New Methods to Evaluate the Effect of Conventional and Modified Crosslinking Treatment for Keratoconus

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“Discovery consists of seeing what everybody has seen and thinking what nobody has thought”

Albert Szent-Györgyi

Dedicated to my children – FILIP and EMMA

Tillägnad mina barn – FILIP och EMMA
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Abstract

**Background:** Today corneal crosslinking with ultraviolet-A photoactivation of riboflavin is an established method to halt the progression of keratoconus. In some cases, when the refractive errors are large and the visual acuity is low, conventional corneal crosslinking may not be sufficient. In these cases it would be desirable with a treatment that both halts the progression and also reduces the refractive errors and improves the quality of vision.

**Aims:** The aims of this thesis were to determine whether mechanical compression of the cornea during corneal crosslinking for keratoconus using a sutured rigid contact lens could improve the optical and visual outcomes of the treatment, and also to find methods to evaluate the effect of different corneal crosslinking treatment regimens.

**Methods:** In a prospective, open, randomized case-control study, 60 eyes of 43 patients with progressive keratoconus, aged 18-28 years, planned for routine corneal crosslinking, and a corresponding age- and sex-matched control group was included. The patients were randomized to conventional corneal crosslinking (CXL; n=30) or corneal crosslinking with mechanical compression of the cornea during the treatment (CRXL; n=30).

Biomicroscopy, autorefractometry, best spectacle corrected visual acuity, axial length measurement, Pentacam® HR Scheimpflug photography, pachymetry, intraocular pressure measurements and corneal biomechanical assessments were performed before treatment (baseline) and at 1 month and 6 months after the treatment.

One of the articles evaluated and compared the optical and visual outcomes between CXL and CRXL, while the other three articles focused on methods to evaluate treatment effects. In Paper I, the corneal light scattering was manually quantified from Scheimpflug images throughout the corneal thickness at 8 measurements points, 0.0 to 3.0 mm from the corneal centre, in patients treated with CXL. In Paper IV the corneal densitometry (light scattering) was measured with the Pentacam® HR software, in 4 circular zones around the corneal apex and at 3 different depths of the corneal stroma, in both CXL and CRXL treated corneas. Paper III quantified the biomechanical effects of CXL in vivo.

**Results:** Corneal light scattering after CXL showed distinctive spatial and temporal profiles and Applanation Resonance Tonometry (ART) -technology demonstrated an increased corneal hysteresis 1 and 6 months after CXL. When comparing the refractive and structural results after CXL and CRXL, CRXL failed to flatten the cornea, and the treatment did not show any benefits to conventional CXL treatment, some variables even indicated an inferior effect. Accordingly, the increase in corneal densitometry was also less pronounced after CRXL.

**Conclusions:** Analysis of corneal light scattering/densitometry shows tissue changes at the expected treatment location, and may be a relevant variable in evaluating the crosslinking effect. ART -technology is an in vivo method with the potential to assess the increased corneal hysteresis after CXL treatment. By refining the method, ART may become a useful tool in the future. Unfortunately, CRXL does not improve the optical and visual outcomes after corneal crosslinking. Possibly, stronger crosslinking would be necessary to stabilize the cornea in a flattened position.
# Abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AL</td>
<td>Axial lengths</td>
</tr>
<tr>
<td>ART</td>
<td>Applanation Resonance Tonometer/ry</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BSCVA</td>
<td>Best spectacle corrected visual acuity</td>
</tr>
<tr>
<td>CCT</td>
<td>Central corneal thickness</td>
</tr>
<tr>
<td>CDVA</td>
<td>Corrected distance visual acuity</td>
</tr>
<tr>
<td>CH</td>
<td>Corneal hysteresis (measures corneal viscoelasticity)</td>
</tr>
<tr>
<td>CH_ART</td>
<td>CH measured with ART -technology</td>
</tr>
<tr>
<td>CH_ORA</td>
<td>CH measured with ORA®</td>
</tr>
<tr>
<td>CRF</td>
<td>Corneal resistance factor (measures corneal rigidity)</td>
</tr>
<tr>
<td>CRF_ORA</td>
<td>CRF measured with ORA®</td>
</tr>
<tr>
<td>CRXL</td>
<td>Corneal reshaping and crosslinking (advanced, modified CXL)</td>
</tr>
<tr>
<td>CRXL_ctrl</td>
<td>Control group to patients treated with CRXL</td>
</tr>
<tr>
<td>CT_min</td>
<td>Corneal thickness at the thinnest point</td>
</tr>
<tr>
<td>CXL</td>
<td>Corneal crosslinking (conventional, routine)</td>
</tr>
<tr>
<td>CXL_ctrl</td>
<td>Control group to patients treated with CXL</td>
</tr>
<tr>
<td>D</td>
<td>Dioptre/s</td>
</tr>
<tr>
<td>DK value</td>
<td>Oxygen permeability of a contact lens</td>
</tr>
<tr>
<td>GAT</td>
<td>Goldmann Applanation Tonometer/ry</td>
</tr>
<tr>
<td>GSU</td>
<td>Gray scale units</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>IOP_ART</td>
<td>IOP measured with ART -technology</td>
</tr>
<tr>
<td>IOP_cc</td>
<td>Corneal compensated IOP</td>
</tr>
<tr>
<td>IOPg</td>
<td>Goldmann correlated IOP</td>
</tr>
<tr>
<td>IOP_GAT</td>
<td>IOP measured with GAT</td>
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<tr>
<td>IOP_ORA</td>
<td>IOP measured with ORA®</td>
</tr>
<tr>
<td>K1</td>
<td>Keratometry value for flat meridian*</td>
</tr>
<tr>
<td>K2</td>
<td>Keratometry value for steep meridian*</td>
</tr>
<tr>
<td>KC</td>
<td>Keratoconus</td>
</tr>
<tr>
<td>K_max</td>
<td>Maximum keratometry reading/value; maximum corneal curvature*</td>
</tr>
<tr>
<td>K_mean</td>
<td>Mean keratometry value, calculated as (K1+K2)/2*</td>
</tr>
<tr>
<td>logMAR</td>
<td>Logarithm of the minimum angle of resolution (visual acuity scale)</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>ORA®</td>
<td>Ocular Response Analyzer</td>
</tr>
<tr>
<td>PZT</td>
<td>Piezoelectric element</td>
</tr>
<tr>
<td>R_min</td>
<td>Minimum corneal curvature; minimum corneal radius of curvature</td>
</tr>
</tbody>
</table>
SOD = Superoxide dismutase
SphEq = Spherical equivalent, calculated as sphere+cylinder/2

*On a scan from Pentacam® HR, K1 corresponds to the flat corneal curvature measured in a central 3 mm zone, K2 corresponds to the steep curvature and K_{max} corresponds to the steepest point of the anterior corneal surface (Gore et al. 2013).
Original papers

This thesis is based on the following publications (I-IV), which will be referred to by their Roman numerals.

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Keratokonus (KC) är en sjukdom i ögats hornhinna som drabbar ca 1/2000 personer. Vid KC sker en förtunning av vävnaden i hornhinnan, vilket gör att hornhinnan börjar bukta utåt. Detta medför i sin tur brytningsfel och nedsatt syn. Idag är korneal crosslinking (CXL) en etablerad metod för behandling av lätt till måttlig KC. Behandlingen inleds med att ett fotosensiterande ämne, riboflavin, ges i form av upprepade ögondroppar i det aktuella ögat under 30 minuter. Ögat belyses sedan med en lampa, som avger UV-ljus, under ytterligare 30 minuter. Kombinationen gör att kollagenet i hornhinnan förstärks genom en kemisk process, s.k. korsbindning. Hornhinnan blir därigenom mer mekaniskt stabil och sjukdomens förlopp hejdas.

Då en del patienter redan har utvecklat betydande brytningsfel när de kommer för behandling är CXL, som är en i huvudsak förebyggande behandling, ofta otillräckligt. Vid dessa mer avancerade förändringar vore det önskvärt med en behandling som både kan hejda sjukdomens förlopp och minska brytningsfelen.

I denna avhandling utvärderas om en mekanisk kompression av hornhinnan under CXL-behandling kan minska patienternas brytningsfel och därmed också förbättra deras synskärpa, genom att ge en bestående utplaning av hornhinnans utbuktning.

I avhandlingen utvärderas även olika kliniska mätmetoders förmåga att kvantifiera behandlingseffekterna av olika varianter av CXL.

43 patienter (60 ögon), i åldern 18-28 år, med diagnosen KC inkluderades. Patienterna randomiserades till behandling med korneal crosslinking utan (CXL; n=30 ögon) eller med (CRXL; n=30 ögon) mekanisk kompression av hornhinnan. Behandlingarna inleddes med att riboflavin gavs i form av upprepade ögondroppar i det aktuella ögat under 30 minuter, efter det att hornhinnans epitel avlägsnats i lokalbedövning. Vid CRXL syddes sedan en flack, hård kontaktlins fast över hornhinnans yta med fyra stygn för att pressa tillbaka utbuktningen. Med kontaktlinsen på plats bestrålades sedan hornhinnan med UV-ljus för att åstadkomma en korsbindning av kollagenet, med förhoppningen att hornhinnan därigenom skulle “läsas” i det nya, mer utplanade läget. För att kompensera för kontaktlinsens absorption av UV-ljus, förlängdes CRXL-gruppens bestrålningstid med 4 minuter, till totalt 34 minuter. En timme efter behandlingen avlägsnades kontaktlinsen. CXL-gruppen fick samma behandling, frånsett kontaktlinsen och den extra bestrålningstiden. Även en ålders- och könsmatchad kontrollgrupp bestående av friska individer inkluderades.
Patienterna undersöktes före samt 1 och 6 månader efter behandlingen. De variabler som kontrollerades var bästa korrigerade synskärpa med glas, hornhinnans brytkraft, hornhinnans tjocklek, ögats längd, ljusspridningen i hornhinnan, ögontryck, hornhinnans elastiska egenskaper (CRF) och hornhinnans viskoelastiska egenskaper (CH).

Avhandlingen består av fyra publicerade artiklar, varvid resultatet av CXL- och CRXL-behandlingarna utvärderades och jämfördes i Artikel II. De övriga artiklarna har fokuserat på utvärderingar av olika kliniska mätmetoders förmåga att kvantifiera behandlingseffekterna av CXL och CRXL. I Artikel I kvantifierades ljusspridningen i hornhinnan före och efter CXL-behandlingen manuellt. Utifrån fotografier tagna med Scheimpflug kameran analyserades totalt 8 tvärsnitt av hornhinnan, belägna 0, 1, 2 och 3 mm från hornhinnans centrum (synaxeln). I Artikel IV analyserades ljusspridningen i båda behandlingsgrupperna med hjälp av Pentacam® HR software, en automatisk metod som kvantifierar ljuspridningens täthet. Denna metod analyserar hornhinnan utifrån 4 cirkulära zoner, utgående från hornhinnans apex (yttersta punkt), på 3 olika djup. I Artikel III kvantifierades hornhinnans biomekaniska egenskaper (CRF och CH) efter CXL-behandlingen.

Datamaterialet har analyserats utifrån kvantitativ metodik och en signifikansnivå på P<0.05 har använts.

Ljusspridningen efter CXL-behandlingen visade tydliga mönster gällande utbredning och tidsförlopp. Efter CXL-behandlingen kunde också en ökad stabilitet i hornhinnan påvisas med hjälp av Applanation Resonans Tonometri (ART)-teknologi (mätt som en ökning av CH-värdet). Vad gäller CRXL-behandlingen klarade den inte av att “läsa” hornhinnan i det nya, mer utplanade, läget och vid jämförelsen de båda behandlingarna emellan, var CRXL inte lika effektiv som CXL. I överensstämmelse med ovan var också ljusspridningen efter CRXL-behandlingen mindre framträdande.

1. Introduction

1.1. The Cornea
The cornea is a transparent and a clear, dome-shaped structure that covers the front part of the eye. This allows light to refract symmetrically to the crystalline lens and then on towards the retina. An adult cornea is about 0.53 mm thick at the centre (Doughty & Zaman 2000) and becomes thicker with increasing distance from the centre (Jonuscheit et al. 2015). The diameter is about 11-12 mm horizontally and 10-11 mm vertically. With an average radius of curvature on 7.8 mm, the cornea provides approximately 43 dioptres (D) of the total 59 D of refractive power of the human eye.

Because of the corneas importance of transparency, it is a tissue without blood vessels. The cornea receives most of its oxygen and nutrients via diffusion from the tear film (through the outside surface) and by the aqueous humour (through the inside surface).

The human cornea is a structure that consists of three layers with two membranes in between. From the anterior to posterior part of the cornea, these structures are: the epithelium, the Bowman´s membrane, the stroma, the Descemet´s membrane and the endothelium.

The corneal epithelium is a thin layer of tightly packed cells covering the corneal surface. It is normally five cell layers thick and it is easily regenerated if the cornea is injured. If an injury goes deeper into the cornea, it may causes opaque areas with decreased corneal transparency.

The Bowman´s membrane lies just beneath the epithelium and is a membrane composed of collagen. This membrane is very tough and difficult to penetrate and protects the cornea from deeper injuries.

The corneal stroma is the middle layer and it representing the largest part of the corneal thickness. The stroma is consisting of highly regularly arranged lamellae of collagen fibrils and extracellular matrix components (for example proteoglycans) along with keratocytes (fibroblasts). The keratocytes continually maintain the stroma by synthesizing collagen and stromal molecules. The regular arrangement with a constant distance between the lamellae is essential for the corneal transparency.

The Descemet´s membrane is a basement membrane of the corneal endothelium. This membrane is composed mainly of collagen.

The endothelium consists of a one cell layer covering the inside of the cornea. These cells are regulating the fluid and the solute transport between the aqueous humour and the corneal stroma. If endothelium cells are damaged, the cells do not regenerate. The overall cell density will then be reduced which will impacts on the fluid regulation and if the endothelium no longer can maintain a proper fluid balance, stromal swelling will occur. This
may cause corneal edema, opacification and loss of visual function. (American Academy of Ophthalmology 2010-2011).

1.2. Keratoconus
Keratoconus (KC) is a progressive corneal degeneration with decreased rigidity of the corneal structure. The definition of KC as a non inflammatory process has been generally accepted (Krachmer et al. 1984; Rabinowitz 1998), but new research have found increased level of inflammatory mediators such as cytokines (e.g. interleukin 6) in the tear fluid of KC patients (Lema et al. 2009; Jun et al. 2011). These results indicate that KC may involve inflammatory events (McMonnies 2015). Also a reduced level of superoxide dismutase (SOD; Behndig et al. 2001) supports this theory. The role of SOD is to remove reactive oxygen species, known to be associated with inflammatory processes.

1.2.1. Epidemiology
The reported incidence of KC is approximately 1 in 2000 people (Rabinowitz 1998; American Academy of Ophthalmology 2010-2011) and affects both men and women, but the ratio between men/women varies between reports (Bawazeer et al. 2000; Lim & Vogt 2002; Saini et al. 2004; Ertan & Muftuoglu 2008; Jonas et al. 2009). KC often debuts at puberty and progress until the third or fourth decades of life, and then the progression often halts (Krachmer et al. 1984; Rabinowitz 1998). It is most common as an isolated condition, but can coexist with other disorders, for example with Leber’s congenital amaurosis (Elder 1994), Down’s syndrome (Cullen & Butler 1963) and mitral valve prolapse (Sharif et al. 1992). KC is also associated with atopic dermatitis and eye rubbing (Bawazeer et al. 2000; Jafri et al. 2004; Gunes et al. 2014), and about 6-10% of reported cases have a positive family history (Rabinowitz 1998).

1.2.2. Signs and symptoms
The weakness of the corneal structures leads to corneal thinning and an abnormal protrusion. The protrusion causes myopia and irregular astigmatism, affecting the visual quality (Krachmer et al. 1984).

KC is a bilateral disease, although often asymmetrical (Rabinowitz 1998). Initially, it is often unilateral (Krachmer et al. 1984; Kennedy et al. 1986), but many patients develop bilateral KC before the progression halts (Li et al. 2004).

1.2.3. Diagnosis
Corneal topography based on the principles of Placido disc or Scheimpflug imaging (Pentacam® HR; see 3.6.1.) are the most sensitive methods to diagnose and quantify KC and have become gold standard methods to
diagnose and monitor KC (Maeda et al. 1994; Rabinowitz 1998; Gordon-Shaag et al. 2015). By producing a topographic map over the corneal surface and various indices, several quantitative methods have been developed, based on these indices (Gordon-Shaag et al. 2015). By combining different tomography parameters, Pentacam® HR can for example derive Amsler-Krumeich stages (Krumeich et al. 1998; Table 1) and Belin-Ambrósio enhanced ectasia assessment (Belin & Ambrósio 2013).

### Table 1. Classification of keratoconus by Amsler-Krumeich stages.

**Stage is determined if one of the characteristics applies.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Characteristics</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Eccentric corneal steepening  &lt;br&gt; Myopia and/or astigmatism of ( \leq 5.00 ) D  &lt;br&gt; Central ( \bar{K}_{\text{mean}} ) reading ( \leq 48.00 ) D  &lt;br&gt; Vogt’s striae, no scars</td>
</tr>
<tr>
<td>II</td>
<td>Myopia and/or astigmatism ( &gt;5.00 ) to 8.00 D  &lt;br&gt; Central ( \bar{K}<em>{\text{mean}} ) reading ( \leq 53.00 ) D  &lt;br&gt; Absence of scars  &lt;br&gt; ( CT</em>{\text{min}} \geq 400 ) μm</td>
</tr>
<tr>
<td>III</td>
<td>Myopia and/or astigmatism ( &gt;8.00 ) to 10.00 D  &lt;br&gt; Central ( \bar{K}<em>{\text{mean}} ) reading ( &gt;53.00 ) D  &lt;br&gt; Absence of scars  &lt;br&gt; ( CT</em>{\text{min}} = 200 ) to 400 μm</td>
</tr>
<tr>
<td>IV</td>
<td>Refraction not measurable  &lt;br&gt; Central ( \bar{K}<em>{\text{mean}} ) reading ( &gt;55.00 ) D  &lt;br&gt; Central corneal scars, perforation  &lt;br&gt; ( CT</em>{\text{min}} \leq 200 ) μm</td>
</tr>
</tbody>
</table>

\( K_{\text{mean}} \); mean keratometry value, calculated as \((K1+K2)/2\).<br>
\( CT_{\text{min}} \); corneal thickness at the thinnest point.

#### 1.2.4. Treatment options

In early stages of KC, the refractive errors can be corrected with glasses or rigid contact lenses (Tan & Por 2007; Gordon-Shaag et al. 2015). None of these interventions, however, have any effect on the progression of the disease (Tuft et al. 1994; Gordon-Shaag et al. 2015). In late stages keratoplasty may be the only treatment option (Tan & Por 2007; Gordon-Shaag et al. 2015).

In recent years, a technique called corneal crosslinking (CXL) has been introduced as a treatment for KC, particularly for the early stages of the disease. This treatment halts the progression of KC (Gordon-Shaag et al. 2015). In attempts to reduce the refractive errors and improve the visual acuity, combinations of CXL together with phototherapeutic keratectomy (Kymionis et al. 2012), and phototherapeutic keratectomy and intrastromal ring segments (Yeung et al. 2013) have been tried. The effect of these treatments may be insufficient in more severe cases of KC (Park & Gritz...
2013). It is also considered controversial to remove corneal tissue in an already thin cornea with decreased rigidity (Zhang & Zhang 2013).

1.3. Crosslinking
1.3.1. Background
Intermolecular crosslinking is a concept to enhance the rigidity of materials and have been used in bioengineering as a technique to strengthen materials for many years (Snibson 2010). Crosslinking is the creation of bonds, covalent or ionic, that connects one polymer chain to another. A polymer is a chain of monomeric material, for example a synthetic polymer or a biological molecule such as a protein (Sorkin & Varssano 2014). Crosslinking changes the material's physical properties. For example, when a rubber molecule is crosslinked, its flexibility will be decreased and its rigidity and melting temperature will be increased (Jenkins et al. 1996).

The idea behind today’s treatment of KC with corneal crosslinking using ultraviolet (UV) irradiation as a way to stiffen the human cornea came after a visit at a dentist who used UV irradiation to harden synthetic tooth filling material (Snibson 2010).

1.3.2. Corneal crosslinking - three different mechanisms
In a normal cornea, aggregated forms of collagen monomers are strengthened by intermolecular crosslinks. Collagen fibrils are naturally enzymatically crosslinked (by enzyme lysyl oxidase) as a part of their maturation process (Ashwin & McDonnell 2009).

Crosslinking also occurs during normal aging and in diabetes mellitus, where the natural crosslinking increases during a non-enzymatic reaction termed glycation (Maillard reaction; Malik et al. 1992; Sady et al. 1995; Daxer et al. 1998).

When riboflavin molecules are exposed to UV-A light of 370 nm wavelength, they absorb energy and reach an excited state. The process that is oxygen depending (Richoz et al. 2013) can produce either singlet oxygen molecules or superoxide radicals, in part depending on the availability of oxygen (Wollensak 2006; Kamaev et al. 2012). These reactive oxygen species are highly reactive and can induce covalent bonds (crosslinks) between many different molecules including in the corneal stroma, e.g. collagen fibrils, proteoglycans or nucleic acids (Pacifici & Davies 1990; Kolli & Aslanides 2010; Meek & Hayes 2013). In 1997 Spoerl et al. were first to achieve crosslinking of corneal collagen by using riboflavin and UV irradiation in enucleated porcine eyes (Spoerl et al. 1998). Later, this technique was studied in vivo by Wollensak et al. They treated 22 progressive KC patients with photosensitizing riboflavin drops and ultraviolet-A (UV-A) light after epithelium removal. Afterwards, clinical follow-up showed that the progression of KC was stopped in all eyes. In 70% of the eyes they also found
a regression of the refractive error and the maximum keratometry readings ($K_{\text{max}}$; Wollensak et al. 2003a). Today CXL is a safe and efficient routine method to halt the progression of KC (Goldich et al. 2010; Kolli & Aslanides 2010) and the effects of crosslinking treatment can be summarized in two stages: first, a photochemical reaction takes place in the corneal stroma and thereafter a wound healing process (Salomão et al. 2011; Touboul et al. 2012). Corneal haze and a corneal demarcation line after the crosslinking treatment are signs of wound healing responses after the treatment (Wollensak et al. 2003a; Seiler & Hafezi 2006; Mazzotta et al. 2012).

1.3.3. The “Dresden protocol”
The standard treatment protocol, named the “Dresden protocol” was first described in 2003 by Wollensak et al. The protocol included the following steps:

- A topical anesthetic to anesthetize the eye.
- Removal of the central 9 mm of the corneal epithelium.
- Application of topical 0.1% riboflavin (vitamin B2) on the deepithelized surface every 5 minutes during 30 minutes.
- Irradiation with UV-A light (370 nm, 3 mW/cm$^2$) during 30 minutes with continued application of topical 0.1% riboflavin every 5 minutes.
- Application of topical antibiotics and a soft contact lens.

(Wollensak et al. 2003a; Mazzotta et al. 2008).

1.3.4. Biomechanical/physiochemical changes induced by CXL
Today it is generally accepted that CXL acts through an increase in corneal biomechanical strength (Beshtawi et al. 2013), but the exact mechanisms behind CXL are not fully understood. There are several indirect evidences for the presence of crosslinks in the cornea after CXL (Meek & Hayes 2013). Most evidence are supplied in vitro by biomechanical (Wollensak et al. 2003b; He et al. 2010; Knox Cartwright et al. 2012; Dias et al. 2013), thermomechanical (Sperel et al. 2004a), biochemical (Sperel et al. 2004b; Brummer et al. 2011) and structural (Wollensak et al. 2004b) approaches.

Wollensak et al. (2003b) showed a 328.9% increase of the corneal rigidity in human corneas (increase in Young’s modulus by factor of 4.5). This stiffening effect is found to be more pronounced in the anterior layer of the stroma (Kohlhaas et al. 2006) where a reduced swelling behavior also is found (Wollensak et al. 2007). CXL also induces keratocyte apoptosis with lacunar edema in the space where apoptotic keratocytes used to be. The area next to the treated zone has a diffuse edema. The difference in the edema pattern between the zones may explain the formation of the demarcation line, visible at biomicroscopy after CXL (Wollensak & Herbst 2010b). This is followed by disappearance of the corneal edema, keratocyte repopulation
and an increased density of the extracellular matrix (Wollensak et al. 2004a; Seiler & Hafezi 2006; Dhaliwal & Kaufman 2009; Wollensak & Herbst 2010b). These corneal reactions are visible as corneal haze and often cause a slight decrease in visual acuity during the first months after a CXL treatment (Wollensak et al. 2003a; Mazzotta et al. 2012).

By using gel electrophoresis, a formation of aggregated molecules (polymers) of >1000 kDa was found in treated eyes (Wollensak & Redl 2008), which is a proof of the high-molecular-weight collagen polymers forming crosslink bridges in the collagen (Ashwin & McDonnell 2010).

Another indirect evidence of crosslinked collagen in CXL treated corneas is an increased shrinking temperature in the anterior stroma. Hydrothermal shrinking was observed at 75°C in the anterior stroma and at 70°C the posterior stroma (Spoerl et al. 2004a). Furthermore, increased resistance to enzymatic digestion by pepsin, trypsin and collagenases (Spoerl et al. 2004b) and increased collagen fiber diameters (Wollensak et al. 2004b) can be seen after CXL.

### 1.4. Densitometric evaluation of corneal reflectivity

The corneal reactions (visible as corneal haze) after CXL can be estimated by measuring light scattering. Optical coherence tomography (OCT) and Scheimpflug images (Pentacam® HR) can both provide images of the cornea with possibility to estimate the light scattering. OCT uses a longer wavelength with higher tissue penetration compared to the blue light of the Scheimpflug device and it therefore provides a more detailed image of the corneal stroma (Doors et al. 2009). Still, Scheimpflug images are sufficiently detailed for the densitometric measurements needed to quantify corneal haze after CXL (Greenstein et al. 2010; Wittig-Silva et al. 2013).

### 1.5. Corneal properties affecting IOP measurements

Doughty & Zaman (2000) have according to a meta-analysis from 300 data sets estimated the normal central corneal thickness (CCT) to be 534 µm. Several studies have shown that the thickness of the cornea can affect the measurement of the intraocular pressure (IOP). A thick (rigid) cornea generally results in an overestimation of the IOP and vice versa for a thin cornea (Ehlers et al. 1975; Whitacre & Stein 1993; Doughty & Zaman 2000). Accordingly, KC patients often have lower IOP values due to thin corneas (Rask & Behndig 2006).

The curvature of the cornea may also affect the IOP measurements, such that true IOP will in steep cornea be overestimated and in flat cornea underestimated (Whitacre & Stein 1993). A normal anterior corneal radius of curvature is about 7.6–7.9 mm (Dubbelman et al. 2002; Eysteinsson et al. 2002).
Further investigations will be needed into how the increased corneal mechanical stability after CXL treatment affects the IOP readings (Gkika et al. 2012a).

1.6. Biomechanical properties
If a material deforms reversibly under stress, it is defined as elastic. If a material flows when an external force is applied and then does not regain its original shape when the force is removed, the material is defined as viscous. If a material is viscoelastic, has it characteristics of both elasticity and viscosity, resulting in energy dissipation when stress is applied. The energy lost in the process is named “hysteresis” (Sorkin & Varssano 2014; Figure 1).

Figure 1. Schematic illustration of the concepts of elasticity and viscoelasticity.

1.6.1. Biomechanical properties of the normal cornea
The cornea shows both elastic and viscoelastic properties (Dupps & Wilson 2006; Figure 1). The thickness of the corneal stroma is about 90% of the total corneal thickness. The stroma is the main contributor to the corneal strength and consists of both lamellae of collagen fibrils and proteoglycans (American Academy of Ophthalmology 2010-2011; Sorkin & Varssano 2014). The corneal stroma also consists of 80% water, which gives cornea its viscoelastic properties. The water is thought to be the major contributor to the cornea’s ability to absorb energy, corneal hysteresis (CH; Luce 2005; Sorkin & Varssano 2014). The corneal resistance factor (CRF) is an indicator of the cornea’s ability to resist external forces (Ortiz et al. 2007).
1.6.2. Biomechanical properties in keratoconus
Decreased mechanical stability is a cause of the protrusion of the cornea in KC. Andreassen et al. (1980) have shown that KC corneas have about 40% reduction in rigidity when compared with normal corneas. Accordingly, Daxer & Fratzl (1997) have shown that the regularly arranged lamellae of collagen fibrils in normal corneas appear to be altered in KC corneas. These findings indicate altered crosslinks in KC corneas and possibly contribute to the mechanical instability.

1.6.3. Determination of corneal biomechanical properties
Corneal biomechanical properties can be measured in vivo by using the Ocular Response Analyzer (ORA®, CH and CRF; Luce 2005; Luce 2006). With the Applanation Resonance Tonometry (ART) -technology (Eklund et al. 2003; Hallberg et al. 2004; Hallberg et al. 2007; Jóhannesson et al. 2012) it is also possible to measure CH.

ORA® has been useful in detecting biomechanical changes related to corneal refractive procedures (Chen et al. 2010; Ryan et al. 2011), aging (Kamiya et al. 2009; Narayanaswamy et al. 2011) smoking (Hafezi 2009) diabetes mellitus (Goldich et al. 2009; Hager et al. 2009) and Fuchs’ endothelial dystrophy (del Buey et al. 2009). Several studies have also shown significant decrease in CH and/or CRF values in KC corneas when compared with normal corneas (Shah et al. 2007; Fontes et al. 2011; Gkika et al. 2012b). Despite these findings, several studies have failed to measure significant changes in CH and CRF in KC corneas after CXL (Sedaghat et al. 2010; Spoerl et al. 2011; Gkika et al. 2012b; Goldich et al. 2012).

1.7. Summary of the introduction
CXL has become an established routine method to halt progression of KC by increasing the corneal rigidity (Goldich et al. 2010; Kolli & Aslanides 2010), especially in early stages of the disease. In later stages, when the refractive errors are large, there is yet no treatment able to stop the progression of KC, modify the corneal shape and improve the visual quality.

Given the development of the method and the large scale use of CXL in treatment of KC, there is need for tools to evaluate the treatments effects in vivo (Seiler & Hafezi 2006) and for in vivo quantification of the biomechanical status of the cornea.
2. Aims

The aims of this thesis were to determine whether mechanical compression of the cornea during corneal crosslinking for keratoconus using a sutured rigid contact lens could improve the optical and visual outcomes of the treatment. In addition, we aimed to find methods to evaluate the effect of different corneal crosslinking treatment regimens.

The aims were divided into these specific issues:

- ...to compare refractive changes after corneal crosslinking with and without intra-treatment mechanical flattening of the cornea.
  (Paper II)

- ...to quantify and measure the spatial distribution and time course of the superficial light scattering using Scheimpflug photography or Pentacam® HR software.
  (Paper I, Paper IV)

- ...to quantify the biomechanical effects of CXL using ORA and Applanation Resonance Tonometry (ART)-technology.
  (Paper III)

- ...to assess the effect of CXL treatment on IOP measurement values using three different tonometry techniques.
  (Paper III)
3. Materials and methods

The clinical data used in the Papers (I-IV) were collected in a prospective, open, randomized case-control study termed the CRXL-study (Figure 2). Registered at ClinicalTrials.gov as Treatment of keratoconus with advanced corneal crosslinking, NCT number 02425150 (ClinicalTrials.gov. 2015).

3.1. Statistical power

The group size of 60 eyes (30 eyes in each treatment group) was decided using a power analysis. This study design will allow an 80% chance for detection of differences, both between the treatments and between the time points, according to the table below (α=0.05, power=0.80; Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Differences between treatments</th>
<th>Differences between time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSCVA (logMAR)</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>SphEq (D)</td>
<td>1.9</td>
<td>0.8</td>
</tr>
<tr>
<td>K1 (D)</td>
<td>2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>K2 (D)</td>
<td>2.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Kmax (D)</td>
<td>3.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Densitometry (GSU)/light scattering (AU)</td>
<td>3.1</td>
<td>0.6</td>
</tr>
<tr>
<td>IOP_GAT (mmHg)</td>
<td>2.0</td>
<td>0.8</td>
</tr>
<tr>
<td>CHORA (mmHg)</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>CHART (mmHg)</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CRFORA (mmHg)</td>
<td>1.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

BSCVA; best spectacle corrected visual acuity. SphEq; spherical equivalent, calculated as sphere+cylinder/2. K1; keratometry value for flat meridian. K2; keratometry value for steep meridian. Kmax; maximum keratometry value; maximum corneal curvature, obtained with the Pentacam® HR. IOP_GAT; intraocular pressure measured with Goldmann Appplanation tonometry. CHORA; corneal hysteresis measured with Ocular Response Analyzer. CRFORA; corneal resistance factor measured with Ocular Response Analyzer. CHART; corneal hysteresis measured with Applanation Resonance Tonometry -technology.

3.2. Participants

The study comprised 43 patients (60 eyes; 39 males, 4 females), age 18 to 28 years with age- and sex- matched control group (Figure 2).
3.2.1. Patients
Patients with uni- or bilateral progressive KC, planned for routine corneal crosslinking, were recruited to the study from the Department of Clinical Sciences, Ophthalmology, Norrlands University Hospital, Umeå, Sweden, between October 13, 2009 and December 15, 2011.

After inclusion the KC patients were randomly assigned to receiving either conventional corneal crosslinking (CXL; n=30 eyes) using the “Dresden protocol” (Wollensak et al. 2003a; Mazzotta et al. 2008) or a modified treatment we termed corneal reshaping and crosslinking (CRXL; n=30 eyes).

A list of random numbers between 1 and 60 generated by a computer was used for the randomization. The patients were included in running numbers according to the computer-generated list, an even number was treated with CXL and an uneven number was treated with CRXL.

Of the patients who had both eyes included in the study, 13 patients were randomized to CXL in one eye and CRXL in the other eye. Three patients were randomized to CRXL in both eyes, and one patient to CXL in both eyes.

3.2.2. Control subjects
To rule out natural time-dependent changes over time in the variables assessed, we also included a control group of healthy subjects, recruited between November 9, 2009 and May 3, 2012. Each treated eye of a KC patient was matched with one eye of an age- and sex-matched control subject. The control subjects followed the study protocol in every part, except for the CXL- and CRXL-treatment.

3.3. Inclusion/exclusion criteria
The inclusion criteria were patients planned for routine corneal crosslinking with progressive KC documented with repeated keratometry and subjective refraction or Scheimpflug tomography using the Pentacam® HR (Oculus, Inc. Lynnwood, WA, USA). A corneal thickness exceeding 400 µm at the thinnest point after epithelial removal was also required for inclusion.
Ultrasonic pachymetry (ORA®; Ocular Response Analyzer, software version 1.02, Reichert, Inc. Depew, NY, USA) was performed before and after epithelial removal and in borderline cases hypo-osmotic riboflavin was used when deemed necessary (n=1). Inclusion required the KC patients to be between 18 and 28 years, with no history of previous ocular surgery, no corneal abnormalities except KC, and no cognitive insufficiency interfering with the informed consent.

Conversely, exclusion criteria were age under 18 years or over 28 years, previous ocular surgery, any corneal abnormalities except KC, and a cognitive insufficiency interfering with the informed consent.

Patients wearing rigid contact lenses were instructed not to use their lenses for a week before all examinations.

3.3.1. Diagnosis and documentations of progressive keratoconus

The KC diagnosis was based on the Amsler-Krumeich stages (Krumeich et al. 1998) and the “Total Deviation” keratoconus quantification value from the Belin-Ambrósio enhanced ectasia assessment (Belin & Ambrósio 2013), both obtained from the Pentacam® HR measurements.

An altered red fundus reflex and/or an irregular cornea seen as a distortion of the keratometric mires were also required for diagnosis.

All KC patients had a history of progression, documented by repeated keratometry and refraction (17 eyes) or by repeated Pentacam® HR measurements (43 eyes). The 17 eyes documented by repeated keratometry and refraction had a rapid, pronounced KC progression with increasing K values (increasing corneal steepness) and astigmatism with decreasing best spectacle corrected visual acuity (BSCVA) documented by the referring clinic, which is why we chose not to await further progression before inclusion and treatment in these cases.

3.4. Ophthalmologic examinations

The ophthalmologic examinations were preformed immediately before treatment (baseline visit) and at 1 and 6 months after treatment. Each visit included:

- Slit-lamp biomicroscopic examination.
- Autorefractometry (Oculus Park 1; Oculus, Inc.).
- Best spectacle corrected visual acuity (BSCVA) using the ETDRS fast protocol (Camparini et al. 2001).
- Axial length measurement using the partial coherence interferometry based IOL Master 500® (Carl Zeiss Meditec AG., Jena, Germany).
- Corneal topography (e.g. keratometry) by Scheimpflug imaging using Pentacam® HR.
- Corneal tomography (e.g. light scattering) by Scheimpflug imaging using Pentacam® HR.
- Corneal thickness measurements with both Scheimpflug tomography (Pentacam® HR) and ultrasonic pachymetry (Ultrasonic pachymeter included in the ORA®).
- Intraocular pressure by Goldmann Applanation Tonometry (GAT; Haag-Streit, Inc. Bern, Germany), ORA® and ART -technology (see 3.6.4.).
- Corneal biomechanical assessments employing ORA® and ART -technology (see 3.6.4.).

3.5. Treatments

3.5.1. Conventional corneal crosslinking - CXL
The CXL treatment performed at the baseline visit was performed according to the “Dresden protocol” (Wollensak et al. 2003a; Mazzotta et al. 2008) and involved mechanical removal of the central 9 mm corneal epithelium after topical anesthesia with tetracaine hydrochloride. After the epithelial removal the corneal thickness was measured with ultrasonic pachymetry (ORA®) to ensure that the corneal thickness did exceed 400 µm at the thinnest point. In borderline cases, hypo-osmotic riboflavin was used when deemed necessary (n=1, a patient randomized to CXL). Topical 0.1% riboflavin (Ricrolin®, Sooft, Italy) was applied every 3 minutes during 30 minutes, and the cornea was then irradiated with UV-A light for 30 minutes using a solid-state UV-A illuminator (Caporossi/Baiocchi/Mazzotta, X-linker; Costruzione Strumenti Oftalmici, Firenze, Italy). The shutter was set to deliver energy of 3 mW/cm² and the diameter of the irradiation area was 8 mm. During the UV-A irradiation, tetracaine hydrochloride and riboflavin were applied every 5 minutes.

3.5.2. Corneal reshaping and crosslinking - CRXL
In the CRXL group a 12.5 mm semi scleral rigid Boston XO contact lens (Nordic Lenses, Inc. Kista, Sweden; DK value 100.0) was tightly sutured over the cornea with four 6:0 Vicryl stitches (Ethicon Inc.) to the limbus, after the riboflavin installation and before the UV-A irradiation. During the UV-A irradiation, riboflavin was applied every 5 minutes under the contact lens using a blunt cannula.

The back surface curvature of the contact lens was 11.0 mm, corresponding to a corneal refractive power of +33.5 D. The contact lens was used to induce a pronounced corneal flattening during the crosslinking treatment, with the aim to “lock” cornea in this new position. The flattening induced by the contact lens was based on the assumption that the anterior surface of the cornea aligned to the back surface curvature of the lens. We think this assumption is reasonable because of the four stitches were tightly
sutured to the limbal tissue, rendering concentric MD-folds indicating a pronounced corneal flattening. The MD-folds were visible at the initiation of the UV-A treatment and also one hour after the treatment, when the contact lens was removed. UV-metre measurements revealed that the contact lens absorbed 11% of the UV-A light, which was compensated for by increasing the irradiation time to 34 minutes.

3.6. Technical equipment
3.6.1. Pentacam® HR (Papers I+IV)

Paper I, manual method: Each eye was photographed with the Scheimpflug camera using the “25 pictures” program under standardized, mesopic light conditions. The corneal light scattering, in this investigation expressed as arbitrary units (AU) was analysed from two of the Scheimpflug images, with a superior and a temporal camera position, using the “ImageJ” image-analysis program. Using the “Line Tool” of “ImageJ”, the corneal light scattering was measured at approximately 40 points along a line perpendicular to the corneal surface at the visual axis and 1 mm, 2 mm and 3 mm inferior or temporal to the visual axis (Figure 14). Attempts to make analyses at 4 mm were abandoned because of shadowing from eyelids or light reflected from iris in too many cases. Each line measurement was performed in duplicate and the mean value of the two measurements was used for the analysis. Since there were no differences in the increase in the corneal light scattering between the corresponding temporal and inferior measurement points, a mean of the corresponding points was used in the subsequent calculations. The corneal light scattering raw data thus obtained was processed using the Microsoft Excel software (Microsoft Inc.).

To compare the corneal light scattering at different distances from the visual axis, the values need to be corrected for the larger distance from the illumination source of the Scheimpflug camera and for the angle of the corneal surface at the given point. Both of these factors will decrease the illumination and accordingly decrease the corneal light scattering obtained at the point in question. By using Euclidian geometry, individual corrections were calculated for each cornea, based on the working distance of 80 mm of the Scheimpflug camera and the corneal curvature. Similar individual corrections were also calculated for the UV-A irradiation (working distance 54 mm) during the treatment. The distance between the Scheimpflug camera and the eye is within this system approximately 120 mm, which was not corrected for in these calculations. The calculation was based on the facts that the illumination is inversely proportional to the square of the distance from the light source and inversely proportional to the angle of inclination.

The peak corneal light scattering value was assessed for each measurement point. To obtain an overall measure of corneal light scattering
in the optical centre of each cornea, the mean of all peak values within 2 mm from the visual axis was calculated for each cornea.

The individual photographs were also analysed manually for the occurrence of a demarcation line, by a masked observer.

Paper IV, automated method: The corneal densitometry (light scattering), in this investigation expressed as standardized gray scale units (GSU) was measured with the Pentacam® HR software in 4 circular zones around the corneal apex: a central 0-2 mm zone, a 2-6 mm zone, a 6-10 mm zone and a 10-12 mm zone. The corneal densitometry was also measured at 3 different depths of the corneal stroma: in the anterior 120 µm layer, in the posterior 60 µm layer and in the layer between the two former layers (the central layer).

3.6.2. Goldmann Applanation Tonometry (Paper III)
The GAT is considered the gold standard for estimating the intraocular pressure. The method is based on Imbert Fick’s law which states that the IOP is directly proportional to the ratio between the force (F) needed to applanate a predefined contact area (A; Goldmann 1957; Figure 3).

![Figure 3. Imbert Fick’s law.](image)

\[ IOP = \frac{F}{A} \]

GAT has been shown to be dependent on CCT and corneal rigidity in that a thick (rigid) cornea results in an overestimation of the IOP and vice versa for a thin cornea (Whitacre & Stein 1993). Hence, a change in corneal rigidity should be detectable as a change in the measured IOP value.

The GAT IOP measurements (IOP\textsubscript{GAT}) were performed in the central part of the cornea with the tonometer tip prism set both horizontally (horizontally split semi-circles) and vertically (vertically split semi-circles). Each measurement was made in duplicate and the IOP\textsubscript{GAT} was presented as the mean value of all four measurements.

3.6.3. Ocular Response Analyzer (Paper III)
The ORA® is a tonometer using an electro-optical system to measure the corneal response to indentation by a rapid air-pulse when measuring the IOP. It simultaneously measures the biomechanical properties of the cornea; the corneal rigidity (CRF) and the corneal viscoelasticity (CH). The air-pulse moves the cornea inwards past a defined point of applanation to a slight concavity. Before the cornea then returns to its normal curvature, it’s again passes the defined point of applanation. The corneal movements are recorded and the abovementioned parameters CH and CRF are calculated.
The CH represents the difference between the pressure value at the inward applanation (P1) and the corresponding value at the outward applanation (P2). CRF is calculated from the formula (P1 - kP2). The constant k is defined from an empirical analysis of the relationship between CCT and P1 and P2. CRF has been shown to be more strongly associated with CCT than CH. The ORA® also provides two different IOP parameters: Goldmann correlated IOP (IOPg) and corneal compensated IOP (IOPcc). IOPg is the average of the two pressure values at the applanation points. IOPcc is an IOP parameter designed to be less dependent of corneal curvature and CCT (Luce 2006; Figure 4 and 5).

Each eye was measured four times with the ORA® and the mean value of the four measurements was used for IOPcc, CH and CRF (in this study termed IOP_{ORA}, CH_{ORA} and CRF_{ORA}).

Figure 4. A plot of the air puff pressure function and the applanation signal.

Photography and illustration by JB Rehnman
Figure 5. Formulas for estimating the ORA® parameters.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOPcc</td>
<td>$P_2 - kP_1$</td>
</tr>
<tr>
<td>IOPg</td>
<td>$(P_1 + P_2)/2$</td>
</tr>
<tr>
<td>CRF</td>
<td>$P_1 - kP_2$</td>
</tr>
<tr>
<td>CH</td>
<td>$P_1 - P_2$</td>
</tr>
</tbody>
</table>

3.6.4. Applanation Resonance Tonometry -technology (Paper III)
The ART is a recently introduced IOP tonometer (Hallberg et al. 2007; Jóhannesson et al. 2012) based on resonance technology (Eklund et al. 2003). Two types of ART are available on the market, ART® Servo and ART® Manual. Through the use of ART -technology it is possible to analyse the changes in contact area and contact force during the applanation process, which in turn makes it possible to get information about the biomechanical properties of the cornea (Eklund et al. 2003; Hallberg et al. 2004).

Figure 6. The early research prototype of ART Servo used in the CRXL-study.

Photography by P Hallberg

In this study an early research prototype of ART Servo was used (Figure 6). A pipe shaped (30x5 mm, 1 mm wall thickness) piezoelectric element (PZT) (Morgan Electroceramics, Southampton, UK) was moulded in an aluminium container by silicon rubber (Wacker Elastosil RT622 Wacker
Chemie GmbH, München, Germany). To minimise friction forces, the aluminium container was suspended between two flexible support washers, 0.1 mm thickness, in an outer container made of plastic.

At the rear of the sensor, a force transducer, PS-05KD (Kyowa, Tokyo, Japan) was in contact with the aluminium case. A feedback circuit powered the PZT-element in order to sustain the PZT in its resonance frequency (approximately 60 kHz). The contact piece was made of PEEK-plastic (Amersham Bioscience, Umeå, Sweden). The contact surface had a diameter of 5 mm and a radius of curvature of 8 mm. A step motor (Haydon 21H4AC-2.5-907, Couëron, France) was attached to the end of the sensor body (Figure 7).

**Figure 7. Schematic picture of the ART sensor used in the CRXL-study.**

![Diagram of ART sensor](image)

Illustration by P Hallberg

The PZT formed a resonance sensor that measured the frequency shift ($\Delta f$, Hz), which is proportional to the contact area. The contact force (F, mN) was simultaneously measured by a force transducer. The data was sampled continuously at 1000 Hz from the point when the sensor got in touch with the cornea until the sensor was completely removed, *i.e.* during the applanation and removal phases, respectively.

The step motor provided the sensor with a standardized servo-controlled motion based on feedback from both force and area measurements. After sufficient contact area was reached the sensor automatically reversed from cornea. Applanation velocity was set to 4 mm/s and removal velocity was 7 mm/s.

The slope of the curve between force and frequency within certain frequency ranges (*i.e.* contact area intervals) was interpreted as being proportional to the IOP. A calibration constant transformed the slope of the curve into an IOP value in millimetres of mercury (mmHg). The analysis interval for the IOP during the applanation was calculated from data derived
from the analysis interval between 20 to 80 Hz in accordance with the commercially available instrument (Jóhannesson et al. 2012). For choosing the analysis interval during the removal phase an optimisation algorithm was used (Eklund et al. 2003). It stepped through all combinations of start points and interval lengths, increasing with steps of 10 Hz. The optimal interval was determined as the frequency interval that produced the lowest SD between the slope and the reference IOP measured with GAT. This optimisation process showed that the interval between 120 to 60 Hz produced the least SD. This interval was used in the study for measuring removal IOP. (Figure 8a and 8b). The same calibration constant as for the applanation phase was used during the removal phase.

The CH measured with ART -technology (CH\textsubscript{ART}) was calculated as the difference in IOP between the applanation and the removal phase (Hallberg et al. 2004). For each patient four ART measurements were obtained and the mean values for IOP and CH (in this study named IOP\textsubscript{ART} and CH\textsubscript{ART}) were recorded.

**Figure 8a, 8b** shows IOP measurements of left (red; treated) and right (blue; untreated) eye before (8a) and after (8b) CXL treatment. The IOP is calculated from data originating from a specific contact area interval (measured as a frequency shift, $\Delta f$), from applanation start to applanation end, and from removal start to removal end, marked in the figures. Corneal hysteresis (CH) was then calculated as the difference in IOP during applanation and IOP during removal. For the treated left eye the IOP before and after CXL were similar at applanation, whereas there was a difference in IOP (slope) during the removal. The treated left eye shows an increase in CH.

Illustration by G Mannberg
3.7. Ethics
The study was approved by the Regional Ethical Review Board in Umeå, Sweden and was performed in accordance with the Declaration of Helsinki (World Medical Association 2013).

All participants were provided both written and oral information before giving written consent to participate in the study.

The participants in the control group received financial compensation for their participation.

3.8. Statistical methods
Preoperative data were used as baseline values and were compared with data obtained at 1 and 6 months after treatment (Paper I-IV). In Paper IV were also the quotients between the central and the peripheral zones, at different depths, determined for each individual cornea at 1 and 6 months after treatment. In Paper II and III the above measurements were compared with measurements in the control groups.

For statistical comparisons of normally distributed variables, Student’s paired t test was used for analysis between different time points and between the KC patients and their control group. For comparisons of normally distributed data between the two treatment groups, Student’s unpaired t test was used. Data were presented as mean ± standard deviation (SD) or mean ± standard error of mean (SE or SEM).

For statistical comparison for non-normally distributed variables, independent samples Kruskal-Wallis test was used for multiple groups (the Amsler-Krumeich classification in Paper II, III). Non-normally distributed data were presented as median ± interquartile range.

When comparing more than two groups, Bonferroni corrections were applied (Paper I).

The confidence interval (CI) was calculated and presented in Paper III (99% CI) and in Paper IV (95% CI).

To analyse linear relationships between different parameters, correlations were assessed with Pearson’s bivariate correlation analysis for normally distributed variables (Paper I, IV) and with Spearman’s rho for non-normally distributed variables (Paper II).

SPSS, version 18 (SPSS Inc.) or Microsoft Excel software (Microsoft Inc.) were used when processing raw data.

A P value less than 0.05 were considered statistically significant (Paper I-IV).
4. Results

4.1. Overview over demographic data and parameters
Fifty-eight out of 60 eyes with KC were treated and followed up throughout the 6-month period (Table 3; Figure 9). For parameters including in each Paper, see Table 4.

Table 3. Overview over baseline demographic data.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Time period Baseline - 6 months</th>
<th>Participants included</th>
<th>Treatment group</th>
<th>n eyes/P</th>
<th>Sex F/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>13.10.09 - 14.10.10</td>
<td>P</td>
<td>CXL</td>
<td>13/13</td>
<td>0/13</td>
</tr>
<tr>
<td>II</td>
<td>13.10.09 - 05.11.12</td>
<td>P/C</td>
<td>CXL/CRXL</td>
<td>60/43</td>
<td>4/39</td>
</tr>
<tr>
<td>III</td>
<td>13.10.09 - 05.11.12</td>
<td>P/C</td>
<td>CXL</td>
<td>30/29</td>
<td>2/27</td>
</tr>
<tr>
<td>IV</td>
<td>13.10.09 - 31.05.12</td>
<td>P</td>
<td>CXL/CRXL</td>
<td>60/43</td>
<td>4/39</td>
</tr>
</tbody>
</table>

P; patients.
C; control subjects.
CXL; conventional corneal crosslinking.
CRXL; corneal reshaping and crosslinking.
n; number of included eyes/number of included patients, in the treatment group/groups.
Sex; number of included female/male patients, in the treatment group/groups.
F; female.
M; male.

Figure 9. CONSORT Flow Diagram.
The number of eyes with KC enrolled and randomized to the CRXL-study, and KC eyes included in the 1 and 6 months follow-up.
One patient developed a keratitis after CXL but the infection was subsequently successfully treated and the patient remained in the CRXL-study. The patient’s data was excluded in Paper I, but was included in Paper II, III and IV. In Paper I an additional patient was excluded due to technical problems with the Scheimpflug camera at the 6-month visit. However, in Paper IV this patient was included in all analyses except for the densitometry analysis at the 6 months follow-up.

One control subject was included to match each KC patient. If the patient had both eyes included in the CRXL-study, both eyes of the control subject were also included.

In one of the control subjects a forme fruste KC was suspected and the control subject was excluded and replaced with another control subject. One control subject who was included to match both (CRXL) eyes of one patient was lost to the 6 months follow-up.

Table 4. Overview over analysed parameters in Papers I-IV.

<table>
<thead>
<tr>
<th>Parameter;</th>
<th>Paper:</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSCVA/CDVA</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Sphere</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Cylinder</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>SphEq</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Kmax</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Kmean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Rmin</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTmin</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Densitometry/light scattering</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>IOPGAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>IOPORA</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>IOPART</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>CHORA</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CHART</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CRFORA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

BSCVA; best spectacle corrected visual acuity.
CDVA; corrected distance visual acuity.
SphEq; spherical equivalent, calculated as sphere+cylinder/2.
Kmax; maximum keratometry value; maximum corneal curvature, obtained with the Pentacam® HR.
Kmean; mean keratometry value, obtained with the Pentacam® HR, calculated as (K1+K2)/2.
Rmin; minimum corneal radius of curvature, obtained with the Pentacam® HR.
CTmin; Corneal thickness at the thinnest point, obtained with the Pentacam® HR.
AL; axial lengths.
IOPGAT; intraocular pressure measured with Goldmann Applanation tonometry.
IOPORA; intraocular pressure measured with Ocular Response Analyzer.
IOPART; intraocular pressure measured with Applanation Resonance Tonometry -technology.
CHORA; corneal hysteresis measured with Ocular Response Analyzer.
CHART; corneal hysteresis measured with Applanation Resonance Tonometry -technology.
CRFORA; corneal resistance factor measured with Ocular Response Analyzer.
4.2. Baseline Amsler-Krumeich stage and “Total Deviation” value
The baseline Amsler-Krumeich stage was 2 (2-3) in both treatment groups (median and interquartile range; P=0.94). The “Total Deviation” keratoconus quantification value obtained from the Pentacam® HR measurements was 8.5 ± 6.7 standard deviations for the CXL group and 7.4 ± 3.2 standard deviations for the CRXL group (P=0.43).

None of the included control subjects showed any signs of KC according to these gradings.

4.3. Occurrence of a demarcation line
At the slit-lamp examination, a corneal demarcation line was seen in many of the KC patients in both treatment groups at 1 month, but this was not systematically registered in the study protocol.

On Scheimpflug images analysed by a masked observer, a demarcation line was detected at 1 month in 21 eyes (72%) in the CXL group and in 20 patients (69%) in the CRXL group. Similarly, at 1 month, an increased corneal light scattering was a consistent finding in all images after both treatments.

4.4. Refractive results from the CRXL-study (Paper II)
This is a complete analysis including both the KC patients randomized to CXL and CRXL and the healthy control subjects.

Before the treatments maximum keratometry readings (Kmax) and the refractive astigmatism (cylinder) was higher (P<0.01; Table 6), the BSCVA and the minimum corneal thickness (CTmin) was lower (P<0.01; Table 6; Figure 10) in the KC patients than in the corresponding control subjects. This difference between the KC patients and the control subjects persisted throughout the 6-month period (P<0.01; Table 6; Figure 10).

In the KC patients at baseline, the refraction (sphere, cylinder and spherical equivalent (SphEq)), BSCVA, axial lengths (AL), and Kmax and CTmin (obtained with the Pentacam® HR) did not differ between the CXL and CRXL groups (P>0.20).

---

Table 5. Age distribution of keratoconus patients and matched control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>CRXL</th>
<th>Control subjects</th>
<th>CRXLctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXL</td>
<td>CRXL ctrl</td>
<td>CXL</td>
<td>CRXL ctrl</td>
</tr>
<tr>
<td>n (eyes)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean ± SD (years)</td>
<td>23.7 ± 3.1*†</td>
<td>23.4 ± 3.0**#</td>
<td>23.7 ± 3.1†</td>
<td>23.4 ± 3.0**#</td>
</tr>
<tr>
<td>Range (years)</td>
<td>18.1 - 28.0</td>
<td>18.2 - 27.9</td>
<td>18.2 - 27.8</td>
<td>18.8 - 27.8</td>
</tr>
</tbody>
</table>

* P=0.65; CXL vs. CRXL.
† P=0.58; CXL vs. CRXLctrl.
# P=0.61; CRXL vs. CRXLctrl.
Table 6. Visual and refractive outcomes, K\textsubscript{max} and CT\textsubscript{min} at baseline and at 1 and 6 months follow-up after CXL and CRXL treatment, compared to control subjects.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD; P value compared to baseline.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control subjects</td>
<td>CXL</td>
<td>CRXL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>1 month</td>
</tr>
<tr>
<td>n (eyes)</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Sphere (D)</td>
<td>-0.3 ± 2.7</td>
<td>-0.2 ± 2.5; P=0.41</td>
<td>0.1 ± 2.3; P=0.05</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td>-3.1 ± 2.1</td>
<td>-3.3 ± 2.1; P=0.49</td>
<td>-3.1 ± 2.2; P=0.70</td>
</tr>
<tr>
<td>SphEq (D)</td>
<td>-1.9 ± 2.8</td>
<td>-1.8 ± 2.5; P=0.65</td>
<td>-1.4 ± 2.4; P=0.03</td>
</tr>
<tr>
<td>BSCVA (logMAR)</td>
<td>0.19 ± 0.26</td>
<td>0.25 ± 0.23; P=0.09</td>
<td>0.14 ± 0.18; P=0.03</td>
</tr>
<tr>
<td>K\textsubscript{max} (D)</td>
<td>53.1 ± 4.9</td>
<td>53.9 ± 5.2; P&lt;0.01</td>
<td>52.6 ± 5.2; P=0.02</td>
</tr>
<tr>
<td>CT\textsubscript{min} (µm)</td>
<td>481 ± 41</td>
<td>457 ± 44; P&lt;0.01</td>
<td>470 ± 42; P=0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD; P value compared to patients at the same time point.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control subjects</td>
<td>CXLctrl</td>
<td>CRXLctrl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>1 month</td>
</tr>
<tr>
<td>n (eyes)</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Sphere (D)</td>
<td>-0.3 ± 1.9; P=0.51</td>
<td>-0.7 ± 1.9; P=0.36</td>
<td>-0.7 ± 1.9; P=0.15</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td>-0.9 ± 1.9; P=0.09</td>
<td>-0.9 ± 1.9; P=0.10</td>
<td>-0.9 ± 1.8; P=0.30</td>
</tr>
<tr>
<td>SphEq (D)</td>
<td>-0.14 ± 0.07; P&lt;0.01</td>
<td>-0.18 ± 0.06; P&lt;0.01</td>
<td>-0.18 ± 0.06; P&lt;0.01</td>
</tr>
<tr>
<td>BSCVA (logMAR)</td>
<td>44.5 ± 1.4; P&lt;0.01</td>
<td>44.5 ± 1.4; P&lt;0.01</td>
<td>44.5 ± 1.4; P&lt;0.01</td>
</tr>
<tr>
<td>K\textsubscript{max} (D)</td>
<td>553 ± 33; P&lt;0.01</td>
<td>553 ± 32; P&lt;0.01</td>
<td>554 ± 32; P&lt;0.01</td>
</tr>
<tr>
<td>CT\textsubscript{min} (µm)</td>
<td>562 ± 24; P=0.01</td>
<td>563 ± 27; P&lt;0.01</td>
<td>560 ± 23; P=0.01</td>
</tr>
</tbody>
</table>

SphEq: spherical equivalent, calculated as sphere+cylinder/2.
BSCVA: best spectacle corrected visual acuity.
K\textsubscript{max}: maximum keratometry value; maximum corneal curvature, obtained with the Pentacam® HR.
CT\textsubscript{min}: corneal thickness at the thinnest point, obtained with the Pentacam® HR.

At 1 month a small, insignificant decrease in BSCVA was seen for CXL (P=0.09; P=0.56 for CRXL; Table 6; Figure 10). A significant increase in K\textsubscript{max} (P<0.01) were seen after both treatments (Table 6). Also a significant decrease in CT\textsubscript{min} was observed 1 month after CXL treatment, but after CRXL no corresponding corneal thinning was seen (P<0.01 for CXL; P=0.43 for CRXL; Table 6).

At 6 months a significant improvement in both BSCVA and K\textsubscript{max} were seen after CXL when comparing with the baseline value (P=0.03; P=0.02; Table
6; Figure 10), whereas the small improvement seen in the CRXL group was insignificant (P=0.20; P=0.06; Table 6; Figure 10). The SphEq was also significantly improved from baseline to 6 months after CXL treatment (+0.5 ± 1.1 D), but after CRXL treatment the SphEq showed an insignificant negative development (-0.5 ± 2.1 D), which differed significantly from the improvement seen after CXL (P=0.04). Comparing with the corresponding control subjects, the CXL group did not change in SphEq during the 6-month period (P=0.30; Table 6), whereas the impairment seen in the CRXL group was significant (P=0.02; Table 6). The corneal thinning seen after CXL at 1 month was partially reversed at 6 months (P<0.01 for 1 versus 6 months). In the CRXL group a similar but insignificant change was seen (P=0.06 for 1 versus 6 months).

Figure 10. Mean BSCVA at baseline and at 1 and 6 months follow-up after CXL and CRXL treatment, compared with control subjects.

In accordance with the corneal thinning, the AL decreased after CXL at 1 month (24.28 ± 0.95 to 24.25 ± 0.98, P<0.01) and increased again at 6 month (24.26 ± 0.96, P=0.03). In the CRXL group corresponding but less pronounced changes were seen (P=0.01 and P=0.81, respectively).
No correlation was found between the degree of KC estimated from the baseline “Total Deviation” keratoconus quantification value and the change seen in BSCVA, $K_{\text{max}}$ and SphEq at 6 months after CXL and CRXL ($P>0.20$).

4.5. Corneal light scattering after CXL (Paper I)
This study involved 13 eyes of 13 KC patients (all men) randomized to CXL and enrolled; 2 were excluded (see 4.1.). The result is therefore based on an analysis of 11 eyes.

Table 7. Visual outcomes at baseline and at 1 and 6 months follow-up after CXL.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD; $P$ value compared to baseline.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>n (eyes)</td>
<td></td>
</tr>
<tr>
<td>BSCVA/CDVA (logMAR)</td>
<td>11 0.13 ± 0.19</td>
</tr>
</tbody>
</table>

BSCVA; best spectacle corrected visual acuity.
CDVA; corrected distance visual acuity.

At 1 month a significant decrease in BSCVA ($P=0.048$; Table 7) were seen after CXL treatment. The decrease in BSCVA was significantly correlated with the overall ($0+1+2$ mm) increase in corneal light scattering in the
optical centre of the cornea ($R^2=0.41$, $P=0.035$; Figure 11). An improvement in BSCVA was seen at 6 months ($P=0.140$ compared to baseline; Table 7; $P=0.003$ compared to the 1 month values).

At the optical centre of the cornea a significant increase in the overall light scattering was seen from baseline to 1 month ($P<0.001$; Table 8). The light scattering was reduced at 6 months ($P=0.002$ compared to the 1 month values), but was still higher than the baseline values ($P<0.001$ compared to baseline; Table 8).

### Table 8. Overall light scattering at baseline and at 1 and 6 months follow-up after CXL.

<table>
<thead>
<tr>
<th>Patients Parameters</th>
<th>Mean ± SD; P value compared to baseline.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>n (eyes)</td>
<td>CXL</td>
</tr>
<tr>
<td>Light scattering (AU)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>87.5 ± 10.0</td>
</tr>
</tbody>
</table>

AU; arbitrary units.

The increase in corneal light scattering at 1 month was more pronounced at the visual axis than at 1 or 2 mm from the visual axis. Three millimetres from the visual axis, only a slight increase in corneal light scattering was seen. These differences also remained after the values were corrected for the distance from the illumination source of the Scheimpflug camera and the angle of the corneal surface at the measurement points (see 3.6.1.). After applying factors correcting for these putative errors the means of the maximum increases in light scattering at 0, 1, 2 and 3 mm from the visual axis were shown in Figure 12 ($P<0.002$ when comparing 0 mm to 1 and 2 mm and when comparing 3 mm to all other measurement points). These differences also remained when the values were corrected for the reduced irradiation from the UV-A light according to the same principle (data not shown).

At 6 months, there were no longer a significant difference in maximum corneal light scattering between the visual axis and the 1 and 2 mm measurement points (Figure 12), but there was still significantly less light scattering at 3 mm ($P=0.018$ when comparing 3 mm to all other measurement points; Figure 12).
Figure 12. The maximum increases in corneal light scattering at 1 and 6 months after CXL treatment at 0, 1, 2 and 3 mm from the visual axis (mean ± SD).

In the superficial 70 µm of the cornea, corresponding to the corneal epithelium, the corneal light scattering was unaltered from baseline values both 1 and 6 months after treatment (Figure 13).

Figure 13. The mean increases in corneal light scattering at 1 and 6 months after CXL treatment at different corneal depths 0, 1, 2 and 3 mm from the visual axis.
At 1 month an increase in the corneal light scattering was seen from approximately 80-240 µm depths at all measurement points. At 6 months this peak was still remaining, albeit reduced, and a second peak of increased corneal light scattering, seen as a demarcation line, occurred between 240-340 µm (P=0.037 compared to 1 month values; Figure 13 and 14).

No increased corneal light scattering was seen deeper than 340 µm at either time point (Figure 13).

Figure 14. Example of Scheimpflug photographs showing the spatial distribution of the corneal light scattering before (baseline) and at 1 and 6 months after CXL treatment.

The measurement points (0, 1, 2 and 3 mm) are indicated in the photographs.

Note the increased corneal light scattering at 1 month and the demarcation line at 6 months.

4.6. Corneal light scattering after CXL and CRXL (Paper IV)
In this study the whole material of KC patients randomized to CXL or CRXL were included. At baseline all 60 eyes were analysed. At 1 month, 29 eyes were analysed in the CXL group and 28 in the CRXL group. At the 6-month
visit 29 eyes in each group were analysed, except for the densitometry analysis in the CXL group, where one more eye was excluded due to technical problems with the Scheimpflug camera (see 4.1.).

At baseline there were no differences between the treatment groups (P>0.05; Table 5, 9 and 10).

### Table 9. Overview over baseline data for each treatment group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CXL</th>
<th>CRXL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (eyes)</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Sphere (D)</td>
<td>-0.3 ± 2.7</td>
<td>-0.5 ± 2.5</td>
<td>0.81</td>
</tr>
<tr>
<td>Cylinder (D)</td>
<td>-3.1 ± 2.1</td>
<td>-2.9 ± 2.2</td>
<td>0.76</td>
</tr>
<tr>
<td>SphEq (D)</td>
<td>-1.9 ± 2.7</td>
<td>-1.9 ± 2.5</td>
<td>0.91</td>
</tr>
<tr>
<td>BSCVA (logMAR)</td>
<td>0.19 ± 0.25</td>
<td>0.23 ± 0.29</td>
<td>0.52</td>
</tr>
<tr>
<td>Kmax (D)</td>
<td>53.1 ± 4.8</td>
<td>53.9 ± 5.4</td>
<td>0.57</td>
</tr>
<tr>
<td>Kmean (D)</td>
<td>45.9 ± 3.2</td>
<td>46.7 ± 3.5</td>
<td>0.41</td>
</tr>
<tr>
<td>Rmin (µm)</td>
<td>6.4 ± 0.6</td>
<td>6.3 ± 0.6</td>
<td>0.56</td>
</tr>
<tr>
<td>CTmin (µm)</td>
<td>480 ± 40</td>
<td>479 ± 30</td>
<td>0.96</td>
</tr>
</tbody>
</table>

SphEq; spherical equivalent, calculated as (sphere+cylinder)/2.
BSCVA; best spectacle-corrected visual acuity.
Kmax; maximum keratometry value, maximum corneal curvature, obtained with the Pentacam® HR.
Kmean; mean keratometry value, obtained with the Pentacam® HR, calculated as (K1+K2)/2.
Rmin; minimum corneal radius of curvature, obtained with the Pentacam® HR.
CTmin; corneal thickness at the thinnest point, obtained with the Pentacam® HR.

### 4.6.1. Compared to baseline

At 1 month a significant increase in corneal light scattering was seen in all zones and layers, apart from the peripheral 10-12 mm zone in the CXL group (P≤0.04; change from baseline in centre layer, zone 0-2 mm, 7.2 GSU; Table 10). A similar but smaller significant increase in corneal light scattering was seen in zone 0-2 mm and 2-6 mm in all layers, and in zone 6-10 mm in the anterior layer in the CRXL group (P=0.00; change from baseline in centre layer, zone 0-2 mm, 2.2 GSU; Table 10). At 6 months a regression of the corneal light scattering was seen in both groups, but the corneal light scattering seen in the CXL group was still significant higher in all zones and layers, apart from the peripheral 10-12 mm zone, compared to baseline (P≤0.01; change from baseline in centre layer, zone 0-2 mm, 4.0 GSU; Table 10). In the CRXL group, only zone 0-2 mm and 2-6 mm in the anterior layer still showed a significant increase in corneal light scattering compared to baseline (P≤0.04; change from baseline in centre layer, zone 0-2 mm, 0.5 GSU; Table 10).
Table 10. Corneal densitometry (light scattering) and the differences in corneal densitometry, in all zones and layers, in both treatment groups (GSU).

<table>
<thead>
<tr>
<th>Baseline Layer/ Zone mm</th>
<th>CXL (mean ± SD)</th>
<th>CXL*</th>
<th>CRXL (mean ± SD)</th>
<th>CRXL†</th>
<th>Diff. between CXL and CRXL (mean ± SD)</th>
<th>95% CI, diff. between CXL and CRXL</th>
<th>P value, CRXL vs. CXL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
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<td>2-6</td>
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<td>10-12</td>
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<td>6 months</td>
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<td>10-12</td>
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</table>

CXL*: densitometry values are the paired difference between values at 1 month and baseline or 6 months and baseline.

CRXL†: densitometry values are the paired difference between values at 1 month and baseline or 6 months and baseline.
In both treatment groups, the increase in corneal light scattering at 1 month was more pronounced in the central cornea and leveled off towards the periphery. In the CRXL group this difference was less marked. At 6 months the difference in corneal light scattering between central and peripheral cornea was still persisted in the anterior and central layers of the CXL group, whereas it could no longer be detected in the CRXL corneas (Table 10).

### 4.6.2. Comparison between groups

The increase in corneal light scattering in the CXL group was more persistent in the central anterior part of the cornea compared with the CRXL group (regression from 1 to 6 months -2.7 ± 7.1 vs. -5.9 ± 4.8 GSU).

The increase in corneal light scattering in the CXL group was also larger at both 1 and 6 months in the deeper layers of the central zones compared with the CRXL group (difference between the groups at 1 month in the posterior layer, zone 0-2 mm, 2.61 GSU, 95% CI 1.03 to 4.19, P=0.00; difference between the groups at 6 months in the posterior layer, zone 0-2 mm, 1.12 GSU, 95% CI 0.24 to 2.00, P=0.01; Table 10).

### 4.6.3. Correlations

At 6 months the increase in central corneal light scattering showed a correlation with the reduction in $K_{\text{max}}$ at the same time point ($R=-0.31$ for the whole material). If the two treatments were analysed separately, the correlation was stronger ($R=-0.45$ for CXL; $R=-0.56$ for CRXL; a linear model $y=-2.19x + 6.49$ ($R^2=0.21$) was found for CXL and $y=-0.99x + 0.66$ ($R^2=0.31$) was found for CRXL). Similar correlations were also found between the increase in central corneal light scattering and minimum corneal radius of curvature ($R_{\text{min}}$; $R=0.49$ for the whole material).

No correlations were found between the increases in central corneal light scattering at 6 months and the changes in: mean keratometry value ($K_{\text{mean}}$), BSCVA, SphEq or CH (only for the CXL group). CH assessed as the change in CH using ORA® or ART, measured at the same time point.

Comparison of the increase in corneal light scattering, measured with corneal densitometry (Paper IV) with the increased in corneal light scattering measured manually from the Scheimpflug photography (Paper I), showed a strong correlation ($R=0.90$; Figure 15).
Figure 15. Correlation between the changes in densitometry measured in Paper IV and the changes in corneal light scattering measured manually in Paper I.

4.7. IOP and biomechanical outcomes after CXL (Paper III)
In this substudy were all KC patients randomized to CXL and their healthy control subjects included. One patient (a female) discontinued the study after the intervention. She was excluded from all analyses, which left 29 eyes of 28 patients (1 female) to be evaluated (see 4.1.). Furthermore, some measurements were missing due to technical problems with the tonometers (GAT, ORA® and ART).

At baseline, both ORA® and ART showed a significant difference in CH between KC patients and control subjects (-2.67 ± 2.55 mmHg, 99% CI -4.05 to -1.32, P<0.01 for ORA®; -1.09 ± 1.92 mmHg, 99% CI -2.26 to 0.07, P=0.01 for ART; Table 11; Figure 16a and 16b).
At 1 and 6 months after CXL treatment, ART showed a significant increase in CH in KC patients (1.2 ± 2.4 mmHg, 99% CI -0.1 to 2.5, P=0.02 difference between 1 month and baseline; 1.1 ± 2.7 mmHg, 99% CI -0.3 to 2.6, P=0.04 difference between 6 months and baseline; Table 11; Figure 16a), whereas ORA® did not (Table 11; Figure 16b).

The increase in CH after CXL treatment measured with ART leveled off the difference in CH between KC patients and control subjects at 1 and 6 months (P=0.16 for 1 month; P=0.20 for 6 months; Table 11; Figure 16a). The increase in CH after CXL treatment measured with ART leveled off the difference in CH between KC patients and control subjects at 1 and 6 months (P=0.16 for 1 month; P=0.20 for 6 months; Table 11; Figure 16a). The difference measured with ORA® persisted (P<0.01 for 1 month; P<0.01 for 6 months; Table 11; Figure 16b) and the CHOR value were unaltered by CXL treatment. Also, the CRFORA values were unaltered through the study (Table 11).
Table 11. IOP and corneal biomechanical outcomes, CT_{min} and K_{mean} at baseline and at 1 and 6 months follow-up after CXL treatment, compared with control subjects.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mean ± SD; P value compared to baseline (paired t test), the corresponding numbers of eyes are in parentheses, indicating the numbers of eyes available for comparison for that variable.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ORA® IOP_{ORA}</td>
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<tr>
<td>n (eyes) Baseline</td>
<td>28</td>
</tr>
<tr>
<td>n (eyes) 1 month</td>
<td>29</td>
</tr>
<tr>
<td>P value (n)</td>
<td>0.30 (28)</td>
</tr>
<tr>
<td>n (eyes) 6 months</td>
<td>29</td>
</tr>
<tr>
<td>P value (n)</td>
<td>0.67 (28)</td>
</tr>
</tbody>
</table>

Control subjects

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mean ± SD; P value compared to patients at the same time point (paired t test), the corresponding numbers of eyes are in parentheses, indicating the numbers of eyes available for comparison for that variable.</th>
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</thead>
<tbody>
<tr>
<td>n (eyes) Baseline</td>
<td>28</td>
</tr>
<tr>
<td>P value (n)</td>
<td>0.86 (27)</td>
</tr>
<tr>
<td>n (eyes) 1 month</td>
<td>29</td>
</tr>
<tr>
<td>P value (n)</td>
<td>0.08 (29)</td>
</tr>
<tr>
<td>n (eyes) 6 months</td>
<td>29</td>
</tr>
<tr>
<td>P value (n)</td>
<td>0.71 (29)</td>
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</tbody>
</table>

IOP_{ORA}; Intraocular pressure measured with Ocular Response Analyzer.
CH_{ORA}; Corneal hysteresis measured with Ocular Response Analyzer.
CRF_{ORA}; Corneal resistance factor measured with Ocular Response Analyzer.
ORA® ART IOP_{ART}; Intraocular pressure measured with Applanation Resonance Tonometry -technology.
CH_{ART}; Corneal hysteresis measured with Appplanation Resonance Tonometry -technology.
GAT IOP_{GAT}; Intraocular pressure measured with Goldmann Appplanation tonometry.
CT_{min}; Corneal thickness at the thinnest point, obtained with the Pentacam® HR.
K_{mean}; Mean keratometry value, obtained with the Pentacam® HR, calculated as (K1+K2)/2.

In patients, the IOP did not change significantly with any method through the study, except for an increase of 1.0 ± 1.7 mmHg at 1 month with GAT (P<0.01; Table 11). There was a significantly decrease in CT_{min} by -24 ± 26 μm (P<0.01) at 1 month after treatment and by -11 ± 21 μm (P=0.01) 6 months after treatment, when compared to baseline (Table 11). A significant change of -0.6 ± 0.7 D was seen in corneal curvature (measured as K_{mean}) at 6 months after treatment (P<0.01; Table 11).
5. Discussion

Corneal crosslinking with UV-A photoactivation of riboflavin (Wollensak et al. 2003a) is an established routine method to halt the progression of ectasia in KC patients by increasing the corneal rigidity (Goldich et al. 2010; Kolli & Aslanides 2010). Several studies report convincing results (Wollensak et al. 2003a; Wollensak 2006; Raiskup-Wolf et al. 2008; Vinciguerra et al. 2009; Goldich et al. 2012). A few standardized treatment protocols are available, but the optimal treatment protocol has not yet been determined (Touboul et al. 2012; Kymionis et al. 2012; Sorkin & Varssano 2014; Tomita et al. 2014), maybe due to the lack of available tools to evaluate the treatments effects in vivo.

There are a lot of in vitro studies confirming the stiffening effect of the crosslinking treatment in corneal tissue (Spoerl et al. 1998; Wollensak et al. 2003b; Wollensak et al. 2004b; Spoerl et al. 2004a; Kohlhaas et al. 2006; Wollensak & Redl 2008; Knox Cartwright et al. 2012; Dias et al. 2013). It is generally accepted that the effect of the crosslinking treatment is caused by an increase in corneal biomechanical strength, which remains stable over time (Beshtawi et al. 2013). Still, repeated attempts to show an increase in CH in vivo with ORA® after crosslinking treatment have failed (Sedaghat et al. 2010; Spoerl et al. 2011; Gkika et al. 2012b; Goldich et al. 2012).

5.1. The CRXL-study (Paper II)

The idea behind the CRXL-study was to find a treatment able to stop the progression of KC and to modify the corneal shape with improvement of the visual quality in those patients with large refractive errors. In an attempt to create a corneal flattening during the crosslinking treatment we used a 12.5 mm semi scleral rigid contact lens with a back surface curvature of 11.0 mm, corresponding to a corneal refractive power of +33.5 D. The contact lens was used to induce a pronounced corneal flattening during the crosslinking treatment, with the aim to “lock” the cornea in this new position. The selection of lens was based on the assumption that some degree of regression was to be expected.

Eyes with more severe KC have been reported to respond better to corneal crosslinking (Greenstein & Hersh 2013; Yam & Cheng 2013), but in this study no differences in baseline values were found between the treatment groups. Apart from the rigid contact lens in the CRXL group, we tried to keep all other treatment variables equal between the CXL and the CRXL groups, by installing riboflavin under the contact lens and by increasing the irradiation time in the CRXL group to compensate for the blocking effect of the contact lens.
Six months after the treatments we found no correlation between the degree of KC and the treatment effect for either of the treatment groups. After CXL treatment we found significant improvements of the SphEq and BSCVA, and a decrease in $K_{\text{max}}$ at 6 months. This is in accordance with the findings of Goldich et al. (2012). Over a period of 2 years, several studies have shown a tendency for the corneal curvature to continuously decrease (Raiskup-Wolf et al. 2008; Vinciguerra et al. 2009; Goldich et al. 2012), but the mechanism underlying the continuous corneal flattening after corneal crosslinking remains unclear (Goldich et al. 2012). In the present study we saw no significant improvements of the variables examined up to 6 months after CRXL treatment, which also makes subsequent improvements unlikely. The study continues, however, and future analyses will answer this question.

Despite our attempt to install the riboflavin under the contact lens, the riboflavin film may have differed between the treatment groups, because the contact lens in the CRXL group was tightly aligned to corneal surface. This may have affected the riboflavin film and accordingly the outcome. The influence of the riboflavin film on the outcomes of the crosslinking effect has been demonstrated in 2010 (a) by Wollensak et al. An alternate explanation to the inferior treatment effect with CRXL could be that oxygen was blocked by the rigid contact lens and did not reach the stroma, a known feature of rigid contact lenses (Compañ et al. 2014). Also after accelerated corneal crosslinking some studies have reported an inferior effect compared to conventional corneal crosslinking, which may also be attributed to oxygen depletion (Touboul et al. 2012; Kymionis et al. 2014). Whatever the reason for the reduced corneal light scattering in the CRXL group is (lower UV-A effect, reduced riboflavin concentration or oxygen depletion), they all will result in a reduced oxidative stress and thereby a reduced corneal crosslinking effect.

Other attempts to combine crosslinking treatment with other procedures to change the corneal shape have shown various degrees of success (Kanellopoulos & Binder 2007; Coskunseven et al. 2009; Kymionis et al. 2010; Cheema et al. 2012; Kremer et al. 2012; Vega-Estrada et al. 2012), although the contribution of crosslinking to the summarized effect may not have been fully elucidated (Cakir et al. 2013). Similar to us, Vega-Estrada et al. (2012) were unsuccessful in maintaining the flattened corneal shape induced by microwave treatment in KC corneas with crosslinking. Perhaps the molecular nature of the crosslinking effect on the corneal stroma can explain why the crosslinking treatment fails to maintain the changes in corneal shape induced by mechanical compression and microwave treatment, or maybe the regression is dose dependent.
5.2. Corneal light scattering after CXL and CRXL (Papers I+IV)

In Paper I we demonstrated that the alterations in corneal light scattering after CXL treatment are readily assessable with non-invasive Scheimpflug photography.

The increased corneal light scattering seen after the CXL treatment was not uniformly distributed through the entire thickness of the cornea. In the superficial 70 µm, corresponding to the epithelium, no increase in corneal light scattering could be demonstrated, which aligns well to a previous in vivo confocal microscopy investigation showing complete epithelial restitution two weeks after treatment (Knappe et al. 2011).

In the stroma, the treatment effect was reduced with increasing corneal depth, in accordance with the Lambert-Beer law (Wollensak et al. 2010a). At 1 month after treatment, our findings with a more pronounced increase in corneal light scattering in the superficial stroma, gradually diminishing down to zero at 240 µm depth is in accordance with this principle and indicates that the corneal light scattering is proportional to the level of irradiation at this time point. Our results are also in accordance with confocal microscopy findings demonstrated by Knappe et al. (2011). They showed that in the early healing phase, the space where the apoptotic keratocytes used to be (keratocytes apoptosis is caused by the crosslinking treatment; see 1.3.4.), are replaced by “large, strongly hyperreflective structures” in the anterior and middle corneal stroma. At about 4 months, in connection with the return of the keratocyte population, these structures gradually lose their hyper reflectivity. These findings correlate well with our findings of increased corneal light scattering.

At 6 months after treatment a significant increase in corneal light scattering occurred at the depth of 240-340 µm. This finding corresponds to the “demarcation line” described by Seiler & Hafezi. The demarcation line does not appear to be directly correlated to the irradiation effect or to the degree of keratocyte apoptosis, such as the earlier, more superficial light scattering. Seiler & Hafezi have assumed that this demarcation line represents “differences in the refractive index and/or reflection properties of untreated versus X-linked corneal stroma” (Seiler & Hafezi 2006). Our impression is that our finding is indeed a demarcation line. Behind this line no effect on the corneal stroma can be detected.

The UV-A light source used in the CRXL-study, expect to have a homogeneous irradiation in the whole 8 mm diameter of the treated area and before each treatment, the effect of the UV-A light beam was verified with a UV-metre. Despite this, there were significant differences in the light scattering at the visual axis, compared to 1-2 mm from the visual axis at 1 month, with more pronounced light scattering at the visual axis. These differences cannot be explained by artifacts induced by the UV-A light or the illumination source of the Scheimpflug camera, nor can it be attributed to
differences in irradiation due to the corneal curvature, since we have made individual corrections for each cornea, based on the distances of the UV-A light and the Scheimpflug camera.

At the corneal apex the collagen structure is different in KC corneas compared to normal corneas (Radner et al. 1998). KC corneas also have a defective defence against reactive oxygen species, which is even less efficient in the central part of the cornea (Kenney et al. 2000; Behndig et al. 2001; Chwa et al. 2008). Irradiation of riboflavin molecules with UV-A light is known to generate superoxide radicals (Segura-Aguilar 1993; Viggiano et al. 2003), and extracellular SOD, which is a major corneal scavenger of superoxide radicals exists in lower amounts in the central cornea (Behndig et al. 2001). It is possible that structural and biochemical differences between the corneal centre and the periphery in KC may contribute to the light scattering patterns seen in Paper I.

In Paper IV we showed that 6 months after treatment the increase in corneal light scattering are related to the degree of corneal flattening. We also showed that the increase in corneal light scattering, measured with corneal densitometry (Paper IV) shows a strong correlation to the increased corneal light scattering measured manually from the Scheimpflug photography (Paper I). In Paper IV our impression is that the demarcation line often seen after crosslinking treatment (Seiler & Hafezi 2006) is a part of what we measured as an increase in corneal densitometry, since it occurs at approximately the same depth and has a similar time course as the increase of corneal light scattering in Paper I. Furthermore, the increased corneal densitometry coincides with the zone where the biomechanical stability is believed to increase after crosslinking treatment (Wollensak et al. 2003b; Kohlhaas et al. 2006).

In accordance with the results in Paper II, where we showed that the refractive effects of CRXL were inferior to those of CXL in some respects, Paper IV showed that the increase in corneal densitometry was also less pronounced in the CRXL group. Even if the increase in corneal densitometry is lower after CRXL, the peripheral cornea shows more densitometry increase relative to the corneal centre. Perhaps the corneal flattening induced by the rigid contact lens caused a more uniform irradiation pattern during the CRXL treatment, compared to the CXL treatment, where the cornea was more steep and protruding.

In the CXL group, the increased densitometry goes deeper in the cornea and has a more prolonged time course, compared to the CRXL group. This finding is in accordance with the finding of a deeper demarcation line after conventional corneal crosslinking compared to accelerated corneal crosslinking reported by Kymionis et al. (2014). It may be that the oxygen tension in the corneal stroma is higher during conventional crosslinking treatment.
5.3. Corneal hysteresis after CXL (Paper III)

In Paper III we demonstrated an increase in CH in vivo with ART after CXL treatment for KC. At baseline we found a significant difference in CH between KC patients and control subjects with both ART and ORA®, but after CXL treatment we only found a significant increase in CH with ART. The CH* value was unaltered after CXL treatment, in accordance with previous studies (Sedaghat et al. 2010; Spoerl et al. 2011; Gkika et al. 2012b; Goldich et al. 2012).

ART utilizes resonance technology and it is based on the assumption of constant acoustic impedance in the corneal tissue. Theoretically, it would be possible that the corneal acoustic impedance could be changed by the CXL treatment, but then also the measured IOP* would have changed accordingly, which did not occur. In addition, CH* was calculated as the difference in IOP between the applanation and the removal phase (Hallberg et al. 2004) and a change in the corneal acoustic impedance would not change this difference, consequently it would not affect CH*.

In viscoelastic materials, the counterforce decreases at lower speed, i.e. the difference in IOP between the applanation and the removal phase decreases and CH at low speed will be smaller. ART measures CH at a lower speed than ORA® and therefore have the CH* lower values.

By using a sensor tip with a radius of curvature of only 10 µm, Murayama et al. (2004) have shown that resonance technology is useful to detect elastic properties of small cell membranes. The sensor tip of the ART has a radius of curvature of 8 mm. For measuring crosslinking treatment induced corneal changes, ART would probably benefit from a sensor tip with a steeper curvature (smaller radius of curvature). This would probably increase the sensitivity of the CH* measurements, since the only tissue of interest is the tissue in the corneal centre. The ORA® utilizes a standardized air puff that causes a rather large area of corneal flattening. It is possible that the corneal bending may occur partially outside the treated area, which could lead to difficulties in detecting the stiffening effect from the crosslinking. On the other hand, ORA® shows a significant difference in CH when comparing KC patients with healthy control subjects. In KC patients the thickness of the peripheral cornea is preserved while the central cornea is affected (Fontes et al. 2011). Possibly, the inability of ORA® to detect increased CH after CXL may be caused by the fact that the interlamellar cohesion of the corneal stroma is unaffected by crosslinking, which have been demonstrated by Wollensak et al. (2011). This, however, does not explain the increase in CH seen with ART after CXL.

The decrease in CT* and K* after CXL treatment cannot have contributed to the increase in CH*, because a thinner and flatter cornea would rather have decreased the CH (Narayanaswamy et al. 2011; Hwang et al. 2013). It is generally accepted that a thick (rigid) cornea and a steep
cornea results in an overestimation of the applanation IOP and *vice versa* for a thin cornea and a flat cornea (Whitacre & Stein 1993). Accordingly, KC patients often have lower IOP due to thin corneas (Rask & Behndig 2006). ART and ORA® are two tonometers developed to be less sensitive to different corneal biomechanical properties, such as thickness and curvature (Luce 2005; Hallberg *et al.* 2004). We found that after CXL treatment, $IOP_{ART}$ and $IOP_{ORA}$ were unaltered. At 1 month after CXL treatment $IOP_{GAT}$ was significantly increased compared to baseline, despite the corneal thinning seen at the same time point. We interpret the increased $IOP_{GAT}$ value as a sign of increased corneal rigidity induced by the CXL treatment.
6. Conclusions

To summarize our results, eyes treated with CXL stabilized or improved, and we can confirm the positive results with conventional crosslinking reported by others (Raiskup-Wolf et al. 2008; Vinciguerra et al. 2009; Goldich et al. 2012). After CRXL, however, the results were less convincing. Corneal haze (increased light scattering) was a consistent finding in both treatment groups, which indicates that a crosslinking effect took place in all our subjects. In accordance with the inferior refractive effects of CRXL, we showed that the increase in corneal densitometry was also less pronounced after this treatment.

We also found that the corneal densitometry increase differs in different parts of the cornea and appears to be related to the crosslinking effect. This suggests that the increase in densitometry is a potential indicator of the treatment effect.

The increase in corneal light scattering measured manually showed a strong correlation with the corneal light scattering measured with automated corneal densitometry. Since the automated densitometry method is considerably faster, the automated method has a greater potential to become a clinically useful tool.

Finally, using ART technology, we have been able to assess an increase in CH in vivo after CXL, which has not been demonstrated previously.
7. Future perspectives

Given the widespread use of CXL in today’s KC treatment arsenal, tools capable of quantifying the treatment effect \textit{in vivo} after CXL treatment has a great potential value. Scheimpflug photography and corneal densitometry appear to be useful tools to map the increased corneal light scattering after corneal crosslinking treatment. The light scattering may give valuable information about the corneal response to the crosslinking treatment. It provides an impression of the stromal changes that occur after the treatment and also the depth of the treatment effect. Likewise, a device based on ART - technology can also become a useful tool in future assessment of corneal biomechanical properties after corneal crosslinking treatment.

Further evaluation of the CRXL method and modification of the treatment parameters will be needed. Possibly, the crosslinking effect could be augmented, and the corneal riboflavin concentration and/or the oxygen tension under the rigid contact lens could be increased. It could even be considered to leave the contact lens in the eye for a longer time after the treatment to stabilize the cornea in a flattened position.
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