Regenerative Surgery of the Corneal Stroma for Advanced Keratoconus: 1-Year Outcomes

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• PURPOSE: This study evaluated 1-year safety and efficacy outcomes of corneal stroma cell therapy. Therapy consisted of implanting autologous adipose-derived adult stem cells (ADASc) with or without sheets of decellularized donor human corneal stroma within the stroma of patients with advanced keratoconus.

• DESIGN: This was a prospective interventional nonrandomized series of cases.

• METHODS: Fourteen consecutive patients were selected and divided into 3 experimental groups. Group A patients underwent implantation of autologous ADASc alone (3×10^6 cells/1 mL) (n = 5). Group B patients received decellularized donor 120-μm-thick corneal stroma lamina alone (n = 5). Group C patients had implantation of recellularized donor lamina with 1×10^6 autologous ADASc plus another 1×10^6 cells/1 mL at the time of the surgery (n = 4). Autologous ADASc were obtained by elective liposuction. Implantation was performed in the corneal stroma through a femtosecond-assisted 9.5-mm diameter lamellar dissection with the patient under topical anesthesia. Twelve months of follow-up data are presented.

• RESULTS: No complications were observed during the 1-year follow-up, and full corneal transparency was recovered within 3 months in all patients. No patient lost lines of visual acuity. Corrected distance visual acuity improved 0.231, 0.264, and 0.094 Snellen lines in groups 1, 2, and 3, respectively. In group 1, refractive parameters showed an overall stability, whereas in groups 2 and 3, sphere improved 2.35 diopter (D) and 0.625 D, respectively. Anterior keratometry remained stable (group 1) and improved in groups 2 and 3 (mean improvement of 2D). Corneal aberrometry improved significantly. In optical coherence tomography scans, corneal thickness showed a mean improvement of 14.5 μm (group 1) and 116.4 μm (groups 2 and 3) in the central thickness, and new collagen production was observed at the surgical plane (group 1). Confocal biomicroscopy confirmed the host recellularization of the implanted laminas.

• CONCLUSIONS: Intrastromal implantation of autologous ADASc and decellularized human corneal stroma did not show complications at 1 year of follow-up and were moderately effective for the treatment of advanced keratoconus. (Am J Ophthalmol 2019;203:53–68.

CELLULAR THERAPY OF THE CORNEAL STROMA HAS been gaining interest in the last few years as a potential alternative treatment for corneal stroma diseases such as corneal scarring, dystrophies, and ectasias. Human clinical studies have demonstrated that autologous mesenchymal adipose-derived human stem cells are able to produce corneal collagen when injected into the cornea stroma[1] and that such cells when use together in acellular corneal laminas can increase the thickness of the keratoconic cornea in calculated amounts, preserving corneal transparency and improving vision.[2,3] Such human clinical studies were supported by previous experimental animal studies in which the possibilities of such therapy were confirmed and by the potential advantages that such therapy may have in the treatment of corneal dystrophies and even corneal scarring processes.[4–12]

Corneal ectasias, such as keratoconus, are characterized by progressive thinning, bulging, and distortion of the cornea, with secondary loss of vision due to high, irregular astigmatism.[13] Visual rehabilitation of advanced corneal ectasias requires penetrating or lamellar corneal transplantation techniques, which present several drawbacks, such as graft rejection, failure and slow visual recovery due to high levels of induced postoperative astigmatism in relation to the suture.[13] Also, in many countries, access to donor corneal tissue is limited; approximately 53% of the world's population has no access to corneal transplantation.[14]

As an alternative, tissue engineering of the cornea aims to avoid corneal graft by replacing the diseased corneal tissue by using human stem cells and scaffolds. However, the highly complex structure of corneal stroma has, until now, limited the usefulness of these corneal substitutes in clinical practice due to a lack of either transparency or tissue strength properties.[15] On the other hand, stem cells

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from either ocular or extraocular sources are capable of not only survival and in vivo differentiation into adult human keratocytes but also production of new collagen within the host stroma.[4,5] There is evidence that these cells improve corneal transparency in animal models for corneal dystrophies by corneal stroma remodeling and host keratocyte modulation by paracrine secretion.[6–11] Such cells also show immunomodulatory properties in syngeneic, allogeneic, and even xenogeneic scenarios.[11, 16]

In addition, several corneal decellularization techniques have been described that provide an acellular corneal extracellular matrix.[17] These scaffolds have become more popular in the last few years as they provide a natural environment for the growth and differentiation of cells and are well tolerated even by xenogeneic recipients, because components of the extracellular matrix are generally preserved among species.[12]

Adipose-derived adult stem cells (ADASc) are 1 type of stem cell that has been proposed for corneal cell therapy. Our research group performed an initial animal experiment[4] in which human ADASc were implanted into the corneal stroma of the albino rabbit and were later analyzed by immunohistochemical methods after 6 months. The outcomes demonstrated the presence of human collagen in the rabbit cornea, obviously produced by the implanted ADASc. The corneas were transparent in all cases of the experiment, demonstrating that the newly created collagen was also transparent and not showing any evidence of scarring. Other experiences reported by our group in the experimental animal model demonstrated that decellularized laminas of corneal stroma were better suitable as carriers of the ADASc[12] than other carrier laminas from other biological or synthetic materials.[18] Other studies have confirmed the capability of mesenchymal stem cells to differentiate into keratocytes,[4–9] a finding that suggests that such stem cells could be useful in corneal surgery aiming to improve the corneal cell density in corneal cell therapy and even corneal transparency following trauma and in some corneal dystrophies.[6–11]

Considering all this evidence, our group recently reported for the first time in a phase 1 clinical trial the initial clinical outcomes of ADASc and decellularized corneal laminas used as cellular therapy of corneal stroma in patients with advanced keratoconus, demonstrating excellent preliminary safety levels in terms of lack of complications at 6 month follow-up period and good clinical outcomes in terms of increased corneal thickness, regularity, and transparency.[1–3]

The present paper reports initial, preliminary 1-year outcomes to verify the hypothesis that it is possible to increase the corneal thickness in advanced keratoconus cases and to improve the optical behavior and preserve the corneal transparency of such corneas with consequent improvement in vision. For this purpose, we analyzed and report here the 1-year outcomes of a clinical study of cellular therapy of the corneal stroma in patients with advanced keratoconus following the intrastromal implantation of ADASc alone, decellularized human corneal stromal laminas, or ADASc recellularized human corneal stromal laminas.

METHODS

THE AUTHORS ARE REPUBLISHING THE METHODS INITIALLY reported with the 6-month results of this study. Duplicate publication of the methods was accepted so that the full project would appear in a format suitable for a thesis of the American Ophthalmological Society.

• **STUDY APPROVAL, DESIGN, AND SUBJECTS:** This investigation was designed as an interventional, prospective, non-randomized consecutive series of cases. It was performed with the collaboration of the Research, Development and Innovation Department of Vissum Instituto Oftalmologico de Alicante, Miguel Hernandez University (Alicante, Spain); the Optica General (Saida, Lebanon); the Laser Vision Center (Beirut, Lebanon); and the Reviva Research and Application Center (Middle East Hospital, Beirut, Lebanon). The Institutional Review Board Ethical committee of Reviva Research and Application Center approved this study. All patients gave informed written consent for each procedure. The study was conducted in strict adherence to the tenets of the Declaration of Helsinki and was registered (Autologous Adipose-Derived Adult Stem Cell Transplantation for Corneal Diseases [A-ADAS-CT-CD]; NCT02932852).

Fourteen patients were selected and enrolled in the study during the 3-month enrollment period and were consecutively divided into 3 study groups. Group 1 patients were treated with autologous ADASc implantation (n = 5 patients); group 2 patients received decellularized human corneal stroma transplantation (n = 5 patients); and group 3 patients received autologous ADASc recellularized human corneal stroma transplantation (n = 4 patients).

Patient recruitment was closed after 3 months. The outcomes of the study were expected to be evaluated and reported at the end of 6 and 12 months after surgery. The study endpoint was established at 1 year after the implantation. Clinical monitoring of the patients for the purpose of safety was established monthly and at 1 week and at 1, 3, 6, and 6 months for the purpose of the other clinical outcomes of the investigation. Interim analysis of the cases was planned to be performed and reported with the 6- and 12-month data. The results from the interim analysis reported by our group performed at 6 months was published previously.[1,2]

Patients were selected by the clinical staff from among patients who were followed for the treatment or eventual surgery for keratoconus by local physicians in Lebanon, as a compassionate alternative for the patients' disease. Data about the potential evolution of the disease in the selected

eyes were inconclusive in most cases due to the lack of previous historical information, especially corneal topography maps. The clinical monitor of the study and the one responsible for the assessment and recording of clinical data was M.E.Z.

- Inclusion criteria: criteria included advanced keratoconus, defined as stage ≥IV according to the Red Temática de Investigación Cooperativa en Salud keratoconus classification[19]; ≥18 years of age; negative for human immunodeficiency virus (HIV), hepatitis B (HBV), and hepatitis C (HCV) serology; and no history of malignancy. Sex was not considered to be relevant for the purpose of patient selection.
- Exclusion criteria: criteria included corrected distance visual acuity (CDVA) (decimal scale) <0.1 in the contralateral eye; active concomitant inflammatory eye disease; other ophthalmic comorbidity, such as cataract, retinal diseases or glaucoma; any previous ocular surgical interventions other than cataract; any previous corneal intervention including collagen cross-link; previous corneal hydrops or central corneal scars; history of cognitive impairments or dementia which might have affected 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 breast feeding. Keratoconus progression status was not considered an exclusion or inclusion criteria.

• AUTOLOGOUS ADASC ISOLATION, CHARACTERIZATION AND CULTURE: The procedure was previously described in a publication from our group.[20,21] Briefly, patients were subjected to standard liposuction under good medical practice conditions. Approximately 250 mL of fat mixed with local anesthesia was obtained, washed in phosphate-buffered saline (PBS), and digested in collagenase I for 40 minutes at 37°C. Then, collagenase was inhibited by adding autologous human serum. Erythrocytes were lysed in erythrocyte lysis buffer (Gibco-Life Technologies, Gaithersburg, Maryland). Then the pelleted cells were cultured in Dulbecco's modified eagle medium with sodium pyruvate and L-glutamine (GlutaMAX; (Gibco), 10% autologous human serum, and 1% penicillin-streptomycin (Gibco) plus 0.2% amphotericin B (Gibco). Cell characterization was performed by $CD34^+CD45^-CD105^+$ labeling and flow cytometry analysis as requested by International Federation of Adipose Therapeutics (IFATS).[22] Then, 60 to 80 hours before surgery, quiescence was induced, reducing the amount of serum to 0.5%. The ADASc were implanted in a physiological status more closely resembling the natural non-proliferative stromal keratocytes, as proliferative stem cells within the corneal stroma could potentially induce stromal scarring or haze. Quiescence as well as absence of apoptosis and aneuploidy were verified by propidium iodide labeling (Invitrogen, Carlsbad, California) and cell cycle analysis.[1–4,12]

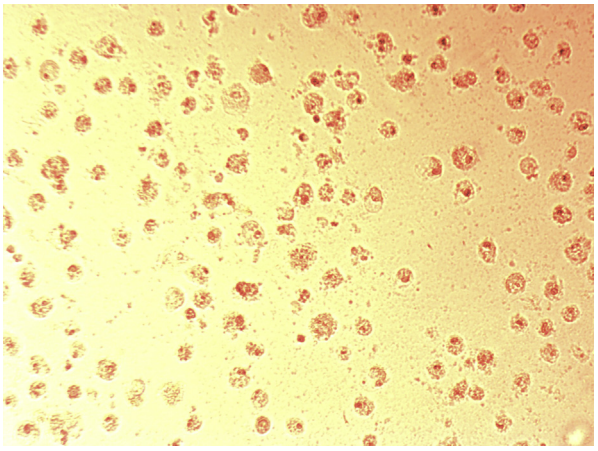

FIGURE 1. Phase-contrast view of autologous adipose-derived adult stem cells (ADASc) after trypsinization and before transplantation (×10 magnification).

In group 1, just before the injection, cells were harvested by trypsinization (Sigma-Aldrich, St. Louis, Missouri). A total of 3×10^6 cells per patient were prepared in saline (Figure 1). This cellular concentration was established according to the observed outcomes of our previous experimental studies and aimed to compensate the expected high cellular loss happening after the implant due to the leakage of the solution.[4,12,18]

In group 3, 24 hours before implantation, the ADASc were harvested by trypsinization (Sigma-Aldrich). An aliquot of 0.5×10^6 cells were cultured on each side of the decellularized corneal stroma lamina for 24 and 12 hours, respectively.

• DECELLULARIZATION AND RECELLULARIZATION OF HUMAN CORNEAL STROMA LAMINAS: Corneal stroma from non-viable transplantation donor human corneas with negative viral serology were used. Corneas were supplied by the eye bank Banco de Ojos para el tratamiento de la Ceguera, Centro de Oftalmología Barraquer (Barcelona, Spain) following Directives 2004/23/EC and 206/17/EC for standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells were are followed.

Corneal laminas from the donor corneas were prepared on an artificial anterior chamber (Barron, Katena Products, Denville, New Jersey). The epithelium was mechanically removed, and the anterior corneal stroma was cut using a 60-kHz IntraLase iFS femtosecond laser (Advanced Medical Optics, Santa Anna, California) in 2 consecutive laminas of 120-μm thickness and 9.0-mm diameter. These samples were subsequently washed in PBS (Sigma-Aldrich) supplemented with a 1% antibiotic-antimycotic solution

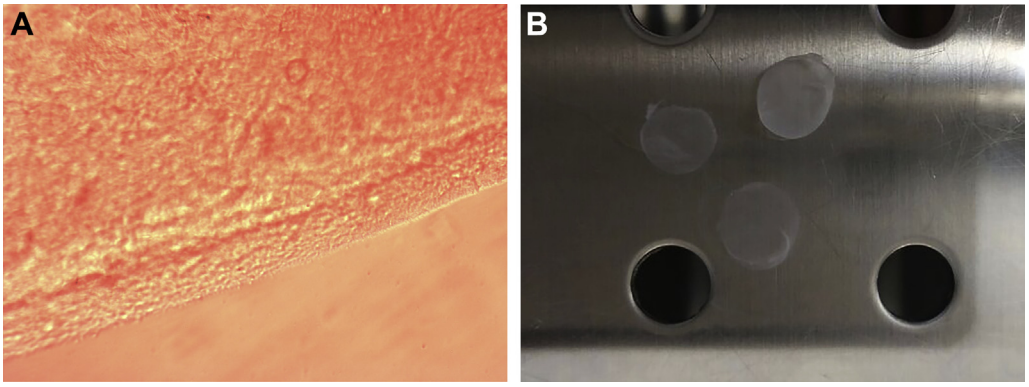

FIGURE 2. (A) Phase-contrast view of a lamina 10 hours after adding ADASc to the first side of the lamina (×10 magnification [photo was taken at the peripheral border]). (B) Macroscopic appearance of the laminas under phosphate-buffered saline in a culture well. ADASc = autologous adipose-derived adult stem cells.

(Gibco). Femtosecond laser parameter settings were equivalent to the ones used for laser-assisted in situ keratomileusis (LASIK) flap dissection, except for an anterior side-cut angle of 360°. With this procedure, 2 laminas were obtained, a superficial one containing Bowman's membrane and a deeper one without. The remaining posterior cornea was discarded. The decellularization protocol was based on previous publications.[12,23] The laminas were immersed in 1% (wt/vol) solution of sodium dodecylsulfate (Sigma-Aldrich) with a protease inhibitor cocktail (P8340; Sigma-Aldrich), and incubated in an orbital shaker (at 75 rpm) for 24 hours at room temperature. Then, the laminas were washed 8 times in PBS with 1% antibiotic-antimycotic solution under 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 under the same conditions at 37°C for 72 hours. Then, the corneal laminas were washed 8 times for 15 mins each in PBS with a 1% antibiotic-antimycotic solution (Figure 2B). Twenty-four hours before implantation, those laminas planned for patients receiving recellularized tissue (group 3) were placed in tissue culture wells for recellularization with ADASc (0.5×10^6 cells were cultured on each side of the lamina) (Figure 2A). Following the recellularization process, the laminas were transferred to the operating room for implantation in PBS. Superficial (laminas with Bowman's membrane) or deep laminas (without) were randomly used for transplantation in groups 2 and 3.

• **SURGICAL PROCEDURE:** *1. Autologous ADASc implantation.* Topical anesthesia was used. The 60-kHz IntraLase iFS femtosecond laser (Advanced Medical Optics) was used in a single-pass mode for the recipient corneal lamellar dissection. An intrastromal lamellar cut of 9.5 mm in diameter was created at half depth of the preoperative thinnest pachymetry point, as measured by the Visante anterior segment optical coherence tomography (OCT) (Carl Zeiss, Jena, Germany). A 30-degree anterior side-cut incision was made. The femtosecond laser parameter settings were similar to the ones used for LASIK. The corneal intrastromal pocket was opened with a Morlet lamellar dissector (Duckworth & Kent, Reading, UK). Then, 3 million autologous ADASc contained in 1 mL were injected into the pocket with a 25-G cannula. Previous to the cellular injection, a 1-mm corneal paracentesis was performed to reduce the intraocular pressure and allow a larger volume to be injected into the stromal pocket. A topical antibiotic and steroid (tobramycin/dexamethasone [Tobradex]; Alcon, Elkridge, Maryland) was applied at the end of the surgery. Corneal sutures were not used.

2. Lenticule implantation. Topical anesthesia with oral sedation was used for all surgeries. As described above, the 60-kHz IntraLase iFS femtosecond laser was used in single-pass mode for the recipient corneal lamellar dissection. The femtosecond laser-assisted corneal dissection ended with a 50-degree anterior side cut as a corneal incision. The corneal intrastromal pocket was then opened, and the lamina was inserted, centered, and unfolded through gentle taping and massaging from the host epithelial surface. As done in the previous group, a temporal limbal paracentesis was performed just before implantation in order to reduce the intraocular pressure. In patients who were receiving a recellularized lamina (group 3), in order to compensate for the expected cellular damage by the implantation process, the pocket was irrigated immediately before and after insertion, using a solution containing an additional 1 million autologous ADASc in 1 mL of PBS. Cells were injected by using a 25-G cannula. The incision was then closed by using 1 interrupted 10/0 Nylon suture. The suture was

removed 1 week after the operation. Topical tobramycin/dexamethasone (Alcon) was 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 Center (Beirut, Lebanon).

• **POSTOPERATIVE CARE AND FOLLOW-UP SCHEDULE:** Topical tobramycin/dexamethasone (Alcon) was used every 6 hours for 1 week, followed by a descending dose of topical dexamethasone 0.1% (Maxidex; Alcon) for 3 more weeks. Patients affected by allergic or vernal conjunctivitis were maintained on the previous antiallergic medication used by the patient as necessary.

For the purpose of safety, the patients were evaluated monthly by the study clinical monitor (M.E.Z.) who recorded any evidence of ocular inflammation, subjective discomfort, or sudden unexpected visual loss. For the purpose of the evaluation of the other clinical parameters, the patients were followed at 1 day and 1 week and at 1, 3, 6, and 12 months postoperatively. The following data were recorded throughout the preoperative assessment, as well as at 1, 3, 6, and 12 months: unaided visual acuity (UVA), corrected distance visual acuity (CDVA), rigid contact lens visual acuity (CLVA), manifest refraction, slit lamp biomicroscopy, fundoscopy, intraocular pressure (IOP), endothelial cell count by specular microscopy (Nidek, Aichi, Japan), corneal topography (Pentacam; Oculus Inc., Wetzlar, Germany), corneal aberrometry (Sirius, CSO, Florence, Italy) with 6-mm pupils, anterior segment OCT-Visante (Carl Zeiss), and corneal confocal biomicroscopy using the Retinal Tomograph-3 (HRT3) Rostock Cornea Module (HRT3; Heidelberg Engineering Inc., Heidelberg, Germany). Intrastromal in vivo keratocyte cell counts were performed as previously described[24]: anterior stroma was defined as the stroma immediately after Bowman's membrane up to the anterior edge of the implanted lamina. Posterior stroma was defined, for the purpose of the study, as the stroma between the posterior edge of the lamina and immediately anterior to Descemet's membrane. The transplanted mid stroma was defined as the tissue 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). Thus, 9 frames per subject were selected for analysis and reviewed by an experienced observer (M.A.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 per square millimeter) was recorded.

• **STATISTICAL ANALYSIS:** Statistical analysis was performed using SPSS version 20.0 software (SPSS Inc., IBM, Armonk, New York) for Windows (Microsoft, Edmond, Washington). Due to the study sample ($n = 14$), non-parametric statistics were used. The Wilcoxon rank sum test was applied to assess the significance of differences between preoperative and postoperative data. For all statistical tests, the same level of significance was used ($P < 0.05$).

RESULTS

FOURTEEN PATIENTS WERE RECRUITED. THEIR MEAN AGE was 33.28 years old (range: 24–49 years of age; mean of 34.2 years of age for group 1, 32.2 years of age for group 2, and 36.25 years of age for group 3). The study sample was composed of 9 females and 5 males (female/male ratio of 2/3 for group 1, 4/1 for group 2, and 3/1 for group 3) as well as 10 right eyes and 4 left eyes.

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 from group 2. This was managed with a bandage contact lens for 1 week, and a full recovery without further complications was observed. Thirteen patients completed the 12-month follow-up. One patient from group 1 was lost after the first postoperative month due to inability to attend further follow-up visits (for reasons unrelated to the study), so that patient was excluded from the subsequent analysis; however, that patient was followed by phone call interviews from the study's clinical monitor (M.A.Z.) on a monthly basis and by another physician who was recruited at the patient's new residence for biomicroscopy and clinical follow-up. No complication had been recorded before the exclusion of this patient, and no subjective negative observation or biomicroscopy or visual complications were described by the recruited ophthalmic professional. The outcomes of the study are summarized in Tables 1 and 2.

• **VISUAL ACUITY:** All patients' visual acuity showed moderate improvement in UVA, CDVA, and CLVA (Figure 3); and no patient experienced any loss of lines in any of these visual parameters. However, the visual improvement patterns were different among the 3 groups. In group 1 (G1), UVA and CDVA improvement was obtained mostly during the first postoperative month, showing an overall stabilization thereafter (mean improvement of 2.1 lines ± 1.3 [$P = 0.07$] and 2.3 lines ± 1.7 [$P = 0.11$] in UVA and CDVA, respectively, 1 year after surgery). On the other hand, best visual acuity with rigid contact lenses (CLVA) showed progressive improvement up to the 12th month (mean total improvement of 2.6 lines ± 1.0 [$P = 0.07$]), although signs of stabilization were already showing after 3 months (Figure 3).

In G2 and G3, visual acuity parameters showed, as expected, an initial worsening within the first

TABLE 1. Visual, Refractive, Keratometric, and Pachymetric Outcomes

	Preoperation mean [median] (range)			1 Month mean [median] (range)			3 Months mean [median] (range)			6 Months mean [median] (range)			12 Months mean [median] (range)		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
UVA (decimal)	0.1 [0.07] (0.05–0.2)	0.07 [0.05] (0.05–0.15)	0.16 [0.12] (0.05–0.33)	0.3 [0.35] (0.1–0.4)	0.11 [0.1] (0.1–0.15)	0.137 [0.12] (0.1–0.2)	0.29 [0.33] (0.15–0.365)	0.21 [0.2] (0.15–0.3)	0.17 [0.17] (0.1–0.233)	0.233 [0.25] (0.1–0.333)	0.25 [0.2] (0.15–0.475)	0.21 [0.15] (0.1–0.45)	0.31 [0.32] (0.15–0.45)	0.24[a] [0.2] (0.1–0.5)	0.25 [0.23] (0.1–0.45)
CDVA (decimal)	0.32 [0.35] (0.2–0.4)	0.17 [0.15] (0.1–0.265)	0.29 [0.32] (0.1–0.4)	0.52 [0.55] (0.2–0.8)	0.17 [0.15] (0.15–0.265)	0.191 [0.2] (0.1–0.266)	0.49 [0.5] (0.2–0.75)	0.29 [0.3] (0.2. 0.425)	0.31 [0.29] (0.15–0.5)	0.481 [0.45] (0.4–0.625)	0.36 [0.4] (0.2–0.5)	0.35 [0.37] (0.1–0.55)	0.56 [0.56] (0.4–0.7)	0.43[a] [0.4] (0.2–0.6)	0.38 [0.44] (0.1–0.55)
CLVA (decimal)	0.51 [0.52] (0.4–0.6)	0.52 [0.57] (0.2–0.85)	0.56 [0.49] (0.4–0.875)	0.65 [0.66] (0.5–0.8)	0.34 [0.36] (0.2–0.475)	0.412 [0.35] (0.2–0.75)	0.74 [0.75] (0.5–0.95)	0.44 [0.45] (0.266–0.65)	0.57 [0.52] (0.45–0.8)	0.762 [0.84] (0.5–0.875)	0.48 [0.5] (0.2–0.7)	0.67 [0.61] (0.55–0.9)	0.77 [0.84] (0.5–0.9)	0.56 [0.52] (0.2–0.85)	0.69 [0.7] (0.5–0.875)
Rx Sphr (D)	−4.06 [−4.37] (−7 to −0.5)	−5.15 [−4.5] (−12 to −1)	−3.81 [−4.12] (−6.75 to −0.25)	−3.69 [−3.5] (−7 to −0.75)	−5.25 [−4.5] (−12 to −1)	−3.38 [−4.12] (−5 to −0.25)	−3.81 [−3.25] (−8 to −0.75)	−3 [−3.5] (−5.5 to −1)	−3.19 [−3.5] (−5.5 to −0.25)	−3.562 [−3.5] (−6.5 to −0.75)	−2.5 [−2.5] (−4 to −1)	−2.94 [−2.5] (−6.5 to −0.25)	−3.56 [−3] (−7.5 to −0.75)	−2.8 [−2] (−5 to −1)	−3.19 [−2.5] (−7.5 to −0.25)
Rx Cyl (D)	−2.94 [−3] (−3.5 to −2.25)	−2.65 [−2] (−5.5 to −1.5)	−3.06 [−3] (−3.25 to −3)	−3.56 [−3.75] (−4.5 to −2.25)	−2.75 [−3] (−4 to −1.5)	−2.937 [−3] (−3.25 to −2.5)	−3.19 [−3.25] (−4 to −2.25)	−2.30 [−2] (−3.25 to −1.25)	−2.87 [−2.75] (−3.50 to −2.50)	−3.25 [−3.25] (−4 to −2.5)	−2.4 [−2] (−4 to −1.25)	−2.87 [−2.75] (−3.5 to −2.5)	−3.5 [−3.25] (−4.5 to −3)	−2.5 [−2.5] (−4 to −1.5)	−2.87 [−3] (−3 to −2.5)
Anterior Km (D)	55.95 [55.5] (47.9–64.9)	60.02 [63.1] (50.5–66.5)	56.83 [57] (47.9–65.4)	56.95 [56] (50.2–65.6)	59.34 [61.9] (49.4–66.3)	55.8 [55.7] (47.5–64.3)	56.1 [55.7] (49.6–63.4)	59.26 [60.4] (52.9–65.1)	55.82 [55.45] (46.9–65.5)	56.82 [55.55] (50.8–65.4)	58.62 [60.6] (48.4–66.4)	55.52 [56.6] (46.7–62.2)	56.65 [55.55] (50.1–65.4)	58.02 [59.7] (48.3–66.2)	55.25 [56.35] (46.5–61.8)
Posterior Km (D)	−8.3 [−8.45] (−9.7 to −6.6)	−9.58 [−10.3] (−11.3 to −7.6)	−8.7 [−8.75] (−10.2 to −7.1)	−8.55 [−8.45] (−10.2 to −7.10)	−9.62 [−10.1] (−11.3 to −7.7)	−8.6 [−8.6] (−10.2 to −7)	−8.37 [−8.45] (−9.8 to −6.8)	−9.64 [−10] (−11.1 to −7.8)	−8.55 [−8.55] (−10.2 to −6.9)	−8.52 [−8.45] (−10.2 to −7)	−9.66 [−10.10] (−11.3 to −7.5)	−8.5 [−8.6] (−9.9 to −6.9)	−8.52 [−8.45] (−10.2 to −7)	−9.64 [−10] (−11.4 to −7.7)	−8.52 [−8.6] (−10 to −6.9)
Kmax (D)	66.3 [64.55] (56.7–79.4)	69.2 [70.4] (59.3–75.5)	66.25 [64.1] (55.6–81.2)	68.8 [65.35] (63.2–81.3)	68.06 [70.9] (56.3–76.9)	65.72 [62.4] (54.3–83.8)	68.32 [64.55] (62.1–82.1)	66.62 [68.6] (56.1–77.1)	65.57 [62.8] (53.8–82.9)	67.95 [64.4] (61.8–81.2)	67.14 [68.3] (54.8–80.3)	63.6 [62.95] (54.1–74.4)	68.32 [64.75] (62.6–81.2)	65 [66.9] (54.8–77.4)	64.67 [66.15] (53.3–73.1)
Topo Cyl (D)	−2.95 [−2.8] (−5.8 to −0.4)	−4.72 [−5.3] (−6.3 to −2.7)	−3.78 [−3.1] (−7.4 to −1.5)	−3.47 [−3.3] (−5.8 to −1.5)	−3.3 [−3.8] (−4.7 to −0.8)	−4.2 [−3.3] (−7.6 to −2.6)	−3.37 [−3.5] (−5.3 to −1.2)	−4.4 [−3.8] (−8.7 to −0.9)	−3.92 [−3.5] (−7.6 to −1.1)	−3.1 [−3] (−5.7 to −0.7)	−4.92 [−4.5] (−11.4 to −0.9)	−3.85 [−3.2] (−8.1to −0.9)	−3.02 [−3.25] (−5.4 to −0.2)	−4.78 [−3.5] (−10.7 to −1.1)	−4.3 [−3.15] (−8.9 to −2)
CCT (μm)	463 [456] (438–503)	389.20 [381] (306–502)	428.25 [459.5] (330–464)	456 [452.5] (435–484)	509 [522] (385–599)	523 [550] (420–572)	465 [456] (439–509)	510.2 [501] (422–617)	544.75 [579] (428–593)	460 [447] (434–512)	517 [509] (427–617)	551 [569.5] (471–594)	456.5 [449] (436–492)	513.6[a] [509] (425–606)	536.75 [563.5] (447–573)

Continued on next page

TABLE 1. Visual, Refractive, Keratometric, and Pachymetric Outcomes (*Continued*)

	Preoperation mean [median] (range)			1 Month mean [median] (range)			3 Months mean [median] (range)			6 Months mean [median] (range)			12 Months mean [median] (range)		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Thinnest point (μm)	405.7 [398.5] (394–432)	360 [364] (255–477)	383.25 [403.5] (275–451)	407.75 [406] (406–438)	468.4 [482] (320–583)	487.75 [515.5] (359–561)	411 [410] (378–446)	472.4 [481] (367–575)	496.25 [532.5] (367–553)	404.5 [402.5] (364–449)	483.6 [485] (370–595)	495.75 [510] (410–553)	405 [408] (364–440)	481.8[a] [481] (368–591)	474.25 [479] (384–555)
Visante CCT (μm)	429.5 [423.5] (407–464)	376.4 [370] (280–482)	417.5 [455.5] (300–459)	428.75 [420.5] (406–468)	497.6 [498] (390–591)	519 [552.5] (393–578)	439.75 [430] (421–478)	496.4 [497] (400–591)	556.25 [578] (428–641)	446 [442] (419–481)	507.4 [503] (426–607)	531.75 [559.5] (440–568)	444 [437.5] (420–481)	501.2[a] [500] (426–597)	525.5 [547.5] (438–569)

ADASC = adipose-derived adult stem cells; CCT = central corneal thickness; CDVA = corrected distance visual acuity; CLVA = contact lens visual acuity; D = diopters; Km = mean keratometry; Kmax = maximum keratometry; Rx Cyl = refractive cylinder; Rx Sphr = refractive sphere; Topo Cyl = topographic cylinder.

Decimal indicates the scale for visual parameters. Data show visual, refractive, keratometric, and pachymetric outcomes for Group 1, autologous ADASC implantation (n = 5); Group 2, decellularized human corneal stroma implantation (n = 5); and Group 3, autologous ADASC recellularized human corneal stroma implantation (n = 4).

[a]Statistically significant ($P \leq 0.05$) differences between the preoperative and 12-month postoperative values for each parameter and for each study group separately.

TABLE 2. Mean Changes From Baseline to 12th Postoperative Month For Visual, Refractive, Keratometric, and Pachymetric Parameters

	12 Months Postoperation to Preoperation		
	Group 1 mean [median] (range)	Group 2 mean [median] (range)	Group 3 mean [median] (range)
UVA (decimal)	0.21 [0.17] (0.1–0.4)	0.17 [0.1] (0.05–0.45)	0.09 [0.05] (0.03–0.25)
CDVA (decimal)	0.23 [0.26] (0–0.4)	0.26 [0.3] (0.05–0.5)	0.09 [0.09] (0–0.2)
CLVA (decimal)	0.26 [0.29] (0.1–0.35)	0.04 [0] (−0.05 to 0.17)	0.13 [0.1] (0–0.32)
Rx Sphr (D)	0.5 [−0.12] (−0.5 to 2.75)	2.35 [0.25] (−0.5 to 10)	0.62 [0.12] (−0.75 to 3)
Rx Cyl (D)	−0.56 [−0.62] (−1.5 to 0.5)	0.15 [0] (−0.5 to 1.5)	0.19 [0] (0–0.75)
Anterior Km (D)	0.70 [0.4] (−0.2 to 2.2)	−2 [−2.2] (−4.9 to 0.9)	−1.58 [−2.15] (−3.6 to 1.6)
Posterior Km (D)	−0.22 [−0.2] (−0.5 to 0)	−0.06 [−0.1] (−0.5 to 0.3)	0.17 [0.1] (−0.2 to 0.7)
Kmax (D)	2.02 [1.3] (−0.4 to 5.9)	−4.20 [−4.5] (−10.3 to 4.4)	−1.58 [0.10] (−10 to 3.5)
Topo Cyl (D)	−0.08 [−0.1] (−1.9 to 1.8)	−0.06 [−0.5] (−4.4 to 4.4)	−0.53 [−0.3] (−1.5 to 0)
CCT (μm)	−6.75 [−9] (−13 to 4)	124.40 [119] (104–157)	108.5 [108] (101–117)
Thinnest point (μm)	−0.75 [7.5] (−30 to 12)	121.20 [115] (113–140)	91 [102] (48–112)
Visante CCT (μm)	14.5 [15] (−19 to 47)	124.8 [119] (114–146)	108 [107] (80–138)

ADASC = adipose-derived adult stem cells; CCT = central corneal thickness; CDVA = corrected distance visual acuity; CLVA = contact lens visual acuity; D = diopters; Km = mean keratometry; Kmax = maximum keratometry; Rx Cyl = refractive cylinder; Rx Sphr = refractive sphere; Topo Cyl = topographic cylinder.

Decimal indicates the scale for visual parameters. Data show mean changes from baseline to the 12th postoperative month for visual, refractive, keratometric, and pachymetric parameters for group 1, autologous ADASC implantation (n = 4); Group 2, decellularized human corneal stroma implantation (n = 5); and Group 3, autologous ADASC recellularized human corneal stroma implantation (n = 4).

postoperative month (related to a mild graft edema), with subsequent progressive improvement over time, during which a net improvement compared with preoperative values was observed within 3 months (for UVA and CDVA) and 6 months (for CLVA) postoperatively (Figure 3). Mean UVA changed from 0.11 (range: 0.05–0.33; Snellen 20/200) to 0.25 (range: 0.1–0.5; Snellen 20/80) at 12 months after surgery ($P = 0.007$; mean improvement of 1.7 lines ± 1.6 [$P = 0.04$] and 1 line ± 1.0 [$P = 0.06$] in G2 and G3, respectively); mean change in CDVA from 0.22 (range: 0.1–0.4; Snellen 20/100) to 0.41 (range: 0.1–0.6; Snellen 20/50; $P = 0.01$; mean improvement of 2.6 lines ± 1.7 [$P = 0.04$] and 0.9 lines ± 0.08 [$P = 0.1$] in G2 and G3, respectively); and mean change in CLVA from 0.54 (range: 0.2–0.87; Snellen 20/40) to 0.62 (range: 0.2–0.87; Snellen 20/32; $P = 0.04$; mean improvement of 0.4 lines ± 0.08 [$P = 0.28$] and 1.3 lines ± 0.13 [$P = 0.1$] in G2 and G3, respectively) (Tables 1 and 2).

• **MANIFEST REFRACTION:** In G1, refractive parameters showed an overall stability (Figure 3), presenting a mean sphere improvement of 0.498 diopters (D) ± 1.5 ($P = 0.90$) 1 year after surgery and a mean cylinder deterioration of −0.56 D ± 0.82 ($P = 0.20$) (Tables 1 and 2).

In G2 and G3, refractive sphere improved from a preoperative mean of −4.55 D (range: −12 to −0.25) to −2.97 D (range: −7.5 to −0.25) 12 months after surgery ($P = 0.23$; mean improvement of 2.35 D ± 4.37 [$P = 0.27$] and 0.62 D ± 1.63 [$P = 0.59$] in G2 and G3, respectively). On the other hand, refractive cylinder remained stable, showing only a mild improvement tendency (Figure 3) from a preopertive mean of −2.83 D (range: −5.5 to −1.5) to a 12-month postoperative mean of −2.66 D (range: −4 to −1.5; $P = 0.46$; mean improvement of 0.15 D ± 0.78 [$P = 1$] and 0.19 D ± 0.37 [$P = 0.31$] in G2 and G3, respectively) (Tables 1 and 2).

• **SLIT LAMP BIOMICROSCOPY:** Before surgery, all corneas were free of posterior stromal or predescemetic scars and presented a clear visual axis. Only 3 patients (1 per group) presented with mild paracentral non-visually significant anterior stromal scars. No postoperative complications including inflammation or rejection signs were recorded throughout the follow-up in all patients.

In G1, corneal transparency was fully recovered within 24 hours after the surgical procedure and continued throughout the whole follow-up period. As described in our previous article,[1] 1 patient from G1 presented with some anterior stromal scars, and a mild improvement of those scars was observed after the third postoperative month, although without a complete resolution up to 12 months postoperatively.

In G2 and G3, the implanted lamina showed a mild early haziness in relation to a mild lenticulare edema during the first postoperative month (which correlated well with the

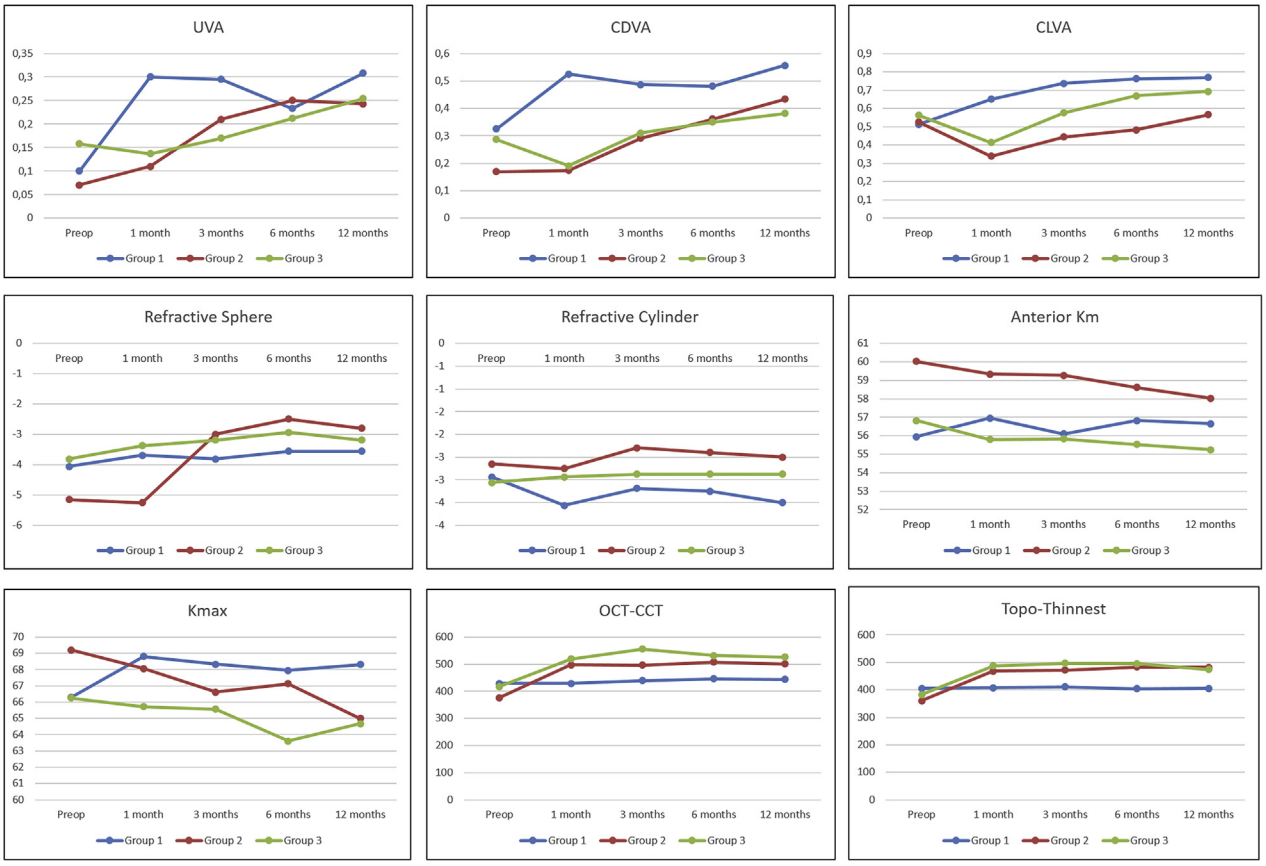

FIGURE 3. Visual, refractive, keratometric, and pachymetric outcomes 12 months after surgery. Central corneal thickness was measured by AS-OCT (OCT-CCT). The thinnest point was measured by Pentacam topographer (Topo-Thinnest). AS-OCT = anterior segment optical coherence tomography; CDVA = corrected distance visual acuity; CLVA = (rigid) contact lens visual acuity; Km = mean keratometry; Kmax = maximum keratometry; OCT-CCT = optical coherence tomography central corneal thickness; UVA = unaided visual acuity.

initial loss in the visual parameters) (Figure 4A and B). Corneal transparency improved progressively throughout the follow-up, showing complete restoration 3 months after surgery in all cases. Twelve months after surgery, no patient presented with significant interface haze or scarring (Figure 4C and D), although some scattered faint patchy "islands" of haze could be observed in most of the G1 and G2 patients, which resulted, however, in no impact on visual outcome (Figure 5). One patient in G1 and another in G2 presented with preoperative anterior stromal scars, but no clinical improvement was observed during the full follow-up period.

• **CORNEAL TOPOGRAPHY:** In G1, all keratometric values were relatively stable (Tables 1 and 2, Figures 3 and 6A). Anterior mean keratometry (Km) remained stable except in 1 patient who presented with deterioration of 2.2 D. However, that patient's topographic anterior cylinder significantly improved (from −2 D preop to −0.2 D at 12 months), explaining the visual improvement observed in that patient. Mean anterior Km changed from 55.95 D (range: 47.9–64.9) to 56.65 D (range: 50.1–65.4) at 12 months after surgery ($P = 0.14$; mean deterioration of 0.7 ± 1.04). Mean anterior astigmatism changed from −2.95 D (range: −5.8 to −0.4) to −3.02 D (range: −5.4 to −0.2) at 12 months after surgery ($P = 0.72$; mean deterioration of −0.08 ± 1.56). Mean anterior maximum keratometry (Kmax) changed from 66.3 D (range: 56.7–79.4) to 68.32 D (range: 62.6–81.2) at 12 months after surgery ($P = 0.14$; mean deterioration of 2.02 ± 2.74). Pachymetric values measured by Pentacam remained stable (Tables 1 and 2, Figures 3 and 6A), without detecting any obvious enhancement in the corneal thickness parameters.

In G2 and G3, an improvement in all anterior keratometric parameters was observed (Tables 1 and 2, Figures 3 and 6B), with a mean anterior Km from 58.6 D (range: 47.9–66.5) preop to 56.78 D (range: 46.5–66.2) at 12 months after surgery ($P = 0.05$; mean improvement of −2D ± 2.35 [$P = 0.13$] and −1.58 D ± 2.31

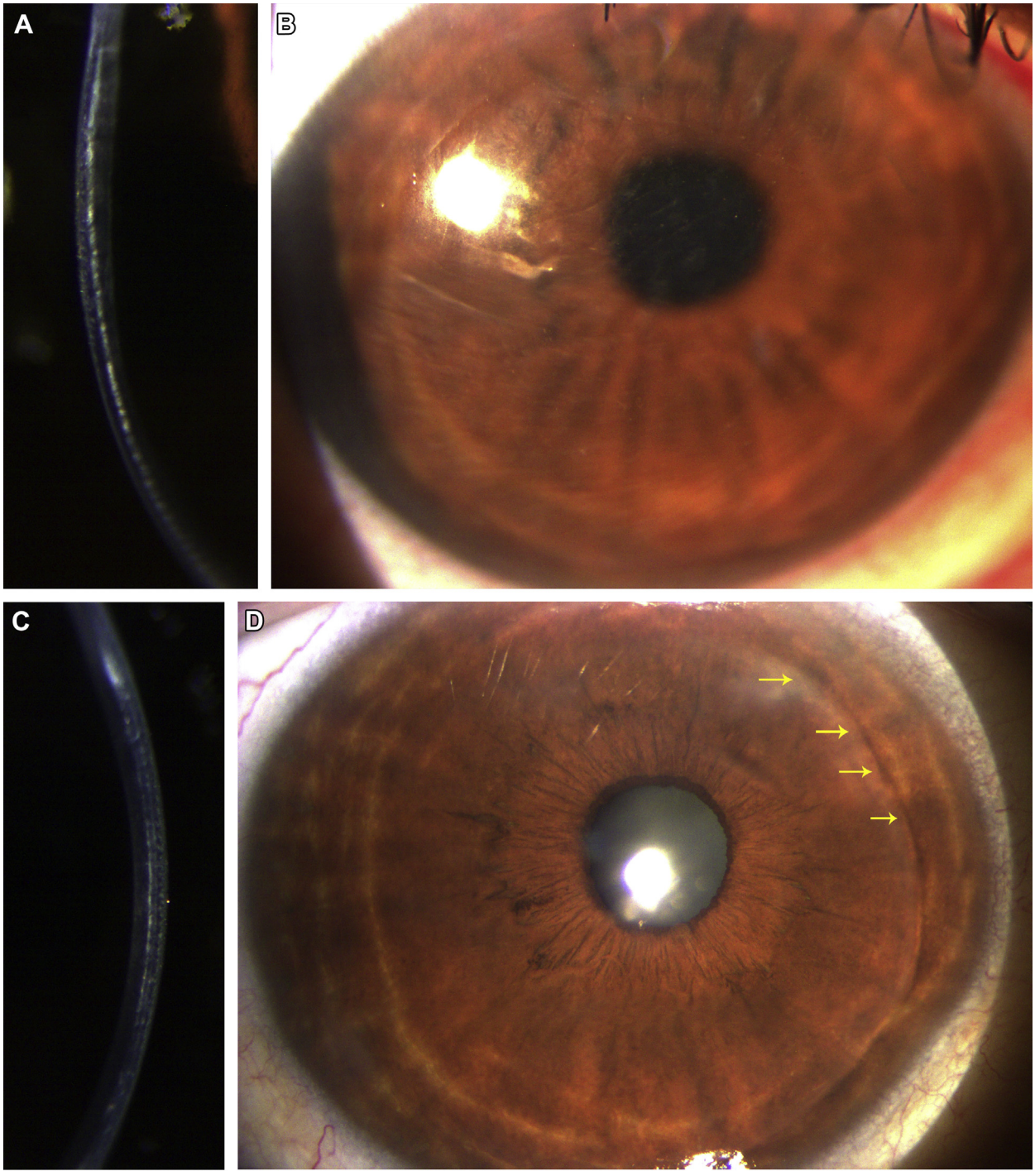

FIGURE 4. Biomicroscopic changes after corneal stroma enhancement. Slit lamp views are from group 2 patient (case 7) showing decellularized lamina transplantation, 1 week (A and B) and 12 months after surgery (C and D) yellow arrows show the lenticule edges.

[$P = 0.27$] in G2 and G3, respectively), and the Kmax changing from 67.89 D (range: 55.6–81.2) to 64.85 D (range: 53.3–77.4; $P = 0.21$; mean improvement of −4.20 D ± 5.82 [$P = 0.13$] and −1.58 D ± 6.16 [$P = 0.26$] in G2 and G3, respectively). We could not demonstrate significant changes in anterior topographic astigmatism: −4.30 D (range: −7.4 to −1.5) preop and −4.56 D (range: −10.7 to −1.1) 12 months postop ($P = 0.52$). As expected, a mean improvement of 120 μm in all thickness parameters was observed in G2 and G3 (Figures 3 and 6B, Tables 1 and 2).

• **CORNEAL ABERROMETRY:** In G1, corneal aberrometry could not be analyzed due to missing preoperative data. In G2 and G3, corneal aberrometry with 6-mm pupil

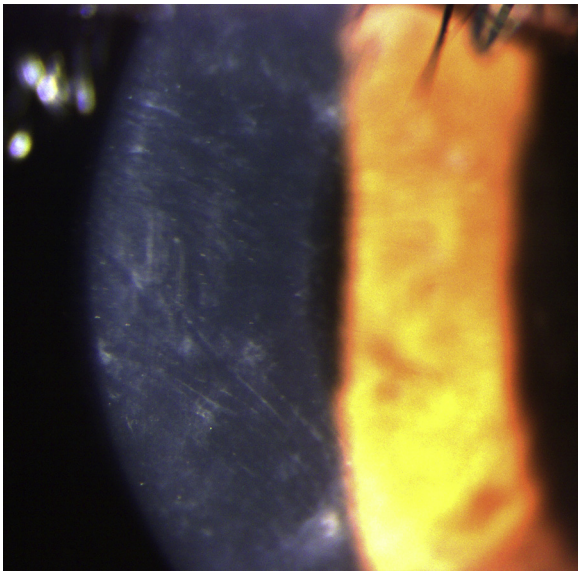

FIGURE 5. Some scattered, faint, patchy "islands" of haze could be observed in most of the group 1 and 2 patients. These areas of haze had no impact on the visual outcome.

demonstrated an important and significant improvement in the spherical aberration, coma, and total higher order aberrations in all patients except 1 patient from G2 who presented the steepest cone of the study sample, and no reliable data could be obtained. We excluded that patient from this analysis to avoid potential bias:

- Spherical aberration: from 1.30 (range: 0.3–2.65) preop to 0.66 μm (range: 0.07–2.05) 12 months after surgery ($P = 0.04$);
- Coma: from 3.49 (range: 1.20–5.82) preop to 2.09 μm (range: 0.53–2.93) 12 months after surgery ($P = 0.07$);
- Total higher order abberations: from 4.14 (range: 1.53–6.13) preop to 3.04 μm (range: 1.33–4.4) 12 months after surgery ($P = 0.24$)

No significant differences between the 6- and 12-month postoperative visits were observed in any of these values.

• **ANTERIOR SEGMENT OCT:** In G1, a mild improvement in the central corneal thickness measured by anterior segment OCT (Visante) was observed in 3 of 4 patients, with a mean increase of 14.5 ± 27 μm at the 12-month visit ($P = 0.47$). This improvement in the thickness occurred after the third month, when (in all patients except for the one without thickness improvement) a hyperreflective (increased optical reflectivity) band at the level of the stromal pocket compatible with an area of new collagen production could be observed (Figure 7A). This area was not homogeneously distributed along the pocket. One year after surgery, 2 of these patients showed a partial normalization of the reflectance of the neo-collagen band (Figure 7B), whereas in 1 patient, its high reflectance was still obvious (Figure 7C).

In G2 and G3, central corneal thickness measured by OCT Visante confirmed the results observed with the topography: mean preop value of 394.66 μm (range: 280–482) and 512 μm (range: 426–597) at 12 months after surgery ($P = 0.008$; mean improvement of 124.8 μm ± 13.44 [$P = 0.04$] and 108 μm ± 24.05 [$P = 0.06$] in G2 and G3, respectively). The transplanted lamina was clearly visible in the corneal OCT, showing a moderate early postoperative hyper-reflectance during the first postoperative month (Figure 8A) in good correlation with the observed mild clinical haze in the implant in the same period of time. After the third postoperative month, the lamina already presented a normal reflectance, equivalent to the surrounding recipient stroma (Figure 8B). In G2 and G3, the findings were equivalent, and no obvious areas of new collagen production were observed in G3.

• **CONFOCAL BIOMICROSCOPY:** In G1, up to postoperative month 3, round-shaped cells were observed in the surgical plane in all cases (Figure 9A). These cells showed a different morphology than the usual dendritic or fusiform shape presented by the anterior and posterior stromal keratocytes (Figure 9B).[1,2] At month 6, these cells at the surgical level already had a fusiform shape and were not different from those observed in other stromal planes.[1] This rounded cellular shape could be considered a landmark for cellular survival through the early postoperative period. We were also able to demonstrate a progressive increase in the cellular density at all measured stromal levels (anterior [$P = 0.07$], mid [$P = 0.07$], and posterior stroma [$P = 0.07$], between preop and 12 months).

In G2 and G3, throughout the first postoperative year, a normal cellular pattern was observed in the anterior and posterior stroma (Figure 9B). The lamina borders were easily visible as a hyper-reflective linear bands in the interface between the normal cellular anterior or posterior stroma and the acellular implanted stroma.[2] The lamina showed a similar acellular appearance in both groups without relevant differences (Figure 9C). Six months after surgery, all patients presented signs of early recellularization with scant isolated cells scattered throughout the lamina. This recellularization of the lamina continued up to the first postoperative year (Figure 9D), which we could demonstrate by a statistically significant increase in the keratocyte density within the lamina, which was more marked in G3 laminas. Moreover, both anterior ($P = 0.008$) and posterior ($P = 0.008$) stroma keratocyte densities also showed a statistically significant progressive increase up to the first year.

This observed tendency for an increase in keratocyte density at the anterior and posterior stroma in all 3 groups could be related to an activation of the host keratocytes,

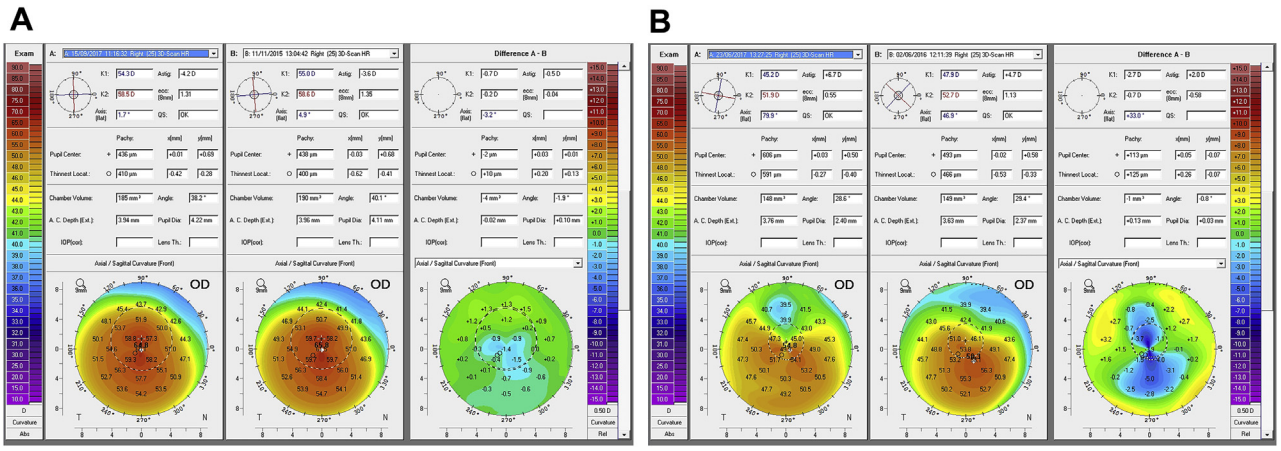

FIGURE 6. (A) Corneal topography (Pentacam) comparison before surgery and 12 months after ADASc implantation (case 4). Note the stability of the keratometric parameters. (B) Topographic changes after corneal stroma enhancement between presurgery and 12 months after surgery (same patient as in Figure 4). Observe the significant flattening of the keratometry and the important improvement in all thickness parameters.

which are responsible for the recellularization observed within the implanted tissue in G2 and G3.

• **OTHER CLINICAL OUTCOMES:** No significant changes in IOP or endothelial cell density were detected in a comparison among preoperative and 6- and 12-month data ($P > 0.05$).

Five patients received superficial laminas that included Bowman's membrane (4 in G2 and 1 in G3), and 4 patients received the deeper lamina without this layer (1 in G2 and 3 in G3). Patients who received superficial laminas that included Bowman's membrane did not show better outcomes in any of the analyzed parameters of the study.

DISCUSSION

THIS PAPER REPORTS THE 1-YEAR OUTCOMES OF THE FIRST human experience with corneal stroma cell therapy. It is the first time that the behavior of autologous stem cells (ADASc in the present study) injected into the corneal stroma demonstrated the appearance of new collagen in the area that corresponds to the injection of the cells, as well as the first time that decellularized human corneal stroma laminas, colonized or not by autologous ADASc, were implanted in clinical cases of keratoconus for therapeutic purposes.

The first important issue that is demonstrated in this study is the lack of complications of the procedure observed in this 1-year clinical study. The use of femtosecond laser to create a stromal pocket at the mid stroma of advanced keratoconus cases is shown in the experience to be feasible and with no negative consequences, for up to 1 year of observation. This finding is consistent with our previous studies in which a large femtosecond laser-assisted corneal lamellar dissection performed approximately at the depth of the ones, followed by the implantation of an intrastromal circular ring, was shown not to be affected by complications either from a visual, topographic, keratometric, or anatomical perspective.[25] The most representative data concerning the safety of the surgery is offered from G1 in which the only injection of cells in the pocket was followed by an immediate, modest but significant improvement, which was maintained throughout the first year of the follow-up. Obviously, this initial response of the cornea indicates the tolerance to this type of surgery as no deterioration was observed in any case in this or the other groups. At this early level of experience, the keratocyte differentiation and the eventual collagen production might not have had any significant influence in the mechanical response of the cornea, so further studies should guarantee the feasibility of this type of surgery from a biomechanical perspective. However, femtosecond laser-assisted pockets of these dimensions were used for the implantation of intracorneal ring segments without any negative impact, either immediate or late, in the operated keratoconic corneas, even though in this experience an intracorneal foreign body was implanted for therapeutic purposes.[26] Moreover, according to previous reports,[27] vertical side cuts through corneal lamellae, rather than horizontal delamination incisions, contributed to the loss of structural integrity during LASIK flap creation, so a minimal impact on corneal biomechanics may be expected after the creation of a lamellar pocket such as the one performed in this clinical study.

In the current experience, the good tolerance and the possibility of increasing corneal thickness by the implantation of decellularized layers of corneal stroma created by femtosecond laser were confirmed. To induce a calculated

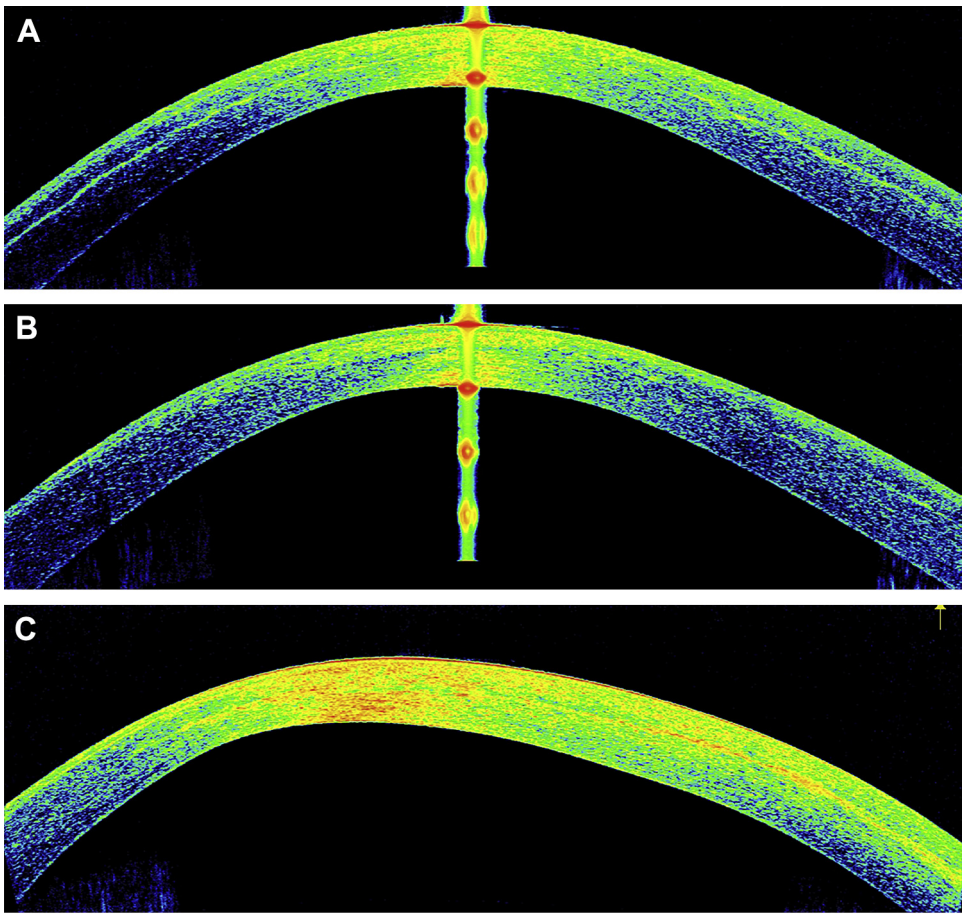

FIGURE 7. Corneal OCT views after ADASc implantation (case 3). (A) Note the hyperreflective (increased optical reflectivity) band of neo-collagen at the level of the stromal pocket 6 months after surgery (case 1). (B) Same patient one year after surgery, showing a partial normalization of the reflectance of the neo-collagen band. C: Another patient (case 4) shown 1 year after surgery, where the high reflectance of the neo-collagen band remains. OCT = optical coherence tomography.

increase in the thickness of the cornea is feasible by using these femto laser-dissected and decellularized laminas that can be implanted without any negative impact on the biomechanics, vision, and especially corneal anatomy. Corneal irregularity has been demonstrated to be improved, albeit modestly, by the reduction of corneal aberrometry, as observed in this study. Therefore, we can deduce that the calculated increase in corneal thickness induced by the use of these laminas can be used on a therapeutic basis without risk for the corneal transparency and benefits the corneal regularity and topography. The refractive sphere of these patients improved as well, which is in agreement with the flattening of the cornea, which is observed in the keratometric analysis.

Probably the most relevant feature is the maintenance of corneal transparency as a fact. In no case was corneal haze or corneal scarring observed at 1 year of follow-up, not even during the experience (with the exception of the early haze seen during the first month after the lamina implantation in G2 and G3). This issue is extremely important because the introduction of heterologous manipulated corneal tissue could be followed by corneal scarring and eventually permanent loss of vision. The reasons for this corneal transparency are probably related to the decellularized character of the laminas as no biological interaction is happening between this foreign tissue of allogenic origin and the local host tissue cells. These findings demonstrate our previous experimental studies in which human decellularized corneal stroma laminas were well tolerated in the experimental animal model.[12] Furthermore, the presence of autologous ADASc in the laminas of G3 could be of further relevance for the maintenance not only for the corneal transparency but also for the decrease of previous corneal scars that could be present in these cases, a fact that has been described previously in the experimental animal model[6–11] and that we could also observe in 1 patient from G1.[1] This isolated observation of a significant biomicroscopy decrease in the scars present at the cornea preoperatively might have been related to the capability of the stem cells to improve corneal transparency by their

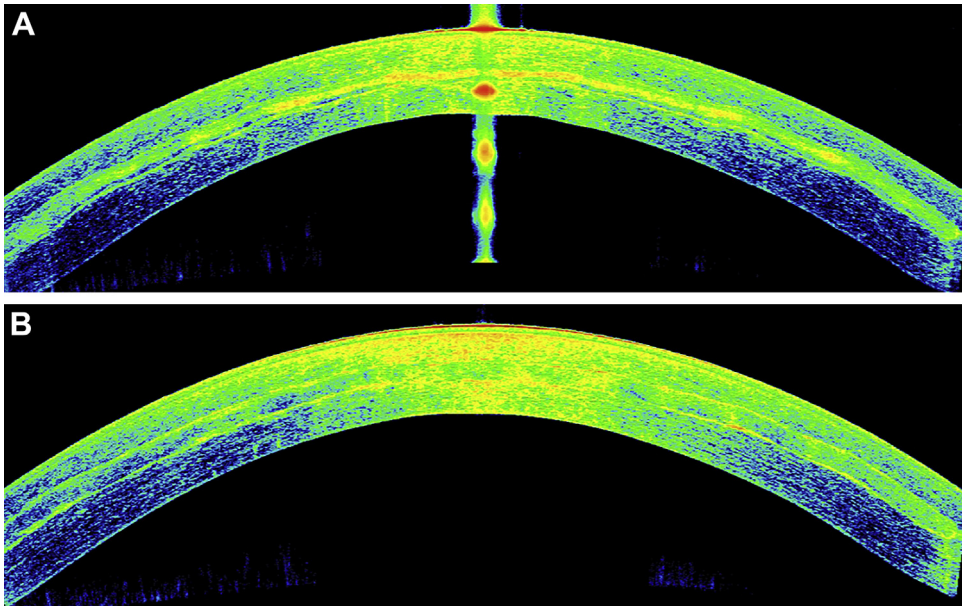

FIGURE 8. Cornea OCT Visante images after corneal stroma enhancement (same patient as in Figures 4 and 6B). (A) One month after surgery. (B) One year after surgery. Note the normalization of the early postoperative increased reflectance of the lamina. OCT = optical coherence tomography.

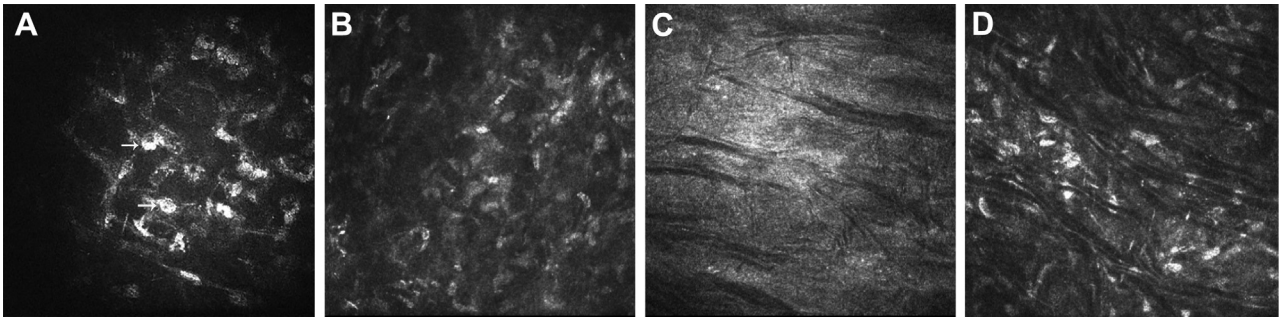

FIGURE 9. Corneal confocal biomicroscopy views. (A) Surgical plane from a group 1 patient (ADASc implantation) at 1 month after surgery (case 1). Stem cell survival is confirmed by the presence of cells showing a more rounded shape (white arrows). (B) Corneal stroma anterior to the implanted lamina; note the fusiform shape of adult keratocytes (case 8). (C) Group 2 patient with implanted lamina showing a complete acellular pattern 3 months after surgery (case 8). (D) Intense recellularization signs in a group 3 lamina 12 months after surgery (case 14).

capability to reorganize previously diseased tissue and the production of new normal collagen. Further studies performed in non-transparent and scarred corneas are necessary to confirm the therapeutic possibility of this new surgery.

Of particular relevance is the observation of a positive biological response from the host keratocytes to the decellularized laminas, especially in those implanted with autologous ADASc. In G2 (treated with decellularized laminas with no addition of ADASc) the increase in the corneal thickness, maintaining the corneal transparency, was parallel to the observation of an increase in the cellularity of both the anterior and the posterior parts of the corneal stroma. Therefore, the decellularized corneal lamina was not only able to maintain transparent conditions, increasing the corneal thickness, but there was also a positive cellular response of the host keratocytes by proliferating and even invading the implanted tissue, which indicates its biological activation (Figure 9). This finding was even more pronounced in those laminas implanted with ADASc, although no statistically significant differences between G2 and G3 could be demonstrated, likely related to the small study sample.

In G1 (treated with ADASc alone), the confocal biomicroscopy findings are also remarkable. Initially, the ADAS cells could be identified as rounded and regular in shape, whereas later, during the experience, these cells progressively transformed into a fusiform shape and showed

an identical appearance to the normal keratocytes of the human cornea (Figure 9).[1] An increase in the anterior and posterior stroma keratocyte densities could also be observed in G1 patients, so not only does the presence of the corneal lamina induce a positive biological response in the cornea, but also this healing response can be enhanced by the inclusion of ADAS cells, which may contribute not only to the biological tolerance of the decellularized corneal lamina but also to the maintenance of its transparency and integration into the host cornea.

The presence or absence of Bowman's membrane in the implanted laminas was not relevant to the results in our study, although the small sample limited the significance of this observation.[28]

As the first-year data from this study demonstrate, we are opening a new area of corneal surgery and corneal research. The use of parts of the cornea for the purpose of a therapeutic increase in corneal thickness in debilitated and thin keratoconic corneas is a new concept for research. Corneal clinical parameters were all maintained or improved, and no deterioration was observed in any of the cases of the present study. Based on the outcomes of the present investigation, the use of autologous stem cells, particularly ADASc, for therapeutic purposes in keratoconus or other corneal dystrophies or even corneal scars related to other causes could be relevant as these cells behave well in the corneal stroma, do not cause opacities and do not induce any inflammatory reaction.

The information in this report requires further confirmation. The limitations in this study are are related to the small numbers of cases included in each group. Clinical pilot investigations of new therapies are difficult to perform and impose many limitations in terms of administration and approbation and ethics. The statistical analysis shows trends, and the outcomes should be considered within the context of the statistical limitations created by the limited number of cases of each group. However, despite these limitations, for the first time, we can offer new information which, even though it requires further confirmation, opens a new area for the clinical use of stem cells and a new type of corneal stroma therapy. The benefits of this are evident. Corneal dystrophies may have treatment alternatives that are different from those for corneal transplantation. Early treatment of these diseases with stem cells of autologous or allogenic origin could be an option even in early stages. In advanced stages, the many problems of corneal transplantation, such as corneal tissue availability, long recovery times, and unpredictable results, could be eliminated. The presence of corneal scars could have an option with the use of these mesenchymal stem cells as an alternative treatment. The most relevant disease whose treatment could benefit most from the results of this study is indeed keratoconus. Keratoconus is the most frequent and important corneal dystrophy due to its sociological impact. The frequency of keratoconus is indeed relevant because it is the primary cause of corneal transplantations in the young population.[13] All these patients could benefit from this new type of surgical therapy, which can be more accessible, does not depend on viable human corneal tissue, and does not present the biological hazards related to allogenic tissue with allogenic cells.

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