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Long-term effects of adipose-derived stem cells for the treatment of bilateral limbal stem cell deficiency

Running head: Long-term effects of ASCs in bilateral LSCD

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ABSTRACT

Purpose: To determine the safety and feasibility of human autologous adipose tissue-derived adult mesenchymal stem cells (ASCs) for ocular surface regeneration in patients with bilateral limbal stem-cell deficiency (LSCD).

Methods: A phase IIa clinical trial was designed (https://Clinicaltrials.gov, NCT01808378) with 8 patients, 3 of whom had aniridia, 2 meibomian glands diseases, 2 multiple surgeries and 1 chronic chemical injury. The therapeutic protocol was as follows: 6-mm of central corneal epithelium was removed, 400,000 ASCs were injected into each limboconjunctival quadrant, 400,000 ASCs were suspended over the cornea for 20 minutes, and finally the cornea was covered with an amniotic membrane patch.

Results: No adverse events were detected after a mean of 86,5 months of follow-up. One year after surgery, 6 of the 8 transplants were scored as successful, five patients had improved uncorrected visual acuity (mean of 12 letters), two patients presented epithelial defects (also present at baseline) and the mean percentage of corneal neovascularization was of 28.75% (36.98%, at baseline). Re-examination 24 months after treatment disclosed preserved efficacy in 4 patients. At the last visit (after a mean of 86,5 months of follow up) epithelial defects were absent in all patients although improvement in all of the variables was only maintained in patient 3 (meibomian glands agenesia).

Conclusion: ASCs are a feasible and conservative therapy for treating bilateral LSCD. Therapeutic effect differs between etiologies and diminishes over time.

KEYWORDS: adipose stem cells, clinical trial, corneal epithelium regeneration, corneal neovascularization, limbal stem cell deficiency, mesenchymal stem cell .

INTRODUCTION

Patients with bilateral total limbal stem cell deficiency (LSCD) do not have a cell source for autologous cultivated limbal transplantation which is the gold therapy for long-term restoration and renewal of the corneal epithelium in eyes with unilateral LSCD [1-5].

In bilateral ocular surface disorders transplantable cultivated epithelial sheets are obtained from cadaver donors, a living-related eye [6] or autologous oral mucosal epithelial cell sheet [7]. Without immunosuppressive therapy, the vast majority of allogeneic corneal epithelial transplantation usually fails due to immunological rejection [8] and has a success rate that tends to decrease gradually over time, with a graft survival rate of 40% at 1 year and 33% at 2 years [9]. Cultivated mucosal epithelial transplantation (COMET) results have showed a clinical success rate between 53%-70% despite recurrent epithelial defects (22%) and long term reconjunctivalization [7,10-12] as well as some degree of neovascularization in all grafted eyes [13]. Autologous adipose-derived stem cells (ASCs) are a mesenchymal cell type (MSC) that is isolated from the vascular-stromal fraction of adipose tissue. The advantages of ASCs over the usual sources (such as bone marrow MSC) are its accessibility, high performance (due to the large volume of MSCs isolated in extractions) and easy expansion, thereby avoiding lengthy procedures with associated risks to the genetic material [14-16]. Clinical experience with these cells in ophthalmic diseases is still limited. Regeneration of the corneal stroma by ASCs was assessed in an experimental study in 2008 [16] and the first case of a patient with a persistent sterile corneal epithelial defect treated with a topical application of ASCs was reported in 2012 [17]. Promising results have been recently published in seven patients with dry eye disease and Sjögren's syndrome, treated with a single transconjunctival injection of allogeneic ASCs into the lacrimal gland [18].

Similar to MSC, it has been hypothesized that the therapeutic effect of ASCs may be due to their immunoregulatory and anti-inflammatory properties [19-20]. Therefore, the use of ASCs is an encouraging approach for enhancing repair or regeneration of damaged tissue [15] in an environment unfavorable for healing.

The target of this clinical trial is to analyze the safety of autologous ASC in bilateral LSCD, and to obtain preliminary results about efficacy in different etiologies that generate bilateral LSCD.

MATERIAL AND METHODS

1. Design

This phase IIa noncomparative clinical trial was designed to evaluate the safety and feasibility of ASCs for LSCD. Taking into consideration this primary endpoint and the innovative nature of our study, we estimate a minimum sample size of 8 patients.

The Ethics Committee of La Paz University Hospital (Madrid, Spain) and the Spanish Agency of Medicinal Products and Medical Devices (AEMPS), in accordance with current legislation, approved the trial. This study was performed according to the amended Declaration of Helsinki and was registered at Clinicaltrials.gov (NCT 01808378).

2. Patients

Patients gave written informed consent before entering the study. Patients aged 18 years or older were eligible for the study if they had ~~total~~ bilateral LSCD diagnosed with a slit-lamp examination by the same ophthalmologist. Only one eye in each patient (the most severely affected) was treated with ASCs. We characterized the LSCD using biomicroscopic examination (the patients were diagnosed by the complete disappearance of the palisades of Vogt and subepithelial neovascularization from the limbus, stippled late fluorescein staining in a vortex pattern, or a combination thereof) and completed the diagnosis with corneal impression cytology [21] ~~and~~ or with the detection of the MUC5AC transcript in corneal epithelium [22]. Exclusion criteria were as follows: history of neoplasia in the last 5 years, allergy to local anesthetics, administration of another experimental drug in the past 90 days, administration of tacrolimus or cyclosporine within the past 4 weeks, any medical and psychiatric condition, diagnosis of congenital or acquired immunodeficiencies, major surgery or trauma in the last 6 months, pregnant and breastfeeding.

3. Treatment procedure

3.1. Isolation, culture, and characterization of autologous adipose-derived stem cells

Adipose tissue was obtained by liposuction. ASCs were harvested from the subcutaneous fat tissue of each patient for autologous use. Adipose-derived stem cell isolation, culture, and cryopreservation was also performed according to a protocol approved by AEMPS in the manufacturing facility of Hospital Gregorio Marañón (Madrid, Spain; manufacturer authorization no: AEMPS-20090211-TA, according to the PEI 04-031; Product in Clinical Research), and according to Spanish and European legislation (ASC production is only permitted in conditions of good manufacturing practices). ASCs were characterized according to legislative requirements during the trial. Nevertheless, potency studies of the cryopreserved leftover cells have been performed. These assays have demonstrated their ability to secrete VEGF, IL-10, MCP-1 MCP-4 MMP-1 GDNF, among other proteins involved in inflammatory processes; in addition to activating fibroblast healing in a scratch model. ASCs were obtained exclusively by collagenase digestion and culture with Dulbecco's Modified Eagle Medium plus 10% fetal bovine serum and 1% ampicillin/streptomycin. After washing extensively and removing cells attached to the plastic, the laboratory data on cell differentiation to osteoblast, chondrocytes, and adipocytes was checked and flow cytometry was performed with positive (CD 27, 44, 90 and 105) and negative markers (CD 34, 45 and 73) before the ASCs could be considered ready for the patients (according to the EMEA/CHMP4/10869/2006 cell therapy guide and phenotype according to the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy [23]). Cell cultivation and expansion continued in an authorized procedure until the required number of cells for implantation (dose) was obtained. For quality-control and logistical reasons, the doses of cells were cryopreserved in liquid N_2 (30% cell death: producer data). At least 1 week before the date of implantation, cells were thawed and cultured. For administration, cells were suspended in a sterile balanced saline solution with 1% human albumin (Octapharma, Madrid, Spain) at 2×10^6 cells/2ml. Samples were taken before release to examine viability, DNA stability, and pathogen controls (analysis performed by the producer).

3.2. Transplantation of the cells onto the eye

Syringes containing 2 x10^6 cells per 2mL of balanced saline solution were prepared. The surgical procedure was done under retrobulbar anesthesia. The 6-mm central corneal epithelium was removed, and the subconjunctival injection was performed in the limbal conjunctiva using a 25G needle, with 400.000 ASCs/0.4mL per quadrant (in 4 quadrants) reaching a total of 1.6x10^6 ASCs per eye in the limbal area. A total of 1.6mL of ASC dilution was injected subconjunctivally. The distribution of this volume within the subconjunctival space varied depending on the conjunctival scarring, but it was sufficient to fill the entire subconjunctival limbal space. To prevent any ASC spillage, pressure was applied to the entry hole using a sponge.

The last 400.000 ASCs/0.4mL ASCs were suspended in a Coronet long handled trephine (Network Medical, London, UK) on the surface of the de-epithelized central cornea for 20 minutes. Lastly, an amniotic membrane (AM) patch was stitched with a running 10-0 nylon suture avoiding the limbal area. At the end of the procedure, a therapeutic contact lens was placed on the ocular surface for protection. Inflammation control was achieved using topical steroids. To prevent bacterial infections, antibiotics were applied topically.

4. Trial outcomes

4.1. Safety

The clinical trial was designed to analyze the safety of the procedure, which was based on the incidence of adverse events (AEs) and serious adverse events (SAEs) that occurred during the patients' follow-up. Quality of life was evaluated one year after treatment using the 12-Item Short Form Survey (SF-12) index. Overall subjective comfort was checked by a questionnaire using a face score consisting of 9 faces, each showing a different expression. Patients were asked to select which face best described the current condition of their eyes during all visits [24].

4.2. Efficacy

During the clinical evaluation we tested subjective pain, assessed the visual acuity (VA) using Early Treatment Diabetic Retinopathy Study (ETDRS) charts, tested for the presence or absence of corneal epithelial defects through fluorescein staining, staged the conjunctival hyperemia on the Efron scale, and performed Schirmer test without anesthesia. Corneal neovascularization (CNV) was quantified in digital pictures by the ImageJ software, calculating the affected area from the total corneal area of a particular eye. One observer who did not participate in any biologic or clinical procedure assessed the slit lamp pictures.

The endpoint for efficacy was measured 1 year after the graft procedure using the following criteria: improvement in VA, absence of pain, absence of corneal epithelial ulcers and improvement in neovascularization through a direct observation using the slit lamp examination with and without fluorescein test. Prior to surgery and at the 12-month visit, all patients underwent impression cytology, Schirmer's test, measurement of intra-ocular pressure, digital images and fundoscopy. Patients were examined during the first year at follow-up visits scheduled for 1,4,8,12,24, and 52 weeks after cell implantation according to the clinical trial. After the end of the study, follow up was extended and re-examination was carried at two different points: 24 months after treatment and an exploration conducted during the last hospital visit.

Statistical analysis

Categorical variables are listed as absolute and relative frequencies. For the continuous variables we employed the mean, standard deviation, median and range. The Wilcoxon test was employed to compare the temporal progression and a before-after paired test. Univariate logistic regressions were performed to assess the variables related to the success of the surgery. Data were analyzed using SPSS v.20.0 software, and the significance was set at $p<.05$.

RESULTS

1. Patients

Eight patients recruited from the ophthalmology departments of La Paz University Hospital, Clínico University Hospital and Fuenlabrada Hospital (Madrid, Spain) were treated between November 2012 and November 2014. Six patients were men, and the mean of age was 38.0 (±9.26) years. The etiologies for the LSCD were aniridia in 3 patients (37.5%) (patients 1,5 and 6), meibomian gland-related disease in 2 patients (25%) (patient 3 had primary meibomian glands agenesia and patient 4 had chronic rosacea blepharitis), iatrogenic in 2 patients (25%) (patients 7 and 8) and chronic chemical injury in 1 patient (12.5%) (patient 2) (Table 1). Six of the patients had a Holland classification of IIa. Of the 3 patients with aniridia, all were Mackman stage 2 at the baseline visit. In terms of LSCD severity, 4 of the patients were mild (50%). The mean conjunctival hyperemia score (on an Efron scale of 0-4) was 1.75 (range 0- 3).

In all cases, the liposuction and cell culture were performed without any incident, and the cell application was performed without complications.

Mean last follow up was 86,5 months after treatment (Patient 1: 116 months; Patient 2: 116 months; Patient 3: 114 months; Patient 4: 33 months; Patient 5: 99 months; Patient 6: 61 months; Patient 7: 81 months; Patient 8: 72 months).

2. Trial outcomes

1. Safety

There were no mild or severe AEs related to the ASCs applications, thereby achieving the safety objective. This result was valid for a mean of 86,5 months after follow-up (33 to 116 months).

During the follow-up period after the end of the trial (after 1st year), patient 7 experienced an adverse event due to an associated disease; the patient´s previous glaucoma gradually worsened, and a glaucoma surgery was performed 13 months after the ASCs treatment. Also, patient 4 experienced a massive stromal graft rejection of a deep anterior lamellar keratoplasty (DALK). Since the DALK was performed 21 months after the ASCs transplantation (the immunosuppressive treatment consisted of local corticosteroids) we did not consider this rejection related to the ASCs treatment. Finally, in patient 8 a DALK was performed 21 months

after ASCs and presented a retinal detachment 44 months after ASCs treatment, significantly reducing the patient´s vision.

Regarding subjective comfort, the mean baseline comfort index was 4.2 which improved to 3 at 12 months. Patients 5 and 8 could not distinguish the faces at baseline visit, and both graded as 3 after the treatment at 12-month visit. The patient with caustication (patient 2) was the only who worsened (from grade 4 to 5).

The overall results of the SF-12 test (physical health index 0.46 at the baseline visit and 0.91 at the final visit, and mental health index of 1 at the baseline visit and 0.50 at the final visit) were not statistically significant (Table 2).

2. **Efficacy**

The trial outcome was regarded as a success based on a composite criterion (absence of pain, improvement in VA, absence of corneal epithelial ulcers and improvement of neovascularization) in 5 (62,5%) of 8 patients assessed at 1 year after the implantation (patient 1 with aniridia, patient 3 with congenital meibomian agenesia, patient 4 with ocular rosacea, patient 5 with aniridia and patient 8 with multiple surgeries). In the univariate logistic-regression analysis, the only variable related to failed grafts at 12 months was the grade of inflammation (Table 3). When considering the 8 patients assessed 2 years after implantation, the number of improved patients decreased to 3 (50% success rate) (patients 3, 4, and 8). The final percentage of successful transplants at the last visit was of only 1 patient (12,50%) (patient 3). If we exclude the VA parameter (as it is interfered by associated ocular conditions) from the composite criterion for success, three cases (3, 4 and 7), 37,50% met the clinical criteria of successful treatment at final follow up (mean 86,5 months). The trial protocol is summarized in Figure 1.

To determine the effect of the procedure, we analyzed the following parameters:

2.1. **Visual acuity**

Of the 8 patients, 5 improved their uncorrected visual acuity, with a mean of 12 (12.31) letters, and 6 presented an improved best corrected visual acuity (BCVA) with pinhole, with a mean of 14 (11,51) letters, at

the 12-month visit. At the 24-month visit, patients 3,4 and 8 had improved BCVA with a mean of 22 (17.21) letters, whereas only patient 3 preserved an improved visual acuity at last follow up (improvement of 15 letters in BCVA, 114 months after ASCs). However, it must be considered that visual results worsened in patient 7 due to an exacerbation of optic neuropathy requiring glaucoma surgery 13 months after treatment, in patient 4 due to a DALK surgery followed by a stromal rejection 21 months after ASCs treatment and in patient 8 due to a retinal detachment three years after treatment (Figure 2).

2.2. Pain

Pain data at baseline indicated that 4 patients experienced recurrent pain episodes and 2 patients felt that the pain often interfered with their activities. At 12 months from treatment, 2 patients reported having pain coinciding with the presence of an epithelial defect (patients 6 and 7). At 24 months from treatment, only slight pain was found in one of the patients (patient 1), coinciding with epithelial defect. This same patient referred mild pain in both eyes at the last visit despite absence of epithelial defect (Table 4).

2.3. Corneal neovascularization

The only quantitative parameter analyzed was corneal neovascularization, which refers to the superficial corneal vessels, taking into account that they can be secondary to conjunctivalization or pannus, both of which are criteria for the definition of LSCD. The mean (+SD) neovascularization percentage at the baseline visit was 36.98% (25.7) of the corneal area which decreased to 28.75 % (32.0) by the 12-month visit (p=0.579; 95% CI: -22.892-39.352). During the following visits, mean neovascularization gradually increased to 30,03% by the 24 month visit and to 47,07% at the last follow up (Table 4).

2.4. Epithelial defect

Of the 8 patients, 6 had a history of epithelial defect before starting the study, and 2 patients had an epithelial defect at study inclusion (patients 6 and 7). These same patients presented an epithelial defect at month 12 but not after 24 months or at the last follow up. Two patients with aniridia (1 and 5) presented small epithelial

defects, inferior to 1 mm size, at 24 months. To sum up, none of the patients presented epithelial defect at the last follow up visit (Table 4).

The mean time to epithelial defect closure after surgery was 9.25 weeks (7.92) except for the aniridia group, where mean time was 15.33 weeks.

3. **Corneal transparency**

Baseline data indicates that all patients had corneal opacity, observed during biomicroscopy. One observer that did not participate in any biological or clinical procedures assessed the patients' slit lamp pictures. By the 12-month follow-up, corneal transparency had improved in 5 of 7 patients (the picture of patient 5 couldn´t be evaluated due to poor quality). If we analyze by condition, improvement was observed in 1 patient with aniridia (patient 1), 2 patients with meibomian (patients 3 and 4) and 2 patients with LSCD secondary to multiples surgeries (patients 7 and 8). At the end of the follow-up, corneal opacity increase was observed in 2 patients (patient 1 and 2, both 116 months of follow up) due to worsening of the limbic deficiency (Figure 3, supplementary material).

4. Postoperative LSCD

The follow-up cytology was requested during the visits at months 3, 6, and 12. However, some patients declined to undergo this test.

In one patient (case 1), the baseline corneal cytology was negative, but the histopathological analysis of the corneal epithelium performed on the day of ASC transplantation showed conjunctivalization. Conversely, this was the case in patients 4 and 8, where corneal cytology showed conjunctival goblet cells while histological analysis did not observed those cells.

Cytology improved in patients 1, 3, 5, and 8 at some point during the follow-up. The cytological disappearance of LSCD signs (absence of conjunctival goblet cells) coincided with an improvement in biomicroscopic signs of LSCD and pain relief in cases 3 and 5. Patient 1 also showed clinical and cytological improvement in the first 6 months of the study (cytology at month 12 could not be performed).

It is worth noting that conjunctival globet cells were not observed in the corneas of recipients 4 and 8 analyzed after a DALK performed in both cases 21 months after the ASC transplantation.

Table 5 (supplementary material) provides specific information about the patients and the cytology results at different follow-up time points. It also shows how these results are related to the observed changes in biomicroscopic signs of LSCD during the study.

DISCUSSION

Our study demonstrated that applying ASCs subconjunctivally and under an AM is a safe procedure, with no related adverse effects. The patient with severe LSCD caused by chronic caustication was the only who experienced an increase in corneal neovascularization and a decrease in visual acuity after the procedure. However, we believe that this event was due more to the surgical removal of the corneal fibrovascular tissue than to the direct effect of the ASCs. An additional safety issue that we could not evaluate is biodistribution; as we are injecting cells in the subconjunctival vascular area, the remote possibility of distribution through the bloodstream exists.

Furthermore, we have investigated the safety and viability of ASCs in bilateral LSCD by means of a new surgical approach: injecting ASCs in the subconjunctival area and denuded cornea instead of a cultivated cell sheet as previously reported in trials with cultured oral mucosal stem cells. These ASC, when topically applied, have been shown to improve reepithelialization in an experimental model of caustication previously [25]. The COMET procedure is commonly employed to address total and severe bilateral LSCD, leading to a stable ocular surface in 70.8% of cases and improved visual acuity in 68.2% of eyes [26]. On 2021, the first-ever ex vivo cultivated oral mucosal epithelial cell transplantation (COMET) product for the treatment of LSCD, named Ocural®, was approved as a regenerative medicine product by the Pharmaceuticals and Medical Devices Agency, the regulatory agency in Japan responsible for the approval and supervision of pharmaceuticals and medical devices [27]. However, a notable complication observed frequently in eyes undergoing COMET was the occurrence of corneal epithelial defects [13]. It is worth mentioning that, although the majority of our patients maintained an epithelial defect-free condition during the long-term follow-up, seven of our cases did

not present severe total LSCD, unlike those treated in other studies using COMET. For this reason, it is plausible that residual limbal epithelial stem cells had the capacity to regenerate the de-epithelialized central corneal area after surgery in our cases. Additionally, the utilization of amniotic membrane may have played a role in facilitating the observed recovery in our patients.

To fully address the efficacy of ASC transplantation, a control group would be necessary. While the initial benefits in terms of conjunctivalization or neovascularization were lost in the majority of patients, it is worth mentioning that the treated eye in case 3, which initially had worse conditions compared to the untreated eye, has maintained long-term good transparency with limited conjunctivalization in the periphery. In contrast, the untreated eye, which had LSCD limited to the superior periphery, now exhibits diffuse corneal involvement. (Figure 4, supplementary material).

Performing more frequent cytology examinations would have allowed us to assess not only the treatment's effect on the presence or absence of LSCD but also the evolution of inflammation and the persistence of the implanted cells. Cytology is not a risk-free technique and can cause epithelial defects in patients with fragile ocular surfaces. We believe this was the main reason for patients declining to undergo this test. Unfortunately, the availability of MUC5AC detection ceased in our center, limiting its use for complementing the initial diagnosis of LSCD in some cases.

~~In our study, the group that experienced the most significant improvement of symptoms, visual acuity, and corneal transparency was patients with LSCD secondary to meibomian gland diseases.~~

~~Additionally, substantial improvement was observed in the iatrogenic LSCD regarding corneal transparency and vascularization. Pain and presence of corneal ulcer resolved in the long term for both patients.~~

~~This approach was clearly insufficient for our most severe case, the patient with chronic caustication.~~

The univariate logistic-regression analysis showed that failures were associated only with inflammation (p=0.019) (Table 4.) However, due to the small sample size, it is important to exercise caution when interpreting the results of our univariate logistic regression analysis. Additionally, given the limitations of the sample size, multivariate analysis could not be performed.

Probably, the chronic inflammation of the patient´s severely damaged receiving bed microenvironment where the ASCs were injected did not allow for cell action. We should have addressed the severity and the prime causes for the chronic conjunctival inflammation before applying the ASC treatment. Concerning aniridia, previous studies report a limited duration of donor limbal stem cell grafts, and the results of studies on stem cells harvested from other sites in the patient (e.g., the oral mucosa) are so far inconclusive [26]. In our study, the therapeutic effect of ASCs in patients with aniridia was questionable; on one hand, the response was torpid and poor in patients 5 and 6 (with epithelial defects throughout the follow-up and little improvement in other parameters) but initially very good in patient 1 in whom we did not employ AM patch. However, after one year, this patient developed a worsening of vision, corneal transparency, and corneal vascularization with persistent pain in both eyes (Figure 4, supplementary material). Also, the re-epithelization time after the procedure was 6 weeks longer in the patients with aniridia.

Despite an optimal initial response in some patients (as described above), deterioration is observed over time in VA, neovascularization, and corneal transparency. However, all patients except patient 1 remained ulcer and pain-free in the long term (mean of 86,5 months after treatment with ASCs). It is important to us that the treatment is minimally invasive. Although more trials are needed to investigate the best cell dose required, we propose repeating the doses every 3-6 months, as this could enhance the anti-inflammatory and repairing effect we observed in a number of our patients. Furthermore, conditions that have shown a greater benefit from this treatment could guide patient selection in futures trials: chronic meibomian gland-associated diseases and iatrogenic LSCD. A future second step will be the use of allogenic ASCs. Thus, a pool of ASCs

could be available for use for acute inflammatory conditions associated with the development of LSCD. In this line, the efficacy of allogeneic bone marrow mesenchymal stem cells (BM-MSCs) transplantation have been tested in human eyes in one trial showing similar results as allogeneic cultivated limbal epithelial transplantation (CLET) to treat patients with total and/or severe LSCD [28]. In this trial BM-MSCs were cultivated on an amniotic membrane and transplanted onto the ocular surface.

Our team selected ASCs because this cells can be easily obtained from low invasive liposuction aspirates rendering a high number of multipotent stem cells unlike BM-MSCs. Currently, no clinical trials using ASCs as a cell therapy for LSCD have been published.

~~Finally, numerous studies have shown that cells have different properties depending on the patient's pathology, age, sex, and lifestyle [28]. Therefore, another aspect that will have to be analyzed in the future is the possibility of taking advantage of the absence of MHC-II in the different MSCs to optimize the drug with cells from selected donors and to analyze the potency of the cells for each case.~~

CONCLUSION

We have demonstrated that ASCs are a safe and feasible therapy for treating bilateral limbic associated keratopathy and that the treatment is a conservative procedure for improving, this deficiency in some patients with non severe LSCD. ~~Our study cannot prove the therapeutic effect of this approach due to the small number of patients treated; however,~~ The procedure seems to have better therapeutic efficacy in inflammatory conditions with some reservoir of limbal stem cells (meibomian gland-associated diseases) and decreased the pain level as well as the presence of epithelial defects in the long term. The most significant limitation of our study is the small sample size and the absence of a control group. These biases prevent us from concluding with certainty whether the implanted stem cells are the only responsible for the benefit and which subgroup of patients may benefit the most from this innovative treatment.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [ABB], upon reasonable request.

Figure Captions:

Figure 1. Flow-chart of clinical trial with clinical evolution by specific pathologies of patients.

Comparison of exploration at 12, 24 and mean 86,5 months with baseline. (Numbers in parentheses refer to the patients' ID).

Figure 2. Visual acuity results. BCVA expressed in number of letters measured with pinhole using ETDRS at 1 m.

Patients 1,4,5,7: initial improvement with decrease of VA from the 12th month (Patient 4 due to stromal rejection of DALK in the 21st month. Patient 7 due to exacerbation of optic neuropathy in the 13th month). Patient 3: improvement of VA maintained throughout the whole follow-up. Patients2 and 6: no improvement of visual acuity. Patient 8: improvement of VA after 12 months due to DALK performed in the 21sst month with subsequent loss of VA after retinal detachment in the 44th month.

Figure 3. Ocular surface and corneal opacity evolution from 8 patients undergoing ASCs transplantation.

Patient 1. Baseline 1.A + 1.B: peripheral CNV and opacity. Diffuse late staining and irregularity of fluorescein. End of Study (12 months) 1.C + 1.D: Improvement in central corneal transparency and central epithelial regularity. End of follow up (116 Months) 1.E + 1.F: Recurrence of corneal diffuse conjunctivalization and increase in diffuse opacification.

Patient 2: Baseline 2.A + 2.B: diffuse conjunctivalization and epithelial irregularity: an island of central transparency is preserved. End of study (12 months) 2.C + 2.D: Stable slit lamp exploration. End of study (116 months) 2.E + 2.F: worsening LSCD and corneal opacification

Patient 3. Baseline 3.A + 3.B: diffuse irregular epithelium and conjunctivalization plus peripheral nodules. End of study (12. months) 3.C + 3.D : Smooth central epithelium and corneal transparency. End of follow up (114 months) 3.E + 3.F: Persistence of corneal central transparency with superior and inferior conjunctivalization.

Patient 4. Baseline 4.A + 4.B: sectorial inferior conjunctivalization and diffuse panus. End of study (12 months) 4.C + 4.D: Manifest improvement of CNV, regularity of the corneal epithelium and transparency. End of follow up (33 months) 4.E: neovascularization with lipidic exudation invading corneal graft after rejection. 4.F smooth epithelium without LSCD signs after DALK in blue cobalt (21 months)

Patient 5. Baseline 5.A + 5.B: Moderate diffuse opacification and peripheral conjunctivalization. Epithelial central defect before treatment. End of study (12 months) 5.C + 5.D: Mild opacification secondary to subepithelial amniotic membrane and mild peripheral CNV. Smooth central epithelium. End of follow up (99 months) 5.E + 5.F: Worsening of the condition , significant diffuse surface irregularity.

Patient 6. Baseline 6.A + 6.B: peripheral CN and mild diffuse opacity. End of study (12 months) 6.C + 6.D: Paracentral persistence of subepithelial amniotic membrane, with similar CNV and corneal opacity. End of follow up (61 months) 6.E + 6.F: Paracentral superior corneal opacification secondary to subepithelial amniotic membrane and central stromal haziness.

Patient 7. Baseline 7.A + 7.B: sectorial inferior conjunctivalization and diffuse corneal epithelial irregularity. End of study (12 months) 7.C + 7.D: Smooth central epithelium maintained at the end of the study. End of follow up (81 months) 7.E + 7.F: Smooth central epithelium maintained at the end of the follow up .

Patient 8: Baseline 8.A + 8.B: diffuse CNV and epithelial irregularity. End of study (12 months) 8.C + 8.D: Smooth central epithelium and central corneal transparency. End of follow up (72 months) 8.E +8.F: Mild corneal haze without CNV in corneal graft at last visit, with more regular epithelium comparing with baseline but worsening comparing with the end of study.

Figure 4. LSCD outcomes in treated vs untreated patients.

Digital photographs under diffuse illumination with white light as well as cobalt blue light are shown. Patients 4,7 and 8 have been excluded as we lack photographs of the untreated eye and patient 7 because is a monocular case.

Patient 1:

- 3A Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- 3B Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- 3C + 3D Improvement of the treated eye and improvement in the staining limited to the periphery.114 months of follow-up.
- 3E + 3F Worsening of the untreated eye with progression towards the center and late staining affecting the cornea diffusely. 114 months of follow-up.

Patient 2:

- 2A Basal photograph of the treated eye (OD) showing total conjunctivalization except the central area.
- 2B Basal photograph of the untreated eye (OI) showing peripheral conjunctivalization.
- 2C + 2D Worsening of the treated eye. 116 months of follow-up.
- 2E + 2F Stabilization of the untreated eye. 116 months of follow-up.

Patient 3:

- 3A Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- 3B Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- 3C + 3D Improvement of the treated eye in diffuse light and improvement in the staining limited to the periphery. 114 months of follow-up.
- 3E + 3F Worsening of the untreated eye with progression towards the center. Late staining affecting the cornea diffusely. 114 months of follow-up.

Patient 5:

- 5A Basal photograph of the treated eye (OD) showing grayish epithelium affecting the entire cornea and peripheral vascularization.
- 5B Basal photograph of the untreated eye (OI) showing clear central cornea.
- 5C + 5D Stabilization of the corneal opacity in the treated eye. 99 months of follow-up.
- 5E + 5F Diffuse mild irregular staining in the treated eye after follow-up. 99 months of follow-up.

Patient 6:

- 6A Basal photograph of the treated eye (OI) showing peripheral CN and mild diffuse opacity.
- 6B Basal photograph of the untreated eye (OI) showing diffuse mild opacification.
- 6C + 6D Worsening of the treated eye with diffuse late corneal staining. 61 months of follow up.
- 6 E + 6F Worsening of the untreated eye with increased corneal opacification and late staining affecting diffusely the cornea. 61 months of follow up.

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CONFLICT OF INTEREST STATEMENT:

Dr. M.Garcia-Arranz applied for 2 patents related to Adipose derived mesenchymal stem cells titled "Identification and isolation of multipotent cells from non-osteochondral mesenchymal tissue" (WO 2006/057649) and "Use of adipose tissue-derived stromal stem cells in treating fistula" (WO 2006/136244). The remaining authors have no other financial or competing interests to declare.

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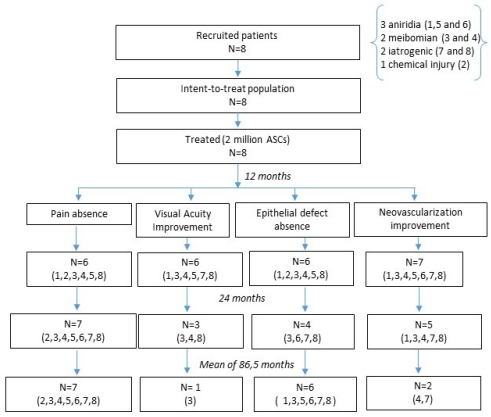

Figure 1. Flow-chart of clinical trial with clinical evolution by specific pathologies of patients. Comparison of exploration at 12, 24 and mean 86,5 months with baseline. (Numbers in parentheses refer to the patients' ID).

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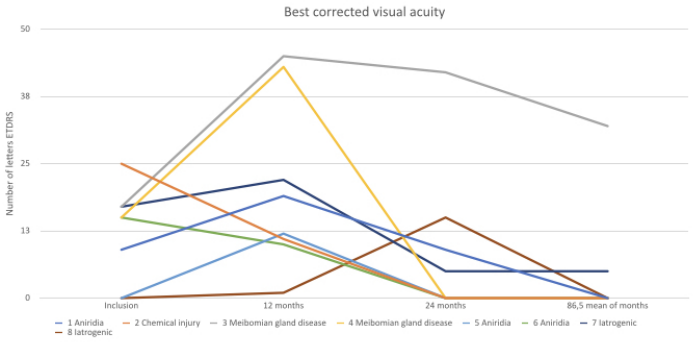

Figure 2. Visual acuity results. BCVA expressed in number of letters measured with pinhole using ETDRS at 1 m.
Patients 1,4,5,7: initial improvement with decrease of VA from the 12th month (Patient 4 due to stromal rejection of DALK in the 21st month. Patient 7 due to
exacerbation of optic neuropathy in the 13th month). Patient 3: improvement of VA maintained throughout the whole follow-up. Patients2 and 6: no improvement of
visual acuity. Patient 8: improvement of VA after 12 months due to DALK performed in the 21st month with subsequent loss of VA after retinal detachment in the 44th
month.

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Table 1. Demographic and Clinical Characteristics at Baseline

Patient ID	Sex	Age, years	Treated eye	Holland Classification	Baseline Disease	Previous Surgeries
1	Male	21	Left	IIA	Congenital aniridia	None
2	Male	36	Right	IIB	Corneal burn	None
3	Female	47	Left	IIA	Meibomian gland agenesis	None
4	Male	33	Left	IA	Rosacea keratitis	None
5	Female	47	Right	IIA	Congenital aniridia	CS
6	Male	41	Right	IIA	Congenital aniridia	CS, SIOL, GS
7	Male	48	Right	IIA	Iatrogenic, nystagmus	CS
8	Male	39	Left	IIA	Iatrogenic	CS, RD, GS

Abbreviations: CS, cataract surgery; SIOL, secondary intraocular lens implant; GS, glaucoma surgery; RD, retinal detachment.

Table 2. Summary of Short form (SF-12) results

Patient ID		Baseline visit	Final visit	Wilcoxon test (p<0.05) Baseline visit / Final visit
1 (A)	Physical Health	54,207	56,840	0.917
2 (CI)		44,425	31,708	
3 (M)		47,064	36,261	
4 (M)		54,026	59,683	
5 (A)		39,721	42,997	
6 (A)		36,655	48,619	
7 (I)		40,550	48,619	
8 (I)		44,377	48,811	
Patient ID		Baseline visit	Final visit	Wilcoxon test (p<0.05) Baseline visit / Final visit
1 (A)	Mental Health	55,963	47,675	0.753
2 (CI)		52,248	58,344	
3 (M)		26,852	59,488	
4 (M)		36,281	40,298	
5 (A)		34,946	38,241	
6 (A)		63,682	51,722	
7 (I)		58,378	51,722	
8 (I)		57,187	57,374	

Summary of SF-12 results on physical and mental health. Red numbers indicate positive changes from baseline to final visit.

Abbreviations: A, aniridia; CI, chemical injury; M, meibomian disease; I, Iatrogenic.

Table 3. Summary of Clinical Results

Patient ID	Pain			Epithelial defect			Corneal neovascularization, %		Adverse effect/Time (months) after ASCs treatment
	Inclusion	1 year	2 years	Inclusion	1 year	2 years	Inclusion	1 year	
1	Presence	Absence	Presence	Mild	No ulcer	Mild	75.44	63.79	Pain, epithelial defect (24 months)
2	Absence	Absence	Absence	No ulcer	No ulcer	No ulcer	62.14	86.74	Corneal neovascularization recurrence (2 months)
3	Presence	Absence	Absence	Mild	No ulcer	No ulcer	8.91	0.32	None
4	Presence	Absence	Absence	No ulcer	No ulcer	No ulcer	41.51	1.39	Lamellar graft rejection (21 months)
5	Presence	Absence	Absence	Mild	No ulcer	Mild	4.98	0.33	Pain, epithelial defect (24 months)
6	Absence	Presence	Absence	Mild	Mild	No ulcer	30.43	28.16	Epithelial defect (12 months)
7	Presence	Presence	Absence	Mild	Mild	No ulcer	54.03	28.24	Pain, epithelial defect (12 months), increased intraocular pressure
8	Absence	Absence	Absence	Mild	No ulcer	No ulcer	18.4	14.89	None

VARIABLE	SUCCESSFUL GRAFTS (n=5)	FAILED GRAFTS (n=3)	P-value on Univariate Analysis	Odds Ratio (95% CI) on Univariate Analysis
Mean age, years (SD)	38.6 (12.26)	38.33 (2.52)	0.964	
Male sex, n (%)	3 (50%)	3 (50%)	0.464	2.00 (0.899-4.452)
LSCD etiology			0.315	
Aniridia	2 (66.7%)	1 (33.3%)		
Meibomian gland disease	2 (100 %)	0 (0%)		0.250 (0.008-7.452)
Other	1 (33.3%)	2 (66.7%)		4.00 (0.134-119.23)
Aniridia etiology for LSCD			0.851	0.750 (0.038-14.972)
No, n (%)	3 (60%)	2 (40%)		
Yes, n (%)	2 (66.7%)	1 (33.3%)		
Previous surgery			0.465	3.00 (0.150-59.890)
None, number of eyes (%)	3 (75%)	1 (25%)		
One or more surgeries, number of eyes (%)	2 (50%)	2 (50%)		
LSCD severity*			0.465	0.333 (0.017-6.654)
Mild	2 (50%)	2 (50%)		

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Moderate + severe	3 (75%)	1 (25%)		
Mean Efron score for severe inflammation, score (SD)	1.00 (0.707)	3.00 (0.000)	0.019	
Mean time from biopsy to transplantation, days (SD)	31.00 (10.20)	35 (17.58)	0.691	
Previous epithelial defects			0.673	0.500 (0.019-12.898)
No, n (%)	1 (50%)	1 (50 %)		
Yes, n (%)	4 (66.7%)	2 (33.3%)		

Table 4: Demographic and clinical characteristics among subjects with successful and failed ASCs grafts.

Table 5: Evolution of Limbal Stem Cell Deficiency (LSCD) in patients treated with Adipose-derived Stem Cells (ASCs) at 12 months and the last follow-up visit, including cytological and histopathological findings.

Patient ID	LSCD severity	Follow up (months)	Central conjunctivalization (late fluorescein corneal staining or invasion of the corneal surface by conjunctival tissue) solo hablamos de conjuntivalizacion central por que solo se elimina el epitelio conjuntival central			Epithelial Defect			Impresion citology or MUC5AC			Histopathological analysis of the excised corneal epithelial tissue during the ASC implantation	Histopathological analysis of the excised cornea during Deep Lamellar Anterior Keratoplasty
			Inclusion	12 months	Last follow up	Inclusion	12 months	Last follow up	Inclusion	3-6 mes	12 months		
1	Moderate-severe	116	Total conjunctivalization	Improvement: Peripheral conjuntivalization	Worsening: Total conjunctivalization	Mild	No ED	No ED	LSCD absence, inflammatory cells	LSCD absence, implanted ASC cells.	The patient could not attend the scheduled appointment.	Conjunctival epithelium with citokeratines 8/18/19. Absence of globet cells.	
2	Moderate-severe	116	Total conjunctivalization	NO significative change	NO significative change	No ED	No ED	No ED	No colaboration, intense photophobia and tearing: refuses			Conjuntival epithelium with citokeratines 8/18/19. Absence of globet cells.	
3	Moderate-severe	114	Total conjunctivalization	**Improvement: Sectorial peripheral conjuntivalization**	Improvement: Sectorial peripheral conjuntivalization	Mild	No ED	No ED	LSCD	LSCD absence, inflammatory cells	**LSCD absence, no inflammatory cells**		
4	Mild	33	Sectorial conjunctivalization and diffuse pannus.	Improvement: Peripheral pannus, late fluorescen staining negative (16 meses)	Worsening: Diffuse pannus	No ED	No ED	No ED	LSCD	The patient could not attend the scheduled appointment.	The patient could not attend the scheduled appointment.	The small fragment of corneal epithelial and stromal tissue does not show the presence of goblet cells.	Keratoplasty performed 20 months after ASC: Epithelial edema, fibrosis and stromal vascularization are observed. Goblet cells are not identified
5	Mild	99	Peripheral conjunctivalization, significant surface irregularity	**Improvement: Peripheral conjunctivalization, mild surface**	Worsening: Peripheral conjunctivalization, significant surface irregularity	Mild	No ED	No ED	MUCSAC +	LSCD, inflammatory cells	**LSCD absence, no inflammatory cells**	Fragment of corneal epithelial tissue with goblet cells.	
6	Mild	61	Peripheral conjunctivalization, mild surface irregularity	**NO significative change**	NO significative change	Mild	Mild	No ED	MUC5AC +	The patient could not attend the scheduled appointment.	**LSCD, inflammatory cells**		
7	Mild	81	Sectorial conjunctivalization	NO significative change	NO significative change	Mild	Mild	No ED	LSCD, inflammatory cells	The test was not performed due to the patient having an epithelial defect.	The patient could not attend the scheduled appointment.		
8	Moderate-severe	72	Total conjunctivalization	**Improvement: Peripheral conjuntivalization**	Peripheral conjunctivalization, mild surface irregularity	Mild	No ED	No ED	LSCD	LSCD absence	**LSCD presence**	Corneal epithelial tissue does not show the presence of goblet cells.	Keratoplasty performed 21 months after ASC: corneal stroma and Bowman's layer are partially covered by epithelium, with mild and focal stromal fibrosis. Goblet cells are not

Figure 3. Ocular surface and corneal opacity evolution from 8 patients undergoing ASCs transplantation.

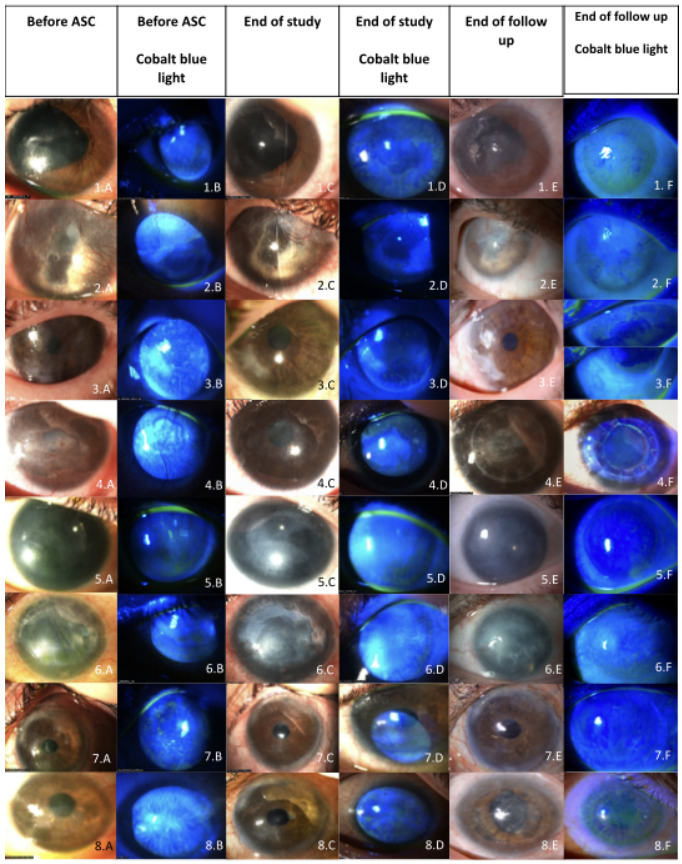

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Figure 3. Ocular surface and corneal opacity evolution from 8 patients undergoing ASCs transplantation.

Patient 1. Baseline 1.A + 1.B: peripheral CNV and opacity. Diffuse late staining and irregularity of fluorescein. **End of Study (12 months) 1.C + 1.D:** Improvement in central corneal transparency and central epithelial regularity. **End of follow up (116 Months) 1.E + 1.F:** Recurrence of corneal diffuse conjunctivalization and increase in diffuse opacification.

Patient 2: Baseline 2.A + 2.B: diffuse conjunctivalization and epithelial irregularity: an island of central transparency is preserved. **End of study (12 months) 2.C + 2.D:** Stable slit lamp exploration. **End of study (116 months) 2.E + 2.F:** worsening LSCD and corneal opacification

Patient 3. Baseline 3.A + 3.B: diffuse irregular epithelium and conjunctivalization plus peripheral nodules. **End of study (12. months) 3.C + 3.D :** Smooth central epithelium and corneal transparency. **End of follow up (114 months) 3.E + 3.F:** Persistence of corneal central transparency with superior and inferior conjunctivalization.

Patient 4. Baseline 4.A + 4.B: sectorial inferior conjunctivalization and diffuse panus. **End of study (12 months) 4.C + 4.D:** Manifest improvement of CNV, regularity of the corneal epithelium and transparency. **End of follow up (33 months) 4.E:** neovascularization with lipidic exudation invading corneal graft after rejection. 4.F smooth epithelium without LSCD signs after DALK in blue cobalt (21 months)

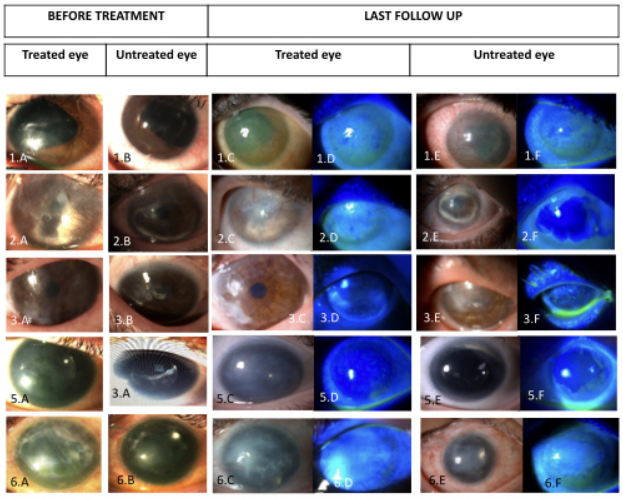
Patient 5. Baseline 5.A + 5.B: Moderate diffuse opacification and peripheral conjunctivalization. Epithelial central defect before treatment. **End of study (12 months) 5.C + 5.D:** Mild opacification secondary to subepithelial amniotic membrane and mild peripheral CNV. Smooth central epithelium. **End of follow up (99 months) 5.E + 5.F:** Worsening of the condition , significant diffuse surface irregularity.

Patient 6. Baseline 6.A + 6.B: peripheral CN and mild diffuse opacity**. End of study (12 months) 6.C + 6.D:** Paracentral persistence of subepithelial amniotic membrane, with similar CNV and corneal opacity. **End of follow up (61 months) 6.E + 6.F:** Paracentral superior corneal opacification secondary to subepithelial amniotic membrane and central stromal haziness.

Patient 7. Baseline 7.A + 7.B: sectorial inferior conjunctivalization and diffuse corneal epithelial irregularity. **End of study (12 months) 7.C + 7.D:** Smooth central epithelium maintained at the end of the study. **End of follow up (81 months) 7.E + 7.F:** Smooth central epithelium maintained at the end of the follow up .

Patient 8: Baseline 8.A + 8.B: diffuse CNV and epithelial irregularity. **End of study (12 months) 8.C + 8.D:** Smooth central epithelium and central corneal transparency. **End of follow up (72 months) 8.E +8.F:** Mild corneal haze without CNV in corneal graft at last visit, with more regular epithelium comparing with baseline but worsening comparing with the end of study

Figure 4: LSCD outcomes in treated vs untreated patients.



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Digital photographs under diffuse illumination with white light as well as cobalt blue light are shown. Patients 4,7 and 8 have been excluded as we lack photographs of the untreated eye and patient 7 because is a monocular case.

Patient 1:

- **3A** Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- **3B** Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- **3C + 3D** Improvement of the treated eye and improvement in the staining limited to the periphery.114 months of follow-up.
- **3E + 3F** Worsening of the untreated eye with progression towards the center and late staining affecting the cornea diffusely**.** 114 months of follow-up.

Patient 2:

- **2A** Basal photograph of the treated eye (OD) showing total conjunctivalization except the central area.
- **2B** Basal photograph of the untreated eye (OI) showing peripheral conjunctivalization.
- **2C + 2D** Worsening of the treated eye. 116 months of follow-up.
- **2E + 2F** Stabilization of the untreated eye**.** 116 months of follow-up.

Patient 3:

- **3A** Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- **3B** Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- **3C + 3D** Improvement of the treated eye in diffuse light and improvement in the staining limited to the periphery. 114 months of follow-up.
- **3E + 3F** Worsening of the untreated eye with progression towards the center. Late staining affecting the cornea diffusely**.** 114 months of follow-up.

Patient 5:

- **5A** Basal photograph of the treated eye (OD) showing grayish epithelium affecting the entire cornea and peripheral vascularization.
- **5B** Basal photograph of the untreated eye (OI) showing clear central cornea.
- **5C + 5D** Stabilization of the corneal opacity in the treated eye. 99 months of follow-up.
- **5E + 5F** Diffuse mild irregular staining in the treated eye after follow-up. 99 months of follow-up.

Patient 6:

- **6A** Basal photograph of the treated eye (OI) showing peripheral CN and mild diffuse opacity.
- **6B** Basal photograph of the untreated eye (OI) showing diffuse mild opacification.
- **6C + 6D** Worsening of the treated eye with diffuse late corneal staining. 61 months of follow up.

- **6 E + 6F** Worsening of the untreated eye with increased corneal opacification and late staining affecting diffusely the cornea. 61 months of follow up.

Dear Editor,

Please find enclosed the review of our manuscript titled: “Long-term effects of adipose-derived stem cells for the treatment of bilateral limbal stem cell deficiency”. We have amended the manuscript in response to the reviewers’ comments and suggestions, and we hope you find the manuscript suitable for publication in your journal.

Please find our responses and the changes in the manuscript **(in blue)** to the reviewer’s comments below.

Response to Reviewing Editor:

Please add a statement on how the sample size was determined and if it was adequate.

Author; We agree with the reviewing editor and a sentence has been included in the section “Material and methods, 1. Design”. In the section “Conclusion” it is already stated that the size was adequate to evaluate the safety of autologous ASC but insufficient to prove the therapeutic effect of this approach.

1. ***Design***

This phase IIa noncomparative clinical trial was designed to evaluate the safety and feasibility of ASCs for LSCD. Taking into consideration this primary endpoint and the innovative nature of our study, we estimate a minimum sample size of 8 patients.

Response to Reviewer 1:

The manuscript “Long-term effects of adipose-derived stem cells for the treatment of bilateral limbal stem cell deficiency” determine the safety and feasibility of human autologous adipose tissue-derived adult mesenchymal stem cells (ASCs) for bilateral LSCD. The authors studied a phase IIa noncomparative clinical trials with 8 patients. It is a new and interesting study and has very long term follow-up. However, there are some points to be concerned.

Author; Thank you very much for reviewer’s support.

-1-

Page 5, Line 55, regarding the isolation, culture and characterization of ASCs, how the authors prove the stem cell property and qualification of adipose tissue.

Author; We agree with the reviewer and a paragraph has been included in the section “Material and methods, 3. Treatment procedure, 3.1 Isolation, culture, and characterization of autologous adipose-derived stem cells”

ASCs were characterized according to legislative requirements during the trial. Nevertheless, potency studies of the cryopreserved leftover cells have been performed. These assays have demonstrated their ability to secrete VEGF, IL-10, MCP-1 MCP-4 MMP-1 GDNF, among other proteins involved in inflammatory processes; in addition to activating fibroblast healing in a scratch model.

-2-

Page 7, Line 50-55, the authors mentioned that prior to surgery and at the 12-month visit, all patients underwent impression cytology, Schirmer's test,..... and re-examine again at 24 months and last visit. However, in the result, there is no any information about impression cytology in text or table, which would be very helpful to evaluate the efficacy of the treatment.

Author; We agree with the reviewer and a new paragraph has been added titled "4. Postoperative LSCD" in the section "Results. 2. Trial outcomes".

4. Postoperative LSCD

The follow-up cytology was requested during the visits at months 3, 6, and 12. However, some patients declined to undergo this test.

In one patient (case 1), the baseline corneal cytology was negative, but the histopathological analysis of the corneal epithelium performed on the day of ASC transplantation showed conjunctivalization. Conversely, this was the case in patients 4 and 8, where corneal cytology showed conjunctival goblet cells while histological analysis did not observed those cells.

Cytology improved in patients 1, 3, 5, and 8 at some point during the follow-up. The cytological disappearance of LSCD signs (absence of conjunctival goblet cells) coincided with an improvement in biomicroscopic signs of LSCD and pain relief in cases 3 and 5. Patient 1 also showed clinical and cytological improvement in the first 6 months of the study (cytology at month 12 could not be performed).

It is worth noting that conjunctival globet cells were not observed in the corneas of recipients 4 and 8 analyzed after a DALK performed in both cases 21 months after the ASC transplantation.

Table 5 (supplementary material) provides specific information about the patients and the cytology results at different follow-up time points. It also shows how these results are related to the observed changes in biomicroscopic signs of LSCD during the study.

-3-

Page 7, Line 50, the authors wrote " the endpoint for efficacy..... VA, absence of pain, absence of corneal epithelial ulcer and improvement in neovascularization with and without fluorescein staining. The authors lack of criteria to define LSCD especially post operatively. Pain may help to evaluate the efficacy, however it is not the specific for LSCD, so it can not be use for diagnose LSCD. Please clarify the word " neovascularization" because neovascularization is not the same as conjunctivalization.

Neovascularization means the new vessels invade to the cornea, which may be from LSCD or other causes.

Author; Although the biomicroscopic hallmark of partial limbal stem cell deficiency (LSCD) is stippled late fluorescein staining over the cornea, quantifying late fluorescein staining was challenging due to the poor quality of our cobalt blue fluorescein pictures. Therefore, we chose corneal neovascularization area as a quantifiable variable, indicative of improvement in ocular surface following treatment. Following the reviewer's insightful suggestion, we have reexamined the medical records and added a new point specifying the changes in LSCD (biomicroscopic description recorded in the medical records and cytology). "Results. 2. Trial outcomes, 4. Postoperative LSCD"
In addition, we have clarified the definition of corneal neovascularization by adding the following paragraph "Results. 2. Trial outcomes, 2.3.Corneal neovascularization".

2.3. Corneal neovascularization

The only quantitative parameter analyzed was corneal neovascularization, which refers to the superficial corneal vessels, taking into account that they can be secondary to conjunctivalization or pannus, both of which are criteria for the definition of LSCD.

-4-

Please clarify the word "corneal epithelial ulcer" which is not the same as persistent epithelial defect or recurrent epithelial defect. Please use the word to match with the purpose of the authors.

Author; We unified our manuscript to consistently use the term "epithelial defect" since it accurately reflects what we measured and aligns with the terminology used in Krachmer's definition of LSCD.

-5-

Page 10, line 3, trial outcome, the authors mention about the success 5 of 8 patients assessed at 1 year by composit criteria (pain, VA, absence of corneal epithelial ulcers annd improvement of neovascularization). It would be more clearer if the authors could also give the success in term of ASC preventing LSCD by using LSCD criteria such as stabilize corneal surface by looking at absence of epithelial defect, no sign of conjunctivalization by slit lamp and fluorescein staining, impression cytology do not detect MUC5AC transcript in corneal epithelium.

Author; We thank the reviewer for this suggestion. After re-reviewing the medical records and photographs, we have added a new Table "Table 5, supplementary material" and a section on the evolution of LSCD, which captures the changes at the end of the study (month 12) and the latest follow-up visit of each patient (detailed response written in question -2-).

-6-

Page 11, line 32, the authors wrote “These same patient presented an epithelial defect....”. The authors used the word “epithelial defect” NOT “ epithelial ulcer”. This is correct but not consistency with other places.

Author; Thank you for your advice. We have revised the manuscript to use the same word consistently.

-7-

Page 11, line 34, and in Table 4 the authors wrote “.....mild epithelial defect” Please explain this.

Author; We have replaced mild with “small epithelial defects, inferior to 1 mm size” in the section “Trial outcomes. 2. Efficacy. 2.4 Epithelial defect”.

-8-

Table 3: Please specify: what is mild, moderate and severe LSCD severity

Author; We have classified peripheral and sectoral LSCD as mild, and LSCD affecting the entire corneal area as moderate to severe. Due to the sample size, we have grouped moderate and severe LSCD into a single severity stage. We have added this specification to the table 3.

-9-

Figure 2: Please describe the axis Y. (Letter of ETDRS?)

Author; Correct, it is AV measured in letters of ETDRS. We have corrected it in the Figure 2.

-10-

Figure 3: Many photographs of fluorescein staining are not clear, too dark, can not explain the details of staining. If can not adjust the brightness, consider to change the photograph. The last column, the end of follow-up of each case was not the same. May delete “Mean 58.6 months” at the title of column and give the details of last visit in the figure legends.

Author; Thank you for the suggestion, we have made the changes in the figure´s legend. We have tried to present the photographs as clearer as posible. Because of the extension of the Fig3 caption, we have moved this file to supplementary material.

In addition, we have incorporated to the manuscript a paragraph clarifying the follow up of each patient. “Material and methods. 4.Trial Outcomes. 4.2 Efficacy.

-11-

There are some points have to be added in the discussion. In this study, the majority of the cases had mild severity, no total LSCD and had IIB only one case. All cases were partial LSCD which might interfere the result of transplantation. The remaining stem cells of the recipients may repopulate and improve the function after transplantation which might be give the different result from the one who had total LSCD.

As mention that above that the severity of the cases were not severe, but from the figure 3 and the efficacy result, many cases, the clinical manifestations were not quite impressive from the last follow-up. This point should be mention and discuss.

Author; thank you for this insightful point. We have incorporated a paragraph in page 13 and page 15 under the "Discussion" section (blue paragraph) and we have removed the following paragraph (strike through text).

In addition we have removed the word "Total" before "bilateral LSCD". Page 5 "Material and methods. 2.Patients"

Page 13:

It is worth mentioning that, although the majority of our patients maintained an epithelial defect-free condition during the long-term follow-up, seven of our cases did not present severe total LSCD, unlike those treated in other studies using COMET. For this reason, it is plausible that residual limbal epithelial stem cells had the capacity to regenerate the de-epithelialized central corneal area after surgery in our cases. Additionally, the utilization of amniotic membrane may have played a role in facilitating the observed recovery in our patients.

To fully address the efficacy of ASC transplantation, a control group would be necessary. While the initial benefits in terms of conjunctivalization or neovascularization were lost in the majority of patients, it is worth mentioning that the treated eye in case 3, which initially had worse conditions compared to the untreated eye, has maintained long-term good transparency with limited conjunctivalization in the periphery. In contrast, the untreated eye, which had LSCD limited to the superior periphery, now exhibits diffuse corneal involvement.

Performing more frequent cytology examinations would have allowed us to assess not only the treatment's effect on the presence or absence of LSCD but also the evolution of inflammation and the persistence of the implanted cells. Cytology is not a risk-free technique and can cause epithelial defects in patients with fragile ocular surfaces. We believe this was the main reason for patients declining to undergo this test. Unfortunately, the availability of MUC5AC detection ceased in our center, limiting its use for complementing the initial diagnosis of LSCD in some cases.

~~*Additionally, substantial improvement was observed in the iatrogenic LSCD regarding corneal transparency and vascularization. Pain and presence of corneal ulcer resolved in the long term for both patients.*~~

~~*This approach was clearly insufficient for our most severe case, the patient with chronic caustication.*~~

Page 15*: (Figure 4, supplementary material). Also, the re-epithelization time after the procedure was 6 weeks longer in the patients with aniridia.*

-12-

Because of small sample size, univariate logistic-regression analysis interpretation have to aware of, and I guess that multivariate can not perform. This point should be added.

Author; we agree with this suggestion, and we have incorporated this paragraph into the "Discussion" section:

However, due to the small sample size, it is important to exercise caution when interpreting the results of our univariate logistic regression analysis. Additionally, given the limitations of the sample size, multivariate analysis could not be performed.

-13-

The limitation and advantage of this study should be mention for example, small sample size but long term follow-up. However, because this study take nearly 10 years in some cases, does any technique change? This should also mention.

Author; We agree with the reviewer and we changed the "Conclusion" section in this terms:

We have demonstrated that ASCs are a safe and feasible therapy for treating bilateral limbic associated keratopathy and that the treatment is a conservative procedure for improving, this deficiency. Our study cannot prove the therapeutic effect of this approach due to the small number of patients treated; however, The procedure seems to have better therapeutic efficacy in inflammatory conditions with some reservoir of limbal stem cells (meibomian gland-associated diseases) and decreased the pain level as well as the presence of epithelial defects in the long term. The most significant limitation of our study is the small sample size and the absence of a control group. These biases prevent us from concluding with certainty whether the implanted stem cells are the only responsible for the benefit and which subgroup of patients may benefit the most from this innovative treatment.

-14-

Give the advantage and disadvantage of ASCs technique when compare to other techniques. The authors compare with BM-MSCs but not compare with COMET which is also autograft.

Author; We agree with the reviewer and a new paragraph has been added in the section "Discussion" as well new references in the "References" section.

The COMET procedure is commonly employed to address total and severe bilateral LSCD, leading to a stable ocular surface in 70.8% of cases and improved visual acuity in 68.2% of eyes [26-27]. On 2021, the first-ever ex vivo cultivated oral mucosal epithelial cell transplantation (COMET) product for the treatment of LSCD, named Ocural®, was approved as a regenerative medicine product by the Pharmaceuticals and Medical Devices Agency, the regulatory agency in Japan responsible for the approval and supervision of pharmaceuticals and medical devices [28]. However, a notable complication observed frequently in eyes undergoing COMET was the occurrence of corneal epithelial defects [27].

Response to Reviewer 2:

This paper is more like a long-term follow-up (case report of 8 patients) of the effect of ASCs on ocular regeneration post-LSCD. The data shows that the outcome is not very encouraging and most of the patent had poor visual acuity and even neo-vascularisation post-transplant. The number of patients recruited in this clinical trial is very less to make any significant conclusion.

However, it's one of the longest follow up after ASC transplantation in LSCD cases and is worth exploring for a better outcome in future studies.

Author; Thank you very much for reviewer's support.

-1-

The authors claim that they injected 2.0 ml of BSS having ASCs, out of which 1.6mL was injected subconjunctival, was this volume injected fully or there was a spill of extra volume? The authors must elaborate on this in more detail. This volume seems very large.

Author: we have detailed more specifically the injection in "Material and Methods. 3.Treatment procedure. 3.2 Transplantation of cells into the eyes"

A total of 1.6mL of ASC dilution was injected subconjunctivally. The distribution of this volume within the subconjunctival space varied depending on the conjunctival scarring, but it was sufficient to fill the entire subconjunctival limbal space. To prevent any ASC spillage, pressure was applied to the entry hole using a sponge.

-2-

Authors should provide OCT images if available for pre-and post-follow-up for better clarity on the corneal epithelium and stromal thickness and clarity/ haze. Similarity should also add fluorescein images if available as supplements.

Author: We appreciate the author's suggestions. Unfortunately, we do not have OCT images of the patients in the study. However, we have added a figure with cobalt blue-filtered photographs to illustrate the progression in both operated and non-operated eyes. (Figure 4)

-3-

Discussion- Page 12- Line 7-15: Authors either should elaborate on the discussion of MHC-II and the role of MSCs for future application for more clarity or should remove this from the discussion.

Author: Thank you for the point. We have removed this remark from the discussion.

-4-

Fig3- Please arrange SLIT lamp images in sequence from patients 1 to 8 as given in the table. Presently it's all arranged randomly and poorly represented

Author: We appreciate the author's suggestions. We present a new arrangement. Following the advice suggested in the last mail we also have moved the Fig3 to supplementary material.

Response to Editorial Manager, Current Eye Research:

We have included in the following section the explanations, and also the changes of the revised manuscript which complement the last questions of the Editorial Manager. We thanks the Editor for her kind advice.

-1-

Author: **We have insert a data availability statement after the main text:**

Data availability statement

The data that support the findings of this study are available from the corresponding author, [ABB], upon reasonable request.

-2-

Author: We have added the figure captions to the main document, following the main text after "conclusions".

-3-

Author: References have been formatted following the author instructions.

-4-
Upload your response to the decision letter in a Word document.

Author: A word document under the title "Rebbutal Letter 5", with the responses to the reviewers questions and to correct the suggestions of the journal last to mails has been uploaded (supplementary file).

-5- Move table 5 to supplementary material (the table is too large to be included in the main text).

Author: It has been done, with an specification included in the manuscript text.

- 6- Number table 4, provide a title and delete the comment.

Author: We have added the title "*Table 4: Demographic and clinical characteristics among subjects with successful and failed ASCs grafts".* **We have also delete the comment under the table (abbreviations).**

- 7- The figure captions for figure 3 and 4 are far too long. Reduce by at least 50 percent or move the figures to supplementary material.
Author: We have moved both figures 3 and 4 to supplementary material with an specification in the manuscript.

-8- Add the heading 'Data availability statement'.
Author; It has been done, thank you for the remainder.

Long-term effects of adipose-derived stem cells for the treatment of bilateral limbal stem cell deficiency

Running head: Long-term effects of ASCs in bilateral LSCD

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Cristina Vidal Arranz: clinical examination of patients, collection and/or assembly of data, manuscript writing.

Almudena Del Hierro-Zarzuelo: Treatment and clinical examination of patients.

David Díaz Valle: Collection of data, final approval of manuscript.

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ABSTRACT

Purpose: To determine the safety and feasibility of human autologous adipose tissue-derived adult mesenchymal stem cells (ASCs) for ocular surface regeneration in patients with bilateral limbal stem-cell deficiency (LSCD).

Methods: A phase IIa clinical trial was designed (https://Clinicaltrials.gov, NCT01808378) with 8 patients, 3 of whom had aniridia, 2 meibomian glands diseases, 2 multiple surgeries and 1 chronic chemical injury. The therapeutic protocol was as follows: 6-mm of central corneal epithelium was removed, 400,000 ASCs were injected into each limboconjunctival quadrant, 400,000 ASCs were suspended over the cornea for 20 minutes, and finally the cornea was covered with an amniotic membrane patch.

Results: No adverse events were detected after a mean of 86,5 months of follow-up. One year after surgery, 6 of the 8 transplants were scored as successful, five patients had improved uncorrected visual acuity (mean of 12 letters), two patients presented epithelial defects (also present at baseline) and the mean percentage of corneal neovascularization was of 28.75% (36.98%, at baseline). Re-examination 24 months after treatment disclosed preserved efficacy in 4 patients. At the last visit (after a mean of 86,5 months of follow up) epithelial defects were absent in all patients although improvement in all of the variables was only maintained in patient 3 (meibomian glands agenesia).

Conclusion: ASCs are a feasible and conservative therapy for treating bilateral LSCD. Therapeutic effect differs between etiologies and diminishes over time.

KEYWORDS: adipose stem cells, clinical trial, corneal epithelium regeneration, corneal neovascularization, limbal stem cell deficiency, mesenchymal stem cell .

INTRODUCTION

Patients with bilateral total limbal stem cell deficiency (LSCD) do not have a cell source for autologous cultivated limbal transplantation which is the gold therapy for long-term restoration and renewal of the corneal epithelium in eyes with unilateral LSCD [1-5].

In bilateral ocular surface disorders transplantable cultivated epithelial sheets are obtained from cadaver donors, a living-related eye [6] or autologous oral mucosal epithelial cell sheet [7]. Without immunosuppressive therapy, the vast majority of allogeneic corneal epithelial transplantation usually fails due to immunological rejection [8] and has a success rate that tends to decrease gradually over time, with a graft survival rate of 40% at 1 year and 33% at 2 years [9]. Cultivated mucosal epithelial transplantation (COMET) results have showed a clinical success rate between 53%-70% despite recurrent epithelial defects (22%) and long term reconjunctivalization [7,10-12] as well as some degree of neovascularization in all grafted eyes [13]. Autologous adipose-derived stem cells (ASCs) are a mesenchymal cell type (MSC) that is isolated from the vascular-stromal fraction of adipose tissue. The advantages of ASCs over the usual sources (such as bone marrow MSC) are its accessibility, high performance (due to the large volume of MSCs isolated in extractions) and easy expansion, thereby avoiding lengthy procedures with associated risks to the genetic material [14-16]. Clinical experience with these cells in ophthalmic diseases is still limited. Regeneration of the corneal stroma by ASCs was assessed in an experimental study in 2008 [16] and the first case of a patient with a persistent sterile corneal epithelial defect treated with a topical application of ASCs was reported in 2012 [17]. Promising results have been recently published in seven patients with dry eye disease and Sjögren's syndrome, treated with a single transconjunctival injection of allogeneic ASCs into the lacrimal gland [18].

Similar to MSC, it has been hypothesized that the therapeutic effect of ASCs may be due to their immunoregulatory and anti-inflammatory properties [19-20]. Therefore, the use of ASCs is an encouraging approach for enhancing repair or regeneration of damaged tissue [15] in an environment unfavorable for healing.

The target of this clinical trial is to analyze the safety of autologous ASC in bilateral LSCD, and to obtain preliminary results about efficacy in different etiologies that generate bilateral LSCD.

MATERIAL AND METHODS

1. Design

This phase IIa noncomparative clinical trial was designed to evaluate the safety and feasibility of ASCs for LSCD. Taking into consideration this primary endpoint and the innovative nature of our study, we estimate a minimum sample size of 8 patients.

The Ethics Committee of La Paz University Hospital (Madrid, Spain) and the Spanish Agency of Medicinal Products and Medical Devices (AEMPS), in accordance with current legislation, approved the trial. This study was performed according to the amended Declaration of Helsinki and was registered at Clinicaltrials.gov (NCT 01808378).

2. Patients

Patients gave written informed consent before entering the study. Patients aged 18 years or older were eligible for the study if they had bilateral LSCD diagnosed with a slit-lamp examination by the same ophthalmologist. Only one eye in each patient (the most severely affected) was treated with ASCs. We characterized the LSCD using biomicroscopic examination (the patients were diagnosed by the complete disappearance of the palisades of Vogt and subepithelial neovascularization from the limbus, stippled late fluorescein staining in a vortex pattern, or a combination thereof) and completed the diagnosis with corneal impression cytology [21] or with the detection of the MUC5AC transcript in corneal epithelium [22]. Exclusion criteria were as follows: history of neoplasia in the last 5 years, allergy to local anesthetics, administration of another experimental drug in the past 90 days, administration of tacrolimus or cyclosporine within the past 4 weeks, any medical and psychiatric condition, diagnosis of congenital or acquired immunodeficiencies, major surgery or trauma in the last 6 months, pregnant and breastfeeding.

3. Treatment procedure

3.1. Isolation, culture, and characterization of autologous adipose-derived stem cells

Adipose tissue was obtained by liposuction. ASCs were harvested from the subcutaneous fat tissue of each patient for autologous use. Adipose-derived stem cell isolation, culture, and cryopreservation was also performed according to a protocol approved by AEMPS in the manufacturing facility of Hospital Gregorio Marañón (Madrid, Spain; manufacturer authorization no: AEMPS-20090211-TA, according to the PEI 04-031; Product in Clinical Research), and according to

Spanish and European legislation (ASC production is only permitted in conditions of good manufacturing practices). ASCs were characterized according to legislative requirements during the trial. Nevertheless, potency studies of the cryopreserved leftover cells have been performed. These assays have demonstrated their ability to secrete VEGF, IL-10, MCP-1 MCP-4 MMP-1 GDNF, among other proteins involved in inflammatory processes; in addition to activating fibroblast healing in a scratch model. ASCs were obtained exclusively by collagenase digestion and culture with Dulbecco's Modified Eagle Medium plus 10% fetal bovine serum and 1% ampicillin/streptomycin. After washing extensively and removing cells attached to the plastic, the laboratory data on cell differentiation to osteoblast, chondrocytes, and adipocytes was checked and flow cytometry was performed with positive (CD 27, 44, 90 and 105) and negative markers (CD 34, 45 and 73) before the ASCs could be considered ready for the patients (according to the EMEA/CHMP4/10869/2006 cell therapy guide and phenotype according to the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy [23]). Cell cultivation and expansion continued in an authorized procedure until the required number of cells for implantation (dose) was obtained. For quality-control and logistical reasons, the doses of cells were cryopreserved in liquid N_2 (30% cell death: producer data). At least 1 week before the date of implantation, cells were thawed and cultured. For administration, cells were suspended in a sterile balanced saline solution with 1% human albumin (Octapharma, Madrid, Spain) at 2×10^6 cells/2ml. Samples were taken before release to examine viability, DNA stability, and pathogen controls (analysis performed by the producer).

3.2. **Transplantation of the cells onto the eye**

Syringes containing 2 $x10^6$ cells per 2mL of balanced saline solution were prepared. The surgical procedure was done under retrobulbar anesthesia. The 6-mm central corneal epithelium was removed, and the subconjunctival injection was performed in the limbal conjunctiva using a 25G needle, with 400.000 ASCs/0.4mL per quadrant (in 4 quadrants) reaching a total of $1.6x10^6$ ASCs per eye in the limbal area. A total of 1.6mL of ASC dilution was injected subconjunctivally. The distribution of this volume within the subconjunctival space varied depending on the conjunctival scarring, but it was sufficient to fill the entire subconjunctival limbal space. To prevent any ASC spillage, pressure was applied to the entry hole using a sponge.

The last 400.000 ASCs/0.4mL ASCs were suspended in a Coronet long handled trephine (Network Medical, London, UK) on the surface of the de-epithelized central cornea for 20 minutes. Lastly, an amniotic membrane (AM) patch was stitched with a running 10-0 nylon suture avoiding the limbal area. At the end of the procedure, a therapeutic contact lens was placed on the ocular surface for protection. Inflammation control was achieved using topical steroids. To prevent bacterial infections, antibiotics were applied topically.

4. Trial outcomes

4.1. Safety

The clinical trial was designed to analyze the safety of the procedure, which was based on the incidence of adverse events (AEs) and serious adverse events (SAEs) that occurred during the patients' follow-up. Quality of life was evaluated one year after treatment using the 12-Item Short Form Survey (SF-12) index. Overall subjective comfort was checked by a questionnaire using a face score consisting of 9 faces, each showing a different expression. Patients were asked to select which face best described the current condition of their eyes during all visits [24].

4.2. Efficacy

During the clinical evaluation we tested subjective pain, assessed the visual acuity (VA) using Early Treatment Diabetic Retinopathy Study (ETDRS) charts, tested for the presence or absence of corneal epithelial defects through fluorescein staining, staged the conjunctival hyperemia on the Efron scale, and performed Schirmer test without anesthesia. Corneal neovascularization (CNV) was quantified in digital pictures by the ImageJ software, calculating the affected area from the total corneal area of a particular eye. One observer who did not participate in any biologic or clinical procedure assessed the slit lamp pictures.

The endpoint for efficacy was measured 1 year after the graft procedure using the following criteria: improvement in VA, absence of pain, absence of corneal epithelial ulcers and improvement in neovascularization through a direct observation using the slit lamp examination with and without fluorescein test. Prior to surgery and at the 12-month visit, all patients underwent impression cytology, Schirmer's test, measurement of intra-ocular pressure, digital images and fundoscopy. Patients were examined during the first year at follow-up visits scheduled for 1,4,8,12,24, and 52 weeks after cell implantation according to the clinical trial. After the end of the study, follow up was

extended and re-examination was carried at two different points: 24 months after treatment and an exploration conducted during the last hospital visit.

Statistical analysis

Categorical variables are listed as absolute and relative frequencies. For the continuous variables we employed the mean, standard deviation, median and range. The Wilcoxon test was employed to compare the temporal progression and a before-after paired test. Univariate logistic regressions were performed to assess the variables related to the success of the surgery. Data were analyzed using SPSS v.20.0 software, and the significance was set at $p<.05$.

RESULTS

1. **Patients**

Eight patients recruited from the ophthalmology departments of La Paz University Hospital, Clínico University Hospital and Fuenlabrada Hospital (Madrid, Spain) were treated between November 2012 and November 2014. Six patients were men, and the mean of age was 38.0 (±9.26) years. The etiologies for the LSCD were aniridia in 3 patients (37.5%) (patients 1,5 and 6), meibomian gland-related disease in 2 patients (25%) (patient 3 had primary meibomian glands agenesia and patient 4 had chronic rosacea blepharitis), iatrogenic in 2 patients (25%) (patients 7 and 8) and chronic chemical injury in 1 patient (12.5%) (patient 2) (Table 1). Six of the patients had a Holland classification of IIa. Of the 3 patients with aniridia, all were Mackman stage 2 at the baseline visit. In terms of LSCD severity, 4 of the patients were mild (50%). The mean conjunctival hyperemia score (on an Efron scale of 0-4) was 1.75 (range 0- 3).

In all cases, the liposuction and cell culture were performed without any incident, and the cell application was performed without complications.

Mean last follow up was 86,5 months after treatment (Patient 1: 116 months; Patient 2: 116 months; Patient 3: 114 months; Patient 4: 33 months; Patient 5: 99 months; Patient 6: 61 months; Patient 7: 81 months; Patient 8: 72 months).

2. **Trial outcomes**

1. **Safety**

There were no mild or severe AEs related to the ASCs applications, thereby achieving the safety objective. This result was valid for a mean of 86,5 months after follow-up (33 to 116 months).

During the follow-up period after the end of the trial (after 1st year), patient 7 experienced an adverse event due to an associated disease; the patient´s previous glaucoma gradually worsened, and a glaucoma surgery was performed 13 months after the ASCs treatment. Also, patient 4 experienced a massive stromal graft rejection of a deep anterior lamellar keratoplasty (DALK). Since the DALK was performed 21 months after the ASCs transplantation (the immunosuppressive treatment consisted of local corticosteroids) we did not consider this rejection related to the ASCs treatment. Finally, in patient 8 a DALK was performed 21 months after ASCs and presented a retinal detachment 44 months after ASCs treatment, significantly reducing the patient´s vision.

Regarding subjective comfort, the mean baseline comfort index was 4.2 which improved to 3 at 12 months. Patients 5 and 8 could not distinguish the faces at baseline visit, and both graded as 3 after the treatment at 12-month visit. The patient with caustication (patient 2) was the only who worsened (from grade 4 to 5).

The overall results of the SF-12 test (physical health index 0.46 at the baseline visit and 0.91 at the final visit, and mental health index of 1 at the baseline visit and 0.50 at the final visit) were not statistically significant (Table 2).

2. **Efficacy**

The trial outcome was regarded as a success based on a composite criterion (absence of pain, improvement in VA, absence of corneal epithelial ulcers and improvement of neovascularization) in 5 (62,5%) of 8 patients assessed at 1 year after the implantation (patient 1 with aniridia, patient 3 with congenital meibomian agenesia, patient 4 with ocular rosacea, patient 5 with aniridia and patient 8 with multiple surgeries). In the univariate logistic-regression analysis, the only variable related to failed grafts at 12 months was the grade of inflammation (Table 3). When considering the 8 patients assessed 2 years after implantation, the number of improved patients decreased to 3 (50% success rate) (patients 3, 4, and 8). The final percentage of successful transplants at the last visit was of only 1 patient (12,50%) (patient 3). If we exclude the VA parameter (as it is interfered by associated ocular conditions) from the composite criterion for success, three cases (3, 4 and 7),

37,50% met the clinical criteria of successful treatment at final follow up (mean 86,5 months). The trial protocol is summarized in Figure 1.

To determine the effect of the procedure, we analyzed the following parameters:

2.1. Visual acuity

Of the 8 patients, 5 improved their uncorrected visual acuity, with a mean of 12 (12.31) letters, and 6 presented an improved best corrected visual acuity (BCVA) with pinhole, with a mean of 14 (11,51) letters, at the 12-month visit. At the 24-month visit, patients 3,4 and 8 had improved BCVA with a mean of 22 (17.21) letters, whereas only patient 3 preserved an improved visual acuity at last follow up (improvement of 15 letters in BCVA, 114 months after ASCs). However, it must be considered that visual results worsened in patient 7 due to an exacerbation of optic neuropathy requiring glaucoma surgery 13 months after treatment, in patient 4 due to a DALK surgery followed by a stromal rejection 21 months after ASCs treatment and in patient 8 due to a retinal detachment three years after treatment (Figure 2).

2.2. Pain

Pain data at baseline indicated that 4 patients experienced recurrent pain episodes and 2 patients felt that the pain often interfered with their activities. At 12 months from treatment, 2 patients reported having pain coinciding with the presence of an epithelial defect (patients 6 and 7). At 24 months from treatment, only slight pain was found in one of the patients (patient 1), coinciding with epithelial defect. This same patient referred mild pain in both eyes at the last visit despite absence of epithelial defect (Table 4).

2.3. Corneal neovascularization

The only quantitative parameter analyzed was corneal neovascularization, which refers to the superficial corneal vessels, taking into account that they can be secondary to conjunctivalization or pannus, both of which are criteria for the definition of LSCD. The mean (+SD) neovascularization percentage at the baseline visit was 36.98% (25.7) of the corneal area which decreased to 28.75 % (32.0) by the 12-month visit (p=0.579; 95% CI: -22.892-39.352). During the following visits, mean neovascularization gradually increased to 30,03% by the 24 month visit and to 47,07% at the last follow up (Table 4).

2.4. Epithelial defect

Of the 8 patients, 6 had a history of epithelial defect before starting the study, and 2 patients had an epithelial defect at study inclusion (patients 6 and 7). These same patients presented an epithelial defect at month 12 but not after 24 months or at the last follow up. Two patients with aniridia (1 and 5) presented small epithelial defects, inferior to 1 mm size, at 24 months. To sum up, none of the patients presented epithelial defect at the last follow up visit (Table 4).

The mean time to epithelial defect closure after surgery was 9.25 weeks (7.92) except for the aniridia group, where mean time was 15.33 weeks.

3. **Corneal transparency**

Baseline data indicates that all patients had corneal opacity, observed during biomicroscopy. One observer that did not participate in any biological or clinical procedures assessed the patients' slit lamp pictures. By the 12-month follow-up, corneal transparency had improved in 5 of 7 patients (the picture of patient 5 couldn't be evaluated due to poor quality). If we analyze by condition, improvement was observed in 1 patient with aniridia (patient 1), 2 patients with meibomian (patients 3 and 4) and 2 patients with LSCD secondary to multiples surgeries (patients 7 and 8). At the end of the follow-up, corneal opacity increase was observed in 2 patients (patient 1 and 2, both 116 months of follow up) due to worsening of the limbic deficiency (Figure 3, supplementary material).

4. Postoperative LSCD

The follow-up cytology was requested during the visits at months 3, 6, and 12. However, some patients declined to undergo this test.

In one patient (case 1), the baseline corneal cytology was negative, but the histopathological analysis of the corneal epithelium performed on the day of ASC transplantation showed conjunctivalization. Conversely, this was the case in patients 4 and 8, where corneal cytology showed conjunctival goblet cells while histological analysis did not observed those cells.

Cytology improved in patients 1, 3, 5, and 8 at some point during the follow-up. The cytological disappearance of LSCD signs (absence of conjunctival goblet cells) coincided with an improvement in biomicroscopic signs of LSCD and pain relief in cases 3 and 5. Patient 1 also showed clinical and cytological improvement in the first 6 months of the study (cytology at month 12 could not be performed).

It is worth noting that conjunctival globet cells were not observed in the corneas of recipients 4 and 8 analyzed after a DALK performed in both cases 21 months after the ASC transplantation.

Table 5 (supplementary material) provides specific information about the patients and the cytology results at different follow-up time points. It also shows how these results are related to the observed changes in biomicroscopic signs of LSCD during the study.

DISCUSSION

Our study demonstrated that applying ASCs subconjunctivally and under an AM is a safe procedure, with no related adverse effects. The patient with severe LSCD caused by chronic caustication was the only who experienced an increase in corneal neovascularization and a decrease in visual acuity after the procedure. However, we believe that this event was due more to the surgical removal of the corneal fibrovascular tissue than to the direct effect of the ASCs. An additional safety issue that we could not evaluate is biodistribution; as we are injecting cells in the subconjunctival vascular area, the remote possibility of distribution through the bloodstream exists.

Furthermore, we have investigated the safety and viability of ASCs in bilateral LSCD by means of a new surgical approach: injecting ASCs in the subconjunctival area and denuded cornea instead of a cultivated cell sheet as previously reported in trials with cultured oral mucosal stem cells. These ASC, when topically applied, have been shown to improve reepithelialization in an experimental model of caustication previously [25]. The COMET procedure is commonly employed to address total and severe bilateral LSCD, leading to a stable ocular surface in 70.8% of cases and improved visual acuity in 68.2% of eyes [26]. On 2021, the first-ever ex vivo cultivated oral mucosal epithelial cell transplantation (COMET) product for the treatment of LSCD, named Ocural®, was approved as a regenerative medicine product by the Pharmaceuticals and Medical Devices Agency, the regulatory agency in Japan responsible for the approval and supervision of pharmaceuticals and medical devices [27]. However, a notable complication observed frequently in eyes undergoing COMET was the occurrence of corneal epithelial defects [13]. It is worth mentioning that, although the majority of our patients maintained an epithelial defect-free condition during the long-term follow-up, seven of our cases did not present severe total LSCD, unlike those treated in other studies using COMET. For this reason, it is plausible that residual limbal epithelial stem cells had the capacity to regenerate the

de-epithelialized central corneal area after surgery in our cases. Additionally, the utilization of amniotic membrane may have played a role in facilitating the observed recovery in our patients.

To fully address the efficacy of ASC transplantation, a control group would be necessary. While the initial benefits in terms of conjunctivalization or neovascularization were lost in the majority of patients, it is worth mentioning that the treated eye in case 3, which initially had worse conditions compared to the untreated eye, has maintained long-term good transparency with limited conjunctivalization in the periphery. In contrast, the untreated eye, which had LSCD limited to the superior periphery, now exhibits diffuse corneal involvement. (Figure 4, supplementary material).

Performing more frequent cytology examinations would have allowed us to assess not only the treatment's effect on the presence or absence of LSCD but also the evolution of inflammation and the persistence of the implanted cells. Cytology is not a risk-free technique and can cause epithelial defects in patients with fragile ocular surfaces. We believe this was the main reason for patients declining to undergo this test. Unfortunately, the availability of MUC5AC detection ceased in our center, limiting its use for complementing the initial diagnosis of LSCD in some cases.

The univariate logistic-regression analysis showed that failures were associated only with inflammation (p=0.019) (Table 4.) However, due to the small sample size, it is important to exercise caution when interpreting the results of our univariate logistic regression analysis. Additionally, given the limitations of the sample size, multivariate analysis could not be performed.

Probably, the chronic inflammation of the patient´s severely damaged receiving bed microenvironment where the ASCs were injected did not allow for cell action. We should have addressed the severity and the prime causes for the chronic conjunctival inflammation before applying the ASC treatment. Concerning aniridia, previous studies report a limited duration of donor limbal stem cell grafts, and the results of studies on stem cells harvested from other sites in the patient (e.g., the oral mucosa) are so far inconclusive [26]. In our study, the therapeutic effect of ASCs in patients with aniridia was questionable; on one hand, the response was torpid and poor in patients 5 and 6 (with epithelial defects throughout the follow-up and little improvement in other

parameters) but initially very good in patient 1 in whom we did not employ AM patch. However, after one year, this patient developed a worsening of vision, corneal transparency, and corneal vascularization with persistent pain in both eyes (Figure 4, supplementary material). Also, the re-epithelization time after the procedure was 6 weeks longer in the patients with aniridia.

Despite an optimal initial response in some patients (as described above), deterioration is observed over time in VA, neovascularization, and corneal transparency. However, all patients except patient 1 remained ulcer and pain-free in the long term (mean of 86,5 months after treatment with ASCs). It is important to us that the treatment is minimally invasive. Although more trials are needed to investigate the best cell dose required, we propose repeating the doses every 3-6 months, as this could enhance the anti-inflammatory and repairing effect we observed in a number of our patients. Furthermore, conditions that have shown a greater benefit from this treatment could guide patient selection in futures trials: chronic meibomian gland-associated diseases and iatrogenic LSCD. A future second step will be the use of allogenic ASCs. Thus, a pool of ASCs could be available for use for acute inflammatory conditions associated with the development of LSCD. In this line, the efficacy of allogeneic bone marrow mesenchymal stem cells (BM-MSCs) transplantation have been tested in human eyes in one trial showing similar results as allogeneic cultivated limbal epithelial transplantation (CLET) to treat patients with total and/or severe LSCD [28]. In this trial BM-MSCs were cultivated on an amniotic membrane and transplanted onto the ocular surface.

Our team selected ASCs because this cells can be easily obtained from low invasive liposuction aspirates rendering a high number of multipotent stem cells unlike BM-MSCs. Currently, no clinical trials using ASCs as a cell therapy for LSCD have been published.

CONCLUSION

We have demonstrated that ASCs are a safe and feasible therapy for treating bilateral limbic associated keratopathy and that the treatment is a conservative procedure for improving, this deficiency in some patients with non severe LSCD. The procedure seems to have better therapeutic efficacy in inflammatory conditions with some reservoir of limbal stem cells (meibomian gland-associated diseases) and decreased the pain level as well as the presence of epithelial defects in the

long term. The most significant limitation of our study is the small sample size and the absence of a control group. These biases prevent us from concluding with certainty whether the implanted stem cells are the only responsible for the benefit and which subgroup of patients may benefit the most from this innovative treatment.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [ABB], upon reasonable request.

Figure Captions:

Figure 1. Flow-chart of clinical trial with clinical evolution by specific pathologies of patients.

Comparison of exploration at 12, 24 and mean 86,5 months with baseline. (Numbers in parentheses refer to the patients' ID).

Figure 2. Visual acuity results. BCVA expressed in number of letters measured with pinhole using ETDRS at 1 m.

Patients 1,4,5,7: initial improvement with decrease of VA from the 12th month (Patient 4 due to stromal rejection of DALK in the 21st month. Patient 7 due to exacerbation of optic neuropathy in the 13th month). Patient 3: improvement of VA maintained throughout the whole follow-up. Patients2 and 6: no improvement of visual acuity. Patient 8: improvement of VA after 12 months due to DALK performed in the 21sst month with subsequent loss of VA after retinal detachment in the 44th month.

Figure 3. Ocular surface and corneal opacity evolution from 8 patients undergoing ASCs transplantation.

Patient 1. Baseline 1.A + 1.B: peripheral CNV and opacity. Diffuse late staining and irregularity of fluorescein. End of Study (12 months) 1.C + 1.D: Improvement in central corneal transparency and central epithelial regularity. End of follow up (116 Months) 1.E + 1.F:

Recurrence of corneal diffuse conjunctivalization and increase in diffuse opacification.

Patient 2: Baseline 2.A + 2.B: diffuse conjunctivalization and epithelial irregularity: an island of central transparency is preserved. End of study (12 months) 2.C + 2.D: Stable slit lamp exploration. End of study (116 months) 2.E + 2.F: worsening LSCD and corneal opacification

Patient 3. Baseline 3.A + 3.B: diffuse irregular epithelium and conjunctivalization plus peripheral nodules. End of study (12. months) 3.C + 3.D : Smooth central epithelium and corneal transparency. End of follow up (114 months) 3.E + 3.F: Persistence of corneal central transparency with superior and inferior conjunctivalization.

Patient 4. Baseline 4.A + 4.B: sectorial inferior conjunctivalization and diffuse panus. End of study (12 months) 4.C + 4.D: Manifest improvement of CNV, regularity of the corneal epithelium and transparency. End of follow up (33 months) 4.E: neovascularization with lipidic exudation invading corneal graft after rejection. 4.F smooth epithelium without LSCD signs after DALK in blue cobalt (21 months)

Patient 5. Baseline 5.A + 5.B: Moderate diffuse opacification and peripheral conjunctivalization. Epithelial central defect before treatment. End of study (12 months) 5.C + 5.D: Mild opacification secondary to subepithelial amniotic membrane and mild peripheral CNV. Smooth central epithelium. End of follow up (99 months) 5.E + 5.F: Worsening of the condition , significant diffuse surface irregularity.

Patient 6. Baseline 6.A + 6.B: peripheral CN and mild diffuse opacity. End of study (12 months) 6.C + 6.D: Paracentral persistence of subepithelial amniotic membrane, with similar CNV and corneal opacity. End of follow up (61 months) 6.E + 6.F: Paracentral superior corneal opacification secondary to subepithelial amniotic membrane and central stromal haziness.

Patient 7. Baseline 7.A + 7.B: sectorial inferior conjunctivalization and diffuse corneal epithelial

irregularity. End of study (12 months) 7.C + 7.D: Smooth central epithelium maintained at the end of the study. End of follow up (81 months) 7.E + 7.F: Smooth central epithelium maintained at the end of the follow up .

Patient 8: Baseline 8.A + 8.B: diffuse CNV and epithelial irregularity. End of study (12 months) 8.C + 8.D: Smooth central epithelium and central corneal transparency. End of follow up (72 months) 8.E +8.F: Mild corneal haze without CNV in corneal graft at last visit, with more regular epithelium comparing with baseline but worsening comparing with the end of study.

Figure 4. LSCD outcomes in treated vs untreated patients.

Digital photographs under diffuse illumination with white light as well as cobalt blue light are shown. Patients 4,7 and 8 have been excluded as we lack photographs of the untreated eye and patient 7 because is a monocular case.

Patient 1:

- 3A Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- 3B Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- 3C + 3D Improvement of the treated eye and improvement in the staining limited to the periphery.114 months of follow-up.
- 3E + 3F Worsening of the untreated eye with progression towards the center and late staining affecting the cornea diffusely. 114 months of follow-up.

Patient 2:

- 2A Basal photograph of the treated eye (OD) showing total conjunctivalization except the central area.
- 2B Basal photograph of the untreated eye (OI) showing peripheral conjunctivalization.

- 2C + 2D Worsening of the treated eye. 116 months of follow-up.
- 2E + 2F Stabilization of the untreated eye. 116 months of follow-up.

Patient 3:

- 3A Basal photograph of the treated eye (OD) showing grayish thick and vascularized epithelium affecting the entire cornea.
- 3B Basal photograph of the untreated eye (OI) showing peripheral superior conjunctivalization.
- 3C + 3D Improvement of the treated eye in diffuse light and improvement in the staining limited to the periphery. 114 months of follow-up.
- 3E + 3F Worsening of the untreated eye with progression towards the center. Late staining affecting the cornea diffusely. 114 months of follow-up.

Patient 5:

- 5A Basal photograph of the treated eye (OD) showing grayish epithelium affecting the entire cornea and peripheral vascularization.
- 5B Basal photograph of the untreated eye (OI) showing clear central cornea.
- 5C + 5D Stabilization of the corneal opacity in the treated eye. 99 months of follow-up.
- 5E + 5F Diffuse mild irregular staining in the treated eye after follow-up. 99 months of follow-up.

Patient 6:

- 6A Basal photograph of the treated eye (OI) showing peripheral CN and mild diffuse opacity.
- 6B Basal photograph of the untreated eye (OI) showing diffuse mild opacification.
- 6C + 6D Worsening of the treated eye with diffuse late corneal staining. 61 months of follow up.
- 6 E + 6F Worsening of the untreated eye with increased corneal opacification and late staining affecting diffusely the cornea. 61 months of follow up.

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CONFLICT OF INTEREST STATEMENT:

Dr. M.Garcia-Arranz applied for 2 patents related to Adipose derived mesenchymal stem cells titled "Identification and isolation of multipotent cells from non-osteochondral mesenchymal tissue" (WO 2006/057649) and "Use of adipose tissue-derived stromal stem cells in treating fistula" (WO 2006/136244). The remaining authors have no other financial or competing interests to declare.

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