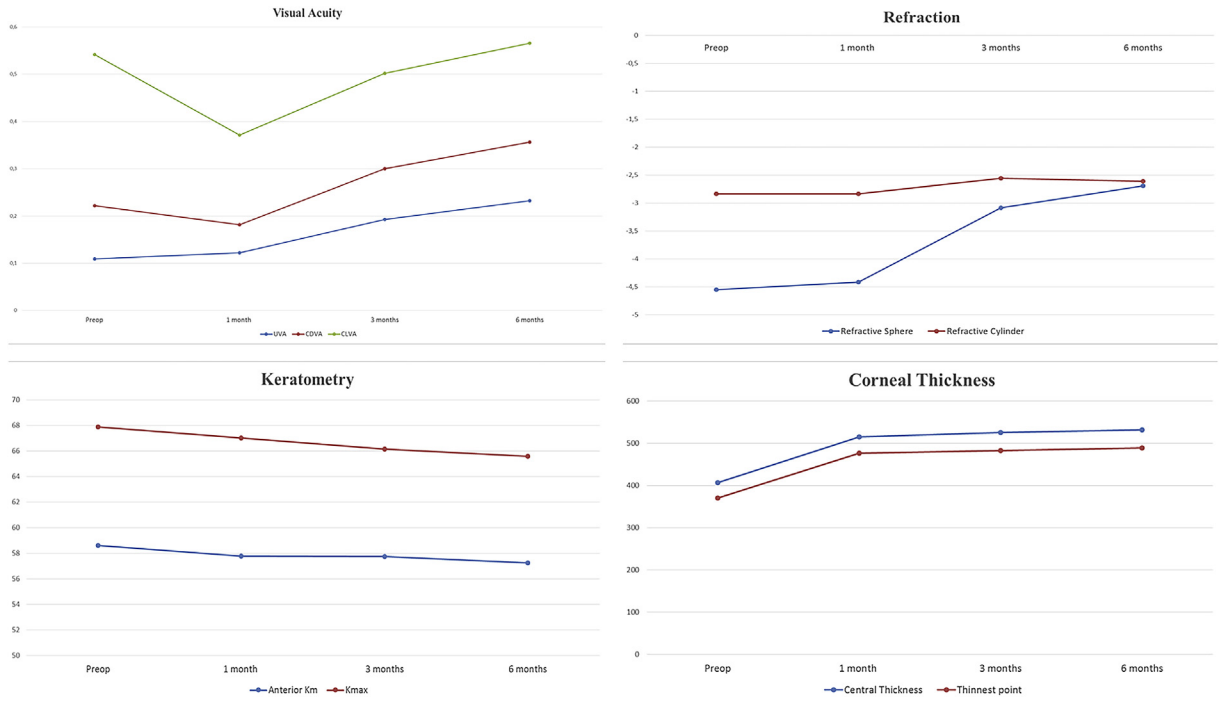

FIGURE 2. Visual (Top left), refractive (Top right), keratometric (Bottom left), and pachymetric (Bottom right) results after 6 months of follow-up. CDVA = corrected distance visual acuity; CLVA = rigid contact lens visual acuity; Kmax = maximum keratometry; Km = mean keratometry; UVA = unaided visual acuity.

right) in all cases. No case showed a residual haze or scarring at the surgical interface 6 months after surgery.

• **CORNEAL TOPOGRAPHY:** An improvement in anterior keratometry was observed (Figure 2, Bottom left and Figure 4, Left), with a mean anterior keratometry (Km) going from 58.6 D (range 47.9–66.5) preoperatively to 57.24 D (range 46.7–66.4) 6 months postsurgery (P = .06), and the maximum keratometry (Kmax) changing from 67.89 D (range 55.6–81.2) to 65.56 D (range 54.1–80.3) (P = .21). Posterior keratometry remained stable, with a preoperative posterior Km of −9.19 (range −11.3 to −7.1) and −9.14 (range −11.3 to −6.9) at postoperative month 6 (P = .61). We could not show significant changes in the anterior topographic astigmatism: −4.30 D (range −7.4 to −1.5) preoperatively and −4.44 (range −11.4 to −0.9) 6 months postoperatively (P = .72). Patient 4 (group 1) preoperatively presented the most advanced cone of the study sample, together with significant anterior stromal paracentral scars not affecting the visual axis. In this patient, we could observe a significant worsening of the anterior keratometry (Kmax from 73 to 80.3 D; anterior cylinder from −6.3 to −11.4 D) in contrast to an improvement of the refractive sphere (from −4.5 to −3.5 D) and cylinder (from −5.5 to −4 D) correlated with an improvement of one line in all visual parameters (Figure 4, Right). This severe keratometric progression may be justified by the presence of stromal scars and may not be real as far as the patient improved clinically. On the other hand, an inferior displacement of the apex of the cone could have induced a central corneal flattening and, subsequently, justify the observed reduction in the refractive sphere. Considering this possible bias, and excluding this case from the analysis, we observed a mean improvement of the anterior Km of 1.51 D (from 57.61 to 56.1 D) and a mean improvement of 3.52 D in the Kmax. No statistically significant differences were observed between groups 1 and 2 (Table 2).

As expected, a mean improvement of 120 μm in all thickness parameters was observed (Figure 2, Bottom right). Central corneal thickness improved from 406.56 (range 306–502) preoperatively to 532.11 μm (range 427–617) 6 months postsurgery (P = .008), and the thinnest point from 370.67 (range 255–477) to 489 μm (range 370–595) (P = .008). No significant differences between groups 1 and 2 were detected (Table 2).

• **CORNEAL ABERROMETRY:** Corneal aberrometry with a 6-mm pupil demonstrated an important and significant improvement in the spherical aberration, coma, and total higher order aberrations in all patients except patient 4 (group 1), in which a worsening of the preoperative aberrations was observed. We excluded this patient from this analysis to avoid potential bias, in the same manner as discussed earlier. Spherical aberration decreased from 1.30 (range 0.3–2.65) preoperatively to 0.678 μm (range

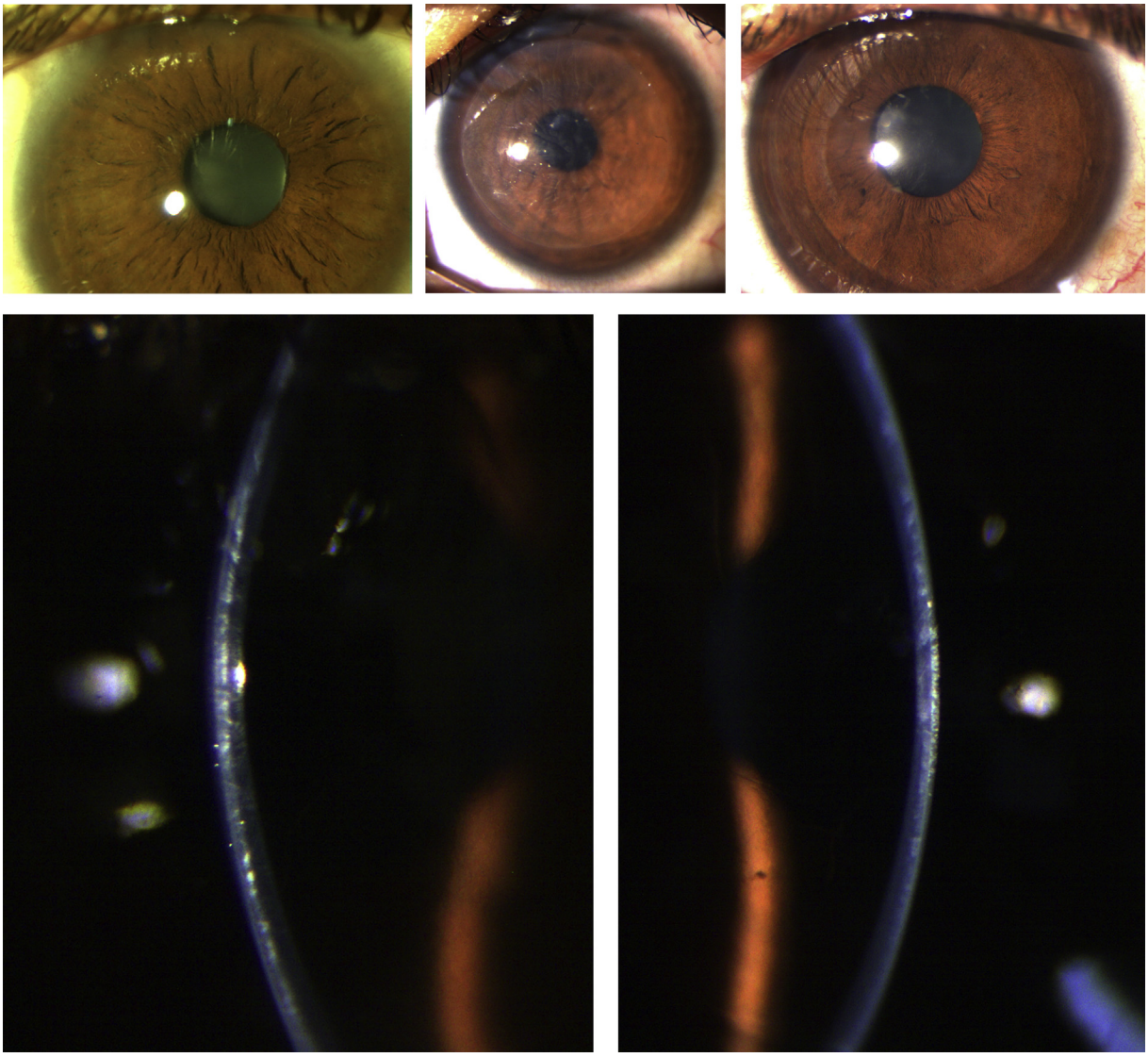

FIGURE 3. Biomicroscopic changes after corneal stroma enhancement. Slit lamp pictures from a patient from group 1 (decellularized lamina transplantation) preoperatively (Top left), 1 week postsurgery (Top middle and Bottom left), and 3 months postsurgery (Top right and Bottom right). Note the complete corneal transparency restoration 3 months postsurgery with a resolution of the initial lenticule haziness observed during the first postoperative weeks.

0.08–2.39) 6 months postsurgery ($P = .018$). Coma decreased from 3.49 μm (range 1.20–5.82) preoperatively to 2.06 μm (range 0.8–3.71) 6 months postsurgery ($P = .23$). Total higher order aberrations decreased from 4.14 μm (range 1.53–6.13) preoperatively to 2.90 μm (range 1.43–4.78) 6 months postsurgery ($P = .31$).

• **ANTERIOR SEGMENT OCT:** Central corneal thickness measured by Visante OCT confirmed the results observed with the topography: a mean preoperative value of 394.66 μm (range 280–482) and 518.22 μm (range 426–607) 6 months after surgery ($P = .08$) (Figure 5, Top right and Bottom right).

The transplanted lamina was clearly visible in the cornea OCT, showing a moderate early postoperative hyperreflectance during the first postoperative month (Figure 5, Middle top left), in good correlation with the observed mild clinical haze in the implant in the same period of time. By the third postoperative month, the lamina already presented a normal reflectance, equivalent to the surrounding recipient stroma (Figure 5, Middle bottom left). In group 2, the findings were equivalent to those observed in group 1, only the lamina borders presented a slightly higher reflectance by the end of the follow-up (Figure 5, Bottom left). No obvious areas of new collagen production were observed.

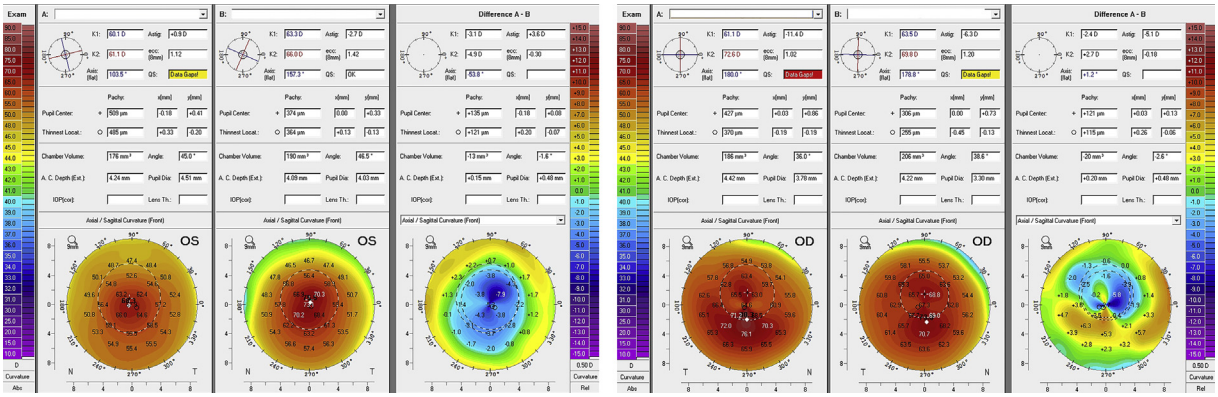

FIGURE 4. Topographic changes after corneal stroma enhancement. (Left) Keratometric change in the same patient shown in Figure 3 preoperatively and 6 months postsurgery. Note the significant flattening of the keratometry and the important improvement in all thickness parameters. (Right) Keratometric change in patient 4 from group 1. This patient had a worsening of anterior keratometry 6 months postsurgery along with refractive, pachymetric, and visual improvement.

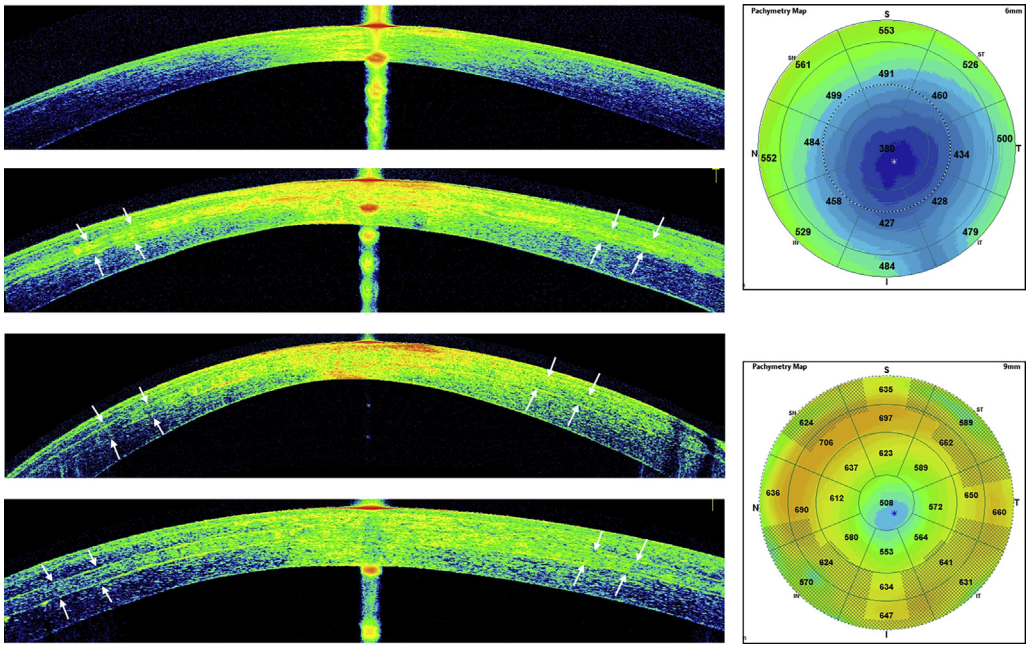

FIGURE 5. Cornea Visante optical coherence tomography images and pachymetric maps from a patient from group 1: preoperatively (Top left and Top right), 1 week postsurgery (Middle top left), and 3 months postsurgery (Middle bottom left and Bottom right). Note the normalization of the early postoperative increased reflectance of the lamina (white arrows) (Middle top left and Middle bottom left) and significant improvement in the pachymetric map (Top right and Bottom right). In patients from group 2, the lamina (white arrows) presented a similar behavior in vivo as group 1 6 months postsurgery (Bottom left), and no obvious areas of newly formed collagen were observed.

• CONFOCAL BIOMICROSCOPY: Throughout the first 3 postoperative months, a normal cellular pattern was observed in the anterior and posterior stroma (Figure 6, Top left). The lamina borders were easily visible as a hyper-reflective linear band in the interface between the normal cellular anterior or posterior stroma and the acellular transplanted stroma (Figure 6, Top middle). The lamina showed a similar appearance in both groups without relevant differences: totally acellular stroma throughout its thickness (Figure 6, Top right and Bottom left). Six months postsurgery, all patients presented early recellularization signs with scanty isolated cells scattered throughout the lamina (Figure 6, Bottom middle). Two patients (1 from each group) showed a higher recellularization of the lamina, with more abundant cells colonizing the implanted stroma (Figure 6, Bottom right).

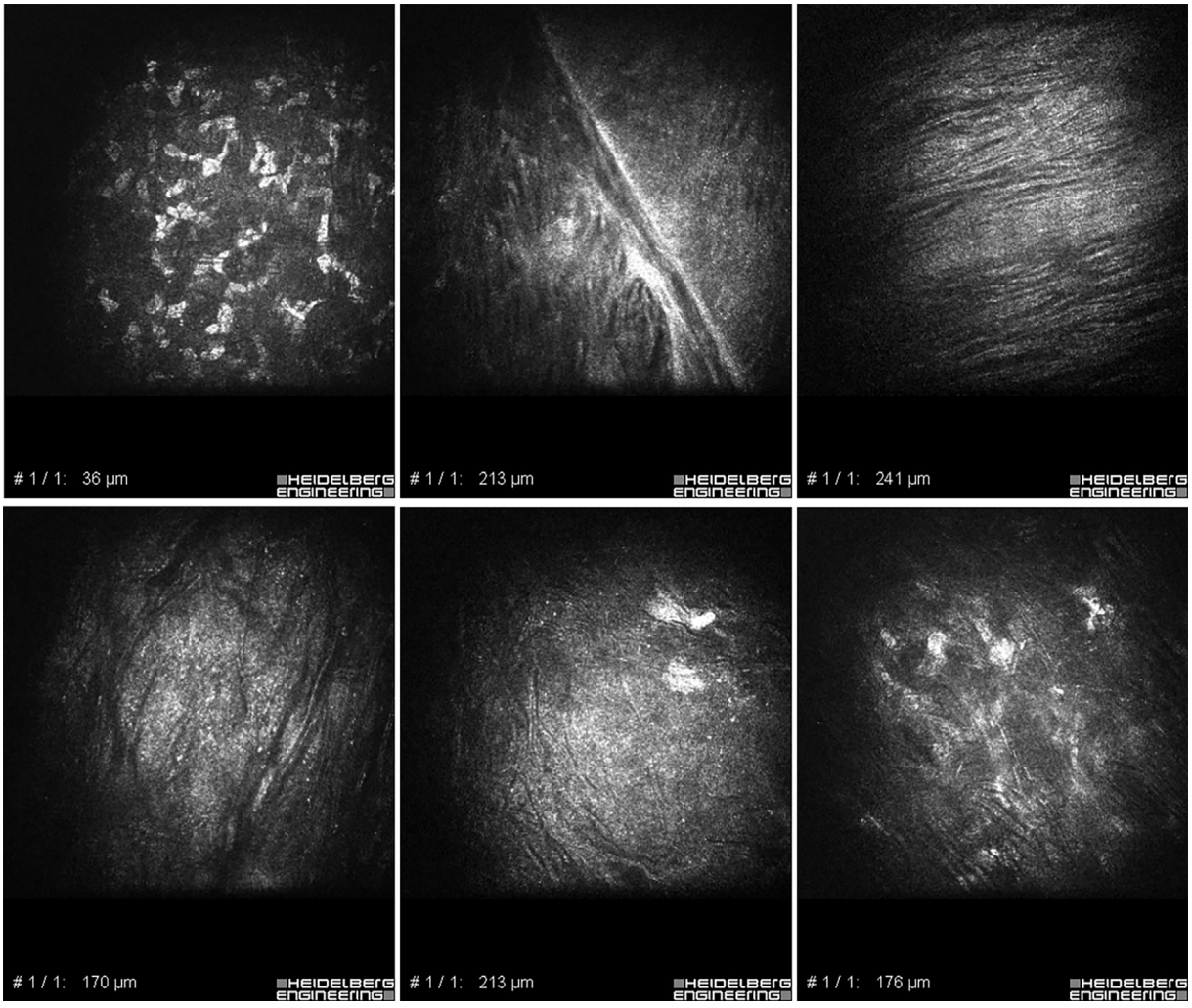

FIGURE 6. Corneal confocal biomicroscopy results from the corneal stroma anterior to the transplanted lamina (Top left), anterior edge of the lamina (Top middle), group 1 implanted lamina showing a complete acellular pattern 3 months postsurgery (Top right), group 2 implanted lamina showing a complete acellular pattern 3 months postsurgery (Bottom left), early recellularization signs in a group 1 lamina 6 months postsurgery (Bottom middle), and a more intense recellularization in a group 2 lamina 6 months postoperatively (Bottom right).

As previously described, keratocyte cellular density (cells/mm^2) was measured preoperatively 6 months postsurgery. Both anterior and posterior stroma keratocyte densities showed a mild increasing tendency, but this was statistically significant in the posterior stroma (anterior stroma from 207 [range 163.3–286.6] to 247 [range 190–356.6] [$P = .21$]; posterior stroma from 186.7 [range 136.6–216] to 238.4 [range 163.3–293.3] [$P = .015$]).

This observed mild increase in the keratocyte density at the anterior and posterior stroma could be in relation to an activation of the host keratocytes that are responsible for the early recellularization observed within the implanted tissue 6 months postsurgery, where the lamina presents a mean keratocyte density of 54.6 cells/mm^2 (range 16–156). No significant differences between groups 1 and 2 were detected (Table 3).

• **OTHER CLINICAL OUTCOMES:** There were no significant changes in intraocular pressure ($P = .213$) or endothelial cell density ($P = .327$).

Five patients received superficial laminas, including Bowman membrane (4 in group 1 and 1 in group 2), and 4 patients received the deeper lamina without this layer (1 in group 1 and 3 in group 2). Patients containing superficial laminas did not have better outcomes in any of the analyzed parameters of the study.

DISCUSSION

KERATOCONUS MANAGEMENT IS IN CONSTANT EVOLUtion, and in the last few years alternative and innovative

TABLE 3. Keratocyte Cellular Density for Groups 1 and 2[a]

	Preoperatively, Mean (Range)		6 Months Postoperatively, Mean (Range)	
	Group 1	Group 2	Group 1	Group 2
Anterior stroma (cells/mm^2)	222.6 (163–286.6)	187.6 (176.6–198)	215.3 (190–273.3)	286.6 (210–356.6)
Posterior stroma (cells/mm^2)	181.8 (136.6–216)	192.8 (174–203.3)	239.1[b] (213.3–265)	237.4 (163–293.3)
Grafted stroma (lamina; cells/mm^2)	N/A	N/A	54.1 (22.5–156)	55.2 (16–117.5)

N/A = not applicable.

[a]Group 1 underwent decellularized human corneal stroma transplantation ($n = 5$) and group 2 underwent autologous adipose-derived adult stem cell recellularized human corneal stroma transplantation ($n = 4$) before and 6 months postsurgery.

[b]Statistically significant ($P \leq .05$) differences between the preoperative and 6-month postoperative values for each parameter and for each study group separately.

approaches have been proposed to minimize the invasiveness of classical corneal transplantation techniques for the most advanced cases.[1] Regenerative medicine of the corneal stroma is a novel and promising line of therapy for keratoconus and other corneal dystrophies.[13] However, its efficacy still remains to be properly established, and an improved stem cell delivery technique is needed. Nevertheless, the demonstrated production of new human extracellular matrix by the transplanted mesenchymal stem cells into the corneal stroma in vivo in animal models,[4–10] and in humans,[13] is not expected to be enough to rehabilitate a severely thinned cornea. In this scenario, we postulated that the addition of decellularized corneal stromal tissue could enhance the previously observed results with cellular therapy alone,[13] because it provides a tectonic support and an ideal environment for the stem cells.[15,22]

The current study presents a novel treatment line for advanced keratoconus cases, with a straightforward and quick surgical technique that can be performed under topical anasthesia and can achieve a partial functional rehabilitation of the advanced ectatic cornea and an almost complete thickness recovery. This potentially allows these corneas to be candidates again for further noninvasive surgical visual rehabilitation techniques. Also, each donor cornea can easily generate up to 3 donor laminas following this model, which could partially solve the world's shortage of donor corneas, improving patient access to corneal transplantation in many parts of the world. Moreover, the decellularization technique using sodium dodecyl sulfate solution is a noncomplex and nonexpensive method that allows the removal of the entire allogeneic donor cellular component from the graft, avoiding any rejection risk, while the remaining extracellular matrix still preserves its normal architecture as has been demonstrated in vitro and in the animal model by our group and other authors.[14,15,22]

In this phase 1 pilot clinical trial, we report for first time, to the best of our knowledge, the implantation of decellularized corneal stromal tissue into the human cornea in vivo. The selected lamina thickness (120 μm) was decided upon in relation to our previous experience in the animal model.[15] We considered this thickness ideal to rehabilitate the advanced keratoconic cornea, and thicker implants may not easily fit within the intrastromal pocket. Moreover, in our previous experimental studies we observed that thin laminas were occasionally damaged during the laboratory tissue processing and, because human stromal collagen presents with a high compaction degree, decellularization was not optimal and presented heterogeneously when thick laminas were processed (unpublished data). All visual parameters moderately improved more than 1 line, achieving statistically significant differences on the unaided visual acuity despite the small study sample. This correlates well with the significant improvement of the refractive sphere and spherical aberration. All anterior keratometric parameters—coma and total higher order aberrations—systematically improved. However, we could not reach statistical significance in the current study because of the small sample size ($n = 9$) analyzed. In fact, a mean improvement of the anterior Km and Kmax of 1.5 and 3.5 D, respectively, can be expected considering our results. All thickness parameters significantly improved over 100 μm, achieving almost normalization of the corneal thickness. As initially postulated, we did not observe any clinical inflammatory signs during the follow-up, and there were no obvious inflammatory cells during confocal biomicroscopy. We only observed a mild lenticule haze the first postoperative month in good correlation with the initial drop in the visual function; this was fully recovered by the third postoperative month in all patients. This lack of interface haze up to postoperative month 6 may be in relation with the acellular category of the implanted tissue and the lack of donor keratocytes whose activation could induce haze. In addition, recellularized laminas did not show postoperative haze because they were colonized initially by ADASCs and not by adult keratocytes. Mesenchymal stem cells have been postulated to avoid or even improve preexisting scars, although no differences in corneal transparency could be demonstrated among groups.[6–9]

On the other hand, within 6 months of follow-up, we were not able to show potential advantages of adding mesenchymal stem cells to the decellularized stromal lamina. Recellularized grafts did not show a faster visual recovery, enhanced outcomes, or areas of new collagen production using corneal OCT. However, we cannot discard the possible advantages of the transplanted ADASCs in the long-term maintenance of the collagen lenticule. In our previous study with ADASCs alone in the same type of patients, we observed a slight improvement in their visual function and in the central corneal thickness, with new collagen production.[13] Additional studies with longer follow-up and larger samples are required to clarify the possible role of the cellular therapy in addition to this type of corneal implant.

Melles and associates recently described the outcomes with the transplantation of the Bowman membrane (BM) into the mid-stroma of advanced keratoconic eyes in a similar fashion as the present study.[23] Taking into account the limitations of our small study sample, we did not find differences between patients receiving laminas containing or not containing the BM. If this preliminary finding is confirmed in additional studies, it would suggest that we could graft several laminas from each donor cornea without compromising the clinical outcome. Our clinical results are not preliminarily better than those reported with the BM transplantation as an isolated layer, although the addition of stroma permits a full restoration of the thickness (that is not achieved with BM only). Our approach opens an exciting field for research to explore how established visual rehabilitation techniques, such as corneal collagen crosslinking and intracorneal ring segments, behave in the advanced keratoconic eye with stromal thickness restoration.

An important finding shown in the current study is the fact that early recellularization signs by the host keratocytes have been observed 6 months postsurgery in all patients. We could not demonstrate this host recellularization of the decellularized laminas in our previous animal studies with 3 months of follow-up.[15] Other authors have previously reported this host keratocyte infiltration into decellularized grafts in animal models for anterior lamellar keratoplasty.[24] To the best of our knowledge, we are reporting for the first time this finding for intrastromal decellularized implants. This host recellularization would allow a complete functionalization of the implanted tissue, which should ensure long-term transparency maintenance of the cornea.

A relevant issue not clarified in the present study is whether the intrastromal implantation of decellularized stromal tissue (with or without stem cells) could halt the natural progressive condition of this disease. Study patients with advanced keratoconic eyes were already candidates for corneal transplantation, and the preoperative progressive status of the disease was not determined. Therefore, further biomechanical studies will be required to answer this question. Nevertheless, all patients but 1 (with the limitations already discussed in this case) presented with stabilization or progressive improvement of the visual and keratometric parameters.

In conclusion, corneal stroma enhancement by decellularized corneal stromal lamina transplantation is a novel technique that could be an alternative for advanced keratoconic eyes compared to classical corneal transplantation techniques. Additional studies with longer durations of follow-up and larger sample sizes are necessary to confirm the preliminary results shown in this pilot clinical trial.

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