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Successful restoration of corneal surface integrity with a tissue-engineered allogeneic implant in severe keratitis patients

Carmen González-Gallardo [a,b], Juliana Martínez-Atienza [c], Beatriz Mataix [d], José Ignacio Muñoz-Ávila [a], J. Daniel Martínez-Rodríguez [e], Santiago Medialdea [e], Antonio Ruiz-García [c,f], Antonio Lizana-Moreno [c,f], Salvador Arias-Santiago [c,f], Manuel de la Rosa-Fraile [g], Ingrid Garzon [b,h], Antonio Campos [b,h], Natividad Cuende [c,i], Miguel Alaminos [b,h,**], Miguel González-Andrades [a,j,*], Rosario Mata [c]

[a] Ophthalmology Clinical Unit of San Cecilio University Hospital, Granada, Spain
[b] Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain
[c] Andalusian Network for the Design and Translation of Advanced Therapies (former Andalusian Initiative for Advanced Therapies) – Fundación Andaluza Progreso y Salud, Junta de Andalucía, Seville, Spain
[d] Ophthalmology Clinical Unit of Virgen Macarena University Hospital, Seville, Spain
[e] Ophthalmology Clinical Unit of Virgen de las Nieves University Hospital, Granada, Spain
[f] Cell Therapy and Tissue Engineering Unit, Virgen de las Nieves University Hospital, Granada, Spain
[g] University Hospital Virgen de las Nieves, Microbiology Service, Emeritus, Spain
[h] Tissue Engineering Group, Department of Histology, Universidad de Granada, Granada, Spain
[i] Andalusian Transplant Coordination, Servicio Andaluz de Salud, Seville, Spain
[j] Maimonides Biomedical Research Institute of Cordoba (IMIBIC), Department of Ophthalmology, Reina Sofia University Hospital and University of Cordoba, Cordoba, Spain

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ABSTRACT

Objectives: Corneal diseases are among the main causes of blindness, with approximately 4.6 and 23 million patients worldwide suffering from bilateral and unilateral corneal blindness, respectively. The standard treatment for severe corneal diseases is corneal transplantation. However, relevant disadvantages, particularly in high-risk conditions, have focused the attention on the search for alternatives.

Methods: We report interim findings of a phase I-II clinical study evaluating the safety and preliminary efficacy of a tissue-engineered corneal substitute composed of a nanostructured fibrin-agarose biocompatible scaffold combined with allogeneic corneal epithelial and stromal cells (NANOULCOR). 5 subjects (5 eyes) suffering from trophic corneal ulcers refractory to conventional treatments, who combined stromal degradation or fibrosis and limbal stem cell deficiency, were included and treated with this allogeneic anterior corneal substitute.

Results: The implant completely covered the corneal surface, and ocular surface inflammation decreased following surgery. Only four adverse reactions were registered, and none of them were severe. No detachment, ulcer relapse nor surgical re-interventions were registered after 2 years of follow-up. No signs of graft rejection, local infection or corneal neovascularization were observed either. Efficacy was measured as a significant postoperative improvement in terms of the eye complication grading scales. Anterior segment optical coherence tomography images revealed a more homogeneous and stable ocular surface, with complete scaffold degradation occurring within 3–12 weeks after surgery.

Conclusions: Our findings suggest that the surgical application of this allogeneic anterior human corneal substitute is feasible and safe, showing partial efficacy in the restoration of the corneal surface.

* Correspondence to: Universidad de Granada, Tissue Engineering Group-Department of Histology, Faculty of Medicine, Avenida de la Investigación 11, Torre A, planta 5ª, E18016 Granada, Spain.
** Correspondence to: Maimonides Biomedical Research Institute of Cordoba (IMIBIC), Avenida Menéndez Pidal s/n, E14004 Cordoba, Spain.
E-mail addresses: malaminos@ugr.es (M. Alaminos), miguel.gonzalez@imibic.org (M. González-Andrades).

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1. Introduction

Corneal diseases stand among the main causes of blindness in the world. Approximately, there are 4.6 million patients worldwide suffering from bilateral corneal blindness, while 23 million patients are affected by unilateral corneal blindness [1,2]. The main treatment applied to severe corneal diseases that cause blindness is corneal transplantation [3]. Nowadays, replacement of the full-thickness cornea by a donor cornea (i.e. penetrating keratoplasty) is becoming a less frequent procedure compared to lamellar transplants where only the damaged region of the cornea is replaced by a donor corneal epithelium and stroma (i.e. anterior lamellar keratoplasty), or by a donor corneal endothelium (i.e. endothelial keratoplasty) [3–5]. Despite representing the most frequent transplant performed worldwide, there are several drawbacks related to keratoplasty: difficulties in the access to donor corneas, microbiological contamination and graft rejection of the implanted tissue. According to global surveys, there is a severe shortage of corneal graft tissue supply, and the demand for donor transplantable tissue is increasing due to population ageing [6]. Postoperative infection after human donor corneal transplantation is an important cause of graft failure that, in some instances, is difficult to prevent or control [7].

Patients with corneal neovascularization or limbal stem cell deficiency (LSCD) are considered as high-risk cases, with a high likelihood of rejection and failure of the transplant. Thus, regular keratoplasties are contraindicated because of the poor prognosis offered to these patients [8,9]. LSCD requires restoration of the limbal stem cell population to promote regeneration of the corneal epithelium. This can be achieved by different techniques: limbal transplants, cultured limbal epithelial transplantation (CLET) [10] or single limbal epithelial transplantation (SLET) [11,12]. In spite of the success of these techniques to restore the corneal epithelium, they are not conceived to regenerate the corneal stroma. If the patient associates LSCD with corneal stromal damage, which is a relatively common finding, limbal restoration has to be followed by stromal replacement using a donor cornea. In this regard, Rama et al., described CLET followed by penetrating keratoplasty, once the limbal cell population was recovered [13]. Another therapeutic strategy applied in patients with severe corneal damage in which keratoplasties are contraindicated is the implantation of a keratoprosthesis. The most used worldwide is the Boston Keratoprosthesis Type I, which requires a donor cornea as carrier to implant the device into the patient́s cornea. Despite its capability to recover visual acuity with better results than repeated keratoplasties, its long-term survival is limited because of the high incidence of glaucoma and other sight-threatening complications [14,15]. In addition, patients affected by corneal injury altering the marginal corneal arcades and the limbal microvasculature may have severe alterations of the limbal stem cell niche, and novel therapeutic alternatives are in need in these complex cases [16].

In this context, tissue engineering emerges as a new therapeutic strategy that aspires to address those drawbacks, facilitating the treatment not only of LSCD, but also of the stromal damage. In this regard, very few tissue-engineered therapies focused on stromal regeneration have reached the clinical setting. Alió et al., transplanted a decellularized femtosecond-laser-cut anterior corneal stroma in 9 patients with keratoconus, and 4 of them received a recellularized stroma with autologous adipose-derived mesenchymal stem cells (MSCs). After 6 months of implantation, patients improved visual acuity and other signs like haze or scarring were not observed [17]. May Griffith́s team evaluated two different acellular stromal substitutes. Firstly, recombinant human collagen (RHC) based acellular artificial corneas were transplanted in 9 patients with keratoconus and one patient with permanent mid-stromal scar. The implants stimulated the regeneration of corneal epithelium, stroma and nerves, showing no signs of rejections without sustained immune suppression [18,19]. Secondly, acellular interpenetrating polymer networks of RHC and 2–methacryloyloxyethyl phosphorylcholine (MPC) were transplanted in patients with recurrent corneal ulcers and erosions, providing relief from pain and discomfort, and restoring corneal integrity [20,21]. Lately, Basu et al. are evaluating the capability of MSCs to promote corneal stromal regeneration by directly implanting those into the patient́s cornea or by indirect contact through a paracrine effect [22,23]. Despite of the partial success of these novel strategies to restore corneal function, none of these approaches can restore the limbal stem cell population.

In this milieu, we aimed to develop an allogeneic tissue-engineered anterior lamellar nanostructured artificial human cornea (ATEAHC) to simultaneously treat LSCD and stromal damage. For that purpose, the Tissue Engineering Group of the University of Granada previously designed, generated and evaluated preclinically an ATEAHC model of bioartificial cornea [24]. This model, called NANOULCOR, consists of a nanostructured fibrin-agarose stromal substitute combined with allogeneic corneal epithelial and stromal cells, and was based on a previous full-thickness animal cornea model developed by the research group in which rabbit corneal epithelial, stromal and endothelial cells were combined with a fibrin-agarose scaffold [25]. NANOULCOR showed promising preclinical results in terms of in vitro and in vivo biocompatibility, biomechanical properties, optical behavior and gene expression [24,26,27]. These results encouraged us for pursuing clinical implementation, despite the complexities and challenges that pose the translation into clinical practice of this new type of drugs [28]. For that purpose, the former Andalusian Initiative for Advanced Therapies [29] (at present Andalusian Network for the Design and Translation of Advanced Therapies), specialized in supporting the development of advanced therapies [28], promoted the present phase I-II clinical trial to evaluate the safety and partial efficacy in humans of this tissue engineered product [30]. The clinical trial is focused on patients suffering from trophic corneal ulcers refractory to conventional treatments, who combined stromal degradation or fibrosis, including those with sequelae of previous ulcers, mainly corneal opacification and/or scarring. Conventional treatments aim to improve the lubrication status of the ocular surface (e.g. artificial tears, contact lens, punctal occlusion, etc.) or to provide the missing trophic factors such as epithelial growth factor or nerve growth factor (e.g. autologous serum, platelet-rich plasma, etc.). When these treatments fail, disease progression may lead to corneal melting and perforation, requiring aggressive treatments to prevent loss of visual function and even the eye loss (e.g. cyanoacrylate glue, anterior lamellar keratoplasty, conjunctival graft or amniotic membrane transplants to preserve the anatomical integrity of the cornea). We hypothesize that NANOULCOR can provide relevant growth factors (mainly those contained in the human plasma, which is one of the main compounds used to manufacture the scaffold), the cellular content (corneal epithelial and stromal cells) and the structural elements (the fibrin-agarose scaffold) to promote the regeneration of the patient́s damaged cornea. Here, we report interim findings of the application of NANOULCOR in the first 5 patients recruited in the initial phase of the trial, after finishing the established 2-year follow up.

2. Materials and methods

2.1. Clinical study design

The present trial (ClinicalTrials.gov Identifier: NCT01765244) was designed to evaluate the safety and efficacy of NANOULCOR in patients with severe ulcerative keratitis and no current effective therapeutic alternative. The trial protocol obtained the authorization from the Spanish Health Authorities and from the Ethics Committee, and all patients provided informed consent for participation before undergoing any trial-related procedures. All study methods comply with the principles of the Declaration of Helsinki (WMA, 2013). Eligibility criteria and study design were previously reported in detail [30]. This article describes the interim results from the application of NANOULCOR in the first 5 patients recruited and followed for a period of 24 months. At the time of submitting this manuscript, the recruitment period is closed, with follow up completed in all participating sites.

2.2. Manufacturing process in GMP facility

Human allogeneic anterior corneal substitutes were manufactured at one of the GMP facilities coordinated by the former Andalusian Initiative for Advanced Therapies, specifically the Cell Production & Tissue Engineering Unit at Virgen de las Nieves University Hospital (Granada, Spain), by adapting to a GMP environment a biofabrication protocol previously designed at the Department of Histology of the University of Granada [24]. Cultured cells were obtained from suitable cadaveric donor samples, previously screened for transmittable diseases. Tissue from corneoscleral rings was mechanically divided into limbus and central cornea, and limbal epithelial cells and corneal keratocytes (stromal cells) were isolated and cultured in a suitable media, until they were cryopreserved. Quality controls at this stage included sterility, viability, karyotype, genetic fingerprint, virus culture, chlamydia, microbiological staining and mycoplasma. Bioengineered corneas were manufactured by using sequential culture techniques of corneal stromal cells in a scaffold made of a mixture of 0.1% agarose and fibrin. Nine days later, limbal epithelial cells were seeded on top and maintained in culture for three weeks, including a two-week period applying air-lifting techniques to promote epithelial differentiation. Once the culture process was completed, corneal substitutes were subjected to plastic compression for partial dehydration (i.e. nanostructuration) to improve the mechanical properties of the construct. Quality controls at this stage included sterility, viability, microbiological staining, mycoplasma and endotoxins analysis. The NANOULCOR product was then transported to the hospital at controlled temperature (0ºC-8ºC), where it was scheduled to be implanted within the next 6 h (Fig. S2).

2.3. Trial intervention and evaluation

The trial protocol obtained the authorization from the Spanish Agency of Medicines and Medical Devices (AEMPS) as well as the Referral Institutional Review Board, and all patients provided written informed consent for participation in the trial before undergoing any study-related procedures [30]. All study subjects analyzed were grafted with a NANOULCOR bioengineered allogeneic anterior human corneal substitute containing adult expanded human limbal epithelial cells and corneal stromal cells embedded in a biocompatible fibrin-agarose matrix. NANOULCOR is considered a tissue engineered medicinal product, as defined in Article 2(1)(b) of Regulation (EC) No. 1394/2007, and was therefore manufactured according to good manufacturing practices for clinical grade medicinal products [24]. The implant was grafted to cover the corneal defect, after its debridement by keratectomy, and sutured to the host cornea using 10–0 nylon suture material. After surgery, trial subjects were evaluated according to the protocol's visits and assessments schedule, including clinical examination aided with slit lamp, anterior segment optical coherence tomography (AS-OCT), vital staining tests (i.e. Schirmer, TBUT, fluorescein and lisamine green) and Cochet-Bonnet esthesiometry. Endpoints were evaluated up for a total of 24 months, with regular visits at days 1–7, weeks 2–4, months 2–24 [30].

Eye complications were assessed using the ocular complications severity scales published by Sotozono et al. and Whitcher et al. [31,32]. According to the grading system proposed by Sotozono et al., complications were categorized as ocular (13 items), corneal (7 items), conjunctival (2 items), and eyelid (4 items) complications, and its 13 components were evaluated and graded on a scale from 0 to 3 according to their severity, adding up to a total maximum score of 39 for the global ocular complication score (in the eyes with the worst prognosis). The maximum scores for corneal, conjunctival, and eyelid complications were 21, 6 and 12, respectively. Corneal complications grading score is comprised of superficial punctate keratopathy (SPK), epithelial defect, loss of the palisades of Vogt (POV), conjunctivalization, neovascularization, opacification, and keratinization components, adding up to a total maximum score of 21, for the most severely affected eyes. Conjunctival complication grading score is composed of two items (conjunctival hyperemia and symblepharon formation), adding up to a maximum total score of 4, for the most severely affected eyes. Eyelid complication grading score is obtained by combining the following 4 items of Sotozono' scale: trichiasis, mucocutaneous junction involvement, meibomian gland involvement and punctal involvement. This sub-component of the scale ranges from 0 to a maximum of 12. On the other hand, the SICCA ocular staining score [31] adds up the fluorescein (corneal component) and lissamine green (conjunctival component) staining scores described to a total maximum score of 12 (in the most severely affected eyes). Ocular surface complications were assessed by one expert ophthalmologist in each participating site. Grading score values were then confirmed using slit-lam pictures by two expert ophthalmologists. In the event of inconsistencies among evaluators, average values were used.

On completion of study visits, patients fulfilled a questionnaire to measure their self-perceived improvement and satisfaction with the outcomes of the research. This questionnaire consisted of a visual analog scale (0−10), to quantify the level of improvement perceived in the symptoms of their ocular disease, as well as a question about their general satisfaction with the treatment received.

To assess corneal repair efficacy of the ATEAHC, all corneal surface events were registered. Patients were monitored for any events throughout follow up and causal relation with the implant was established for each AE. Additional safety evaluations were performed to assess safety: eye fundus and posterior OCT, IOP monitoring, in addition to general clinical assessment/explorations, vital signs, and blood tests. AE were classified according to MedDRA dictionary, and categorized in relation to site (ocular / non-ocular), level of severity and time of occurrence, with special concern in events in severe ocular events occurring 24 h after surgery.

2.4. Statistical methods

Descriptive statistics analyses were conducted using Microsoft Excel Software (2016), the RealStats data analysis tool add-in (Version 7.0.5) [33], as well as Graphpad Prism (version 8). Statistical comparison tests were carried out with non-parametric Wilcoxon two-tailed signed rank tests for paired samples. All statistical tests used a level of significance of 5% ($p < 0.05$) for double tailed comparisons.

3. Results

3.1. Patient characteristics

Between 2014 and 2015, a total of 5 ATEAHC lamellar transplants were performed in 5 eligible subjects (5 eyes), including 2 females and 3 males, age range 28–69 years, with severe trophic corneal ulcers (or their sequelae) refractory to conventional treatments due to neurotrophic keratitis of traumatic (4/5), diabetes mellitus (2/5) or infectious etiology (1/5) (Table 1). All subjects were screened and sequentially enrolled, including a postoperative safety period of 45 days among each inclusion. In all cases, epithelial defects and ulcers persisted despite previous treatments, surgical or non-surgical, with extensive corneal edema and fibrosis, plus moderate (4/5) to mild (1/5) LSCD accompanying the ocular surface disease. The condition was unresponsive to most available treatments, including autologous serum, amniotic membrane graft or donor corneal transplant. The duration of the disease ranged from 1 to 6 years (average 3.8 years). Band calcium keratopathy (2/5), endothelial decompensation (3/5), glaucoma (2/5) and retinal disease (3/5) were present preoperatively as concomitant ocular diseases, indicating that most eyes treated were inflicted with the worst prognosis as well as irrecoverable vision loss. For ethical reasons only one eye was treated in each patient, choosing the eye most severely affected. All patients were followed up for a total of 24 months, with regular visits at days 1–7, weeks 2–4, months 2–24 [30]. No screening

Table 1
Summary of demographic information, baseline characteristics and clinical outcomes of patients treated with NANOULCOR. LSCD and corneal fibrosis severity were graded using Vogt palisades loss and corneal opacity scores, respectively as published by Sotozono et al., 2007 (27). DM: Diabetes mellitus. HSV: Herpes Simplex Virus. Y: yes. N: no. M: months. R: right. L: left. OHT: ocular hypertension. IOP: intraocular pressure. VA: visual acuity. LP: light perception. NLP: no light perception. LPP: light perception with projection.

Case	Age/sex	Etiology	Duration	Eye	Prior Keratoplasty	Other concomitant diseases	Visual Acuity			LSCD			Corneal fibrosis			Follow-up (M)	Adverse reactions
							Pre	12 m	24 m	Pre	12 m	24 m	Pre	12 m	24 m		
S1	51/ male	Trauma	5 Y	R	Y	Retinal detachment, Glaucoma, Keratoconus, Endothelial decompensation	LP	LP	LP	Y/ 2	Y/2	Y/2	Y/ 2	Y/2	Y/2	24	N
S2	67/ female	Trauma / DM	< 1 Y	L	N	Retinal detachment, Glaucoma, Endothelial decompensation	LP	LP	LP	Y/ 2	Y/2	Y/ 1,5	Y/ 2	Y/2	Y/ 1,5	24	Y Corneal leucoma Epithelial defect
S3	69/ female	Trauma	6 Y	L	Y	Myopia magna, Maculopathy, OHT, Cataract, Band keratopathy, Endothelial decompensation	NLP	LP	LPP	Y/ 2	Y/2	Y/2	Y/ 2	Y/2	Y/2	24	Y Epithelial defect
S4	52/ male	HSV infection	5 Y	L	N	Iris synechia	LP	CF	CF	Y/ 2	Y/2	Y/2	Y/ 2	Y/1	Y/1	24	N
S5	28/ male	Trauma /DM	2 Y	L	N	Proliferative retinopathy, Band keratopathy	LPP	LPP	LP	Y/ 1	Y/1	Y/2	Y/ 2	Y/1	Y/1	24	N

failures, lost to follow up or withdrawals were recorded; all 5 patients evaluated were included in the interim analysis (Fig. S1).

3.2. Safety and adverse events

No severe adverse events (AE) were registered in any of the cases. Causal relation with the investigational product could not be fully discarded in four AEs (3 corneal epithelial defects and 1 corneal leucoma; Table 2). No local infections related to the implant were observed. No surgical AE were registered and corneal leucoma was the single AE recorded within 24 h of the intervention (Fig. 1). In fact, NANOULCOR graft is translucent at the time of its application, increasing its transparency in the following weeks after surgery in most patients (Fig. 1).

3.3. Ocular complications

All treated patients have registered a significant decrease in basal ocular complications grading score, using the system described by Sotozono et al. (2007) [32]. Preoperatively, the median ocular complications score grading was 14.5 (range, 13 / 16) (Fig. 2A). At 1–6, 9, 12, 18 and 24 months postoperative, the ocular complication grading score improved significantly ($p < 0.05$, Fig. 2A). Median ocular complications grading score at 6, 9, 12 and 24 months after surgery was 9, 7, 9 and 8 respectively (Tables S1 and S2).

Moreover, all treated patients showed a significant postoperative decrease in the corneal component of the grading score. Preoperatively, the median corneal complication score grading was 11.5 (range, 9 / 13); median corneal complications grading score at 6, 9, 12 and 24 months after surgery was 6, 6, 7 and 6 respectively (Tables S3 and S4). The observed postoperative decrease in the corneal grading score reached statistical significance at all time-points of assessment ($p < 0.05$, Fig. 2B). This change represents a mean relative baseline decrease in the grading scores of − 0.436 [range, − 0.652 / − 0.333], − 0.349 [range, − 0.462 / − 0.217] and − 0.431 [range, − 0.615 / − 0.333] at 6, 12 and 24 months after treatment, respectively (Fig. 3, Table S5). When analyzed separately, the items contributing most significantly to the observed reduction in the corneal grading score were superficial punctate keratopathy (SPK) and corneal epithelial defects (relative baseline score reduction at 6, 12 and 24 months: −0.207 [range, −0.333 / −0.077], −0.207 [range, −0.333 / −0.077] and −0.222 [range, −0.333 / −0.154] and −0.094 [range, −0.154 / 0.000], −0.085 [range, −0.154 / 0.000] and −0.132 [range, −0.167 / −0.100], respectively) (Fig. 3). Corneal complications score remained below the baseline score throughout the 24 months follow-up period. However, a moderate increase was observed in two of the subjects at month 10 after surgery, concurring with the discontinuation of the topical steroids. Conjunctival complication score also registered a decrease under the baseline values in all subjects following surgery. However, statistical significance was

Table 2
Ocular adverse events recorded and classified according to MedDRA dictionary during the 2-year follow-up of the 5 patients studied in this initial phase of the trial.

System Organ Class (MedDRA) Lowest Level Term	No. of cases (No. of causally related cases)
Eye disorders	**20 (4)**
Corneal leucoma	7 (1)
Bullous keratopathy	3 (0)
Band keratopathy	2 (0)
Corneal epithelial defects	3 (3)
Dellen	1 (0)
Eye injury	1 (0)
Ocular hypertension	2 (0)
Allergic conjunctivitis	1 (0)
Infections and infestations	**2** (0)
Herpetic keratitis	1 (0)
Infective keratitis	1 (0)

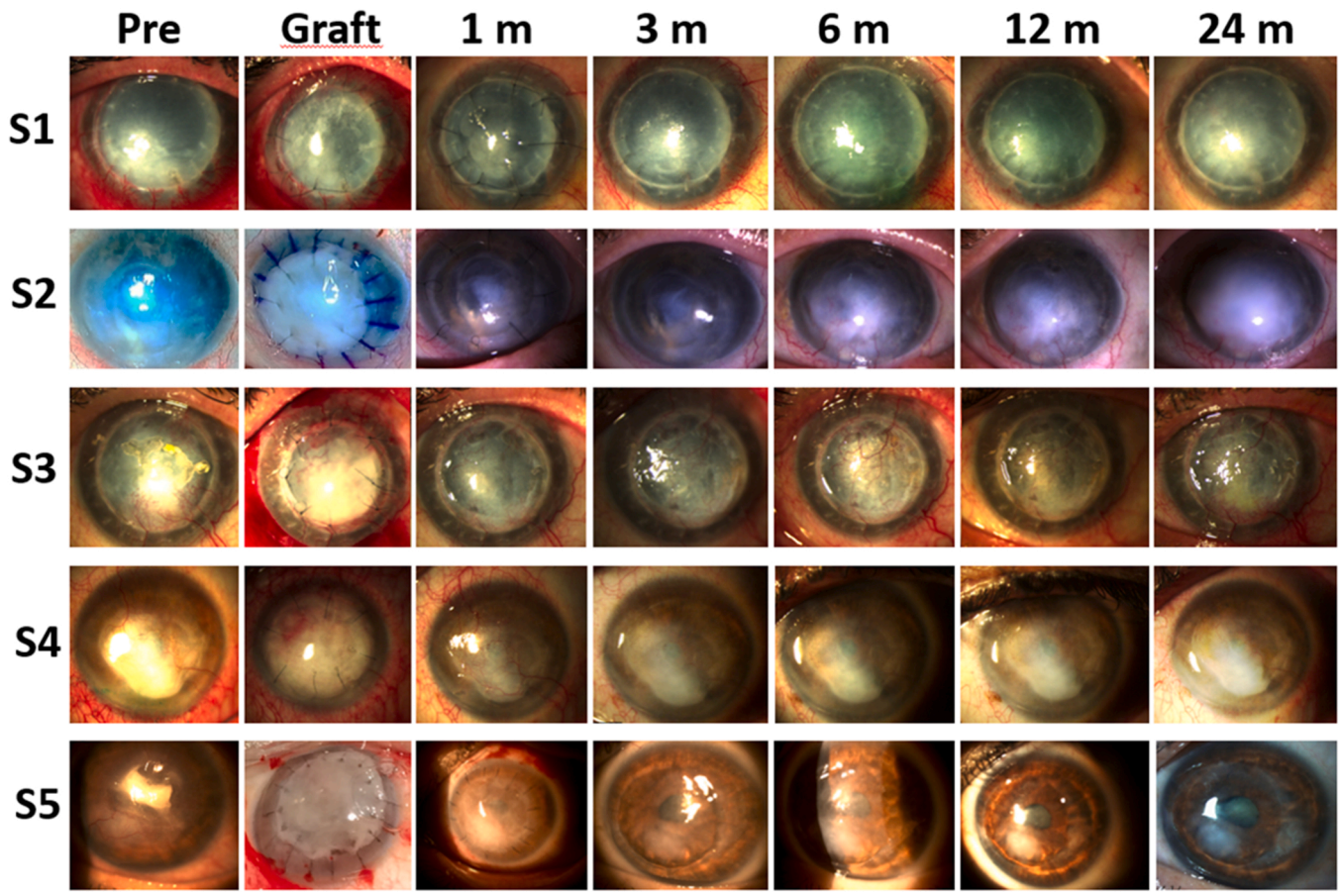

Fig. 1. Clinical progression of implanted eyes observed by slit lamp examination for 24 months follow-up. Left to right: preoperatively (Pre), after 1, 2, 3, 12 and 24 months (m) of follow-up. All five eyes treated with NANOULCOR achieved ocular surface stabilization, with no ulcer recurrence throughout follow up. Subjects S2, S4 and S5 showed a noticeable decrease in corneal stromal opacity, however this gain was only stable throughout 24-months follow up in two of the cases (S4, S5).

only reached at 1–4, 6, 9, 12 and 24 months. Median baseline conjunctival complication score was 2 (range, 1 / 3), decreasing to a median of 0.0 at 6, 9, 12 and 24 months after surgery (Fig. 2C, Tables S6 and S7). Median preoperative eyelid complication score was 2 (range, 0 / 3), changing to 2 (range, 0 / 3), 1 (range, 0 / 2), 1 (range, 0 / 2) and 2 (range, 0 / 2) at 6-, 9-, 12- and 24-months following treatment with NANOULCOR. However, none of the observed changes in the eyelid component of the scale reached statistical significance (Fig. 2D, Tables S8 and S9).

The Whitcher's kerato-conjunctival complications scale (i.e. SICCA OS Score) adds up the corneal and conjunctival items obtained using eye staining tests with fluorescein and lissamine green dye, respectively [31]. Median SICCA-OS Score observed a postoperative decrease at all evaluation time points. However, these changes were only significant at months 1, 2 ($p = 0.0422$), 18 and 24 ($p = 0.0431$) after treatment with the ATEAHC (Fig. 4, Tables S10 and S11).

Corneal sensitivity evaluation was performed at each evaluation time point by Cochet-Bonnet contact esthesiometry. Data showed non-significant postoperative changes in corneal sensitivity (Tables S12 and S13). Tear function evaluation was performed at each trial visit using the Schirmer test, as well as the Tear Breakup Time test. Again, results showed non-significant changes in tear function after receiving treatment with the ATEAHC (Tables S14-S17).

3.4. Postoperative clinical outcomes

Severe ocular surface inflammation with corneal fibrosis was pre-operatively present in all 5 treated eyes. The implant completely covered the corneal surface, and ocular surface inflammation decreased in the months following surgery and remained stable throughout the follow up time. At the 24th postoperative month, SPK and epithelial defects item of Sotozono' grading scale registered an average relative baseline improvement of − 0.222 [range, − 0.333 / − 0.154] and − 0.132 [range, − 0.167 / − 0.100], respectively (Fig. 3C). In all eyes treated with the bioartificial cornea, ocular surface stabilization was achieved, with no events of ulcer relapse, nor rescue surgical treatment needed throughout the follow-up time. No graft detachment or signs of graft rejections were recorded either.

Anterior segment optical coherence tomography (AS-OCT) images indicated that complete scaffold degradation occurred after an average time of 49.6 days (confidence interval [CI], 38.2–61.0) of the intervention (Fig. 5). In this regard, AS-OCT also revealed a more homogeneous surface after the surgery compared to the preoperative status in all eyes, thus proving the efficacy of the ATEAHC in the reconstruction of the corneal surface.

3.5. Patient self-perceived improvement and general satisfaction

All patients referred a mean postoperative improvement on their ocular symptoms of 7.8 (range, 6 / 10) points of the evaluation scale. Eye pain was the symptom that all subjects reported had improved. Visual acuity was also mentioned by one of the subjects (S4). Furthermore, all evaluated subjects claimed to be willing to receive the same treatment if their disease started again (Table S18).

3.6. Visual acuity

Visual acuity was measured in all cases using a low vision

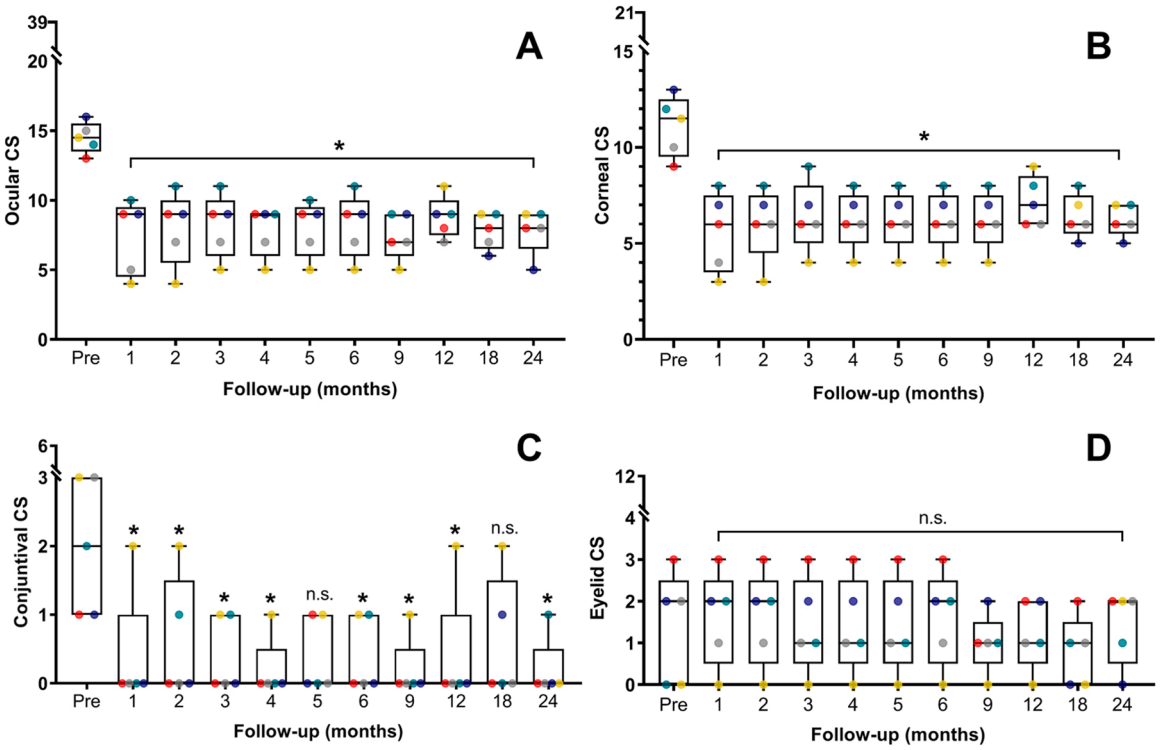

Fig. 2. Preoperative and postoperative scores of ocular complications at 1–6, 9, 12, 18 and 24 months after treatment with NANOULCOR. Eye complications were assessed following the grading system previously reported by Sotozono et al. [32]. The bottom and top lines of each box correspond to the percentiles 25th and 75th, respectively; the line that divides each box indicates the median score value. Colored circles represent individual scores for each patient: S1 – red, S2 – yellow, S3 – teal, S4 – gray, S5 – blue. Complications were categorized as ocular (Panel A), corneal (Panel B), conjunctival (Panel C) and eyelid (Panel D). Changes from baseline score at each postoperative visit were analyzed using the Wilcoxon signed-rank test. CS: complication score. n.s.: non-significant ($p > 0.05$). *: $p < 0.05$. All treated patients showed a significant postoperative decrease in basal ocular, corneal and conjunctival complication scores. No significant changes were observed in the eyelid component of the grading scale (Tables S1–S4 and S6–S9).

semiquantitative scale with five categories: counting fingers (CF), hand motion (HM), light perception with projection (LPP), light perception (LP) and no light perception (NLP). Three out of five patients registered an increase in visual acuity at 24 months after treatment with the ATEAHC (Table 3). These results are however neither statistically significant nor methodologically conclusive, as three out of the five patients evaluated presented deeper ocular alterations that impeded visual acuity recovery. At this early phase of evaluation, the primary trial goal was to test the safety of the NANOULCOR treatment and to improve the patients' symptoms (recurrent ulcers and pain); therefore, patients with very low or null vision, often irrecoverable, were preferred for recruitment. The study design is hence limited for the evaluation of any visual acuity improvements.

3.7. Corneal transparency and fibrosis

Corneal transparency was measured using the opacification component of the previously published ocular complication grading scale [32]. This item can be ranked from 0 to 3, where 0 = clear cornea with iris details clearly visualized, 1 = partial obscuration of the iris details, 2 = iris details poorly seen with pupil margin just visible, and 3 = complete obscuration of iris and pupil details. The ATEAHC product is translucent at the time of surgery. However, a moderate increase in corneal transparency was observed in all patients in the months following transplant with NANOULCOR. Mean relative baseline decrease in corneal opacity was − 0.035 [range, − 0.100 / 0.000], − 0.035 [range, − 0.100 / 0.000] and − 0.044 [range, − 0.100 / 0.000] at 6, 12 and 24 months after treatment, respectively (Fig. 3). However, the gain in corneal transparency remained stable throughout follow up only in two subjects (S4 and S5).

3.8. Clinical progress of concomitant ocular surface diseases

Two out of five patients receiving the implant showed concomitant calcific band keratopathy diagnosed before grafting (Table 1). A temporary clinical improvement of the symptoms was observed in both cases after the surgery. However, this keratopathy relapsed after 10 months in one of the subjects (S5). Endothelial bullous keratopathy, also present in three of the patients, did not improve postoperatively, producing recurrent corneal epithelial bullae (or microbullae) in all cases. This ocular condition was included as exclusion criteria in subsequent amendments to the study protocol.

4. Discussion

The results of our first 5 patients enrolled in this advanced-therapy clinical trial suggest that NANOULCOR can be safely implanted in patients suffering severe trophic corneal ulcers or their sequelae, showing at least, partial efficacy in the restoration of the corneal surface. Our results show that the surgical application of the implant is feasible and safe, with a good tolerance and overall patient satisfaction.

The ATMP evaluated in this trial showed partial efficacy in the restoration of the corneal surface of 5 patients, maintaining the corneal surface integrity. The addition of agarose to a fibrin-based scaffold [25], combined with plastic compression [27], demonstrated its suitability to improve the mechanical properties of the scaffold. This allowed a better handling of the product, and interrupted 10–0 nylon sutures were applied without tearing the implant, avoiding the use of overlying sutures which can cause a delay on epithelial growth [34]. Moreover, the combination of fibrin and agarose led to a slower degradation rate as compared to pure fibrin-based scaffolds. Reabsorption and complete

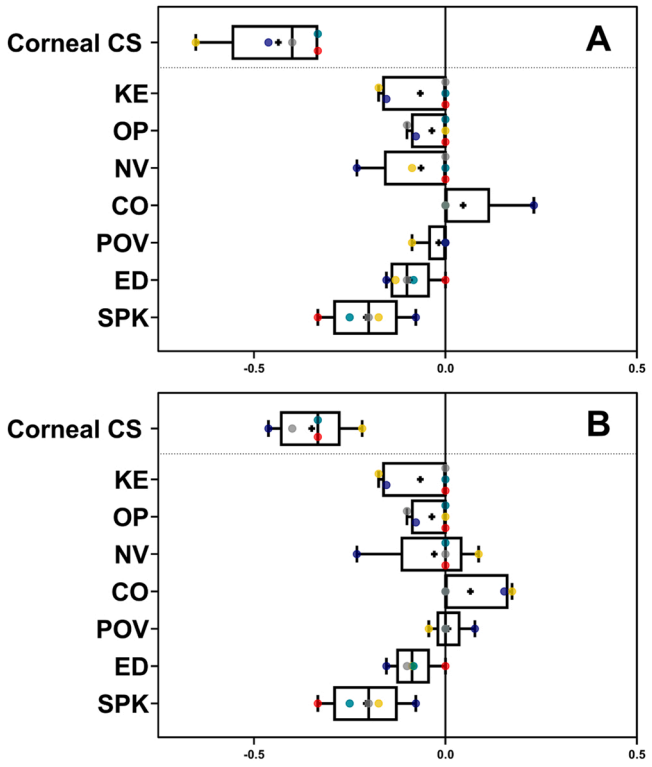

Fig. 3. Relative baseline changes in corneal complication grading score and its [32] at 6 (A) and 12 (B) months after treatment with NANOULCOR. Corneal CS, corneal complications grading score; KE, corneal keratinization; OP, corneal opacity; NV, corneal neovascularization; CO, corneal conjunctivalization; POV, Vogt palisades loss; ED, epithelial defect; SPK, superficial punctate keratopathy. The horizontal lines at the right and left of each box represent the maximum and minimum relative baseline change observed, respectively. The left and right lines of the box correspond to the percentiles 25th and 75th, respectively. The line that divides the box vertically indicates the median, and the "+" sign represents the mean of the baseline decrease in the score. The colored circles represent individual scores for each patient: S1 – red, S2 – yellow, S3 – teal, S4 – gray, S5 – blue. When analyzed separately, the items contributing most significantly to the observed decrease in the corneal complications grading score were superficial SPK and ED (Table S5).

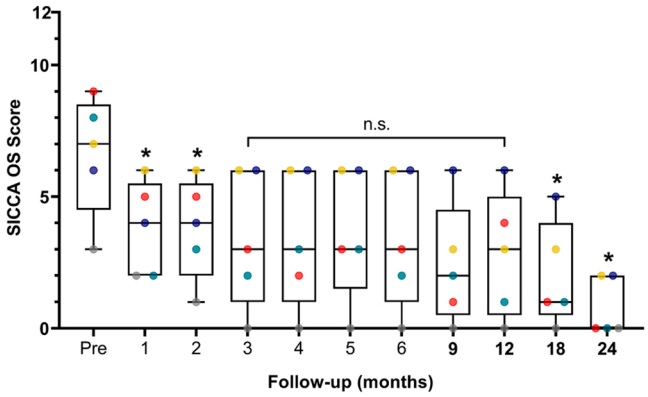

Fig. 4. Preoperative and postoperative scores of keratoconjunctival complications score (SICCA-OS Score [31]) at 1–6, 9, 12, 18 and 24 months following treatment with ATEAHC. This scale can take values from 0, for healthy eyes, to a maximum of 12, for eyes with the worst prognosis The horizontal lines below and above each box represent the maximum and minimum score values, respectively. The bottom and top lines of each box correspond to the percentiles 25th and 75th, respectively. The line that divides each box indicates the median score value. The colored circles represent individual scores for each patient: S1 – red, S2 – yellow, S3 – teal, S4 – gray, S5 – blue. The changes from baseline score at each postoperative visit were analyzed using the Wilcoxon signed-rank test: $p = 0.0422$ (at months 1 and 2), $p = 0.0431$ (at months 18 and 24). n.s.: non-significant. *: $p < 0.05$. SICCA OS score decreased postoperatively with significant changes at months 1, 2 ($p = 0.0422$), 18 and 24 ($p = 0.0431$) following treatment with NANOULCOR (Tables S10 and S11).

biointegration of the fibrin-agarose implant occurred within 3–12 weeks after surgery, largely extending the presence of the scaffold in the treated area in comparison to fibrin adhesives or fibrin scaffolds (i.e. 1 or 2 days for full reabsorption in the cornea) [35].

This was the first time that fibrin-agarose scaffolds were implanted in humans. In agreement with our preclinical results, this novel biomaterial showed excellent biocompatibility both *ex vivo* and in vivo. Our clinical results reported here confirm its biocompatibility, suggesting that NANOULCOR is safe for human use. Only four adverse reactions were registered, and none of them were considered severe. No detachment, ulcer relapse nor surgical re-interventions were registered within 24 months of follow-up. Despite of the presence of two different allogeneic cell populations cultured in the scaffold (i.e. corneal epithelial and stromal cells), no signs of graft rejection, local infection or corneal neovascularization were observed. In this regard, the homeostatic interactions established by the corneal epithelium and the underlying stromal cells may promote a non-inflammatory environment [36,37], in addition to the corticosteroid effect applied by the postoperative treatment during the first 12 months.

The therapeutic efficacy of NANOULCOR was also demonstrated after observing an improvement in terms of the Sotozono and SICCA-OSS scales. The ocular complication scores measured by these scales registered a significant decrease after the intervention. Separated analysis by its components, showed that this decrease originated mainly in the corneal component of the scale, with evident reductions occurring in the superficial punctate keratopathy and epithelial defect items. This might be partly due to the growth factors released from the plasma and the cellular component of the ATMP. It is well known that human plasma contains multiple growth factors, such as EGF or TGF-β, that promote corneal epithelial regeneration [38,39]. Moreover, the presence of a cultured corneal epithelium, optimally differentiated by the presence of stromal cells inside the scaffold, could have facilitated the epithelial regeneration of the patient cornea after its implantation [36,37]. This differentiating capability together with the stimulation of a proregenerative and non-inflammatory environment, would confirm the adequacy of combining two different cell populations in the same ATMP, despite increasing the complexity in terms of manufacturing. This is one of the first complex ATMP organs with two different cell populations clinically evaluated in humans.

Currently, the only approved and commercialized ATMP generated by tissue engineering in Europe is Holoclar®, indicated to perform CLET. Holoclar® is based on autologous limbal epithelial cells cultured on a fibrin scaffold [40]. This requires obtaining a biopsy from a healthy region of the patient limbus, which is shipped to a Good Manufacturing Practices (GMP) facility where the ATMP is produced. The ATMP is finally shipped back to the surgeon for implantation into the patient after excising the corneal pannus. The use of an autologous epithelial cell source may avoid immunotoxic effects, but increases the complexity and the costs of manufacturing, leading to additional hurdles that delay market accessibility [41]. By contrast, allogeneic cells, like the ones used in NANOULCOR, can be manufactured more efficiently, in bigger batches, facilitating the manufacturing process and reducing the production cost. This can be also addressed by applying other approaches like the use of IPs cells [42], MSCs [43] and other cell types, but still the possible related adverse events are uncertain.

The first phase of this study was focused on the evaluation of safety. Consequently, as previously mentioned, patients with very low or null vision, often irrecoverable due to previous ocular disease, were preferred for recruitment. Nevertheless, the study also allowed a preliminary evaluation of partial efficacy in this type of patients. Transparency increased within the first weeks after surgery and three out of

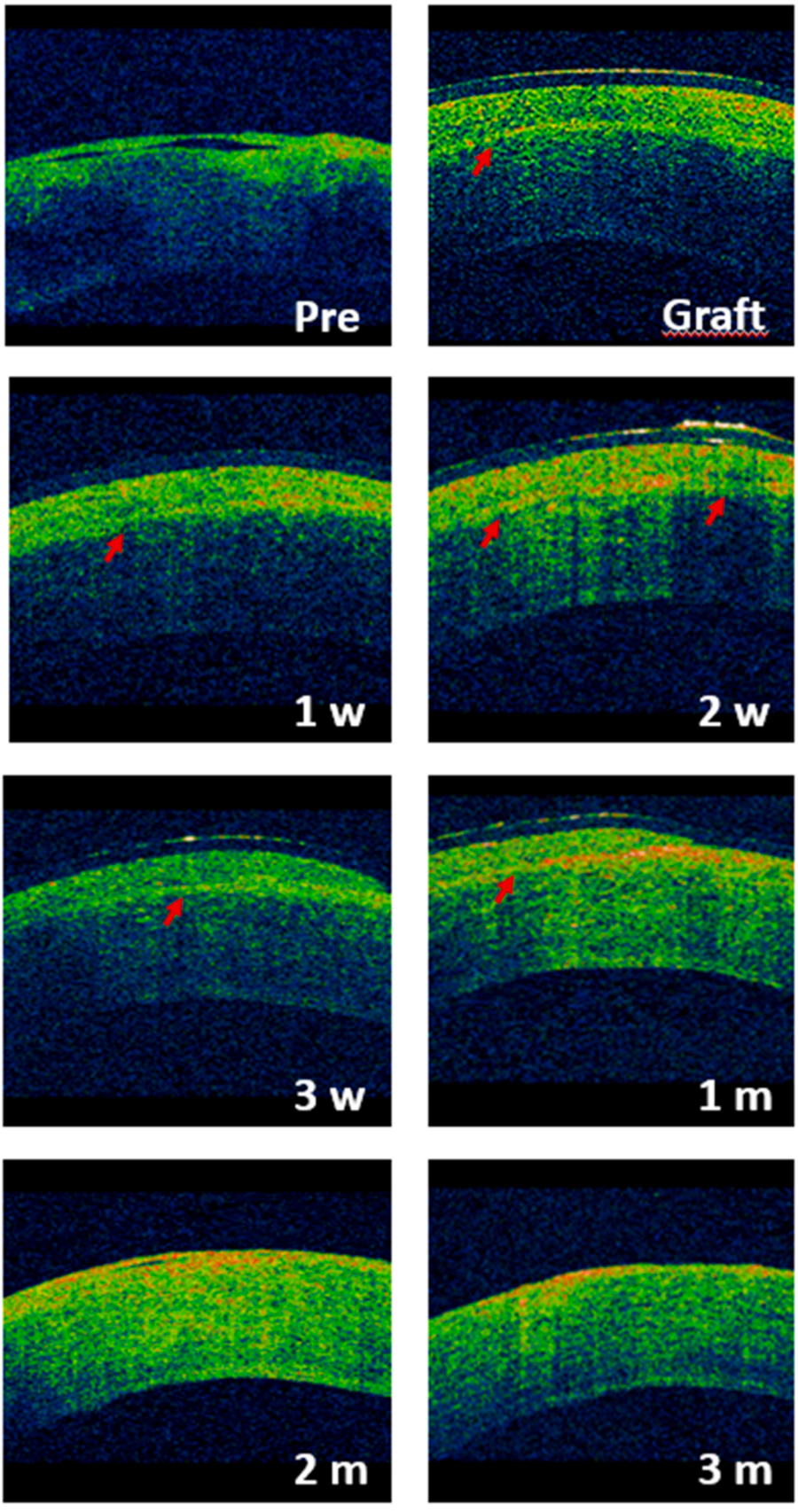

Fig. 5. Anterior segment optical coherence tomography (AS-OCT) imaging of the S1 cornea implanted with NANOULCOR. Left to right and up to down: preoperatively (pre), after 1, 2, 3 weeks (w) and 1, 2 and 3 months (m) of postoperative follow up. Red arrows indicate the lamellar interface. AS-OCT images reveal a more homogeneous and stable surface after treatment with NANOULCOR, with complete scaffold degradation occurring on average within 49.6 days [CI, 38.2–61.0] after surgery. No events of ulcer relapse, graft detachment, nor rescue surgical treatment were captured in any of the subjects throughout a 24 months follow-up period.

Table 3
Progression of visual acuity. Levels of vision: counting fingers (CF), hand motion (HM), light perception and focusing (LPP), light perception (LP) and no light perception (NLP). Two out of three patients registered an improvement over two levels in visual acuity, however results are not statistically significant.

FOLLOW UP	S 1	S 2	S 3	S 4	S 5
Pre	LP	LP	NLP	LP	LPP
3 months	LP	HM	LP	CF	LP
6 months	LP	HM	LP	CF	LPP
12 months	LP	LP	LP	CF	LPP
24 months	LP	LP	LPP	CF	LP
Post-treatment mode	LP	LP	LP	CF	LP
Visual acuity progression *(baseline* vs *24 months)*	=	=	↑↑	↑↑↑	↓

five patients registered discrete visual acuity improvements. However, this gain in corneal transparency can partially be explained by the removal of corneal fibrotic tissue performed before the administration of the implant. The main advantage offered to the enrolled patients was the possibility of improving their ocular symptoms such as pain and discomfort. In this regard, when asked, all 5 patients referred a significant improvement in their ocular symptoms, including eye pain. This effect was observed in other clinical trials where a non-cellular scaffold was tested [21], indicating that part of the analgesic effect could be due to the corneal excision and lesion removal. Furthermore, all 5 patients expressed their desire to undergo the same treatment if they re-started again.

In conclusion, NANOULCOR is safe and promotes the regeneration of the corneal surface in patients suffering severe trophic corneal ulcers or its sequelae, partially improving corneal transparency and ocular symptoms. As mentioned before, the results showed here are preliminary. We expect to confirm the reported observations after finishing the next phase of the trial, once the next patients receiving either the experimental group (NANOULCOR implant) or the control group (amniotic membrane graft) have completed a 2-year follow up. The recruitment of the trial was closed in February 2020, with follow up still ongoing.

The present study has several limitations. On the one hand, this is a preliminary report showing the initial feasibility and biosafety results of the implant of a bioartificial anterior lamellar cornea in 5 patients, meaning that results should be taken with care until a larger cohort of patients can be analyzed in the future. On the other hand, results should be analyzed at longer periods of time to confirm the biocompatibility of the NANOULCOR technology. Finally, the usefulness of these bioartificial corneas should be determined in other conditions affecting the eye surface, especially in cases with a functional retina in which a possible improvement in visual acuity can be analyzed. Future works should be carried out in the future to determine the real potential of this technology as an ATMP therapy for patients with cornea damage.

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CRediT authorship contribution statement

Carmen Gonzalez-Gallardo: Conceptualization, Methodology, Development, Design of methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Juliana Martinez-Atienza:** Methodology, Development, Design of methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Beatriz Mataix:** Investigation, Writing – review & editing. **José Ignacio Muñoz-Ávila:** Investigation, Writing – review & editing. **J. Daniel Martínez-Rodríguez:** Investigation, Writing – review & editing. **Santiago Medialdea:** Investigation, Writing – review & editing. **Antonio Ruiz-García:** Validation, Formal analysis, Data curation, Writing – review & editing. **Antonio Lizana-Moreno:** Validation, Formal analysis, Data curation, Writing – review & editing. **Salvador Arias-Santiago:** Validation, Formal analysis, Data curation, Writing – review & editing. **Manuel de la Rosa-Fraile:** Validation, Formal analysis, Data curation, Writing – review & editing. **Ingrid Garzon:** Validation, Writing – review & editing. **Antonio Campos:** Conceptualization, Methodology, Development, Design of methodology, Validation, Formal analysis, Data curation, Writing – review & editing, Project administration, Funding acquisition. **Natividad Cuende:** Conceptualization, Methodology, Development, Design of methodology, Writing – review & editing. **Miguel Alaminos:** Conceptualization, Methodology, Development, Design of methodology, Validation, Formal analysis, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Miguel González-Andrades:** Conceptualization, Methodology, Development, Design of methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Rosario Mata:** Conceptualization, Methodology, Development, Design of methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Conflict of interest statement

Dr. Campos, Dr. Alaminos, Dr. Muñoz-Ávila and Dr. González-Andrades are inventors of issued patents P200930625 and P200930943, broadly relevant to the work. Remaining authors declare that they have no competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the

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