Graefe's Archive for Clinical and Experimental Ophthalmology (2024) 262:2569–2577
https://doi.org/10.1007/s00417-024-06451-8

CORNEA

Real-time monitoring of riboflavin concentration using different clinically available ophthalmic formulations for epi-off and epi-on corneal cross-linking

Marco Lombardo[1,2] · Sebastiano Serrao[1,2] · Giuseppe Massimo Bernava[3] · Rita Mencucci[4] · Mario Fruschelli[5] · Giuseppe Lombardo[1,3]

Received: 4 December 2023 / Revised: 27 February 2024 / Accepted: 8 March 2024 / Published online: 19 March 2024

Abstract

Purpose To assess the feasibility of theranostics to determine the riboflavin concentration in the cornea using clinically available ophthalmic formulations during epithelium-off (epi-off) and transepithelial (epi-on) corneal cross-linking procedures.

Methods Thirty-two eye bank human donor corneas were equally randomized in eight groups; groups 1 to 3 and groups 4 to 8 underwent epi-off and epi-on delivery of riboflavin respectively. Riboflavin ophthalmic solutions were applied onto the cornea according to the manufacturers' instructions. The amount of riboflavin into the cornea was estimated, at preset time intervals during imbibition time, using theranostic UV-A device (C4V CHROMO4VIS, Regensight srl, Italy) and expressed as *riboflavin score* (d.u.). Measurements of corneal riboflavin concentration (expressed as μg/cm^3) were also performed by spectroscopy absorbance technique (AvaLight-DH-S-BAL, Avantes) for external validation of theranostic measurements.

Results At the end of imbibition time in epi-off delivery protocols, the average *riboflavin score* ranged from 0.77 ± 0.38 (the average corneal riboflavin concentration was 213 ± 190 μg/cm^3) to 1.79 ± 0.07 (554 ± 103 μg/cm^3). In epi-on delivery protocols, the average *riboflavin score* ranged from 0.17 ± 0.01 to 0.67 ± 0.19 (corneal riboflavin concentration ranged from 6 ± 5 μg/cm^3 to 122 ± 39 μg/cm^3) at the end of imbibition time. A statistically significant linear correlation ($P \leq 0.05$) was found between the theranostic and spectrophotometry measurements in all groups.

Conclusions Real-time theranostic imaging provided an accurate strategy for assessing permeation of riboflavin into the human cornea during the imbibition phase of corneal cross-linking, regardless of delivery protocol. A large variability in corneal riboflavin concentration exists between clinically available ophthalmic formulations both in epi-off and epi-on delivery protocols.

Keywords Riboflavin · Corneal cross-linking · Theranostics

✉ Marco Lombardo
mlombardo@visioeng.it

✉ Giuseppe Lombardo
giuseppe.lombardo@cnr.it

1 Studio Italiano di Oftalmologia, Via Livenza 3, 00198 Rome, Italy

2 Vision Engineering Italy Srl, Via Livenza 3, 00198 Rome, Italy

3 CNR-IPCF, Istituto per i Processi Chimico-Fisici, Viale F. Stagno D'Alcontres 37, 98158 Messina, Italy

4 SOD Oculistica, AOU Careggi, Università di Firenze, Largo Brambilla 3, 50134 Florence, Italy

5 Sezione Oftalmologia, Policlinico Santa Maria alle Scotte, Università di Siena, Dip. Scienze mediche-chirurgiche e neuroscienze, Viale Bracci, 53100 Siena, Italy

Key messages

What is known:

- In the epi-off CXL protocol, riboflavin is expected to enrich homogeneously the corneal stroma regardless of the specific ophthalmic formulation used.
- In the epi-on CXL protocol, riboflavin is unable to diffuse efficiently into the corneal stroma through the intact epithelium.

What is new:

- Theranostics enables the operator to have a real-time assessment of the permeation of riboflavin into the corneal stroma during CXL treatment, regardless of the delivery strategy or the specific formulation of riboflavin used.

Introduction

Corneal cross-linking (CXL) has been established as an elective treatment option for patients with progressive keratoconus for more than 15 years in Europe and since 2016 also in the USA [1–4]. Clinical studies have reported an average K_{max} flattening (i.e., the maximum keratometry point of anterior corneal topography) of -1.0 ± 0.8 D at 1-year follow-up after standard treatment protocol, which requires for epithelial debridement (i.e., epithelium-off or epi-off protocol) [1–4]. Although effective for stabilizing the cornea, the epithelium-off CXL protocol has been associated with a 1.3% risk of sight-threatening severe adverse events from infectious keratitis, corneal burn, and corneal scarring and up to 20% risk of moderate adverse events (i.e., corneal haze, corneal infiltrates). [2, 5]

In an effort to accelerate visual rehabilitation and reduce both postoperative patient discomfort and the risk of adverse events associated with epithelial debridement, the concept of transepithelial (or epi-on) delivery of riboflavin has gained increasing interest among investigators in the last decade.

Based upon the knowledge that the epithelium does not block UV-A light radiation for efficient photo-activation of stromal riboflavin [6–8], most of research has focused on the development of ophthalmic formulations for improving the permeation of riboflavin through the intact epithelium. Previous investigations have found that dextran-enriched riboflavin formulations have limited ability to penetrate through the intact epithelium [9, 10]. As a result, dextran-free riboflavin ophthalmic solutions have been developed to facilitate the passage of riboflavin across the intact epithelium. Hydroxypropyl methycellulose (HPMC) has been often used as vehicle and additional chemical agents, such as benzalkonium chloride (BAC), ethylenediamine tetraacetic acid (EDTA), trometamol (Tris), vitamin E-TPGS (α-Tocopheryl Polyethylene Glycol Succinate), and sodium chloride (NaCl), have been added to enhance the penetration of riboflavin through the intact corneal epithelium. Despite these efforts, concerns still remain about the clinical efficacy of transepithelial CXL protocols [11]; the limits of epi-on delivery protocols have been associated with the relatively high molecular weight of riboflavin (340 Da), the decreasing permeability of the corneal epithelium to molecules greater than 180 Da, and the inability to understand, during the imbibition phase of CXL, the effective penetration of the therapeutic substance into the corneal stroma. As riboflavin is essential to the CXL process, acting both as a photosensitizer for activating the formation of new cross-linking chemical bonds among stromal proteins and as an absorber of UV-A light to prevent damage to corneal endothelium and internal ocular structures, the effectiveness of the therapy relies on the adequate absorption of riboflavin into the corneal stroma [12–15]. Recently, measurement of riboflavin concentration into the cornea has been attained non-invasively by exploiting theranostics, which is an emerging technology that combines therapy and diagnostics [16–18]. This approach involves the use of riboflavin not only as a therapeutic agent in CXL, but also as a probe for spectroscopic measurement of its concentration in the cornea. By harnessing theranostics, clinicians can simultaneously treat and measure riboflavin concentration non-invasively during CXL [16–18].

In this study, we used a theranostic UV-A device to measure and compare riboflavin stromal penetration in eye-bank human donor tissues following application of several clinically available riboflavin ophthalmic formulations developed for epithelium-off and/or transepithelial delivery protocols for corneal cross-linking. Spectrophotometry measurements were performed on the same corneal samples and analyzed for external validation of theranostic measurements.

Materials and methods

Thirty-two eye bank human sclero-corneal tissues from different donors were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy). The samples were shipped to the laboratory in 6% dextran-enriched corneal storage medium and were used for experiment within 8 h. Inclusion criteria included an endothelial cell density (ECD) > 1600 cells/mm^2; exclusion criteria included history of corneal pathologies, traumas, or eye surgery. The study adhered to the tenets of Declaration of Helsinki for the use of human tissues.

At the beginning of experiment, central corneal thickness (CCT; µm) was measured in each corneal tissue using anterior segment optical coherence tomography (HS-100, Canon, Japan). Measurement of CCT was also repeated at the end of experiment.

The sclero-corneal tissues were randomly and equally divided, using random number generation sequence in SPSS statistical software (SPSS Inc., ver. 17, IBM, US), into eight treatment groups, as described below:

- (Group 1-Off) Epithelium-off kit including 20% dextran-enriched 0.1% riboflavin and dextran-free 0.1% riboflavin delivery: following complete debridement of corneal epithelium with an Amoils' brush, riboflavin eye drops containing 0.146% riboflavin 5-phosphate and 20% dextran (Photrexa Viscous, Glaukos corp., USA) were applied onto the stromal surface every 2 min for 30 min. If the corneal thickness at the end of imbibition time was below 400 µm, a dextran-free 0.146% riboflavin 5-phosphate solution (Photrexa, Glaukos corp., USA) should be applied every 5 to 10 s until the corneal thickness increased to at least 400 µm.
- (Group 2-Off) Epithelium-off 0.22% riboflavin delivery: following complete debridement of corneal epithelium with an Amoils' brush, riboflavin eye drops containing 0.30% riboflavin 5-phosphate (equivalent to 0.22% riboflavin base; RitSight, Regensight srl, Italy) were applied onto the stromal surface every 20 s for 15 min.
- (Group 3-Off) Epithelium-off 0.1% riboflavin sodium phosphate delivery: following complete debridement of corneal epithelium with an Amoils' brush, eye drops containing 0.1% riboflavin 5-phosphate (Riboker, Iromed Group srl, Italy) were applied onto the stromal surface every 30 s for 15 min.
- (Group 4-On) Transepithelial 0.1% riboflavin delivery: Vitamin E TPGS-enriched 0.1% riboflavin (Ribofast, Iromed Group srl, Italy) eye drops were applied onto the cornea with intact epithelium every 10 s for 10 min.
- (Group 5-On) Transepithelial kit including HPMC-enriched 0.22% riboflavin and 0.25% riboflavin delivery (ParaCel kit, Glaukos Corp. USA): ParaCel Part 1 (i.e., the HPMC-enriched riboflavin) was applied onto the anterior surface of epithelium-intact corneas every 90 s for 4 min; this was followed by the application of ParaCel Part 2 eye drops every 90 s for additional 6 min.
- (Group 6-On) Transepithelial HPMC-enriched 0.25% riboflavin delivery (Mediocross TE, Glaukos Corp., USA): the eye drops were applied onto the anterior surface of epithelium-intact corneas every minute for 20 min.
- (Group 7-On) Transepithelial 0.22% riboflavin delivery: 0.30% riboflavin 5-phosphate solution (equivalent to 0.22% riboflavin base; RitSight, Regensight srl, Italy) eye drops were applied onto the anterior surface of epithelium-intact corneas every 20 s for 20 min.
- (Group 8-On) Transepithelial 0.1% riboflavin sodium phosphate: 0.1% riboflavin 5-phosphate solution (Riboker Iromed Group srl, Italy) was applied onto the corneal surface with intact epithelium every 30 s for 10 min.

The riboflavin ophthalmic formulations were applied onto the corneal samples according to the manufacturers' instructions for use, as summarized in supplementary Table 1. Application of riboflavin was done using the same methodology in all tissues, as follows: each tissue was placed in an artificial anterior chamber (AAC, Coronet, Network Medical Products Ltd, North Yorkshire, UK) pressurized with the AAC filled with 0.9% sodium chloride; the AAC was connected, through tubing, to a column manometer in order to maintain intracameral pressure within a physiological range during experiment. The samples undergoing epithelium-off protocol were de-epithelialized immediately before commencing the experiment using an Amoils' brush (Innovative Excimer Solutions Inc., Toronto, Canada). The corneal surface of samples undergoing epithelium-on protocol was gently washed with balanced salt solution before each optical measurement.

Data acquisition and analysis

The theranostic UV-A device (C4V CHROMO4VIS, Regensight srl, Italy) was used to measure the concentration of riboflavin in the cornea during the application phase of CXL treatment. At pre-set time intervals (i.e., every 5 min), the theranostic device irradiated the cornea with 3 mW/cm^2 UV-A power density for a few seconds; thereafter, it processed corneal fluorescence image and provided the operator with a real-time measure estimating the corneal riboflavin concentration, as previously described [16–18]. Sensitivity of the method is 10 µg/cm^3. The corneal riboflavin concentration estimated by analysis of theranostic images was expressed using an imaging biomarker, i.e., the *riboflavin score*, which was calculated for each corneal sample using a 2nd-order polynomial function that proportionally depends

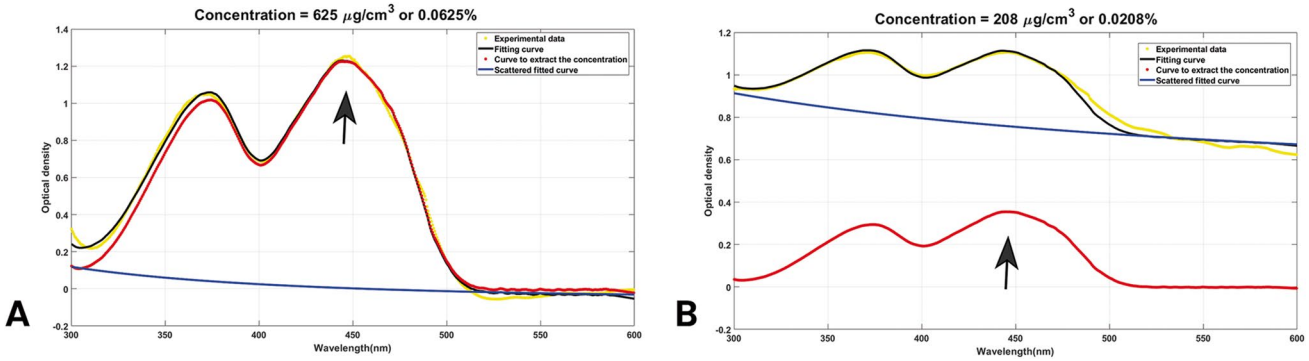

Fig. 1 The figure displays the optical density experimental data ($A(\lambda)$; yellow curve), the analytical curve fit ($A_p(\lambda)$; black curve) with Eq. 1 (see the "Materials and methods" section) together with the contributions to the fit (i.e., riboflavin absorption, $cA_W s$, red curve, and corneal scattering, $a\lambda^{-n} + b$, blue curve). The corneal riboflavin concentration was estimated at 445-nm wavelength (arrows). Two representative samples are shown: **A** after 15 min and **B** after 20 min of imbibition with 0.22% riboflavin base ophthalmic solution (i.e., group 2-Off and group 7-On respectively)

on the green intensity signal acquired by the RGB camera of the theranostic device [16]. The *riboflavin score* was expressed in dimensionless unit (d.u.) [16–18].

A spectrophotometer (Avaspec 2048L, Avantes BV, Apeldoorn, The Netherlands) was used for measuring the spectrum of absorbance (or optical density) of each sample at the same intervals of theranostic measurements. The light beam (200- to 2500-nm range) was generated by a deuterium-halogen lamp (AvaLight-DH-S-BAL, Avantes BV, Apeldoorn, The Netherlands) and collimated using a 6.0-mm diameter lens with 8.7-mm focal length. The light was delivered to the corneal surface using a 600-μm diameter solar resistant optical fiber with 0.22 numerical aperture (FC-UV-600–2-SR, Avantes BV, Apeldoorn, The Netherlands). The resulting beam was focused at the front surface of the central cornea with a 2.0-mm diameter. After passing through the cornea, the light beam was collected and focused by a lens, with the same aforementioned characteristics, on an analogous solar resistant optical fiber to a spectrophotometer (Avaspec 2048L, Avantes BV, Apeldoorn, The Netherlands) connected to a computer for data acquisition and processing. Before each experiment on a corneal sample, the setup was calibrated by acquiring dark

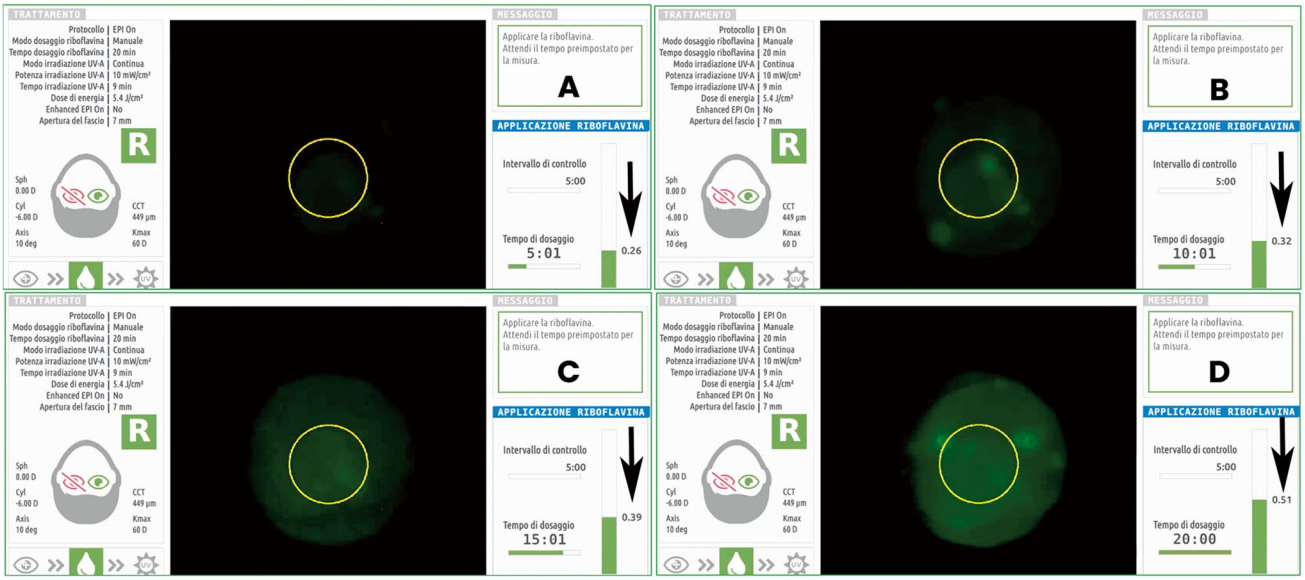

Fig. 2 At pre-set time intervals during the imbibition phase of corneal cross-linking, the theranostic UV-A devices provide a real-time estimation of riboflavin concentration into the cornea under treatment. The theranostic device tracks the amount of riboflavin diffusing into the corneal tissue and calculates the *riboflavin score* (d.u.) providing a measure of the riboflavin in the cornea by analysis of the green fluorescence emitted by the tissue. In **A**, **B**, **C**, and **D**, the screenshots of the UV-A device's display showing (arrows) the values of the *riboflavin score* at 5 min, 10 min, 15 min, and 20 min of the imbibition phase in a corneal sample of group 7-On respectively

Table 1 Corneal riboflavin concentration measured by spectrophotometry and theranostic UV-A device (*riboflavin score*) during epi-off imbibition protocols

Time	Corneal riboflavin concentration (μg/cm^3)			*Riboflavin score* (d.u.)		
	Group 2-Off (RitSight)	Group 1-Off (Photrexa kit)	Group 3-Off (Riboker)	Group 2-Off (RitSight)	Group 1-Off (Photrexa kit)	Group 3-Off (Riboker)
5 min	325 ± 108	48 ± 21	168 ± 50	1.36 ± 0.04	0.42 ± 0.31	0.86 ± 0.31
10 min	519 ± 116	89 ± 71	270 ± 36	1.69 ± 0.05	0.64 ± 0.33	1.25 ± 0.30
15 min	554 ± 103	138 ± 109	367 ± 38*	1.79 ± 0.07 (R = 0.77; P = .05)	0.71 ± 0.36	1.48 ± 0.21** (R = 0.71; P = .05)
30 min	-	213 ± 190*,**	-	-	0.77 ± 0.38**,*** (R = 0.98; P = .02)	-

$^{*}P < 0.05$ RitSight-Riboker and Riboker-Photrexa

$^{**}P < 0.01$ RitSight-Photrexa (corneal riboflavin concentration)

$^{**}P < 0.01$ RitSight-Riboker and Riboker-Photrexa (*riboflavin score*)

$^{***}P < 0.001$ RitSight-Photrexa

R is the Pearson correlation coefficients between the two optical techniques used for estimating corneal riboflavin concentration in each study group

and white signals. The dark signal was obtained by turning off the lamp; the white signal was acquired with the light beam passing through a quartz plate (MICQ, Shanghai Wechance Industrial Co Ltd, China), where the corneal samples were placed for spectrophotometry measurements. All collected data were expressed as spectrum of absorbance (i.e., the optical density). The absorbance spectrum acquired for each sample at each time interval (corresponding to the theranostic measurement) was therefore referenced with respect to the calibration curve, as described in previous studies [6, 9, 16]. An absorbance spectrum $A(\lambda)$ was measured for each cornea in the 300–600-nm wavelength range over the central cornea (Fig. 1). The extinction coefficient of riboflavin at 445-nm wavelength (λ) ($\varepsilon_{445} = 12{,}500\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1}$) was determined by measuring the absorbance of several riboflavin dilutions, ranging from 5×10^{-6} to 3.5×10^{-4} g/cm^3 using a quartz cuvette with 0.5-mm path (StonyLab Scientific, USA) [6, 13, 19]. The experimental results $A(\lambda)$ were fitted with the analytical expression [6]:

$$A_p(\lambda) = c_{\mathrm{ribo}} A_W s + a\lambda^{-n} + b \quad (1)$$

where A_w is the unitary riboflavin absorbance, s is the central corneal thickness (CCT), the quantity $a\lambda^{-n} + b$ takes into account the scattering contribution of the stroma, and the c_{ribo} is the riboflavin absorbed by the tissue. The unknown quantities a, b, c_{ribo}, and n were obtained by minimizing the sum of the squares of the residual $r(\lambda) = A_p(\lambda) - A(\lambda)$ for each corresponding wavelength. The riboflavin concentration,

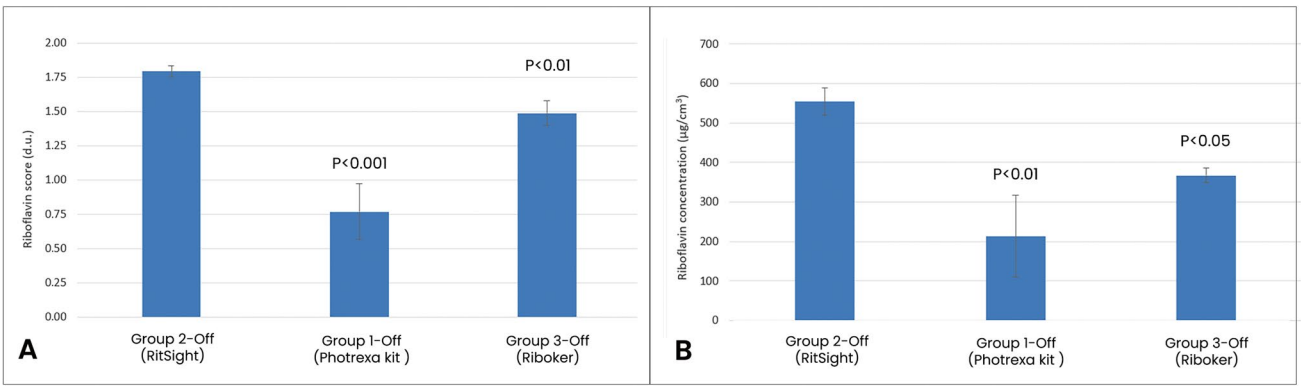

Fig. 3 Histograms showing the average (bars = SD) riboflavin concentrations assessed, at the end of imbibition time, into the corneal samples undergoing epithelium-off delivery of riboflavin by theranostic UV-A device (**A** showing the *riboflavin score*) and spectrophotometry (**B** showing corneal concentration). The two panels are shown for direct comparison of measurements estimated by theranostics and spectrophotometry. Group 2-Off (RitSight; 15-min application time) achieved a statistically significant greater amount of corneal riboflavin in comparison with group 1-Off (Photrexa kit; 30 min; $P < 0.01$) and group 3-Off (Riboker; 15 min; $P < 0.05$) in both optical methodologies

Table 2 Corneal riboflavin concentration measured by spectrophotometry and theranostic UV-A device (*riboflavin score*) during epi-on imbibition protocols

Time	Corneal riboflavin concentration (μg/cm^3)					*Riboflavin score* (d.u.)				
	Group 7-On (Rit-Sight)	Group 6-On (MedioCross TE)	Group 4-On (Ribofast)	Group 8-On (Riboker)	Group 5-On (Paracel kit)	Group 7-On (Rit-Sight)	Group 6-On (MedioCross TE)	Group 4-On (Ribo-fast)	Group 8-On (Riboker)	Group 5-On (Paracel kit)
5 min	32 ± 16	0 ± 0	5 ± 9	10 ± 6	26 ± 9	0.25 ± 0.04	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.02	0.24 ± 0.07
10 min	62 ± 21	1 ± 2	7 ± 12	18 ± 3	55 ± 11*	0.34 ± 0.14	0.17 ± 0.01	0.17 ± 0.03	0.20 ± 0.03	0.31 ± 0.07* (R = 0.99; P = .003)
15 min	95 ± 29	3 ± 3	14 ± 9*,**,***	33 ± 6*,**	-	0.47 ± 0.17	0.17 ± 0.01	0.20 ± 0.08* (R = 0.97; P = .01)	0.24 ± 0.05* (R = 0.87; P = .01)	-
20 min	122 ± 39	6 ± 5***	-	-	-	0.67 ± 0.19 (R = 0.99; P = .001)	0.17 ± 0.01* (R = 0.87; P = .05)	-	-	-

$^{*}P < 0.05$ RitSight-Paracel kit; Riboker-Ribofast; Paracel-Riboker

$^{**}P < 0.01$ RitSight-Riboker; Paracel-Ribofast

$^{***}P < 0.001$ RitSight-MedioCross; RitSight-Ribofast; Riboker-MedioCross; Paracel-MedioCross

$^{*}P < 0.05$ RitSight-MedioCross, RitSight-Ribofast, RitSight-Riboker; RitSight-Paracel kit; Riboker-MedioCross; Paracel-MedioCross (*riboflavin score*)

R is the Pearson correlation coefficients between the two optical techniques used for estimating corneal riboflavin concentration in each study group

c_{ribo}, was determined for l = 445 nm and expressed as μg/cm^3 (100 μg/cm^3 = 0.01%).

Statistics

In this study, data were given as mean ± standard deviation (SD). Sample size calculation was performed to detect a mean difference of 20 μg/cm^3 (SD ± 10 μg/cm^3) between the average corneal riboflavin concentration of the study groups in pairwise comparison, at a significance level of 5%, a power of 80%, and an allocation ratio of 1:1. Minimum sample size was $n = 4$ for each study group.

Baseline parameters between all groups were compared using the Tukey test for pairwise comparisons. The Pearson correlation coefficient (two-tailed test of significance) was calculated in order to estimate linear correlation between the estimates of corneal concentration of riboflavin calculated with the two independent optical methods. The Student *t*-test was performed to compare data acquired with each optical technique between pairs of groups. Statistical analysis was performed using SPSS statistical software (SPSS Inc., ver. 17, IBM, USA) and $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using SPSS statistical software (SPSS Inc., ver. 17, IBM, USA) and $P < 0.05$ was considered as statistically significant.

Results

Baseline data of corneal samples are summarized in supplementary Table 2; there were no statistically significant differences in any donor corneal parameter between groups either at baseline or at the end of treatment. A representative case of the functioning of the theranostic device during the imbibition phase of corneal cross-linking is shown in Fig. 2.

In epithelium-off delivery protocols, the average corneal riboflavin concentration at the end of imbibition time ranged from 213 ± 190 to 554 ± 103 μg/cm^3; the *riboflavin score* ranged from 0.77 ± 0.38 to 1.79 ± 0.07. Table 1 and Fig. 3 provide a comprehensive overview of the theranostic and spectrophotometry measurements recorded in the three study groups of epithelium-off delivery protocols. A statistically significant linear correlation was found between measurements of the two optical methods (Table 1). Dextran-free riboflavin ophthalmic formulations (group 2-Off and group 3-Off) achieved a higher corneal concentration than the dextran-enriched riboflavin solution (group 1-Off) at all time points of measurements. Since the corneal thickness was above 400 μm in all cases, the Photrexa ophthalmic solution (i.e., the kit product without dextran) was not applied, as recommended in the manufacturer's instruction for use. Group 2-Off (RitSight) achieved a statistically significant greater

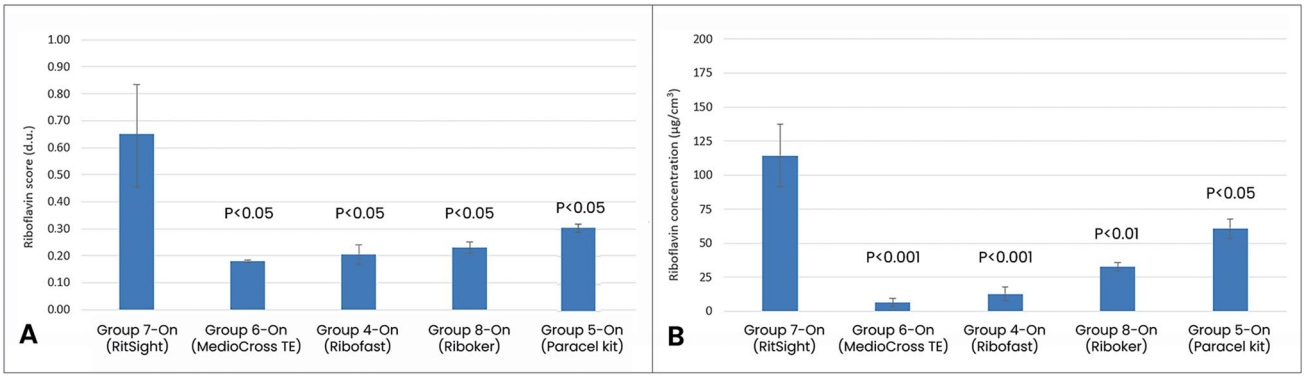

Fig. 4 Histograms of the average estimates of riboflavin concentration (bars = SD) assessed, at the end of imbibition time, into the corneal samples undergoing transepithelial delivery of riboflavin by theranostic UV-A device (**A** showing the *riboflavin score*) and spectrophotometry (**B** showing corneal concentration). Group 7-On (RitSight; 20-min application time) achieved a statistically significant greater amount of corneal riboflavin in comparison with all other groups (MedioCross TE, 20 min; Paracel kit, 10 min; Riboker, 15 min; and Ribofast, 15 min; $P<0.05$) in both optical methodologies. Significant differences in corneal riboflavin concentration were found between groups 4-On, 5-On, 6-On, and 8-On (see Table 2)

amount of corneal riboflavin than group 1-Off ($P<0.01$) and group 3-Off ($P<0.05$), whereas group 3-Off achieved more corneal riboflavin concentration than group 1-Off ($P<0.05$).

In transepithelial delivery protocols, the average corneal riboflavin concentration at the end of imbibition time ranged from 6 ± 5 to 122 ± 39 µg/cm^3; the *riboflavin score* ranged from 0.17 ± 0.01 to 0.67 ± 0.19. Table 2 and Fig. 4 provide a summary of the theranostic and spectrophotometry measurement outcomes in the five study groups of epithelium-on delivery protocols. A statistically significant linear correlation of riboflavin estimates was found between the two optical methods in all study groups (Table 2). Group 6-On, which used the HMPC-enriched riboflavin solution (MedioCross TE), had the lowest concentration of riboflavin in the cornea; while group 7-On (RitSight) achieved statistically significant greater amount of corneal riboflavin than all other epi-on groups (Table 2).

Discussion

Ensuring the proper permeation of riboflavin into the corneal stroma during CXL is crucial for maximizing treatment safety and efficacy for every patient [1–4, 11, 14, 15]. Epithelium removal is the standard and most frequently used method to facilitate penetration of riboflavin into the corneal stroma; the lack of methodologies for improving efficient and predictable epi-on delivery of riboflavin has so far limited the widespread clinical adoption of transepithelial CXL protocols. In the present study, the authors used a new theranostics technology implemented in a UV-A medical device to assess th e amount of corneal riboflavin during the imbibition phase of CXL. They tested various ophthalmic formulations used in clinical practice with the aim to understand the riboflavin absorption in the cornea during treatment. Eye bank human donor tissues were randomized to receive either epithelium-off or transepithelial delivery of riboflavin using ophthalmic formulations manufactured for the indication of use. The study results showed that there were significant differences in riboflavin corneal concentrations between different products after the imbibition period both in epi-off and epi-on delivery protocols.

In epi-off protocols, the dextran-enriched solution (group 1-Off; 30-min soaking time) performed worse compared to dextran-free solutions, while the highly concentrated riboflavin formulation (group 2-Off; 15-min soaking time) achieved the highest concentration of riboflavin in the corneal stroma. The presence of dextran could potentially impede the movement of riboflavin molecules into the stromal layers of the cornea, as reported in clinical studies. [20–22].

The results of epi-on delivery protocols have shown a limited penetration of riboflavin within the imbibition time recommended by the manufacturers in all study groups, except for group 7-On (0.22% riboflavin base solution). The delivery of riboflavin in group 7-On was on average 4.5 times higher compared to other epi-on ophthalmic solutions tested in this study. The HPMC-enriched solution (group 6-On) had the lowest performance; this finding evidenced that the inclusion of any viscoleastic agent in a riboflavin ophthalmic formulation could potentially impede the diffusion of riboflavin through the intact epithelium. Group 5-On had a dual transepithelial delivery system that includes a HPMC-enriched formulation and a HPMC-free formulation; these formulations are applied sequentially onto the cornea before UV-A light irradiation, thus minimizing the detrimental impact of the viscoelastic substance on riboflavin diffusion through the intact epithelium. The results of the present study were equivalent to previous reports assessing

transepithelial permeation of riboflavin solutions in porcine corneas [23, 24]; in which the ParaCel kit (group 5-On) and MedioCross TE (group 6-On) products have shown little and no penetration of riboflavin into the stroma through the intact epithelium respectively. Previous reports have already evidenced the high variability in corneal riboflavin permeation between different ophthalmic formulations [24]. The results of this study confirmed that epi-on protocols vary widely in their ability to achieve a sufficient dose of riboflavin in the corneal stroma, which could potentially explain the variability in clinical outcomes of transepithelial CXL procedures [11, 25–27]. Understanding and addressing this variability is important to improve the delivery and efficacy of epi-on treatment procedures.

A significant linear correlation was found between spectrophotometry [6, 23], which is the standard methodology to estimate corneal riboflavin concentration, and theranostics, regardless of the riboflavin delivery protocol. This result confirmed the accuracy of the novel, non-invasive, methodology to estimate the amount of riboflavin into the human cornea under treatment. [16–18]

During the last decade, efforts have been done to improve delivery of riboflavin into the stroma through the intact epithelium. The corneal epithelium contains 5–7 cellular layers with the superficial layer sealed by tight junctions, which provide a semi-permeable barrier to substances of increasing size. To overcome this barrier, viscoelastic-free riboflavin solution and the addition of enhancers have been developed to promote the permeability of riboflavin through the epithelium. Despite these efforts, authors [6, 24] have shown that neither the removal of dextran from the riboflavin formulation nor the addition of Tris, EDTA, NaCl, or BAK could be alone sufficient to significantly enhance the penetration of riboflavin across an intact epithelium. On the contrary, hypotonicity has been shown to be a major factor, by creating an osmotic gradient, to permit the penetration of riboflavin through the tight junctions between epithelial cells [28, 29]. The increase of riboflavin concentration of the imbibition solution has been shown to be an additional successful strategy to improve stromal permeation through the intact epithelium and reduce the time of imbibition [30–32]. Finally, prolonging the imbibition time, although less acceptable in a clinical environment, would be a strategy to improve transepithelial delivery of riboflavin. Increasing the frequency of drop application does not necessarily correlate with increased permeation of riboflavin into the corneal stroma; the primary packaging and delivery system of eye drops can affect the efficacy of riboflavin permeation. The RitSight product consists of a PE-LD bottle with calibrated dropper tip that regulates the drop size, thus playing a significant role in ensuring consistent dosing and absorption of riboflavin, while all the other products consist of pre-filled syringe with cannula that does not allow to regulate the amount of drop size. Overall, the reported difference in the varying degrees of effectiveness in delivering riboflavin to the corneal stroma emphasized the need for careful consideration of the formulation's composition and properties. An amphiphilic formulation with the right buffer and stabilizer systems can help overcome barriers, such as the electrostatic repulsion between the anionic riboflavin 5-phosphate and the negatively charged surface of the cornea, which reduces its epithelial penetration. In addition, it would allow greater penetration of riboflavin through the lipophilic epithelium and the hydrophilic corneal stroma.

A limit of the present work may include the lack of measurement of all the clinically available riboflavin formulations; further studies on assessing corneal iontophoresis delivery are ensured in order to test an active strategy for transepithelial delivery of riboflavin [33].

In conclusion, as theorized by previous authors [14], real-time monitoring of corneal riboflavin concentration could be advantageous for optimizing CXL therapeutic effect and solving current uncertainties for improving safety and efficacy of CXL treatment on a personal basis. Authors have demonstrated that the main mechanism of the riboflavin/UV-A CXL treatment is the direct interaction between riboflavin triplets and reactive groups of stromal proteins, which leads to the cross-linking of the stromal proteins through radical reactions [12]. This implies that, in ambient environment (i.e., 21% partial pressure of oxygen), the amount of riboflavin into the stroma and the role of type I mechanism are predominant for the formation of additional chemical bonds between stromal proteins [6, 13]. In this view, the use of real-time assessment of riboflavin concentration during CXL treatment has been proven to be effective in improving corneal tissue strength in both epi-off and epi-on CXL procedures with accuracy and precision [17, 18]. By utilizing a *riboflavin score* threshold, the theranostic UV-A device has shown to accurately activate UV-A light for the photo-activation of corneal riboflavin, resulting in a reliable corneal stiffening effect, regardless of the application protocol and ophthalmic formulation used [14, 17, 18].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00417-024-06451-8.

Acknowledgements We are thankful to Davide Camposampiero, Andrea Grassetto, and Diego Ponzin (Veneto Eye Bank Foundation, Venezia Zelarino, Italy) for their work in providing donor tissues for research purpose.

Funding This study was in part funded by Lazio Innova in the framework of FESR 2021–2027 programme with grant n. A0613-2023–077528 and by PO FESR 2014–2020. Action 1.1.5, project NUSTEO, CUP: G68I18000700007—application code 08CT2120090065.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Marco Lombardo and Giuseppe Lombardo are co-inventors on issued family patent (IT102019000011985, CN114126650, and US20230131004) related to this work. Marco Lombardo, Giuseppe Lombardo, and Sebastiano Serrao are equity owners in Regensight srl. Mario Fruschelli, Rita Mencucci, and Giuseppe Massimo Bernava have no conflict of interest.

References

1. Meiri Z, Keren S, Rosenblatt A, Sarig T, Shenhav L, Varssano D (2016) Efficacy of corneal collagen cross-linking for the treatment of keratoconus: a systematic review and meta-analysis. Cornea 35(3):417–428
2. Serrao S, Lombardo G, Lombardo M (2022) Adverse events after riboflavin/UV-A corneal cross-linking: a literature review. Int Ophthalmol 42(1):337–348
3. Hersh PS, Stulting RD, Muller D, Durrie DS, Rajpal RK, United States Crosslinking Study Group (2017) United States multicenter clinical trial of corneal collagen crosslinking for keratoconus treatment. Ophthalmology 124(9):1259–1270
4. Chunyu T, Xiujun P, Zhengjun F, Xia Z, Feihu Z (2014) Corneal collagen cross-linking in keratoconus: a systematic review and meta-analysis. Sci Rep 4:5652
5. Koller T, Mrochen M, Seiler T (2009) Complication and failure rates after corneal crosslinking. J Cataract Refract Surg 35:1358–1362
6. Lombardo M, Pucci G, Barberi R, Lombardo G (2015) Interaction of ultraviolet light with the cornea: clinical implications for corneal crosslinking. J Cataract Refract Surg 41(2):446–459
7. Tao X, Yu H, Zhang Y et al (2013) Role of corneal epithelium in riboflavin/ultraviolet-A mediated corneal cross-linking treatment in rabbit eyes. Biomed Res Int 2013:624563
8. Koppen C, Gobin L, Tassignon M-J (2010) The absorption characteristics of the human cornea in ultraviolet-A crosslinking. Eye Contact Lens 2:77–80
9. Lombardo M, Micali N, Villari V et al (2015) Ultraviolet A - visible spectral absorbance of the human cornea after transepithelial soaking with dextran-enriched and dextran-free riboflavin 0.1% ophthalmic solutions. J Cataract Refract Surg 41:2283–2290
10. Baiocchi S, Mazzotta C, Cerretani D, Caporossi T, Caporossi A (2009) Corneal crosslinking: riboflavin concentration in corneal stroma exposed with and without epithelium. J Cataract Refract Surg 35:893–899
11. Ng SM, Ren M, Lindsley KB, Hawkins BS, Kuo IC (2021) Transepithelial versus epithelium-off corneal crosslinking for progressive keratoconus. Cochrane Database Syst Rev (3):CD013512
12. Kamaev P, Friedman MD, Sherr E, Muller D (2012) Photochemical kinetics of corneal cross-linking with riboflavin. Invest Ophthalmol Vis Sci 53:2360–2367
13. Sel S, Nass N, Pötzsch S et al (2014) UVA irradiation of riboflavin generates oxygen-dependent hydroxyl radicals. Redox Rep 19:72–79
14. Schumacher S, Mrochen M, Wernli J, Bueeler M, Seiler T (2012) Optimization model for UV-Riboflavin corneal cross-linking. Invest Ophthalmol Vis Sci 53:762–769
15. Lin J-T, Cheng D-C (2017) Modeling the efficacy profiles of UV-light activated corneal collagen crosslinking. PLoS ONE 12(4):e0175002
16. Lombardo G, Villari V, Micali N et al (2018) Non-invasive optical method for real-time assessment of intracorneal riboflavin concentration and efficacy of corneal cross-linking. J Biophotonics 11(7):e201800028
17. Lombardo G, Bernava GM, Serrao S, Lombardo M (2022) Theranostic-guided corneal cross-linking: pre-clinical evidence on a new treatment paradigm for keratoconus. J Biophotonics 15(12):e202200218
18. Lombardo G, Bernava GM, Serrao S, Roszkwoska AM, Lombardo M (2023) Predicting corneal cross-linking treatment efficacy with real-time assessment of corneal riboflavin concentration. J Cataract Refract Surg 49(6):635–641
19. Lombardo M, Micali N, Villari V, Serrao S, Lombardo G (2016) All-optical method to assess stromal concentration of riboflavin in conventional and accelerated UV-A irradiation of the human cornea. Invest Opthalmol Vis Sci 57(2):476–483
20. Wollensak G, Iomdina E (2009) Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement. J Cataract Refract Surg 35:540–546
21. Rosenblat E, Hersh PS (2016) Intraoperative corneal thickness change and clinical outcomes after corneal collagen crosslinking: standard crosslinking versus hypotonic riboflavin. J Cataract Refract Surg 42(4):596–605
22. Ehmke T, Seiler TG, Fischinger I, Ripken T, Heisterkamp A, Frueh BE (2016) Comparison of corneal riboflavin gradients using dextran and HPMC solutions. J Refract Surg 32(12):798–802
23. Rubinfeld RS, Stulting RD, Gum GG, Talamo JH (2018) Quantitative analysis of corneal stromal riboflavin concentration without epithelial removal. J Cataract Refract Surg 44:237–242
24. Hayes S, Morgan SR, O'Brart DP, O'Brart N, Meek KM (2016) A study of stromal riboflavin absorption in ex vivo porcine corneas using new and existing delivery protocols for corneal cross-linking. Acta Ophthalmol 94:e109–e117
25. Al Fayez MF, Alfayez S, Alfayez Y (2015) Transepithelial versus epithelium-off corneal collagen cross-linking for progressive keratoconus: a prospective randomized controlled trial. Cornea 34(Suppl 10):S53-56
26. D'Oria F, Palazón A, Alio JL (2021) Corneal collagen cross-linking epithelium-on vs. epithelium-off: a systematic review and meta-analysis. Eye Vis (Lond) 8(1):34
27. Zhang X, Zhao J, Li M, Tian M, Shen Y, Zhou X (2018) Conventional and transepithelial corneal cross-linking for patients with keratoconus. PLoS ONE 13(4):e0195105
28. Kaur IP, Smitha R (2002) Penetration enhancers and ocular bioadhesives: two new avenues for ophthalmic drug delivery. Drug Dev Ind Pharm 28:353–369
29. Raiskup F, Pinelli R, Spoerl E (2012) Riboflavin osmolar modification for transepithelial corneal cross-linking. Curr. Eye Res 37(3):234–238
30. O'Brart NAL, O'Brart DPS, Aldahlawi NH, Hayes S, Meek KM (2018) An investigation of the effects of riboflavin concentration on the efficacy of corneal cross-linking using an enzymatic resistance model in porcine corneas. Invest Ophthalmol Vis Sci 59:1058–1065
31. Franke MAD, Landes T, Seiler TG et al (2021) Corneal riboflavin gradients and UV-absorption characteristics after topical application of riboflavin in concentrations ranging from 0.1 % to 0.5%. Exp Eye Res 213:108842
32. Iseli HP, Popp M, Seiler T, Spoerl E, Mrochen M (2011) Laboratory measurement of the absorption coefficient of riboflavin for ultraviolet light (365 nm). J Refract Surg 27:1952–2001
33. Lombardo G, Serrao S, Lombardo M (2020) Comparison between standard and transepithelial corneal crosslinking using a theranostic UV-A device. Graefes Arch Clin Exp Ophthalmol 258(4):829–834