Full length article

A double-crosslinked nanocellulose-reinforced dexamethasone-loaded collagen hydrogel for corneal application and sustained anti-inflammatory activity

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Abstract

In cases of blinding disease or trauma, hydrogels have been proposed as scaffolds for corneal regeneration and vehicles for ocular drug delivery. Restoration of corneal transparency, augmenting a thin cornea and postoperative drug delivery are particularly challenging in resource-limited regions where drug availability and patient compliance may be suboptimal. Here, we report a bioengineered hydrogel based on porcine skin collagen as an alternative to human donor corneal tissue for applications where long-term stability of the hydrogel is required. The hydrogel is reinforced with cellulose nanofibers extracted from the Ciona intestinalis sea invertebrate followed by double chemical and photochemical crosslinking. The hydrogel is additionally loaded with dexamethasone to provide sustained anti-inflammatory activity. The reinforced double-crosslinked hydrogel after drug loading maintained high optical transparency with significantly improved mechanical characteristics compared to non-reinforced hydrogels, while supporting a gradual sustained drug release for 60 days in vitro. Dexamethasone, after exposure to crosslinking and sterilization procedures used in hydrogel production, inhibited tube formation and cell migration of TNFα-stimulated vascular endothelial cells. The drug-loaded hydrogels suppressed key pro-inflammatory cytokines CCL2 and CXCL5 in TNFα-stimulated human corneal epithelial cells. Eight weeks after intra-stromal implantation in the cornea of 12 New-Zealand white rabbits subjected to an inflammatory suture stimulus, the dexamethasone-releasing hydrogels suppressed TNFα, MMP-9, and leukocyte and fibroblast cell invasion, resulting in reduced corneal haze, sustained corneal thickness and stromal morphology, and reduced overall vessel invasion. This collagen-nanocellulose double-crosslinked hydrogel can be implanted to treat corneal stromal disease while suppressing inflammation and maintaining transparency after corneal transplantation.

Statement of significance

To treat blinding diseases, hydrogel scaffolds have been proposed to facilitate corneal restoration and ocular drug delivery. Here, we improve on a clinically tested collagen-based scaffold to improve mechanical robustness and enzymatic resistance by incorporating sustainably sourced nanocellulose and dual chemical-photochemical crosslinking to reinforce the scaffold, while simultaneously achieving sustained release of an incorporated anti-inflammatory drug, dexamethasone. Evaluated in the context of a corneal disease model with inflammation, the drug-releasing nanocellulose-reinforced collagen scaffold maintained the cornea’s transparency and resisted degradation while suppressing inflammation postop-
1. Introduction

According to the World Health Organization, 2.2 billion people suffer from vision impairment globally, with at least 45% of cases being preventable, or yet to be addressed [1]. One of the major diseases causing preventable blindness in its advanced stages is the corneal disease keratoconus [2]. Keratoconus is a disease of progressive corneal thinning and worsening of refraction due to steepening curvature of the cornea. The disease affects millions globally, with the heaviest burden in Africa, the Middle East and Asia [3–5]. As keratoconus progresses and is not amenable to conservative treatments, invasive surgical approaches are required, and in advanced stages, corneal transplantation is often necessary. A major global shortage of donor corneas, however, translates into a large burden of preventable blindness, predominantly in low and middle-income countries [2]. Thus, an alternative to donor tissue is needed that can be used to treat keratoconus and be applicable in areas of the world where eye banking and cornea donations are not easily accessible. Moreover, as surgery must be accompanied by administration of postoperative medications to avoid inflammation and tissue rejection, an additional challenge is optimal delivery of drugs to the eye in resource-strapped regions.

The most common form of pharmacological treatment delivery is topical preparations in the form of eye drops, ointments, and gels [6–9]. Although topical preparations are generally cost-effective, non-invasive and easy to use [7], the blink reflex, high tear turnover rate and the lacrimal drainage result in low bioavailability of topical drugs [6–8]. It is estimated that only 20% of the original applied dose is delivered to the cornea [7], even with 100% compliance. In areas of limited resources and with large rural populations, minimizing hospital visits and the need for additional follow-ups due to poor compliance and postoperative complications would aid in addressing the burden of low vision in these regions. To overcome issues of low bioavailability and poor patient compliance, alternative drug delivery systems such as drug-eluting therapeutic contact lenses (CLs) [8–11] and intracorneal injections [8–12] have been proposed. In particular, antibiotics and corticosteroids are the most common drugs used in the treatment of corneal diseases and attempts have been made to optimize delivery with the aid of biomaterials and delivery approaches [10–16]. Notably, biomaterial-enabled nanoparticles including liposomes, dendrimers, polymeric nanoparticles, niosomes, nanosuspensions and hydrogels have been tested for corneal drug delivery [8]. Among these, hydrogels are well known for their sustained drug release, increased retention time and their ability to respond to stimuli that allows for ease of administration [6].

The major limitation, however, in addressing advanced corneal disease in low and middle-income regions is the limited global supply of human donor corneas. This has led to the development of bioengineered alternatives to human corneas, with collagen derived from different sources being the most well-studied and the only alternative to human donor reaching human studies [17–19]. We have previously reported on hydrogel-based corneal implants made from porcine skin collagen (called the ‘bioengineered porcine construct’, BPC) [20–23]. The BPC was successfully loaded with recombinant human nerve growth factor and sustained drug release was achieved in vitro for up to two months [20]. More recently, we reported a novel cell-free double crosslinked BPC (called BPCDX) with enhanced mechanical strength by virtue of double chemical and photochemical stabilization [23]. The BPCDX could be safely implanted into the corneal stroma of 20 patients with advanced keratoconus without native tissue removal. BPCDX restored vision to blind subjects in regions of dire need, using a simpler and safer method than current transplantation, while achieving equivalent outcomes and avoiding the need for tissue donation. The idea of this biomaterial is that it will be stable in the long-term following implantation, and will not degrade but rather augment the thin cornea, and thereafter maintain the new corneal thickness. Topical steroids, however, were necessary to administer to the eye following implantation of the BPCDX. In resource-strained regions and where risk of low compliance exists, a corticosteroid integrated within the implant could avoid the need for repeated application of topical eye drops postoperatively. Moreover, even though BPCDX is mechanically more robust than earlier versions, the mechanical properties of human donors are still superior [23].

The first aim of this study was therefore to further enhance the stability of the BPCDX hydrogel and its mechanical properties by mechanically reinforcing it with highly purified cellulose nanofibers (CNF) extracted from the Ciona intestinalis sea invertebrate. Cellulose is the most abundant natural polymer and nanocellulose-based biomedical materials have become popular in recent years due to outstanding mechanical properties, low cost, biocompatibility, availability and sustainability [24].

The second aim of this study was to develop an alternative administration route for dexamethasone in the cornea by loading this new class of CNF-reinforced double-crosslinked hydrogel with dexamethasone (called BPCDX-CNF-Dex) for sustained release. Dexamethasone is a well-known corticosteroid that has recently received renewed attention for its potent properties in suppressing the cytokine storm that arises in patients with severe Covid-19 [25]. We have previously shown that this cytokine storm, triggered by surgery or underlying corneal disease, is also effectively suppressed in the rat cornea by topical dexamethasone treatment, where the chemokines CCL2 and CXCL5, both involved in immune cell recruitment, were among the most suppressed factors [26,27]. Topical corticosteroids are the mainstay of treatment following all corneal transplantations and given the challenges of topical administration and risk for graft rejection, they are typically used for years following high-risk transplantations and around 30% of surgeons combine topical with systemic corticosteroids [28]. The immunosuppressive effects of dexamethasone delivery via subconjunctival implants and via topical application have been shown to result in significant reduction of allograft rejection and neovascularization in rabbit models [29], however, to our knowledge this is the first time dexamethasone has been delivered as anti-inflammatory therapy via a corneal implant.

The double-crosslinked nanocellulose-reinforced dexamethasone-loaded bioengineered porcine collagen hydrogels were evaluated for cell compatibility in vitro, optical transparency, mechanical properties, and drug release profile. BPCDX-CNF and BPCDX-CNF-Dex hydrogels were then implanted in rabbit corneas in a model of postsurgical corneal inflammation to evaluate the effect of the BPCDX-CNF-Dex implants on corneal thickness, trans-
parenchyma, inflammation and corneal neovascularization following implantation surgery, and to evaluate its resistance to degradation within an inflamed corneal environment.

2. Materials and methods

2.1. Fabrication of nanocellulose-reinforced bioengineered corneal implants loaded with dexamethasone

Medical grade purified freeze-dried type I porcine dermal collagen (SE Eng. Co., S. Korea) was dissolved in PBS at room temperature to form a 5% collagen solution. The collagen solution was then exposed to a controlled vacuum evaporation, and then mixed with 1% medical-grade CNF (from *Cliona intestinalis*, Ocean Tunicet AS, Norway) at 1% CNF to collagen ratio to emulate the natural collagen-proteoglycan scaffolding of the ECM. Crosslinkers including 1-[3-(Dimethylamino) propyl]-3-ethylcarboxydiimide methiodide (EDCM), N-hydroxysuccinimide (NHS), and riboflavin (vitamin B2) (Care Group, Vadodara) were added each at 1% (w/v) ratios. In order to functionalize the nanocellulose to enable collagen-nanocellulose linkages, the nanocellulose was TEMPO-oxidized, which enabled the nanocellulose to link with collagen fibrils by covalent bonding of aldehyde groups on the oxidized nanocellulose with primary amine groups of collagen (mediated by EDC-NHS coupling) [30,31]. The solution was mixed thoroughly and dispensed into custom curved molds with spacers to result in hydrogels with a thickness of 200 μm. This thickness, although thinner than the implants that would be used in patients [23], was chosen to be of comparable size and also ensure that the rabbit eyes could accommodate and support the implants. Also, the addition of such a thickness to the rabbit eyes, would result in a corneal thickness, after some expected dehydration, of around 550 μm, which is close to the average normal corneal thickness in humans. Samples were then cured at room temperature. Removal from molds was achieved by immersion in PBS for one hour at room temperature. Finally, the samples were rinsed with sterile PBS to extract any reaction residues. Following this chemical crosslinking, a second photochemical crosslinking of samples was performed by instilling onto the sample 0.1% riboflavin solution in 20% dextran and exposing to 365 nm UV light with an irradiance of 3 mW/cm² for 30 min while rinsing with riboflavin solution every 3 min [32]. A final UVC sterilization step of the hydrogels at 245 nm for 7 s was performed to ensure that the gels are medical-grade material suitable for transplantation. Both the CNF reinforcement and the photochemical crosslinking steps were employed to improve the hydrogels’ mechanical strength, resistance to degradation, and long-term stability. The result is an entirely natural, chemical-free, transparent hydrogel.

For dexamethasone loading into BPCDX-CNFD hydrogels, the commercially available eye drops Aspoto-Maxidex with dexamethasone concentration 1 mg/mL (Aspoto-Maxidex 1 mg/mL Novartis Sverige AB, Kista, SE) was lyophilized to make a powder that we then dissolved in an MES buffered saline solution, which could be done up to 2 mg/mL while retaining transparency. Dexamethasone 2 mg/mL was added in the biopolymer mixture solution and the rest of the process was performed as described above. All BPCDX-CNFD and BPCDX-CNFD-Dex were formulated and manufactured under GMP conditions by Naturalens AB and LinkoCare Life Sciences AB, Linköping, Sweden.

2.2. Material characterization for BPCDX-CNFD and BPCDX-CNFD-Dex hydrogels

BPCDX-CNFD and BPCDX-CNFD-Dex hydrogels (n ≥ 3) were characterized for their mechanical properties and for collagenase degradability, water content, optical properties and microstructure according to the quality control Standard Operating Procedures (SOPs) of Naturalens AB and LinkoCare Life Sciences AB, Linköping, SE. In addition, BPCDX hydrogels (n = 22) reported earlier were used for the comparison of the mechanical properties of the earlier generation hydrogel formulation [23] with the current one. Light transmission measurements were made at visible light wavelengths of 450 nm and 600 nm using a USB4000 UV–Vis Spectrophotometer (Mettler Toledo, Stockholm, SE). Water content of hydrogels was determined by a Mettler Toledo moisture analyzer (HX204). The formulas used for the determination of the water content percentage, the swelling ratio and the crosslinking index are given in the supplementary methods section. For the collagenase degradation assay, samples were equilibrated to pH 7.4 by incubation for one hour in 5 mL of 0.1 M tris-HCl buffer with 5 mM calcium chloride at 37 °C. Samples were digested by subsequent addition of 1 mg/mL type I collagenase (from *Clostridium histolyticum*), resulting in a final collagenase concentration of 17 μg/mL. At intervals of 2 h, 4 h and 8 h of incubation, samples were removed from the solution and their residual mass was weighed. Percentage residual mass was determined by the ratio of initial sample weight to the weight at each time point. Mechanical testing was performed and the main mechanical properties including ultimate tensile strength, strain, energy to break and elastic modulus were measured by tensile testing until material failure in an Instron Automated Materials Testing System (Model 5943) with a load cell of 50 N capacity and pneumatic metal grips at a crosshead speed of 10 mm/min. The tensile strength, tensile strain, energy at maximum load and Young’s modulus were measured and automatically calculated and recorded data by the system BlueHill software (Version 2). For the microstructural analysis of the implants, scanning electron microscopy (SEM) was performed using a Hitachi SEM Model S-2250 N microscope at different magnifications. Before loading with dexamethasone, BPCDX-CNFD hydrogels were removed from the PBS solution, immediately frozen to −80 °C overnight, and lyophilized for 5–6 h. Implants were then fixed for SEM analysis according to the instructions, sputter-coated with gold layers for 60 s at 0.1 bar vacuum pressure.

2.3. In vitro dexamethasone drug release from BPCDX-CNFD-Dex hydrogels

Six BPCDX-CNFD-Dex hydrogels were incubated in individual tubes, each containing 2 mL of sterile PBS, and maintained at 37 °C for a period of 60 days. At the sampling timepoint intervals of 6 h, 24 h, 3 d, 5 d, 10 d, 20 d, 30 d, 40 d, 50 d and 60 d, a sample from each tube was measured by UV spectrophotometry for absorbance at 240 nm for the determination of dexamethasone concentration. More details on the procedure are given in the supplementary methods document.

2.4. Cell culture conditions

Human umbilical vein endothelial cells (HUCVECs) were grown in Growth Medium according to the EGM-2 BulletKit™ formulation (CC-3162, Lonza Group Ltd, Basel, CH). The cells were incubated at 37 °C in 5% CO₂ in a humidified incubator, with culture medium replaced on a 48-hour basis and cell confluence always maintained below 80%. Commercially available primary human corneal epithelial cells (HCECs) were purchased (EpiGro™ Human Corneal Epithelial Cells, Merck KGaA, Darmstadt, DE) and cultured with EpiGro™ Ocular Epithelia Complete Media (Merck KGaA), at a density of 5000 cells/cm² in a 5% CO₂ humidified incubator at 37 °C.
2.5. Calcein AM fluorescent staining

Calcein acetoxyethyl ester (Calcein AM, C3099, Invitrogen, ThermoFisher) staining was used to visualize live cells under fluorescence. Details on the protocol are in the supplementary methods document.

2.6. Tube formation assay

5 × 10⁴ HUVECs per P48 well were seeded on top of polymerized GelTrex matrix. Four wells were designated for each of the 3 experimental conditions: control (normal growth medium), dexamethasone (15 μg/mL in growth medium), and irradiated dexamethasone (15 μg/mL in growth medium). After 24 h, the culture was stained with Calcein AM and imaged. The number of branches was measured in the ImageJ software (NIH, Bethesda, MD, USA) using the Angiogenesis analyzer plugin [33]. A detailed description of the protocol used is given in the supplementary methods document.

2.7. Cell migration assay

Ibidi migration assay 3-well silicone inserts (ibidi GmbH, Gräfelfing, DE) were used on a P6 well plate to create consistent 500 μm-width gaps between cell populations for the measurement of the rate of gap closure. 2 × 10⁴ HUVECs per well, were seeded into each insert well. Cells were incubated for 24 h and then stimulated for with 10 ng/mL TNFα. All the gaps were photographed under brightfield in an EVOS® FL microscope at the 0 h, 10 h and 20 h timepoints. After the 20 h timepoint, cells were stained with Calcein-AM, and representative photos were acquired from all gaps. The total gap area was measured using ImageJ software (FIJI distribution v1.52n). More details on the protocol and conditions used are found in the supplementary methods document.

2.8. Gene expression analysis

For the analysis of CCL2, CXCL5, MMP9, TIMP1, TIMP3 and VEGFA expression under dexamethasone treatment, 3 × 10⁵ HUVECs were seeded into each well of a P6 plate and incubated for 48 h followed by stimulation with 10 ng/mL TNFα. Detailed procedures, primers and reagents are described in the supplementary methods document.

2.9. Biocompatibility of BPCDX-CNFD and BPCDX-CNFD-Dex hydrogels

The pre-cast-to-8mm-diameter hydrogels were rinsed in PBS, equilibrated in EpigRO growth medium for 2 h in a humidified cell culture incubator, placed in a P48 well and allowed to adhere to the bottom of the well for 2 h at 37 °C. Primary HCECs were seeded at a density of 10⁵ cells/well in the P48 wells with adhered BPCDX-CNFD or BPCDX-CNFD-Dex hydrogels, and incubated for 1 h prior to adding additional medium to reach a final volume of 200 μL of growth medium per well. Growth medium was refreshed every 48 h and cells were maintained in culture for 16 days. On day 16, the cells were washed with PBS, covered with fresh growth medium, stained with Calcein AM and imaged as described above.

2.10. Implantation of biomaterials in a rabbit model of corneal inflammation and neovascularization

All animal experimental procedures were conducted after receiving approval by the Linköping Animal Research Ethics Committee (Application no. 1411) and following the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the Use of Animals in Ophthalmic and Vision Research.

12 male New Zealand white albino rabbits weighing 3–3.5 kg were randomized into two groups: a group receiving dexamethasone-loaded implants (group: BPCDX-CNFD-Dex, n = 6) and a control group of six rabbits receiving the non-loaded hydrogel (group: BPCDX-CNFD, n = 6). Operations were performed under general anesthesia (anesthetic details, as well as post-operative treatment detailed in supplementary methods document)

An Intralase iFS 150 kHz femtosecond laser (Abbott Medical Optics, CA, USA) was used to create central circular cuts in the corneal stroma of the right eye of all rabbits. Specifically, the laser created an 8.2 mm diameter circular lamellar cut 150 μm below the surface of the corneal epithelium, and an arc-shaped access cut subtending an angle of 50° and extending to the epithelial surface (Fig. 1). Directly prior to implantation, the 200 μm-thick hydrogels were manually trephined to yield implants of 8 mm diameter. Implants were then manually inserted in the corneal stroma through the access cut using surgical forceps. To induce postoperative inflammation leading to neovascularization, 4 surgical sutures (10–0 Nylon, Mani Inc., Japan) were placed intrastromally, parallel to the circular cut and located between the implant and the limbus, immediately following biomaterial insertion (Fig. 1).

At 8 weeks postoperative, operated eyes were clinically examined by in vivo imaging and photographic image documentation. Following the examinations, an anterior chamber paracentesis was done in operated eyes of all animals. Using a 1 mL syringe with a 26-gauge needle and insertion parallel to the iris plane at 9 o’clock in the right eye. 100 μL of aqueous humor were collected, flash frozen to −80 °C and saved for aqueous humor cytokine analysis. All animals were euthanized at 8 weeks postoperative, by pentobarbital overdose via injection in the ear vein while under deep anesthesia. The corneas were then excised for further analyses.

2.11. Postoperative clinical evaluation

In vivo live imaging of the operated cornea was performed pre-operatively and at the 8-week follow-up using a laser-scanning in vivo confocal microscope (IVCM) with corneal imaging module (HRT3-RCM; Heidelberg Engineering, Heidelberg, Germany). Morphological comparisons between dexamethasone and control groups were made in terms of subbasal nerves, inflammatory cell infiltration, thickness of anterior stromal haze, and endothelial cell density.

Anterior segment optical coherence tomography (AS-OCT; Optovue, Fremont, CA, USA) was performed to evaluate the morphology of the implant and surrounding cornea and to determine the thickness of the cornea before-, immediately after- and 8 weeks after operation. After bringing the cornea into focus, images of the central cornea were captured, and the average corneal thickness in a 2 mm central zone was automatically calculated and recorded.

The intraocular pressure of both operated and untouched corneas in all animals was measured at 8 weeks by a handheld rebound tonometer (iCare IC100; Medilens Nordic AB, Sweden) while under general anesthesia.

Operated eyes were additionally examined under an operating microscope and corneal haze/opacities and corneal neovascularization were graded according to the scale given in Table 1.

Neovessels infiltrating into the implants were manually marked in each photographic image of the cornea. ImageJ software was used to calculate the percentage area of vascularization in the cornea. All manual markings were performed by two independent observers in a masked manner and the mean values of the two measurements were used for statistical analysis.
2.12. Analysis of inflammatory cytokines in aqueous humor

Aqueous humor samples, collected and frozen at 8 weeks postoperatively, were sent to Tebu-Bio SAS (Le Perray-en-Yvelines, France) for analysis using the quantibody multiplex ELISA-based quantification method for rabbit cytokines. Protein levels were quantified in each sample relative to standard normal curves for the following cytokines: TNFα, NCAM-1, and MMP-9.

2.13. Histology - immunohistochemistry

Following euthanasia, all operated corneas were excised and fixed in 4% paraformaldehyde solution, embedded in paraffin blocks, and sectioned at 4 μm thickness. Details on tissue processing, sectioning, staining and microscopy imaging, as well as antibodies used and their concentrations are given in the supplementary methods section.

2.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc. CA 92037 USA). The data is presented using mean values and standard deviation (SD). The independent t-test was used for comparing data from two groups. One-way analysis of variance (ANOVA) using Tukey’s multiple comparison test was performed when comparing data from multiple groups. A two-tailed p-value < 0.05 was considered significant.

3. Results

3.1. Biodegradability and physical characterization of BPCDX-CNDF and BPCDX-CNDF-Dex hydrogels

The optical and mechanical properties of the dexamethasone loaded and control hydrogels are shown in Fig. 2. Although the incorporation of dexamethasone in BPCDX-CNDF-Dex reduced the visible light transparency of the material as measured at 200 μm thickness compared to the BPCDX-CNDF-
Fig. 2. Hydrogel material properties. A. Light transmission through the hydrogels at visible wavelengths (n = 3), overlaid against published values of human cornea light transmission [34,35] and BPCDX transmission [23]. B. Photograph of hydrogels over printed type, illustrating material transparency. Ctrl: BPCDX-CNF, Dex: BPCDX-CNF-Dex.
C-F. Mechanical properties of BPCDX [23], BPCDX-CNF and BPCDX-CNF-Dex hydrogels (n = 3). G. Water content of hydrogels (n = 3). H. Swelling ratio of hydrogels (n = 3).
I. Degradation of hydrogels under collagenase digestion over an 8 hr period (n = 9, 3, 2 for BPCDX, BPCDX-CNF and BPCDX-CNF-Dex, respectively). J. Dexamethasone in vitro cumulative release from BPCDX-CNF-Dex over a 60 day period, determined by photometric absorption, n = 6. A Richards’ 5 parameter logistic curve fit is overlayed on the chart. K. Scanning electron microscopy images of the anterior surface (left panel), the posterior surface (middle panel) and the cross-section (right panel) of BPCDX-CNF. Data presented as mean values ± SEM. * for p < 0.05, ** for p < 0.01, *** for p < 0.001, n/s for non-significant, p ≥ 0.05.
Dexamethasone material still maintained a higher level of transparency compared to the human cornea, with over 85% transmission (Fig. 2A) and did not render any skewed color gamut or perceivable light scattering when viewed against a white background and high contrast objects (Fig. 2B). Reinforcement of BPCDX with CNF from Ciona intestinalis resulted in improved mechanical properties compared to BPCDX properties reported previously (Fig. 2C–F). In particular, BPCDX-CNFX had both higher tensile stress (797.9 kPa vs 455.8 kPa, \( p = 0.0002 \)) and energy at maximum load (38.3 kPa vs 22.86 kPa, \( p = 0.0187 \)) compared to BPCDX, and also a higher Young’s modulus (10.81 GPa vs 6.29 GPa, \( p = 0.0002 \)), while optical and other properties were unaffected. Incorporating dexamethasone in the hydrogels did not have any significant impact on the material strength, with a lack of significant difference between BPCDX-CNFX vs BPCDX-CNFX-Dex materials regarding Young’s modulus, or tensile stress, strain and energy per unit volume under maximum load (Fig. 2C–F). Water content, which defines the percentage of water in the hydrogel and is an indirect measure of the degree of crosslinking of the hydrogel, was unaffected by the addition of dexamethasone (Fig. 2G). Hydrogels had a water content of about 87%, with a calculated cross-linking index of 1.136 ± 0.003 for BPCDX, 1.142 ± 0.014 for BPCDX-CNFX and 1.125 ± 0.033 for BPCDX-CNFX-Dex (non-significant differences). The swelling ratio (709 ± 70.3 vs 834.3 ± 153 for BPCDX-CNFX vs BPCDX-CNFX-Dex, respectively), was also non-significantly different between groups (Fig. 2H). Enzymatic degradation of both BPCDX-CNFX and BPCDX-CNFX-Dex hydrogels by collagenase was much faster than the human cornea but with no apparent differences between the two (Fig. 2I). SEM analyses indicated that the hydrogels presented a porous structure with a highly intertwined thin fiber network with porous regions randomly distributed between the fibers (Fig. 2K).

3.2. Drug release over time

To evaluate the drug release kinetics of the dexamethasone-loaded hydrogels, we measured the cumulative release of dexamethasone from BPCDX-CNFX-Dex hydrogels in vitro. This also served to determine the optimal dexamethasone concentration that would model the behavior of the implants in cell culture studies. The released drug amount followed a 5 parameter logistic Richards’ curve model, with an asymptote of approximately 49.2 μg (Fig. 2J). The initial drug release was about 8.2 μg (16.2% of total amount) within the first 6 h and 10.3 μg (20.4%) in the first 24 h. Half of maximum release (50%) was reached during day 9. The cumulative released amount would further increase to about 31.3 μg during the relatively rapid release phase (first ten days), where 61.8% of the drug had been released. A slower, gradual release continued until the experimental endpoint at day 60. For subsequent in vitro experiments, we used the concentration of 15 μg/ml, determined by the release data at the 10 day timepoint.

3.3. Cell migration, tube formation and proinflammatory cytokine expression in dexamethasone treated HUVECs

HUVECs were used to model the vascular endothelial cells that form new blood vessels in the cornea, under stimulation and concurrent dexamethasone treatment. Vascular endothelial cells are sensitive to the cytokine gradient in the cornea and can rapidly transform to a proliferative and migratory phenotype in an inflammatory environment [36]. In the cell migration assay, treatment of TNFα-stimulated HUVECs with dexamethasone resulted in inhibition of cell migration whereas in non-treated controls, the stimulated HUVECs migrated into the gap within 20 h, such that the gap was no longer visible (Fig. 3A, B). No difference was observed in the migration inhibition between UV-irradiated and non-irradiated dexamethasone.

Tube formation in HUVECs 24 h after stimulation with TNFα was inhibited by treatment with dexamethasone. Irregular linear formations were observed, but without typical tube-like structures. Conversely, HUVECs in the control group formed capillary-like tubular structures at the same time point (Fig. 3C). The tube-like structures were significantly longer in the control group compared to the dexamethasone group (\( p = 0.029 \)). Additionally, dexamethasone disrupted the formation of meshes. This disturbance in tube formation was similar with UV-irradiated or non-irradiated dexamethasone treatment. Protein and gene expression of the stimulated cells indicated suppression of CCL2 and CXCL5 pro-inflammatory chemokines with dexamethasone treatment relative to stimulated but untreated cells (Fig. 3E, F). In order to verify that irradiation of dexamethasone with UV under the crosslinking conditions does not affect its biological activity, we performed qPCR measurements on targets known to be affected by dexamethasone in HUVECs. For all the tested targets, no difference was observed regarding the UV irradiation status of the drug, with both treatments significantly upregulating TIMP1, MMP9 and VEGFA (Fig. 3G–J).

3.4. Biocompatibility of BPCDX-CNFX-Dex biomaterials with primary human corneal epithelial cells

As a major source of pro-inflammatory cytokines and chemokines in the cornea is the corneal epithelium, we sought to determine whether dexamethasone-loaded implantable materials could impact the ability of human corneal epithelial cells to release important chemokines during an inflammatory response. Both BPCDX-CNFX-Dex and BPCDX-CNFX samples were populated with human corneal epithelial cells in vitro and maintained for 16 days in culture. Both materials supported viable and adherent epithelial cells (Fig. 4A). Following TNFα stimulation, expression of CCL2 and CXCL5 in HCECs cultured on BPCDX-CNFX-Dex was significantly decreased relative to HCECs cultured on control non-loaded BPCDX-CNFX (Fig. 4B, \( p = 0.01 \) for both chemokines).

3.5. In vivo implantation and evaluation in a rabbit corneal inflammation model

All femtosecond laser procedures were completed without incident and biomaterials were successfully inserted in the corneal stroma through the femtosecond laser access cut without complications. Four sutures were also successfully placed intrastromally in a circumferential pattern parallel to implants outside the implanted zone as in Fig. 1.

In vivo and ex vivo images of all operated corneas in this study are provided in Fig. 5A. Of the operated corneas, 10 of 12 were either transparent or had only slight opacity with the iris details posterior to implanted corneas clearly visible at 8 weeks. Both corneas with significant haze were from the BPCDX-CNFX control group; however, ordinal scoring of opacification did not indicate a difference between the groups (Fig. 5B). BPCDX-CNFX-Dex implanted corneas had a lower total number of invading vessels which were mainly restricted to the periphery of the cornea, around the sutures and outside the implanted zone (Fig. 5A, C–E). Only one BPCDX-CNFX-Dex-implanted cornea had vessels entering the implanted zone, compared to four corneas in the BPCDX-CNFX group at 8 weeks (Fig. 5C). The difference between treatment and control groups at 8 weeks however, did not reach significance (Fig. 5C–E).

Measurement of intraocular pressure at 8 weeks revealed a significantly higher pressure (9.4 ± 1.4 mmHg) in the eyes with BPCDX-CNFX-Dex implants relative to eyes receiving the BPCDX-CNFX material (5.4 ± 0.8 mmHg, \( p = 0.03 \); Fig. 5F). This result confirmed the sustained delivery of dexamethasone, which is known
to increase intraocular pressure. The intraocular pressure of non-operated eyes was 3.5 ± 0.2 mmHg. Only in the BPCDX-CNF-Dex group was the difference between operated and non-operated eyes statistically significant (p = 0.004).

All implants were examined with AS-OCT directly after operation and at 8 weeks. The gross morphology of the surrounding tissue observed with OCT was not affected by the surgical technique or the implant type and all implants remained in place after 8 weeks (Fig. 6A). Although most of the corneas were clinically transparent with no haze formation (Fig. 5A), AS-OCT revealed a modest hyper-reflectivity in the implanted region and the surrounding stroma (Fig. 6A) which was, however, of no clinical
Fig. 4. Hydrogel biocompatibility. A. Representative images of corneal epithelial cells grown on BPCDX-CNF and BPCDX-CNF-Dex hydrogels after 16d of in vitro culture. B. Gene expression profile for CCL2 and CXCL5 in primary human corneal epithelial cells grown on BPCDX-CNF or BPCDX-CNF-Dex after TNFα stimulation (n = 4 per group). Data are presented as mean values ± SEM. * indicates p < 0.015.

Fig. 5. Corneal opacity, corneal neovascularization and intraocular pressure after implantation of BPCDX-CNF. A. Photographs of the operated eye at 8 weeks follow-up for the BPCDX-CNF and BPCDX-CNF-Dex groups. In vivo images were taken prior to sacrifice, while ex vivo images of the cornea were taken immediately after cornea extraction. Black arrows indicate vessels invading the cornea. B. Score of corneal haze at 8 weeks. C-E. Neovascularization assessment at 8 weeks, presented as percentage of area vascularized, assigned score to each cornea and number of vessels reaching the implant. F. Intraocular pressure of the right (operated eye) and the left non-operated eye at 8 weeks. Data are presented as individual values, overlayed with mean group value ± SEM. * for p < 0.05, ** for p < 0.01, *** for p < 0.001.
importance (haze score ≤ 1) in 10 out of 12 operated corneas (Fig. 5B) at 8 weeks. The central corneal thickness directly after operation was significantly increased compared to preoperatively in both groups (p = 0.0047 for group Control, p = 0.0006 for group Dex), accounting for the extra material added (Fig. 6B, C). At 8 weeks however, the BPCDX-CNF-Dex-implanted corneas maintained a significant thickness increase while the BPCDX-CNF-implanted corneas had a mean thickness not significantly different from preoperative, with half the rabbits in the BPCDX-CNF group (n = 3) exhibiting a thickness decrease, indicating some post-operative thinning. No correlation was found in pairwise comparisons of corneal thinning and haze. While the central corneal thickness increase in BPCDX-CNF implanted corneas was not maintained at 8 weeks (Control pre-op vs 8 weeks post-op p = 0.30), in BPCDX-CNF-Dex-implanted corneas the thickness increase was maintained relative to the preoperative thickness (BPCDX-CNF-Dex group pre-op vs 8 weeks post-op p = 0.003) (Fig. 6C).

3.6. In vivo and ex vivo morphological examination of the cornea

Corneal layers were visualized in vivo pre- and post-operatively by in vivo confocal microscopy (IVCM). Preoperatively, parallel-running subbasal nerves were observed, while the anterior stroma consisted of regularly distributed quiescent keratocytes, and the endothelium was clearly visible and consisted of a regular pattern of hexagonal cells (Fig. 7A). Using the femtosecond laser intrastromal surgical technique, the endothelium remained intact following operation in both groups (Fig. 7A). In the BPCDX-CNF-Dex group, the endothelial cell density as determined by IVCM did not have any detectable difference postoperatively relative to the pre-
operative level (Fig. 7B). Despite a tendency towards lower density post-operatively, the difference in BPCDX-CNf controls was also non-significant. The BPCDX-CNf group, however, had significantly reduced endothelial cell density relative to the BPCDX-CNf-Dex group (p = 0.035).

Interestingly, IVCM has the capacity to detect sub-clinical levels of haze that impact the collagen organization within the ECM. In 4 out of 6 BPCDX-CNf-implanted corneas, an anterior corneal haze obscured the anterior keratocytes (Fig. 7A, C). No anterior haze was found in the BPCDX-CNf-Dex group and keratocytes were more easily visible, although some increase in reflectivity was present indicating keratocyte activation (Fig. 7A). In both BPCDX-CNf and BPCDX-CNf-Dex, fewer nerves were visible at 8 weeks postoperatively compared to preoperatively. In the BPCDX-CNf-Dex group, 5 of 6 corneas had detectable sub-basal nerves while the respective count in BPCDX-CNf was 3 out of 6 (Fig. 7D). Inflammatory cells were observed in the implants and surrounding stroma in the BPCDX-CNf group. In BPCDX-CNf, macrophages were found in 2 out of 6 cases, identified by oval-formed cells with dark nuclei [21,37], while inflammatory stromal cells of rounded and elongated form were present in the anterior stroma in 5 of 6 cases. By contrast, no inflammatory cells were observed in the stroma of the BPCDX-CNf-Dex group with IVCM (Fig. 7E). This inflammation in the BPCDX-CNf group versus no detectable inflammation in BPCDX-CNf-Dex was statistically significant (Contingency Fisher’s exact test p = 0.0152). Moreover, the BPCDX-CNf group presented with implants that contained what appeared to be a leukocytic infiltrate (Fig. 7A). Vessels observed macroscopically (Fig. 5A, C) were also visualized with IVCM in both groups (Fig. 7A). However, the vessels in the BPCDX-CNf-Dex group were mostly restricted to the peripheral region around the sutures with 1 of 6 corneas having vessels entering the implanted zone, while in BPCDX-CNf, vessels were observed in the central corneal region in 4 of 6 cases (Fig. 5A, C and Fig. 7A).

3.7. Inflammatory cytokines and markers in aqueous humor

An ELISA-based anterior chamber cytokine analysis was performed as an indirect measure of sustained inflammatory activity in the cornea 8wks postoperatively (Fig. 8A). The concentra-
tion of TNFα was significantly suppressed in the BPCDX-CNF-Dex group relative to the BPCDX-CNF group (p = 0.0055). NCAM-1 (neural cell adhesion molecule 1), a putative marker of the health of corneal endothelial cells [38], did not differ between groups. The protease MMP-9, however, was also suppressed in the BPCDX-CNF-Dex group, corroborating the finding of implant degradation in the BPCDX-CNF group, versus intact implants in the BPCDX-CNF-Dex group.

3.8. Histology of the explanted corneas

H&E staining revealed that implants were still present after 8 weeks, however, in the BPCDX-CNF group the hydrogel material was partially degraded with segments visible and large portions of degraded implant (Fig. 8B). The BPCDX-CNF-Dex group had intact implant borders, while the BPCDX-CNF group had degraded implant borders (Fig. 8C). In addition, collagen surrounding the BPCDX-CNF implants exhibited disrupted organization. In the BPCDX-CNF-Dex group, implants were intact without signs of degradation, with regular appearance of surrounding collagen. Inflammatory cell infiltration was evaluated by immunostaining against CD45. CD45+ leukocytes were not found in the BPCDX-CNF-Dex group but were present in the stroma of corneas with BPCDX-CNF implants (Fig. 8C). Furthermore, degradation was observed in both central and peripheral parts of the BPCDX-CNF implants, whereas the BPCDX-CNF-Dex implants and implant edges remained intact (Fig. 8C). Immunostaining against α-SMA indicated that myofibroblasts were present in both groups but the BPCDX-CNF group had considerably more α-SMA positive cells that were mostly localized in close apposition to the implants.

4. Discussion

In this study, we successfully improved the mechanical characteristics of earlier formulations of an implantable bioengineered porcine collagen hydrogel [BPC] [20–22] and BPCDX [23] by incorporating cellulose nanofibers (CNF) extracted from the sea invertebrate Ciona intestinalis to stiffen the hydrogel. The BPCDX implanted in human corneas with advanced keratoconus exhibited resistance to degradation in vivo and the increase in corneal thick-
ness was sustained for at least 2 years after implantation, but the tensile strength and Young's modulus as measured in laboratory tests did not match the human cornea [23]. It is still unknown whether the ideal biomaterial should be as strong as the human cornea, and matching all the mechanical properties of the native cornea may be not necessary for bioengineered tissue to be optimally effective. At this stage of the technology development however, the native tissue properties are still benchmarks for the bioengineered materials to be compared against and be targeted towards. To mimic the natural extracellular matrix of the corneal stroma, which is mainly composed of collagen and proteoglycans [39], cellulose nanofibers (CNFs) were added to the medical-grade porcine collagen to introduce new chemical bonds between collagen fibrils and CNF similar to collagen-proteoglycan interactions in the cornea. Cellulose-based materials are promising for bioengineering because of their diversity and versatility of biochemical and biophysical characteristics [24,40], but only a few reports analyze their suitability in corneal applications [41–43]. More specifically, bacterial nanocellulose (BNC) has been studied as a possible carrier to support limbal stem cell culture and transplantation [43] or as an alternative to amniotic membrane [42]. Cellulose nanocrystal (CNC)-reinforced Poly(vinyl alcohol) (PVA) hydrogels have been studied for their in vitro biocompatibility with human corneal epithelial cells but further in vitro and in vivo studies are required before their suitability as contact lens or materials for corneal regeneration can be determined [41]. To our knowledge, this is the first time that CNF extracted from Ciona intestinalis, a sustainable source of CNF obtained from aquaculture, is used in corneal bioengineering both in vitro and in vivo in an animal model. Notably, the CNF-reinforced double-crosslinked hydrogel had a Young's modulus comparable to that of the human cornea, with significantly enhanced mechanical properties relative to the BPCDX. Moreover, the biomaterials and methods used to develop the BPCDX-CNFDex are fully natural, minimizing the potential for toxicity while improving biocompatibility, as demonstrated by supporting growth of human corneal epithelial cells in vitro. The porous nature of the hydrogels [20,22] was not altered by adding CNF as shown by electron microscopy. We have previously demonstrated that after in vivo implantation of porous hydrogels [20,22] cells migrate into the bulk of the porous biomaterials through the pores or by expressing enzymes that degrade the hydrogel, thus facilitating cell population within the hydrogel and new collagen production by these invading cells [22].

Due to challenges associated with steroid eye drops such as low bioavailability and patient non-compliance, patient-friendly methods of sustained steroid delivery have been proposed as a means of improving the treatment of anterior ocular inflammation. Drug eluting contact lenses [11], polymeric films [10,15] and nanofibers [13] have been used to deliver dexamethasone, but only for 1–7 days in vivo. Collagen has previously been used as an ocular drug delivery system, as degradable shields [6], or within composite hydrogels [44,45], but with short drug delivery times of hours to days. In a previous study, we demonstrated that collagen-based hydrogels could be successfully loaded with NGF with sustained drug release for up to two months in vitro [20]. Here, for the first time, we loaded dexamethasone within the reinforced BPCDX hydrogel (BPCDX-CNFDex) and analyzed the effects of dexamethasone released by the hydrogels in vitro and in vivo in a model of postoperative inflammation. The irradiation of dexamethasone with ultraviolet-A light did not affect its inhibitory properties in vitro and did not appear to affect its anti-inflammatory properties in vivo. BPCDX-CNFDex was mechanically stronger than BPC [22] and BPCDX [23] while still maintaining transparency, even after dexamethasone loading.

In contrast to the short delivery times in the prior studies mentioned above, dexamethasone release from BPCDX-CNFDex was sustained for at least two months in vitro with continued effect in vivo. An initial release burst of 10 days was observed in vitro, followed by weeks to months of sustained and gradually tapered steroid delivery, which gives the desired clinical effect. The release profile from BPCDX-CNFDex would be ideal in cases following corneal transplantation as it offers rapid initial release when the inflammatory response and rejection risk following surgery is highest, followed by more gradual and sustained release over a longer period when rejection risk still exists but is much lower. Given the proper models of drug release, which can predict the concentration at the limit plateau, a desired drug concentration can be achieved in a gradual manner and sustained for prolonged periods. However, the time point when the hydrogels release the total amount of loaded dexamethasone is still unknown as the study duration of both in vitro and in vivo studies was two months. Ideally, the amount of dexamethasone in the aqueous humor (in vivo pharmacokinetics) should be determined at various time points [10] until dexamethasone is no longer detected. Based on the present results, the optimal loading with dexamethasone that would enable release over a predetermined period while maintaining transparency could in principle be found. Nevertheless, we confirmed the biological activity of the released dexamethasone by significantly decreased expression of the pro-inflammatory chemokines CCL2 and CXCL5 in HCECs grown on BPCDX-CNFDex hydrogels relative to BPCDX-CNFDex. Sustained suppression of TNFα and MPP9 in the aqueous humor of the eyes implanted with BPCDX-CNFDex after two months further demonstrates the long-term bioactivity of the released steroid. BPCDX-CNFDex hydrogels inhibited blood vessel growth into the implants, likely due to the effect on vascular endothelial cell migration, tube formation and significant downregulation of pro-inflammatory cytokines such as CCL2, CXCL5 and TNFα. These findings are in accordance with earlier studies exploring the effect of dexamethasone in inflammatory corneal conditions [26,29]. Although there was significant suppression of pro-inflammatory markers and inflammatory cells in this study, only a tendency towards reduced neovascularization and suppressed central vessel invasion in the BPCDX-CNFDex group was observed. This is likely due to the limited study group size and the use of small peripheral sutures which resulted in only a modest neovascular response, even in the control group. Future studies aiming to evaluate the effect of dexamethasone release not only in inflammation but also in neovascularization should ideally use more aggressive models such as alkali burn injury [13] to trigger a stronger pro-angiogenic corneal environment.

Nevertheless, the model was aggressive enough to trigger the release of enzymes such as MPP9 in vivo, resulting in BPCDX-CNFDex implant degradation, myofibroblast recruitment to the implant site, and collagen degradation and reorganization. By contrast, BPCDX-CNFDex implants remained intact without signs of degradation, and OCT and histology examinations confirmed a maintained corneal thickness. Microscopically, dexamethasone resulted in significant suppression of inflammatory cell migration and myofibroblast-induced corneal haze, thereby suppressing clinically-visible inflammation that could potentially lead to rejection of the graft. Although in our in vitro enzymatic degradation studies both materials exhibited similar degradability properties, resistance to degradation and thinning of the BPCDX-CNFDex implants in vivo, confirmed by both OCT and histology, can be attributed to a reduced extracellular matrix degrading enzymatic activity, which we also partially documented with the suppression of MPP9, and reduced inflammatory cell invasion.

Endothelium had similar morphology and density compared to preoperatively in both groups indicating that neither operation, which is a pure intrastromal technique, nor dexamethasone release from the implants affected the endothelial cells. This was also reflected by NCAM-1 expression in the anterior chamber that did
not differ between groups and relative to untouched eyes. Multiple studies have demonstrated that different administration routes of dexamethasone in the eye have most often no or beneficial effect on corneal endothelial cells depending on underlying pathology [46–48]. Moreover, we have previously demonstrated that endothelium remains intact after this minimally invasive surgical technique that enables pure intrastromal implantation of hydrogels [23].

The eyes implanted with dexamethasone-loaded hydrogels had sustained increase in intraocular pressure relative to controls. This increase could be explained by the significantly thicker corneas in the dexamethasone group compared to controls two months after operation due to the known effect of corneal thickness in intraocular pressure measurements, with thicker corneas resulting in falsely higher intraocular pressures [49]. However, higher intraocular pressure as a side-effect due to dexamethasone release from the implants cannot be excluded. In that case, whether this level of increase in rabbits is clinically significant or how this would translate to humans is unknown and would require further investigation. Nevertheless, a pilot study on the safety of dexamethasone-releasing subconjunctival implants in human patients did not report any significant IOP increase or other adverse event related to the implant [29,50]. A clinically significant IOP increase, if it would arise, could be treated with common pressure-lowering medications or implant removal. However, as the amount of dexamethasone released was shown to taper off substantially within two months, a detrimental long-term effect of drug release is not expected.

In this study hydrogels were implanted without removing part of the stroma, resulting in thicker corneas directly after operation. This increased thickness was maintained up to two months in the BPCDX-CNF-Dex hydrogels. This surgical technique is intended to treat a wide range of corneal diseases, from ectatic corneal conditions like keratoconus to challenging cases of corneal melting or even perforations due to infections, neurotrophic keratitis and surgical/chemical injury to the cornea which require restoration of corneal thickness and integrity in combination to anti-inflammatory therapy [51]. In low-risk cases with preserved angiogenic privilege, such as keratoconus or pellucid marginal degeneration, a dexamethasone loaded implant could augment the corneal thickness without tissue removal and without the need of self-administering drops after operation. In high-risk cases with deeper scarring and vessels, thicker BPCDX-CNF-Dex hydrogels could either augment the corneal stroma or replace the opaque part, based on our earlier studies showing that femtosecond laser-assisted keratoplasty enables removal of different proportions of the corneal stroma and that hydrogels can be manufactured with different thicknesses and diameters [20–22]. Thicker implants would incorporate a greater total amount of dexamethasone that is desirable in such cases. Finally, besides the use of the present material as a corneal implant, applications as a contact lens-like onlay could be envisioned, where the drug-eluting properties could be used for therapeutic drug delivery.

Among the limitations of this study is that the time point of total dexamethasone release is still unknown due to the short duration of both in vitro and in vivo studies. The small study groups and the inflammatory surgical model that induced only a mild vascular response even in the control group did not allow us to compare the degree of neovascularization and haze formation between the groups even though a tendency towards reduced neovascularization was observed in the dexamethasone group. The IOP increase in the dexamethasone group and its clinical importance need to be further elucidated. If this alternative administration route of dexamethasone is considered to replace the traditional topical route in form of drops, a direct comparison of these two delivery methods in term of efficacy and complications needs to be performed in future studies. Finally, the long-term stability of the BPCDX-CNF needs to be determined. Given the present results, the need for long-term shelf life studies is warranted, to evaluate material parameters before and after storage, and drug activity in the longer term.

In conclusion, we demonstrate a transparent hydrogel composed of collagen, reinforced by nanocellulose fibers and strengthened by combined chemical and photochemical crosslinking (BPCDX-CNF). BPCDX-CNF is a sustainably sourced, natural bio-material composed of porcine dermal collagen as a byproduct of the food industry, while CNF is produced by sustainable aquaculture. This confers the advantages of abundance, renewability and biocompatibility. Moreover, BPCDX-CNF can be successfully loaded with dexamethasone that can effectively suppress inflammation for at least two months following implantation, potentially providing a way to overcome the need for postoperative dexamethasone eye drops following surgery. Corneal transparency could thus be maintained with a simplified surgical procedure and drug delivery method applicable to low-resource areas where alternatives to human donor corneas, standard invasive surgeries, and need for postoperative medications are required.

Declaration of Competing Interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Neil Lagali, Mehrdad Rafat, Anthony Mukwaya, Marco Belisario reports financial support was provided by European Commission. Mehrdad Rafat reports a relationship with LinkoCare Life Sciences AB that includes: board membership and equity or stocks. Shideh Tabe reports a relationship with LinkoCare Life Sciences AB and that includes: board membership. Beatrice Pecho reports a relationship with Memira Eye Center Scandinavia that includes: employment. Mehrdad Rafat reports a relationship with Naturalens AB that includes: board membership. Shideh Tabe reports a relationship with NaturaLens AB that includes: board membership.

Data availability statement

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons.

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Supplementary materials


References

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