The assessment of disease progression in keratoconus and corneal crosslinking in thin corneae

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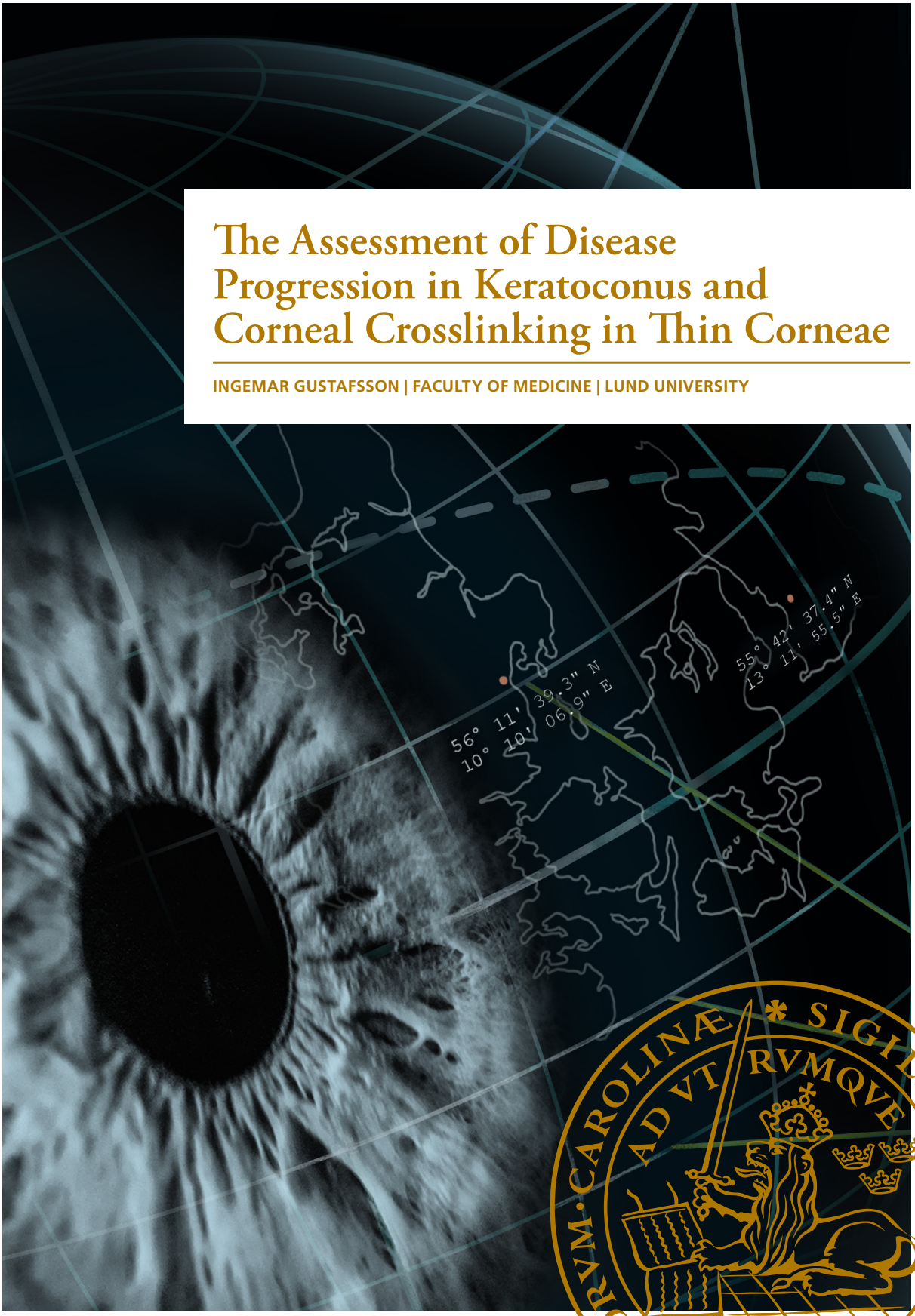

The Assessment of Disease Progression in Keratoconus and Corneal Crosslinking in Thin Corneae

The early diagnosis of progressive keratoconus is of fundamental importance for timely referral to corneal crosslinking in order to preserve visual acuity. This thesis describes how progression can be detected at an earlier stage, and how overdiagnosis, and thus unnecessary crosslinking, can be avoided using currently available equipment. This thesis also describes how thin corneae can be treated with corneal crosslinking.

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The Assessment of Disease Progression in Keratoconus and Corneal Crosslinking in Thin Corneae

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Keratoconus generally manifests in adolescents, and can progress leading to severely impaired vision. The risk of progression is inversely correlated to age; thus younger patients are at higher risk than older ones. Progressive keratoconus can be halted by corneal crosslinking (CXL). The general indication for CXL is progressive keratoconus, although children are commonly treated with CXL upon diagnosis. Tomography is used to assess progression, and the most commonly used system is the Pentacam HR. Measurements made on different visits are compared to determine whether the patient's keratoconus has progressed, and they should be referred for CXL. However, there is no consensus on which parameters should be used, or the change in magnitude of these parameters that indicates progression. An increase in the curvature power of the steepest point on the anterior surface, Kmax, of 1.0 dioptres is commonly used for all patients. However, there is little evidence that this is appropriate. Furthermore, inconsistent results have been presented regarding the magnitude at which progression can be detected. Such studies are often based on determinations of the repeatability of measurements made on one occasion. However, the progression of keratoconus is evaluated from measurements made on different occasions, and it is reasonable to assume that measurements obtained on different days will be subject to greater variation due to the biomechanical instability of corneae affected by keratoconus. Also, it has been suggested in studies that the repeatability of measurements in subjects with more severe keratoconus have poorer repeatability. Another important aspect of keratoconus is that it is a thinning disorder. A minimum corneal thickness of 400 µm has been suggested for the safe performance of CXL. Thus, a significant proportion of keratoconus patients will be excluded from the standard CXL treatment protocol.

Thus, in the first study we elucidated the association between measurement error and disease severity. In the second investigation we investigated the inter-day repeatability and in the third investigation we investigated the Belin ABCD Progression Display. In the fourth study we investigate a protocol in which sterile water was added during the crosslinking procedure to increase the corneal thickness.

The results demonstrated that the measurement error is associated to the disease severity and that limits at which progression is defined should be defined by inter-day measurements. The results also suggest that the diagnosis of progressive keratoconus by the Belin ABCD Progression Display will lead to overdiagnosis of progression. Further, the results suggest that the addition of sterile water is effective in increasing the corneal thickness above the suggested safety limits. These results have important clinical implications. The results demonstrate that limits at which progression is defined should be stratified according to the severity of the disease. Patients with less advanced keratoconus will be underdiagnosed as progressive if commonly used parameters are not stratified according to disease severity. This could lead to delayed referral for CXL, resulting in an avoidable risk of deterioration in vision. Patients with more advanced keratoconus, on the other hand, would be overdiagnosed as progressive, which could lead to unnecessary CXL, thus subjecting the patient to discomfort and possible treatment-associated complications. This risk of overdiagnosis of progression is also relevant when using the Belin ABCD Progression Display. Further, The data suggest that such a simple measure as adding sterile water during corneal crosslinking could enhance the corneal thickness above safety limits.

Key words: keratoconus, corneal crosslinking, Scheimpflug, corneal tomography

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To Lorenza, Alberto and Edvard

Continuous effort – not strength or intelligence
– is the key to unlocking our potential.

Sir Winston Churchill

Abstract

Keratoconus generally manifests in adolescents, and can progress leading to severely impaired vision. The risk of progression is inversely correlated to age; thus younger patients are at higher risk than older ones. Progressive keratoconus can be halted by corneal crosslinking (CXL). The general indication for CXL is progressive keratoconus, although children are commonly treated with CXL upon diagnosis. Tomography is used to assess progression, and the most commonly used system is the Pentacam HR. Measurements made at successive visits are compared to determine whether the patient's keratoconus has progressed, and they should be referred for CXL. However, there is no consensus regarding which parameters should be used, or the change in magnitude of these parameters that indicates progression. An increase in the curvature power of the steepest point on the anterior surface, Kmax, of 1.0 dioptres is commonly used for all patients. However, there is little evidence that this is appropriate. Furthermore, inconsistent results have been presented regarding the magnitude at which progression can be detected. Such studies are often based on the repeatability of measurements made on one occasion. However, the progression of keratoconus is evaluated from measurements made on different occasions, and it is reasonable to assume that measurements obtained on different days will be subject to greater variation due to the biomechanical instability of corneae affected by keratoconus. Furthermore, studies have indicated that the repeatability of measurements in subjects with more severe keratoconus is poorer. Another important aspect of keratoconus is that it is a thinning disorder. A corneal thickness of 400 μm has been suggested as the minimum for the safe performance of CXL. Thus, a significant proportion of keratoconus patients will be excluded from the standard CXL treatment protocol.

The first study thus elucidated the association between measurement error and disease severity. The inter-day repeatability of measurements and the reliability of the Belin ABCD Progression Display in diagnosing progression were the subjects of the second and third studies. Finally, a protocol in which sterile water was added during the crosslinking procedure, to increase the corneal thickness, was investigated.

The results showed that the measurement error is correlated to the disease severity, and that the limits used to define progression should be based on inter-day measurements. The results also suggest that the diagnosis of progressive keratoconus using the Belin ABCD Progression Display will lead to the

overdiagnosis of progression. The results of the final study suggest that the addition of sterile water is effective in increasing the corneal thickness above the suggested safety limit.

These results have important clinical implications. The first is that the limits at which progression is defined should be stratified according to the severity of the disease. Patients with less advanced keratoconus will be underdiagnosed as progressive if commonly used parameters are not stratified according to disease severity. This could lead to delayed referral for CXL, resulting in an avoidable risk of deterioration in vision. Patients with more advanced keratoconus, on the other hand, would be overdiagnosed as progressive, which could lead to unnecessary CXL, thus subjecting the patient to discomfort and possible treatment-associated complications. There is also a risk of overdiagnosis of progression when using the Belin ABCD Progression Display. Furthermore, the results suggest that such a simple measure as adding sterile water during corneal crosslinking could increase the corneal thickness above the current safety limit and thus permit subjects with thin corneae to undergo crosslinking.

Papers Included in this Thesis

This thesis is based on four papers, which are referred to in the text by their Roman numerals.

I. Gustafsson I, Bergström A, Myers AC, Ivarsen A, Hjortdal J
Association between Keratoconus Disease Severity and Repeatability in Measurements of Parameters for the Assessment of Progressive Disease
PLoS One 2020; 15 (2): e 0228992O

II. Gustafsson I, Bergström A, Cardiakides A, Ivarsen A, Hjortdal JØ
The Interday Repeatability of Parameters for the Assessment of Progressive Disease in Subjects with Less Advanced Keratoconus
Am J Ophthalmol 2021 Jan. 7; 225: 38- 46

III. Gustafsson I, Faxén T, Vicente A, Bergström A, Ivarsen A, Hjortdal JØ An Inter-day Assessment of the ABC Parameters in the Evaluation of Progressive Keratoconus
Sci Rep 2021 Aug 6;11(1):16037

IV. Gustafsson I, Cardiakides Myers A, Ivarsen A, Hjortdal JØ
Retrospective Analysis of the Effects of Using Sterile Water in Addition to Hypoosmolar Riboflavin during Corneal Collagen Crosslinking for Keratoconus
J Cataract Refract Surg 2017 Mar. 43 (3): 426-427

Abbreviations

"A"	Anterior curvature of the 3 mm zone over the thinnest point in the Belin ABCD Progression Display
ARK	Auto-refracto-keratometer
"B"	Posterior curvature of the 3 mm zone under the thinnest point in the Belin ABCD Progression Display
"C"	Thickness at the thinnest point on the cornea in the Belin ABCD Progression Display
CI	Confidence interval
CV	Coefficient of variation
CXL	Corneal crosslinking
DALK	Deep anterior lamellar keratoplasty
HPMC	Hydroxypropyl methylcellulose
ICC	Intraclass correlation coefficient
K1	Curvature power of the central flat meridian
K2	Curvature power of the central steep meridian
Kmax	Curvature power of the steepest point on the anterior surface
MCT	Minimum corneal thickness
PK	Penetrating keratoplasty
PL	Prediction limit
R	Repeatability coefficient
Rmin	Curvature power of the steepest point on the posterior surface
S_w	Within-subject standard deviation
UVA	Ultraviolet A
IQR	Inter-quartile range
OCT	Optical coherence tomography
SD	Standard deviation

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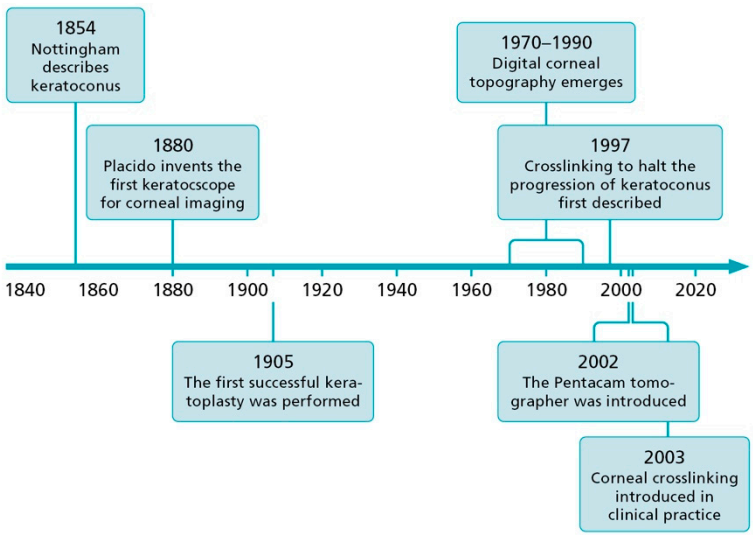
GENERAL INTRODUCTION

The term “keratoconus” was used for the first time in 1854, by John Nottingham in his comprehensive description of the condition, and this publication is commonly considered to be the first publication on keratoconus[1]. Important diagnostic and therapeutic strategies have been developed since then, as summarized in the timeline below[2-4].



A brief description of the anatomy and physiology of the cornea

The cornea[5, 6] is responsible for most of the refraction of incoming light, corresponding to approximately 43 dioptres (D). The lens then focuses the light on the fovea through appropriate accommodation, according to the distance to the observed object. The cornea also protects the eye from various kinds of stress and trauma.

The cornea is highly innervated, helping to identify threats such as microbial infection and foreign bodies.

The cornea measures approximately 11 mm vertically and 12 mm horizontally. The thickness is typically 500 µm at the centre, and increases towards the periphery. The cornea is divided into five layers and membranes: the epithelium and its basal membrane, Bowman's layer, the stroma, Descemet's membrane and the endothelium (Figure 1).

The epithelium is approximately 45 µm thick and is a stratified, non-keratinized, squamous epithelium composed of 4-6 cell layers. The superficial cells contain the glycocalyx, which interacts with the mucin from the goblet cells to provide a smooth tear film, and thus an optimal refractive surface. The superficial cells are strongly connected, while the basal cells are strongly attached to the underlying basal membrane by hemidesmosomes. The epithelium is important in protecting the eye against UV irradiation, microbes and other external effects. Below the epithelial basal membrane is Bowman's layer. This is acellular, and is made up of collagen. Its role is to reinforce the cornea.

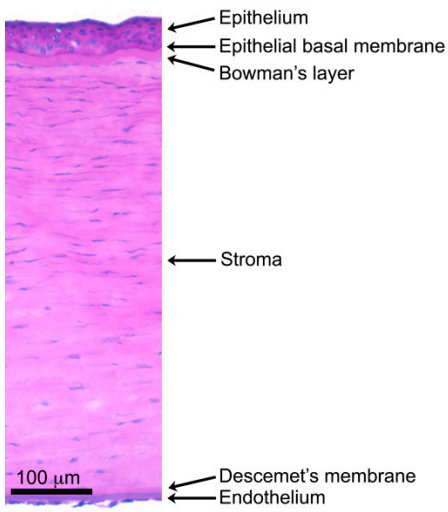


Figure 1. Histological cross-section of a normal cornea. (Photo courtesy of André Vicente MD, PhD.)

The stroma makes up approximately 80-85% of the cornea. The stroma contains collagen fibres arranged in bundles called fibrils. These fibrils are arranged parallel to each other and form 200-250 layers that make up the stroma. The layers are arranged orthogonally to each other. This arrangement ensures that the cornea is transparent, and affords it strength. The stroma also contains keratocytes that synthesize collagen and maintain the extracellular matrix.

Descemet's membrane is made up of collagen fibres. The underlying endothelium continuously produces collagen, and Descemet's membrane thus becomes thicker with age. The endothelium consists of a monolayer of hexagonal cells without mitotic activity[4]. The cell density of the endothelium decreases from approximately 4000 cells/mm^2 at birth, at a rate of 0.6% per year. These cells are important ion transporters creating a hypertonic aqueous humour. This leads to an osmotic gradient that is responsible for maintaining a degree of hydration of 80% in the stroma, which is necessary to maintain the transparency of the cornea.

Pathophysiology of keratoconus

Corneae affected by keratoconus have lower biomechanical strength than normal corneas[7], and the intraocular pressure causes protrusion at the weakest part. This biomechanical weakening is attributed to collagen degradation by proteolytic enzymes in combination with a reduction in proteinase inhibitors[8]. A reduction in collagen-producing keratocytes has also been reported[9]. Together, these factors lead to a negative balance between the production and degradation of the proteins, leading to a thinner and weaker cornea. Apart from a reduction in collagen lamellae, a pathological distribution of the protein lamellae contributes to the thinning of the cornea[10]. This redistribution of lamellae could be attributed to reduced inter-lamellar adhesions[11], weaker interlamellar interlacing and weaker interlacing with Bowman's membrane[12]. It is intuitive to associate this reduced interlamellar strength with the effects of atopy and eye rubbing in the development of keratoconus. Although atopic disease is 7 times more frequent in these patients[13], keratoconus can develop independently of eye rubbing[14].

The pathophysiology behind keratoconus is intriguing, and not yet fully understood. Keratoconus is not always an isolated disease entity, but is sometimes associated with syndromes such as Leber's congenital amaurosis[15], Turner's syndrome[16] and Down's syndrome[17]. Furthermore, keratoconus appears to be associated with endocrine and metabolic factors[18], and has been clinically associated with pregnancy and thyrotoxicocis[19, 20], hormone replacement therapy[21] and *in vitro* fertilization[22]. Keratoconus has also been reported in association with several ophthalmic disorders, both corneal and non-corneal[23]. The pathogenesis of keratoconus requires further investigation, including genetic studies. In a large study performed recently, 36 genomic loci were found to be associated with keratoconus, 31 of which were described for the first time[24]. This could be important in elucidating the development of keratoconus.

Epidemiology

The prevalence of keratoconus has been investigated in several studies worldwide, however, significant differences have been found between countries. One of the lowest rates of prevalence was found in the United States[25], 0.17 in 1000 inhabitants, and one of the highest in Iran[26], with a rate of 40 in 1000. From a European perspective, data from the Netherlands[27] suggest 2.65 in 1000, while data from Denmark[28] suggest 0.86 in 1000. No epidemiologic data from Sweden are currently available. Recently, a study carried out in Australia suggested a prevalence of keratoconus of 1.2%, which is one of the highest reported so far[29]. Some investigations have suggested that both sexes are equally affected by keratoconus[30-32], while others have suggested a predominance of keratoconus in males, of 60.6%[27] and 66.9%[13]. A predominance among males has also been seen at our clinic when recruiting participants in previous and ongoing investigations. To ensure that the recruitment of subjects was not skewed from a gender perspective, information was obtained from the National Swedish Patient Register. Between 2014 and 2018, 1756 patients were diagnosed with keratoconus at the Skåne University Hospital. The proportion of *males* ranged from 73 to 79% over this period, reflecting the gender distribution among the recruited patients[33]. A number of factors can affect the reported prevalence in different countries, such as the use of more precise instruments and differences in the way in which keratoconus is defined. Genetic differences between populations could also explain some of the differences, however, a recent publication suggested remarkable genetic consistency regarding the genes associated with keratoconus across different ethnic groups[24].

Clinical features

Keratoconus is considered to be a bilateral, asymmetric disease causing different degrees of irregular astigmatism with reduced and distorted visual acuity that can have a significant impact on the quality of life[34, 35]. In general, keratoconus develops within the first two decades of life, and is associated with a risk of progressive steepening and thinning of the cornea, and thus progressive visual deterioration (Figure 2). Younger age at diagnosis is associated with a higher risk of progression[36, 37], and the need for penetrating keratoplasty (PK)[38]. Progression usually ceases or slows down at a certain age, and clinically, progression is rarely seen after the age of 40 years.

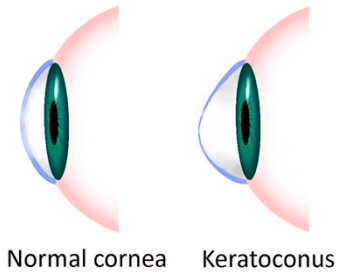


Figure 2. The illustration on the left shows a normal cornea, while that on the right shows thinning of the cornea with resulting protrusion and irregular shape in a subject with keratoconus. (Courtesy of Jenny Hult MD).

Diagnosis

Moderate to advanced keratoconus can generally be detected during a slit-lamp examination, revealing stromal thinning, conical protrusion, an iron line (Fleischer ring) close to the cone area, and discreet striae (Vogt's striae) in the deep stroma that disappear on digital pressure[39]. Stromal scars and prominent corneal nerves may also be visible[23]. In advanced cases, Munson's sign or Rizzuti's sign can be present[40]. In less advanced keratoconus, the anamnesis and best-corrected visual acuity can suggest underlying keratoconus, however, definitive diagnosis relies on examination using corneal topography or tomography[41]. In the absence of modern tomographic equipment, a Placido-based instrument or a Javal keratometer can reveal keratoconus through distorted images[42].

There is no consensus on the diagnostic criteria of keratoconus. In 2015, The Global Consensus on Keratoconus and Ectatic Diseases[43] suggested that the diagnosis of keratoconus depended on: 1) abnormal posterior elevation, 2) abnormal corneal thickness distribution, and 3) clinical noninflammatory thinning, and that tomography was the best and most widely available diagnostic tool for the diagnosis of early keratoconus (i.e. a Scheimpflug-based imaging device or optical coherence tomography (OCT)). The most commonly used tomographic system is the Pentacam HR[44] (a Scheimpflug device). The Pentacam HR has two kinds of software that can be used to detect keratoconus, namely the "four maps refractive display"[41] (Figure 3) and the "Belin-Ambrosio Enhanced Ectasia Display"[45] (Figure 4). The latter has improved capacity to detect cases of mild keratoconus[46].

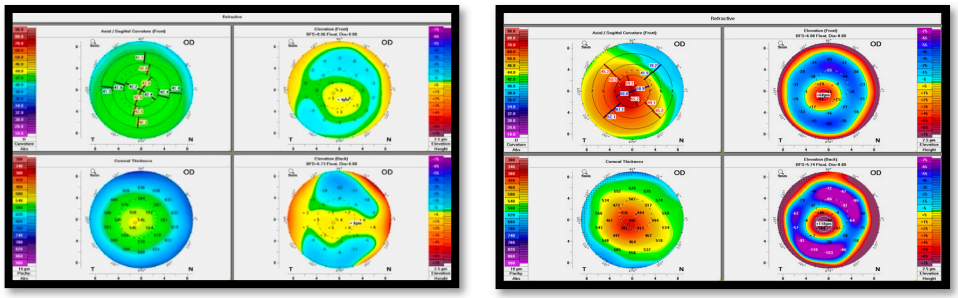


Figure 3. The four maps refractive display in the Pentacam HR. The left figure shows measurements of a normal cornea, and that on the right a cornea with keratoconus. (Photos by the author unless stated otherwise).

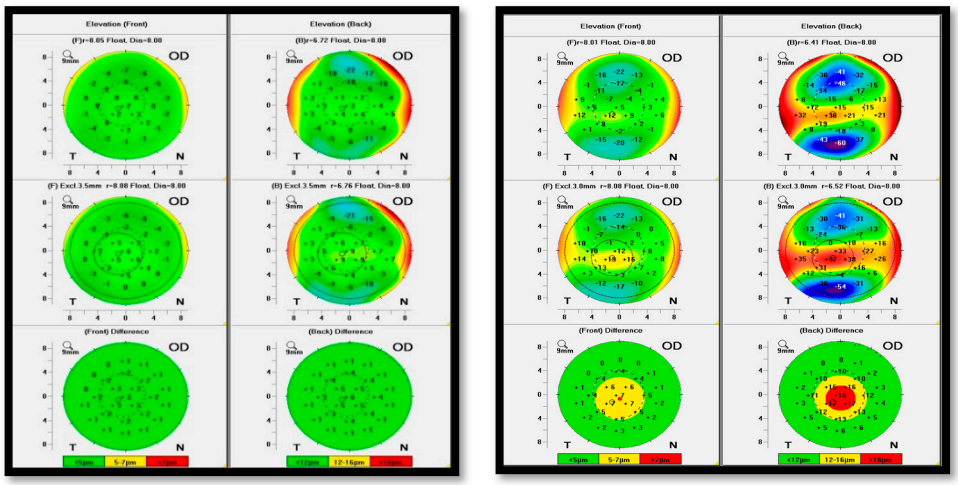


Figure 4. The Belin-Ambrosio Enhanced Ectasia Display in the Pentacam HR. The left figure shows results from a normal cornea, and that on the right results from a cornea with keratoconus. The upper images show the two elevation maps (left image – front elevation, right image – back elevation) from the four maps refractive display. The centre images are from the enhanced ectasia display (left image – front elevation, right image – back elevation). The ectatic area is more evident in the enhanced ectasia display. The lower images show the difference between the enhanced ectasia display and the four maps refractive display.

The use of biomarkers has been suggested for the early diagnosis of keratoconus[47]. Biomarkers could indicate prophylactic treatment by corneal crosslinking before the indication of keratoconus by corneal tomography. Biomarkers could also serve as a screening tool prior to refractive surgery to avoid future complications.

Indications for treatment with corneal crosslinking

In general, the indication for corneal crosslinking (CXL) is documented or perceived progressive keratoconus[43]. The US FDA also suggests progression as the indication for treatment with CXL[48]. It is suggested that children and adolescents undergo CXL upon diagnosis due to the risk of rapid progression[49]. The risk of progression is inversely correlated to age[36, 37, 38], and it is well known from clinical practice that progression can be rapid in young patients. Unfortunately, there is no consensus on the diagnosis of progression. The Global Consensus on Keratoconus and Ectatic Diseases[43] suggested that a consistent change in at least two of the following parameters, where the magnitude of change was above the measurement error of the testing system, was suggestive of progression: 1) steepening of the anterior surface, 2) steepening of the posterior surface, and 3) thinning and/or an increase in the rate of change of corneal thickness from the periphery to the thinnest point. However, neither specific parameters, nor a specific change in magnitude of these, was suggested.

In the same year as the Global Consensus on Keratoconus and Ectatic Diseases was published (2015), a new system was introduced for the classification of keratoconus disease severity (The Belin ABCD Grading System)[50], and later also for the detection of progressive disease (The Belin ABCD Progression Display)[51]. Software for these is incorporated in the Pentacam HR, allowing the analysis of the anterior curvature (A), the posterior curvature (B) and the corneal thickness (C) in a 3 mm zone centred around the thinnest point on the cornea. D represents visual acuity, and can be added at the clinician's discretion. It has been suggested that it is better to measure the parameters A and B over an area instead of at a single point on the cornea, as in the case of curvature power of the steepest point on the anterior surface (Kmax) and curvature power of the steepest point on the posterior surface (Rmin)[51]. Measurements obtained over time are presented graphically by the software, and so-called "gates" represent limits at which progression is suggested.

Treatment of keratoconus

Contact lenses

The use of rigid contact lenses is important in the management of keratoconus as they improve visual acuity by correcting the irregular shape of the cornea. Development in recent years has allowed for the correction of more advanced keratoconus and rendered the lenses more tolerable. In fact, contact lenses can delay or reduce the need for corneal transplantation[52].

Corneal crosslinking

The use of CXL for the stabilization of keratoconus was suggested in 1997 following a study on an animal model[2], and the first clinical results were presented in 2003[4]. The method induces crosslinks between the collagen fibrils and between the fibrils and the extracellular matrix in the cornea, thus increasing its biomechanical strength, and preventing further progression of the disease[53, 54]. To achieve this, the cornea is soaked in riboflavin, followed by irradiation with UVA light (365 nm). Riboflavin acts as a photoreactor and induces free radicals, followed by the creation of covalent crosslinks[55]. Riboflavin also provides a shield against excessive radiation, which could damage the endothelial cells[55]. However, the riboflavin molecule is too large to pass through the corneal epithelium[56], so this must be abraded in order to allow the riboflavin to reach the cornea[57, 58]. In the original Dresden Protocol of 2003, isoosmolar riboflavin was instilled in the de-epithelized cornea, followed by radiation of 3 mW/cm^2 for 30 minutes[4]. A treatment protocol using radiation of 9 mW/cm^2 (10 min treatment time) was later developed to reduce the treatment time. This has since been followed by higher fluence rates of 18 mW/cm^2 (5 min treatment time) and 30 mW/cm^2 (3 min treatment time)[59]. However, it has been found that the induction of crosslinks is oxygen dependent, and too intense irradiation will be less effective in inducing crosslinks due to the rapid initial depletion of oxygen, leading to poorer clinical outcome[60-63]. In order to circumvent the problem of early oxygen depletion, it has been suggested that oxygen be supplied during CXL[64], or that pulsed UVA irradiation be used[65]. The latter would ensure slower oxygen consumption such that the stroma would be naturally replenished with oxygen during the treatment. The Dresden protocol, with a fluence of 3 mW/cm^2, does not appear to benefit from the addition of oxygen, probably because of the low fluence, and thus lower oxygen consumption per unit time[66].

To avoid endothelial damage due to the effects of UVA radiation and free oxygen radicals, it has been suggested that the minimum thickness of the cornea for CXL is 400 µm[55, 67]. An isoosmolar solution of riboflavin containing dextran (402.7 mOsmol/L)[4] has been used In the Dresden protocol (the osmolarity of the cornea is approximately 420 mOsml/L)[68]. However, this solution, combined with the evaporation of water from the de-epithelized cornea, was sometimes found to cause a reduction in the thickness to less than 400 µm[69-71], thus exposing the endothelium to risk. Reduction in the corneal thickness is associated with the treatment time using protocols employing an isoosmolar solution of riboflavin with dextran[69, 72, 73], so, from this point of view, shorter treatment protocols are preferable. The reduction in corneal thickness was found to be less when an eyelid speculum was not used during riboflavin instillation[74], and when the eyelids were kept closed between drops when administering riboflavin[75]. The physiologic effect on corneal hydration when eyelids are kept closed has been described previously[76]. However, the corneal thickness could still be reduced below 400 µm. In one study, the corneal thickness of the investigated cohort decreased to less than 400 µm after approximately 15

minutes' irradiation[74]. In order to maintain the corneal thickness during CXL, thus making more subjects eligible for CXL, a hypoosmolar riboflavin solution[77] (310 mOsmol/L) was introduced in 2009[78]. However, the swelling of the cornea due to the hypoosmotic nature of the solution is thought to be temporary, and was not maintained throughout the 30 minutes of UVA irradiation[75]. In order to avoid a reduction in the corneal thickness during CXL and to render more patients with thin corneas eligible for CXL, a riboflavin solution based on hydroxypropyl methylcellulose (HPMC) has been suggested, and positive results have been reported[79, 80]. However, pre-clinical data suggest only superficial UVA absorbance and increased corneal volume, which could reduce the crosslinking effect[77, 81]. Long-term clinical follow-up and more pre-clinical data are thus required. So-called "epithelium-on" or "epi-on" CXL techniques have been developed for the purpose of maintaining the corneal thickness during CXL. Retaining the epithelium makes the cornea thicker *per se*, and should reduce the evaporative effect during CXL[70]. Theoretically, retaining the epithelium could also reduce pain during the procedure, and the risk of microbial keratitis following CXL[82]. However, as the riboflavin molecule is too large to pass through the epithelium[57] "enhancers" such as benzalkonium chloride or EDTA must be added[83, 84]. These disintegrate the epithelium making it more porous[83]. It can thus be debated whether the epithelium actually remains "on" the cornea, and whether this process actually reduces pain or the risk of infection. Pre-clinical concerns have also been raised in connection with epi-on protocols as the epithelium may reflect some of the UVA light[85, 86] and the flow of oxygen can be expected to be reduced[87, 88], and less riboflavin will reach the stroma[89], all of which would reduce the effect of CXL. In fact, it has been suggested that epi-on techniques lead to less stiffening of the cornea[84], a more superficial demarcation line[90], and are less effective in halting the progression of keratoconus[90-92]. However, it has also been suggested that epi-on CXL is safer and is associated with fewer side effects[90]. Positive results have also been reported in epi-on investigations[82], although these have been questioned[93]. Iontophoresis-assisted transport of riboflavin into the cornea (denoted i-CXL) is an alternative to the epi-on protocol. A small electric current is used to create an electromotive force that leads the riboflavin into the stroma[94]. However, this method is also associated with the problem of low oxygen diffusion, and possibly lower levels of intra-stromal riboflavin concentration than in so-called "epi-off" techniques[95]. In fact, most investigations suggest that i-CXL is less efficient in halting progression[96-98]. CXL in thin corneae is further discussed in the section "Background of the projects" below.

Several questions remain to be answered in connection with CXL. The first is, how corneas < 400 µm thick can be safely treated, which was investigated in this work. Two other important questions are, how many crosslinks are needed in different cohorts of patients[13], and how long the effect of CXL actually lasts. These are briefly discussed under Future perspectives.

Combination of corneal crosslinking with photorefractive keratectomy

The aim of this technique is to improve the refractive surface of the cornea by laser-assisted photorefractive keratectomy, followed by CXL to stabilize the cornea. Positive refractive outcomes and stabilization of the cornea have been reported[99, 100], but visual loss has also been observed due to haze induced by the combination of photorefractive keratectomy and CXL[101].

Intracorneal ring segments

The use of intracorneal ring segments in patients with keratoconus was described in 2000[102], and the US FDA approved their use in 2004 through a humanitarian device exemption. It has been suggested that intracorneal ring segments are indicated in patients with less advanced keratoconus who have contact lens intolerance, or when a modest increase in uncorrected and best-corrected visual acuity is desired[103]. Corneal flattening and improved vision have been reported[103], however, this surgical technique is also associated with risks such as intracorneal infection and erosion of the anterior surface by the ring segments[103, 104].

Corneal transplantation

Corneal transplantation is indicated when keratoconus has reached the point at which contact lenses cannot improve visual acuity, or when contact lenses cannot be worn due to high corneal steepness. Surgery involves a full-thickness graft (i.e. penetrating keratoplasty) or deep anterior lamellar keratoplasty (DALK), the latter leaving the patient's endothelium and Descemet's membrane intact. Surgery is generally successful in terms of graft survival and in improving the patient's visual acuity. However, surgery is associated with both short- and long-term risks, most importantly, the risk of graft rejection. The endothelium is the most common tissue in the cornea against which graft rejection occurs, and there is thus a clear advantage in performing DALK. Indeed, the five-year risk of graft rejection is 50% in PK, but only 1-2% in DALK[105]. However, no difference has been found between the visual outcome following PK and DALK[105]. The use of both PK and DALK in the treatment of keratoconus has decreased in the past decade, possibly due to better contact lenses[52] or the use of CXL[106], or a combination of both (Figure 5).

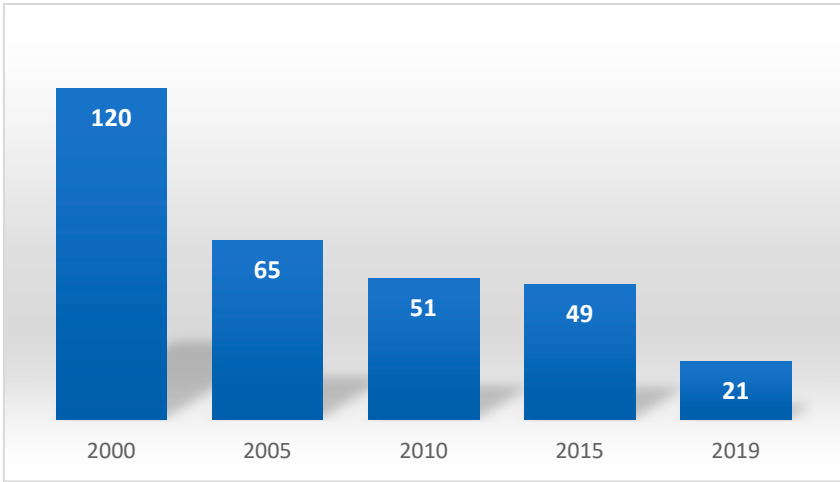


Figure 5. The number of penetrating keratoplasties performed to treat keratoconus in Sweden in five-year intervals during the past 20 years. The final period ended in 2019 due to the SARS COVID-19 pandemic. Only one DALK was performed in 2019 and none during the other years. (Data obtained from the Swedish Corneal Transplant Registry, reproduced with due permission.)

MOTIVATION FOR THE STUDIES

Pre-clinical studies have demonstrated that CXL induces crosslinks and increases the stiffness of the cornea[2, 54, 84, 107]. In addition, there is long-standing clinical experience of its efficacy. Nevertheless, the clinical efficacy of CXL in stopping the progression of keratoconus was deemed very low in a Cochrane Review in 2015[44]. The relatively late approval of CXL by the US FDA in 2016 also appears to be due to a lack of robust scientific evidence[108]. The Cochrane Review also commented on the lack of well-performed randomized clinical trials. Furthermore, there is a lack of consensus on the definition of progressive keratoconus, and the evaluation of treatment efficacy, both of which have led to difficulties in performing meta-analyses on the available data[109]. Today, there is more evidence supporting the efficacy of CXL in halting the progression of keratoconus[110, 111], however, the continued lack of consensus on the definition of progressive keratoconus remains, which could delay the introduction of evidence-based science in clinical practice.

The most common definitions of progression are an increase in Kmax of 1.0 D, alone[44], or in addition to changes in other parameters[111] such as an increase of 1.00 D or more in manifest cylinder, or an increase of 0.50 D or more in manifest refraction spherical equivalent. An increase greater than these in one or more of these three parameters was considered a sign of progression in the investigation behind the US FDA approval[111]. However, other parameters have also been used[51]. Several investigators have evaluated the repeatability of measurements of parameters commonly used in the detection of progression, often with conflicting results[112-114]. The inter-operator effect on the repeatability of measurements has been investigated[112, 115], but cannot be considered to explain the incongruences. It has also been reported that the repeatability is poorer in cohorts with more advanced keratoconus than in those with less advanced disease[116-118], although the association between disease severity and the repeatability of the measurements was not analysed *per se*. The effect of time should also be considered. In the abovementioned studies, repeated measurements were made during the same visit (day). The progression of keratoconus is evaluated over time, and it would thus be more relevant to evaluate the repeatability of inter-day measurements. Furthermore, day-to-day changes can be expected due to the low biomechanical strength of the cornea[119], indeed, individuals with keratoconus report fluctuating visual acuity between days. The inter-day perspective in the diagnosis of progression with the Pentacam HR has only been considered in one study[120], which included both keratoconus and post-LASIK

(laser assisted *in situ* keratomileusis) ectasia, and the difference between intra- and inter-day repeatability was not discussed. An inter-day evaluation of parameters commonly used in the diagnosis of progressive keratoconus is thus of considerable interest. This is also relevant regarding the A, B and C parameters in the Belin ABCD Progression Display, as these are based on intra-day measurements[51]. The effect of disease severity should also be considered, as this could affect the measurements. Furthermore, the software in tomographic equipment should allow for the comparison of means of replicates and single measurements on different occasions. This is of particular interest as the comparison of means of replicates will have a significant positive effect on the repeatability of the measurements[112], and consequently the limits at which progression can be detected. The Global Consensus on Keratoconus and Ectatic Diseases[43] suggested that progression should be assessed using Scheimpflug-based equipment or OCT. However, as an auto-refract-keratometer can be used to measure the curvature power of the central flat meridian (K1) and the curvature power of the central steep meridian (K2), it would be interesting to investigate whether such a widely available instrument could be used for the assessment of progressive keratoconus. This equipment has not previously been evaluated in the management of keratoconus, but the measurements obtained have high repeatability in healthy subjects[121].

It has been found that the corneal thickness can be reduced below 400 µm when performing CXL with isoosmolar or hypoosmolar solutions of riboflavin[69], and there are no well-documented treatment protocols available for such patients. A protocol using a UV-permeable contact lens soaked in riboflavin has been suggested, so-called contact-lens-assisted CXL[122]. The thickness of the cornea is artificially increased using this method. However, the possible risk of reflected UVA irradiation (despite using a UV-permeable lens) and the possible reduction in oxygen availability due to the presence of the contact lens must be considered[60, 61, 83, 86, 123, 124]. At our clinic, we have used sterile water during CXL to increase the thickness of the cornea. Sterile water has an osmolarity of 0 mOsmol/L, and a small volume was found to be sufficient to increase the corneal thickness above 400 µm. Only in rare cases was it not possible to maintain the thickness of the cornea above the safe value. It is therefore of interest to evaluate this treatment modality further. To the best of the author's knowledge, no studies have yet been published on the use of sterile water in CXL.

Aims

The main aim of this research was to develop a reliable definition of progressive keratoconus. The other important purpose was to investigate different CXL techniques for the treatment of progressive keratoconus through a randomized clinical trial (ClinicalTrials.gov, Identifier: NCT04427956). However, it was not possible to complete the clinical trial within the time frame of this PhD project, so this thesis is mainly concerned with the assessment and definition of progression. A retrospective analysis of the effects of adding sterile water during CXL was also performed. This is of interest as the addition of sterile water is sometimes necessary during CXL in the ongoing clinical trial.

The specific aims of the studies were as follows.

I. To investigate and describe the association between disease severity and the repeatability of measurements of parameters commonly used in the detection of progressive keratoconus (Paper I)

II. To calculate limits at which progressive keratoconus can be detected based on inter-day measurements, taking the association between disease severity and commonly used parameters into consideration (Paper II)

III. To determine the repeatability of measurements of the parameters A, B and C in the Belin ABCD Progression Display from an inter-day perspective, taking disease severity into consideration (Paper III)

IV. To investigate the effects of adding sterile water during CXL for the purpose of maintaining a sufficient corneal thickness for CXL, ensuring the safety of the procedure (Paper IV)

SUBJECTS AND METHODS

Study I

Subjects ≥ 18 years were consecutively recruited for this study. Keratoconus was diagnosed clinically, and by examination using the Pentacam HR. The sagittal curvature pattern, posterior and anterior elevation maps, and corneal thickness pattern were assessed, together with information from the Belin-Ambrosio Enhanced Ectasia Display. Patients with all stages of keratoconus were included as the purpose of the study was to investigate the association between disease severity and measurement error. However, subjects with corneal scarring were excluded. Patients who had previously undergone CXL, or who had any history of ophthalmic surgery or concomitant eye disorder, were also excluded to avoid bias. Pregnant and breastfeeding women were also excluded. This last exclusion criterion was probably unnecessary in the intra-day perspective, however, in an inter-day perspective it is reasonable, as breastfeeding or pregnancy can make the cornea more elastic and thus prone to changes[19]. The use of contact lenses was discontinued two weeks prior to participation in the study. The optimal time interval for discontinuing the use of contact lenses is not known, nor whether it is necessary, but two weeks was chosen as this seemed reasonable. Only one eye was eligible for inclusion in 24 subjects, and in the remaining 37, computerized randomization was performed to select one eye for inclusion in the study (in total 29 right eyes and 32 left eyes). Only one eye was included to avoid possible paired-organ bias[125]. Fifty-four participants were male, and seven female, and the mean age was 29 years (range 18-49 years).

Four replicate measurements were made by the same examiner (the author) using the Pentacam HR system. Patients were instructed to blink but not to lean back between measurements. Four replicate measurements were then made using the NIDEK ARK-560A auto-refracto-keratometry device under the same conditions and by the same examiner, using auto-alignment mode. Only examinations deemed "OK" by the Pentacam HR system and error-free by the NIDEK ARK-560A instrument were accepted. K1, K2, Kmax, Rmin and the minimum corneal thickness (MCT) were obtained from the Pentacam HR, and K1 and K2 from the NIDEK ARK instrument.

Study II

Patients were enrolled consecutively. The inclusion criteria were identical to those in Study I, with one important difference, namely that only patients with mild to moderate keratoconus were included (stages 1-2 according to the Amsler-Krumeich classification[126]). The reason for this was to focus on patients who had the most to gain from CXL, i.e. those with the most visual acuity to preserve. The exclusion criteria were the same as those in Study I.

Twenty-five patients were enrolled. Only 1 eye was eligible for inclusion in 8 patients due to previous CXL or keratoconus stage > 2. Both eyes were examined in the remaining 17 subjects (right eye first, then left) followed by computerized randomization to select one eye for inclusion in the study, to avoid possible paired-organ bias[125] (12 right eyes and 13 left eyes). Twenty-two participants were male and three were female and the mean age was 27 years (21-45 years).

Healthy controls (n = 25) were enrolled from among medical students and residents in ophthalmology. The inclusion criteria were age ≥ 18 years and no history of any ocular pathology or previous ocular surgery. Pregnant and breastfeeding women were excluded. Ocular pathology was excluded by a clinical examination and by examination using the Pentacam HR. Only 1 eye was eligible for inclusion in 3 patients, due to scarring of the cornea. If both eyes were eligible for inclusion, both were examined and computerized randomization was performed as described above, resulting in 12 right eyes and 13 left eyes. Fourteen participants were male and eleven were female, and the mean age was 29 years (23-41 years).

Measurements were performed (by the author) as described above (Study I) on Day 0, and repeated three days later (Day 3). K1, K2, Kmax, Rmin and MCT were obtained using the Pentacam HR, and K1 and K2 from the NIDEK ARK. Diurnal effects, i.e. “time after awakening”, were not considered. The effects of time after awakening appear to be most prominent within two hours of waking, and were thus not considered significant in the present study[127, 128].

Study III

In this study, the parameters A, B and C in the Belin ABCD Progression Display were extracted from the measurements described in Papers I and II. As in Study I, the association between the measurement error and disease severity was evaluated. The inter-day repeatability and its association with disease severity was then assessed, as in Study II.

Study IV

Patients on whom pre-CXL measurements had been made with the Pentacam HR on the same day as CXL, and who had attended the one-year follow-up visit between the years 2010 and 2015 were identified, and their data analysed retrospectively. In total, twenty-eight patients (32 eyes) were identified as having been treated with hypoosmolar riboflavin and sterile water (6 women, 22 men; mean age 25.8 years ± 5.3 (standard deviation, SD). The baseline median value of Kmax was 54.9 D (interquartile range (IQR) = 9.4 D), and the median thickness at the thinnest point on the cornea was 451 μm (IQR = 42 μm) (range 388 to 537 μm). Fourteen patients (17 eyes) were identified as having been treated with hypoosmolar riboflavin alone (2 women, 12 men; mean age 24.9 ± 6.1 years). The baseline median value of Kmax for these patients was 55.7 D (IQR = 11.5 D), and the median thickness at the thinnest point on the cornea was 465 μm (IQR = 59 μm) (range 374 to 516 μm). No significant differences were observed in the baseline characteristics of the patients.

Statistical methods

Descriptive statistics are given as subject mean, standard deviation (SD), and range. Repeatability was assessed by calculating the within-subject standard deviation (S_w), precision, repeatability coefficient (RC), intraclass correlation coefficient (ICC), and coefficient of variation (CV) with associated confidence intervals (CIs)[129-131].

The limits of agreement were calculated using the replicates and a linear mixed-effect model[132]. Transformed (natural logarithm) data were analysed where appropriate[133]. Differences between coefficients of variation were assessed using a regression test[134] (Study I). Values of K1, K2 and Kmax were divided into three groups based on parameter magnitude to give groups of as equal size as possible (Study I). Bland-Altman plots were used to analyse the agreement between measurements made with the two instruments (Study I)[129, 131]. The sample size required for Study 2 was calculated considering the inter-day repeatability of Kmax as the primary outcome variable[129]. A value of S_w of 0.36 was used based on a previous investigation[135], and the width of the 95% CI was set to 30% of the within-subject standard deviation on each side, resulting in 22 subjects.

Kendall's tau-b was used to analyse correlations between the mean and standard deviation of replicate measurements[131] when evaluating the association between measurement error and disease severity in the intra-day (Study I) and in the inter-day (Study II) scenarios. In addition to Kendall's tau-b, Spearman's rho was also used in Study III to describe this correlation for one-tailed measurements (as used in the Belin ABCD Progression Display. Natural-logarithm-transformed data were analysed when appropriate.

The values obtained for the 4 replicate measurements on Day 0 and Day 3 were averaged for each day, and were used to calculate the inter-day repeatability for the clinical scenario when using the mean value of measurements to assess progression (Studies II & III). When calculating prediction limits in the clinical scenario when single measurements were used to assess progression, the variance between replicate measurements was included in the calculation[132] to provide more accurate results (Studies II & III). In Study IV the descriptive statistics are given as median values and the IQR, and comparisons between groups were made with the Wilcoxon signed-rank test.

IBM SPSS Statistics 22 for Windows (IBM Corporation, Armonk, New York, USA) and SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc., Cary, North Carolina, USA) were used for statistical analyses. A p-value less than 0.05 was considered significant.

Definitions

- Within-subject standard deviation[129] (S_w): The square root of the variance between subjects.
- Precision[129]: $1.96 \times S_w$: The difference between a measurement and the true value should lie below this limit in 95% of the measurements.
- Repeatability coefficient[136] (R): The variation in repeated measurements made on the same subject under identical conditions. The underlying values are assumed to be constant during the measurements. The difference between 2 measurements should lie below this limit for 95% of the pairs of observations.
- Coefficient of variation[129] (CV): S_w divided by the total subject mean.
- Intraclass correlation coefficient[130] (ICC): The variance between subjects divided by the variance between subjects plus the variance within subjects.
- Prediction limit[132] (PL): 95% prediction intervals for differences between 2 future single measurements

Ethical considerations

The Regional Ethics Committee of Lund University, Sweden, approved the studies (No. 2015/373). Written consent was obtained from all subjects in Studies I-III prior to participation. Prior to conducting the retrospective study (IV), at the suggestion of the Ethics Committee, an advertisement was published in a regional newspaper

(Sydsvenska Dagbladet) in order to reach subjects and provide them with the opportunity to opt out of the study. However, no patients contacted us. The studies were conducted at the Department of Ophthalmology at the Skåne University Hospital, Lund, Sweden, according to the Declaration of Helsinki.

Equipment

Technical specifications of the Pentacam HR

The Pentacam HR (version 1.20r10, Oculus Optikgeräte GmbH, Wetzlar, Germany) is the most commonly used tomographer in the diagnosis of keratoconus, and in the assessment of progression[44].

The system employs a monochromatic blue (475 nm) light-emitting diode to illuminate the cornea, and a rotating high-resolution camera captures the reflections according to the Scheimpflug principle[137]. A 3D image of the cornea is then created from 138 000 data points[138]. Images can be captured at a rate of 25 pictures per second or 50 pictures per 2 seconds. In the work described here, the capture rate used was 25 pictures/second as this is faster, and it was deemed that this could facilitate patient compliance in replicate measurements. It has been reported that the repeatability of measurements is marginally better using 25 scans/s than when using 50 scans/2 s in healthy subjects[139]. The system has a second camera that controls eye movements and adjusts the measurements accordingly. This is also used for quality control. If the software in the system deems the measurements acceptable, they are described as “OK”. Suboptimal measurements are indicated in yellow (indicating that the data should be interpreted with caution), while red indicates that the measurements should be repeated.

The NIDEK ARK 560-A

The NIDEK ARK 560-A (NIDEK Co. Ltd., Japan) is a commonly used auto-refracto-keratometer in the clinical setting (however, not in the management of keratoconus). It captures a mire ring on the cornea on which the analysis is based. K1 and K2 are obtained in the central 3.3 mm diameter zone of measurement[140].

RESULTS

The association between keratoconus disease severity and measurement error

Four replicate measurements were made on one occasion using the Pentacam HR and the auto-refracto-keratometer (NIDEK ARK) in one eye of 61 patients with keratoconus stage < 4 (Amsler-Krumeich Classification). The data are presented in Table 1. All the parameters investigated showed an ICC close to 1.0, which means that the variability of the measurements is attributed to differences between subjects, rather than within subjects. This is an important validation of the investigation method, demonstrating that the variability of the measurements can be further evaluated on group level.

The initial assessment was performed in order to investigate the variability of the measurements of each parameter, and to rank them accordingly. The CV, expressed in %, is appropriate for this purpose as it provides a unitless measure of the variability. As can be seen from Table 1, K1 has the lowest CV followed by K2, Kmax, MCT and finally Rmin. The CV in K1 is approximately three times lower than that in Rmin, and approximately two times lower than that of the most commonly used parameter, Kmax. K1 and K2 can be measured using the NIDEK ARK and the Pentacam HR, and a regression test was performed to determine whether there was a significant difference between the CV in K1 and K2 measured by the two instruments. The results showed that there was no significant difference between the CV of K1 or K2 using the two instruments (K1, $p = 0.130$ and K2, $p = 0.498$).

The second assessment was carried out to investigate the association between disease severity and the measurement error. This was done by analysing the correlation between the SD and the magnitude of the measured parameter, using Kendall's tau-b. All the parameters except the MCT and Rmin showed a significant association. Kmax showed the strongest association (Kendall's tau-b = 0.532, $p < 0.001$) followed by K2 (Pentacam HR) (Kendall's tau-b = 0.305, $p = 0.001$) and (ARK) (Kendall's tau-b = 0.320, $p = 0.001$) and K1 (Pentacam HR) (Kendall's tau-b = 0.239, $p< 0.008$) and (ARK) (Kendall's tau-b = 0.230, $p = 0.016$). In order to understand the clinical implications of this association, the patients were divided into three groups based on the values of Kmax, K2 and K1, and the repeatability of the measurements was calculated for each group. Kmax is the most commonly used

parameter in the detection of progression, and was thus of particular interest. The CV is less appropriate to describe the differences between the stratified groups, so R was used, as it describes the limit below which 95% of the differences between measurements are expected to fall. In the group with the lowest disease severity, a difference between measurements could be detected at 0.32 D (95% CI, 0.26-0.37 D), while in the group with the most advanced disease a difference could only be detected at 1.62 D (95% CI, 1.33-1.91 D). The corresponding values for K1 (which showed the least variability) were 0.40 D (95% CI, 0.33-0.47 D) and 0.54 D (95% CI, 0.44-0.63 D) using the Pentacam HR, and 0.34 D (95% CI, 0.28-0.40 D) and 0.70 D (95% CI, 0.58-0.82 D) using the NIDEK ARK.

Table 1. Descriptive statistics and repeatability for the whole cohort, and when the patients were divided into three groups according to disease severity

	n	Mean (SD)[a]	Median (Min–Max)[a]	S_w (95% CI)	Repeatability (95% CI)	ICC	CV (%)	Kendell's tau-b[b]	p[b]
K1 (D), Pentacam HR	61	44.0 (3.13)	43.5 (38.9-0.2)	0.18 (0.16-0.20)	0.51 (0.46-0.56)	0.997	0.41[c]	0.239	0.008
<43.0	20	41.4 (1.32)	41.4 (38.9-42.9)	0.14 (0.12-0.17)	0.40 (0.33-0.47)	0.988	0.35	0.092	0.597
≥43.0 <44.5	20	43.6 (0.38)	43.4 (43.0-44.1)	0.21 (0.1-0.24)	0.57 (0.47-0.68)	0.753	0.47[c]	0.330	0.049
≥44.5	21	46.8 (3.49)	45.3 (44.5-60.2)	0.19 (0.16-0.23)	0.54 (0.44-0.63)	0.997	0.41	0.302	0.057
K1 (D), NIDEK ARK	61	44.8 (3.09)	44.2 (40.7-62.2)	0.19 (0.17-0.21)	0.54 (0.48-0.59)	0.996	0.43[c]	0.230	0.016
<43.5	18	42.3 (0.72)	42.2 (40.7-43.3)	0.12 (0.10-0.15)	0.34 (0.28-0.40)	0.971	0.29	−0.145	0.444
≥43.5 <45.2	20	44.1 (0.42)	44.1 (43.5-44.7)	0.16 (0.14-0.19)	0.46 (0.37-0.54)	0.862	0.37	0.111	0.546
≥45.2	23	47.4 (3.55)	46.7 (45.2-62.2)	0.25 (0.21-0.29)	0.70 (0.58-0.82)	0.995	0.53	0.051	0.746
K2 (D), Pentacam HR	61	47.0 (4.23)	46.1 (41.4-67.8)	0.27 (0.25-0.30)	0.76 (0.68-0.83)	0.996	0.57[c]	0.305	0.001
<44.8	20	43.3 (1.04)	43.6 (41.4-44.8)	0.13 (0.10-0.15)	0.35 (0.29-0.42)	0.985	0.29	−0.154	0.373
≥44.8 <47.8	20	46.3 (0.94)	46.1 (44.8-47.6)	0.21 (0.17-0.24)	0.57 (0.47-0.67)	0.954	0.44[c]	0.338	0.040
≥47.8	21	51.3 (4.32)	50.2 (47.8-67.8)	0.40 (0.33-0.47)	1.11 (0.92-1.31)	0.991	0.78	0.129	0.415
K2 (D), NIDEK ARK	61	47.6 (4.55)	46.6 (41.9-73.6)	0.25 (0.22-0.27)	0.69 (0.62-0.76)	0.997	0.50[c]	0.320	0.001
<45.3	20	44.0 (0.96)	44.4 (41.9-45.2)	0.12 (0.095-0.14)	0.32 (0.26-0.38)	0.986	0.26	0.150	0.405
≥45.3 <48.6	20	46.9 (0.98)	46.6 (45.3-48.3)	0.23 (0.19-0.27)	0.63 (0.52-0.74)	0.949	0.49	−0.091	0.608
≥48.6	21	51.7 (5.38)	50.1 (48.6-73.6)	0.34 (0.28-0.40)	0.95 (0.78-1.12)	0.996	0.66	0.035	0.830
Kmax (D), Pentacam HR	61	52.0 (6.14)	50.6 (42.5-77.1)	0.44 (0.40-0.49)	1.23 (1.10-1.35)	0.995	0.80[c]	0.523	<0.001
<48.2	20	46.2 (1.62)	46.6 (42.5-48.0)	0.11 (0.094-0.13)	0.32 (0.26-0.37)	0.995	0.25	0.281	0.090
≥48.2 <53.9	21	51.1 (2.03)	50.6 (48.2-53.9)	0.48 (0.43-0.56)	1.33 (1.10-1.56)	0.946	0.94	0.298	0.061
≥53.9	20	58.8 (5.27)	57.3 (53.9-77.1)	0.59 (0.48-0.69)	1.62 (1.33-1.91)	0.988	1.00	0.316	0.052
MCT (µm), Pentacam HR	61	485.5 (40.6)	483.0 (394.5-578)	5.11 (4.59-5.63)	14.2 (12.7-15.6)	0.984	1.05	0.134	0.129
Rmin (mm), Pentacam HR	61	4.93 (0.71)	4.93 (2.99-6.29)	0.063 (0.057-0.07)	0.18 (0.16-0.19)	0.992	1.28	−0.153	0.083

[a]Subject mean, [b]Subject SD versus subject mean, [c]Calculated from transformed data. Curvature power of the central flat meridian (K1), curvature power of the central steep meridian (K2), curvature power of the steepest point on the anterior surface (Kmax), minimum corneal thickness (MCT), curvature power of the steepest point on the posterior surface (Rmin).

The inter-day assessment of repeatability in mild to moderate keratoconus

In this study four consecutive measurements were made on Day 0 and again on Day 3, in 25 patients with keratoconus stage 1-2 (Amsler-Krumeich Classification) and 25 healthy controls. Measurements were made with the Pentacam HR and the NIDEK ARK. The results demonstrated a high ICC, close to, or equal to, 1.0, thus suggesting that the variability of the measurements can be attributed to differences between subjects, rather than within subjects. Given the results in Study I, the association between disease severity and measurement error was also assessed. Healthy controls naturally showed no such association, while in patients with keratoconus this association was only clinically and statistically significant for Kmax (Kendall's tau-b = 0.483, p = 0.0001). This association is clearly visible in Figure 6 (keratoconus patients) but not in Figure 7 (healthy controls).

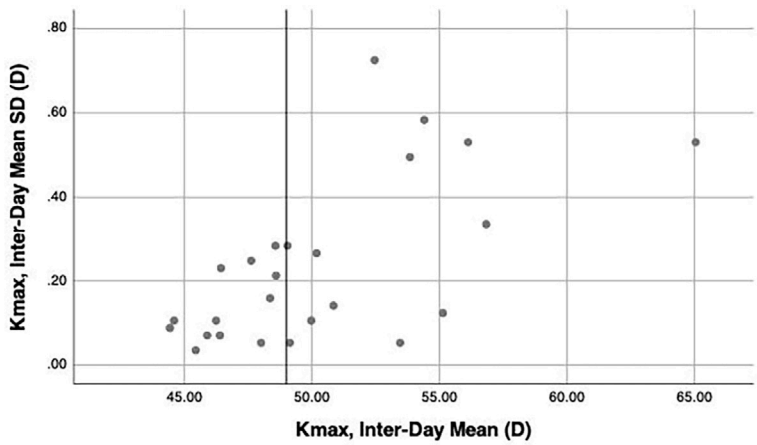


Figure 6. Mean standard deviation (SD) in the curvature power of the steepest point on the anterior surface (Kmax) plotted against the mean inter-day values of Kmax for the keratoconus patients. The reference line indicates the median mean value at 49.0 dioptres (D).

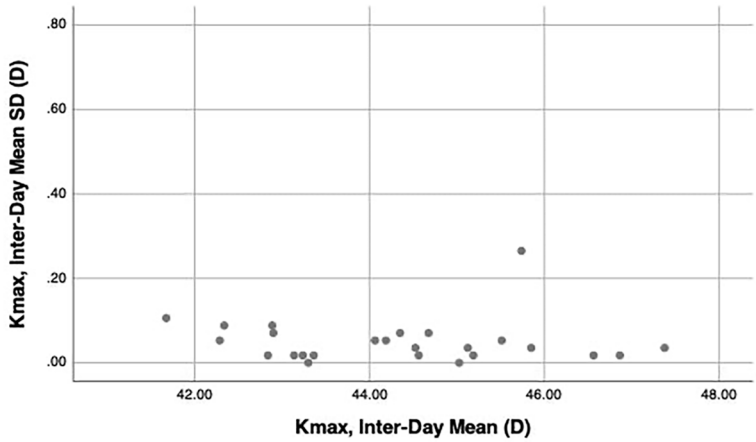


Figure 7. Mean standard deviation (SD) in the curvature power of the steepest point on the anterior surface (Kmax) plotted against the mean inter-day values of Kmax for the heathy controls.

As in the first study, K1 showed the lowest variability in subjects with keratoconus, followed by K2 and Kmax, which showed the same variability. MCT and Rmin showed the highest variability (Table 2). The variability of the measurements was clearly lower for all parameters in the healthy controls. In both groups K1 showed the least variability and MCT and Rmin showed the highest variability. However, the variability in healthy controls must be considered to be very low.

The inter-day repeatability was calculated for two different scenarios, one using the mean of replicates (Table 2), in this case, the mean of four measurements on each occasion, and the other using a single measurement on each occasion (Table 3). When interpreting these results, it is important to bear in mind that an increase of 1.0 D in Kmax is commonly used to detect progression. The results obtained using the mean of four replicate measurements on each occasion (Table 2) suggest that progression can be detected at 0.84 D (95% CI, 0.61-1.07 D). However, when the patients were divided into two groups according to disease severity, progression could be detected at an increase in Kmax of 0.44 D (95% CI, 0.27-0.62 D) in subjects with Kmax < 49 D, and at an increase in Kmax of 1.08 D (95% CI, 0.67-1.50 D) in subjects with Kmax ≥ 49 D.

When using single measurements on each occasion (Table 3) progression could, in general, be detected at an increase of 1.11 D (PL, –0.90-1.11 D). When the patients were divided according to disease severity, progression could be detected at 0.67 D (PL, –0.49-0.67 D) in subjects with Kmax < 49 D and at 1.42 D (PL, –1.19-1.42 D) in subjects with Kmax ≥ 49.

Table 4 presents the intra-day results for Day 0 and Day 3. The intra-day repeatability improved on Day 3 for all keratometric parameters except MCT and Rmin. This suggests a learning effect as patients gained experience of the measurement situation.

Table 2. Descriptive statistics and inter-day repeatability of measurements in the keratoconus patients and healthy controls (mean of 4 replicates)

		Mean (SD)[a]	(Min-Max)[a]	S_w (95% CI)	Repeatability (95% CI)	ICC	CV (%)
K1 (D)							
PC	Keratoconus	43.6 (1.8)	(40.6-47.2)	0.16 (0.11-0.20)	0.44 (0.32-0.56)	0.99 (0.98-1.00)	0.36
	Controls	43.0 (1.3)	(40.9-45.6)	0.04 (0.029-0.051)	0.11 (0.080-0.14)	1.00 (1.00-1.00)	0.093
ARK	Keratoconus	44.4 (1.7)	(42.0-48.3)	0.12 (0.084-0.15)	0.32 (0.23-0.41)	1.00 (0.99-1.00)	0.37
	Controls	43.3 (1.3)	(40.8-46.0)	0.072 (0.051-0.092)	0.20 (0.14-0.25)	1.00 (0.99-1.00)	0.17
K2 (D)							
PC	Keratoconus	46.6 (2.8)	(42.8-55.8)	0.26 (0.19-0.33)	0.72 (0.52-0.92)	0.99 (0.98-1.00)	0.57
	Controls	43.9 (1.4)	(41.4-46.6)	0.063 (0.045-0.080)	0.17 (0.13-0.22)	1.00 (1.00-1.00)	0.14
ARK	Keratoconus	46.6 (2.7)	(43.5-55.6)	0.33 (0.24-0.43)	0.93 (0.67-1.18)	0.98 (0.97-0.99)	0.72
	Controls	44.1 (1.4)	(41.3-46.7)	0.15 (0.11-0.20)	0.43 (0.31-0.54)	0.99 (0.97-0.99)	0.35
Kmax (D)							
PC	Keratoconus all	50.3 (2.03)	(44.4-65.1)	0.30 (0.22-0.39)	0.84 (0.61-1.07)	1.00 (0.99-1.00)	0.57[b]
	Keratoconus < 49 D	46.7 (2.03)	(44.4.2-48.6)	0.16 (0.10-0.22)	0.44 (0.27-0.62)	0.99 (0.96-1.00)	0.34
	Keratoconus ≥ 49 D	53.6 (2.03)	(49.1.2-65.1)	0.39 (0.24-0.54)	1.08 (0.67-1.50)	0.99 (0.97-1.00)	0.73
	Controls	44.3 (5.27)	(41.7-47.4)	0.072 (0.052-0.092)	0.20 (0.15-0.26)	1.00 (0.99-1.00)	0.16
MCT (µm)							
PC	Keratoconus	493.0 (35.1)	(442.3-560.8)	2.92 (2.11-3.73)	8.11 (5.86-10.4)	0.99 (0.98-1.00)	0.63[b]
	Controls	538.8 (23.4)	(497.0-582.6)	2.40 (1.74-3.07)	6.66 (4.81-8.51)	0.99 (0.98-1.00)	0.45
Rmin (mm)							
PC	Keratoconus	5.1 (0.63)	(3.9-6.1)	0.076 (0.055-0.10)	0.21 (0.15-0.27)	0.99 (0.97-1.00)	1.49
	Controls	6.1 (0.24)	(5.6-6.6)	0.023 (0.016-0.029)	0.063 (0.045-0.080)	0.99 (0.98-1.00)	0.37

[a]Subject mean, [b]Calculated using the natural logarithm transformation. Pentacam HR (PC), NIDEK auto-refracto-keratometer (ARK). K1: curvature power of the central flat meridian, K2: curvature power of the central steep meridian, Kmax: curvature power of the steepest point on the anterior surface, MCT: minimum corneal thickness, Rmin: curvature power of the steepest point on the posterior surface.

Table 3. Inter-day differences between single measurements with prediction limits for the keratoconus patients

	Variance components			Mean difference	Lower prediction limit	Upper prediction limit
	$\hat{\tau}^2$	$\hat{\sigma}_1^2$	$\hat{\sigma}_2^2$	$\hat{\alpha}_1 - \hat{\alpha}_2$	$\hat{\alpha}_1 - \hat{\alpha}_2 - 2 \times \sqrt{2\hat{\tau}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$	$\hat{\alpha}_1 - \hat{\alpha}_2 + 2 \times \sqrt{2\hat{\tau}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$
Pentacam HR						
K1 (D)	0.020	0.023	0.019	0.027	−0.55	0.60
K2 (D)	0.058	0.059	0.036	0.018	−0.90	0.94
Kmax (D)	0.078	0.063	0.036	0.10	−0.90	1.11
Kmax < 49 (D)	0.017	0.035	0.015	0.092	−0.49	0.67
Kmax ≥ 49 (D)	0.14	0.089	0.055	0.12	−1.19	1.42
MCT (µm)	5.26	14.16	14.90	−0.17	−12.75	12.41
Rmin (mm)	0.0011	0.0026	0.035	−0.017	−0.42	0.38
NIDEK ARK						
K1 (D)	0.0019	0.034	0.056	−0.043	−0.66	0.57
K2 (D)	0.080	0.10	0.11	−0.14	−1.36	1.08

$\hat{\tau}^2$=squared between-subject mean variance on Day 0 and Day 3. $\hat{\sigma}_1^2$=squared within-subject mean variance on Day 0. $\hat{\sigma}_2^2$=squared within-subject mean variance on Day 3. α̂1-α̂2= difference between means measured on Day 0 and Day 3. K1: curvature power of the central flat meridian, K2: curvature power of the central steep meridian, Kmax: curvature power of the steepest point on the anterior surface, MCT: minimum corneal thickness, Rmin: curvature power of the steepest point on the posterior surface.

Table 4. Descriptive statistics and repeatability of measurements on Day 0 and Day 3 in the keratoconus patients

		Mean (SD)[a]	(Min-Max)[a]	S_w (95% CI)	Repeatability (95% CI)	ICC	CV (%)
K1 (D)							
Pentacam HR	Day 0	43.6 (1.8)	(40.6-47.2)	0.16 (0.11-0.20)	0.44 (0.32-0.56)	0.99 (0.98-1.00)	0.36
	Day 3	43.0 (1.3)	(40.9-45.6)	0.04 (0.029-0.051)	0.11 (0.080-0.14)	1.00 (1.00-1.00)	0.093
NIDEK ARK	Day 0	44.4 (1.7)	(42.0-48.3)	0.12 (0.084-0.15)	0.32 (0.23-0.41)	1.00 (0.99-1.00)	0.37
	Day 3	43.3 (1.3)	(40.8-46.0)	0.072 (0.051-0.092)	0.20 (0.14-0.25)	1.00 (0.99-1.00)	0.17
K2 (D)							
Pentacam HR	Day 0	46.6 (2.8)	(42.8-55.8)	0.26 (0.19-0.33)	0.72 (0.52-0.92)	0.99 (0.98-1.00)	0.57
	Day 3	43.9 (1.4)	(41.4-46.6)	0.063 (0.045-0.080)	0.17 (0.13-0.22)	1.00 (1.00-1.00)	0.14
NIDEK ARK	Day 0	46.6 (2.7)	(43.5-55.6)	0.33 (0.24-0.43)	0.93 (0.67-1.18)	0.98 (0.97-0.99)	0.72
	Day 3	44.1 (1.4)	(41.3-46.7)	0.15 (0.11-0.20)	0.43 (0.31-0.54)	0.99 (0.97-0.99)	0.35
Kmax (D)							
Pentacam HR	Day 0	46.7 (2.03)	(44.4.2-48.6)	0.16 (0.10-0.22)	0.44 (0.27-0.62)	0.99 (0.96-1.00)	0.34
	Day 3	53.6 (2.03)	(49.1.2-65.1)	0.39 (0.24-0.54)	1.08 (0.67-1.50)	0.99 (0.97-1.00)	0.73
MCT (µm)							
Pentacam HR	Day 0	493.0 (35.1)	(442.3-560.8)	2.92 (2.11-3.73)	8.11 (5.86-10.4)	0.99 (0.98-1.00)	0.63[b]
	Day 3	538.8 (23.4)	(497.0-582.6)	2.40 (1.74-3.07)	6.66 (4.81-8.51)	0.99 (0.98-1.00)	0.45
Rmin (mm)							
Pentacam HR	Day 0	5.1 (0.63)	(3.9-6.1)	0.076 (0.055-0.10)	0.21 (0.15-0.27)	0.99 (0.97-1.00)	1.49
	Day 3	6.1 (0.24)	(5.6-6.6)	0.023 (0.016-0.029)	0.063 (0.045-0.080)	0.99 (0.98-1.00)	0.37

[a]Subject mean. Curvature power of the central flat meridian (K1), curvature power of the central steep meridian (K2), curvature power of the steepest point on the anterior surface (Kmax), minimum corneal thickness (MCT), curvature power of the steepest point on the posterior surface (Rmin).

Assessing progression with the Belin ABCD Progression Display

This study was performed as a *post hoc* investigation using the measurements obtained in Studies I & II. As in the case of the first two studies, the values of ICC were close to 1.0. The association between the measurement error and disease severity was statistically significant for the parameters A (Kendall's tau-b = –0.377, p = 0.009) and C (Kendall's tau-b = –0.350, p = 0.016), but not for B (Figure 8). The results are presented in Table 5.

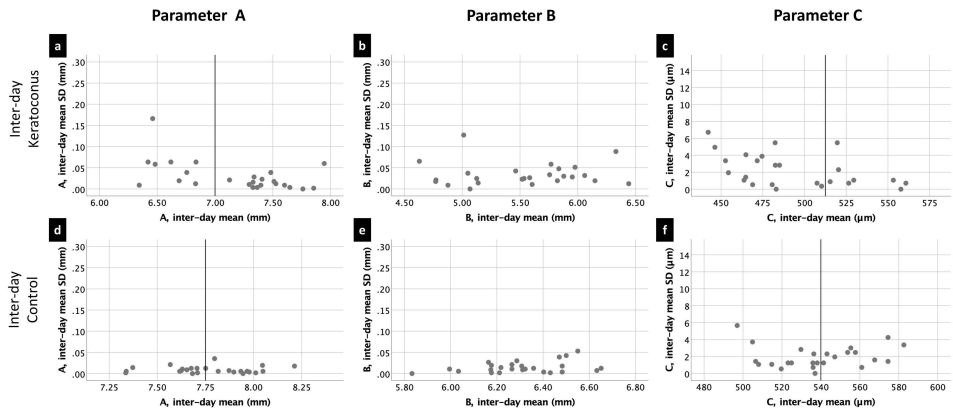


Figure 8. Mean values of inter-day and intra-day measurements of the parameters A, B and C, plotted against the standard deviation. (a) Inter-day measurements of parameter A in subjects with keratoconus, (d) inter-day measurements of A in control patients, (b) inter-day measurements of B in subjects with keratoconus, (e) interday measurements of B in the control group, (c) inter-day measurements of C in subjects with keratoconus and (f) inter-day measurements of C in the control group. Lower values of parameters A and B indicate more severe keratoconus, while lower values of C indicate less severe disease. The vertical lines indicate the median.

The repeatability of the inter-day measurements in patients with keratoconus was best for parameter C (CV 0.60%), followed by A (CV 0.64%), and then B (CV 0.79%). When dividing the keratoconus patients into two groups according to the value of parameter A, those with a value of A below the median showed a repeat-ability that was about 2 times better that those with a value of A above the median value. (It should be borne in mind that *higher* values of A and B indicate more severe disease.) When the patients were similarly divided based on values of parameter C, the repeatability was approximately 2 times better for patients with a value of C

above the median value than for those with a value below the median. (Note that a *lower* value of C indicates more severe disease.)

The results of the calculations of the prediction limits are presented in Table 6. The PLs for single inter-day measurements in patients with keratoconus were –0.19-0.17 mm for parameter A, –0.19-0.16 mm for B, and –12.5-12.9 µm for C. The patients were divided into groups according to the values of the parameters A and C: above and below the median value. The PLs for single inter-day measurements in patients with values of A below the median value were –0.25-0.22 mm, and for those with values above the median value the prediction limits were –0.11-0.081 mm. The corresponding PLs for parameter C were –15.4-14.0 µm for values below the median value and –9.51-11.5 µm for values above the median value.

In a randomized comparison between two measurements (one on Day 0 and one on Day 3) in each patient with keratoconus, 6 (24%) showed progression in at least one parameter. This process was repeated following a new randomization, confirming the initial results that 6 subjects were indicated as having progressive disease.

Table 5. Descriptive statistics and inter-day repeatability of measurements for the keratoconus patients and healthy controls (mean of replicates)

	Mean (SD)[a]	(Min–Max)[a]	Sw (95% CI)	R (95% CI)	ICC (95% CI)	CV (%)	Kendall's tau-b[b]	p[b] (2-tailed)	Spearman's rho[b]	p[b] (1-tailed)
Keratoconus patients (n=25)										
A (mm)										
All	7.17 (0.48)	(6.34-7.95)	0.046 (0.033-0.059)	0.13 (0.092-0.16)	0.99 (0.98-1.00)	0.64	–0.377	0.009	–0.481	0.007
<7.33	6.81 (0.36)	(6.34-7.33)	0.060 (0.037-0.082)	0.17 (0.10-0.23)	0.97 (0.92-0.99)					
≥7.33	7.57 (0.20)	(7.34-7.95)	0.024 (0.015-0.034)	0.07 (0.040-0.094)	0.98 (0.95-1.00)					
B (mm)	5.52 (0.51)	(4.63-6.44)	0.044 (0.032-0.056)	0.12 (0.088-0.16)	0.99 (0.98-1.00)	0.79	0.113	0.427	0.132	0.265
C (μm)										
All	492.7 (35.1)	(442.3-560.8)	2.95 (2.13-3.77)	8.17 (5.91-10.4)	0.99 (0.98-1.00)	0.60	–0.350	0.016	–0.480	0.008
<482.5	463.9 (12.9)	(442.3-482.4)	3.67 (2.20-5.13)	10.2 (6.10-14.2)	0.92 (0.76-0.98)					
≥482.5	519.3 (26.8)	(482.5-560.8)	2.07 (1.28-2.87)	5.75 (3.54-7.96)	0.99 (0.98-1.00)					
Healthy controls (n=25)										
A (mm)	7.77 (0.23)	(7.34-8.21)	0.012 (0.009-0.015)	0.033 (0.024-0.042)	1.00 (0.99-1.00)	0.15	–0.069	0.638	–0.104	0.311
B (mm)	6.31 (0.20)	(5.83-6.65)	0.020 (0.015-0.026)	0.056 (0.041-0.072)	0.99 (0.98-1.00)	0.32	0.189	0.190	0.260	0.105
C (μm)	538.8 (23.4)	(497.0-582.6)	2.34 (1.69-2.98)	6.47 (4.68-8.27)	0.99 (0.98-1.00)	0.43	0.171	0.240	0.209	0.158

[a]Subject mean. [b]Subject SD versus subject mean. A: anterior curvature of the 3 mm zone over the thinnest point (mm), B: posterior curvature of the 3 mm zone under the thinnest point (mm), C: thickness at the thinnest point on the cornea (μm).

Table 6. Inter-day differences between single measurements with prediction limits for all patients with keratoconus, and divided into groups according to severity, together with healthy controls

	Variance components			Mean difference	Lower prediction limit	Upper prediction limit
	$\hat{t}^2$	$\hat{\sigma}_1^2$	$\hat{\sigma}_2^2$	$\hat{\alpha}_1 - \hat{\alpha}_2$	$\hat{\alpha}_1 - \hat{\alpha}_2 - 2 \times \sqrt{2\hat{t}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$	$\hat{\alpha}_1 - \hat{\alpha}_2 + 2 \times \sqrt{2\hat{t}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$
Patients with keratoconus						
A (mm)						
All	0.0015	0.0020	0.0029	-0.013	−0.19	0.17
<7.33	0.0027	0.0035	0.0046	-0.013	−0.25	0.22
≥7.33	0.00039	0.00035	0.0011	-0.012	−0.11	0.081
B (mm)	0.0011	0.0030	0.0024	-0.019	−0.19	0.16
C (μm)						
All	5.34	14.8	14.8	0.18	−12.5	12.9
<482.5	10.2	17.7	16.1	-0.69	−15.4	14.0
≥482.5	0.94	12.0	13.6	0.98	−9.51	11.5
Healthy controls						
A (mm)	0.0001	0.0002	0.0001	0.003	−0.04	0.05
B (mm)	0.0001	0.0007	0.0019	0.003	−0.10	0.11
C (μm)	0.90	17.02	15.51	1.17	−10.6	12.9

[a]Subject mean. [b]Subject SD versus subject mean. Definitions: $\hat{t}^2$ = squared between-subject mean variance between Day 0 and Day 3; $\hat{\sigma}_1^2$ = squared within-subject mean variance on Day 0; $\hat{\sigma}_2^2$ = squared within-subject mean variance on Day 3; $\hat{\alpha}_1 - \hat{\alpha}_2$ = difference between means on Day 0 and Day 3. A: anterior curvature of the 3 mm zone over the thinnest point (mm), B: posterior curvature of the 3 mm zone under the thinnest point (mm), C: thickness at the thinnest point on the cornea (μm).

The addition of sterile water during CXL with hypoosmolar riboflavin

The subjects included in this study were patients treated with CXL during the years 2010 to 2015 who had undergone tomographic examination immediately prior to CXL, and who had completed the 1-year follow-up examination when Pentacam HR measurements were made again. Only subjects who were treated with hypoosmolar riboflavin, alone or in combination with sterile water, were included. The median value of Kmax was statistically significantly reduced in both the hypoosmolar riboflavin group and in the group treated with hypoosmolar riboflavin with the addition of sterile water, at follow-up after 1 year. The change in Kmax was –1.7 D, IQR = 3.25 D ($p = 0.006$) in the group treated with hypoosmolar riboflavin only, and –0.85 D, IQR = 1.35 D ($p < 0.001$) in the group treated with hypoosmolar riboflavin + sterile water (Figure 9). No statistically significant difference was seen between the change in Kmax in the two groups ($p= 0.065$).

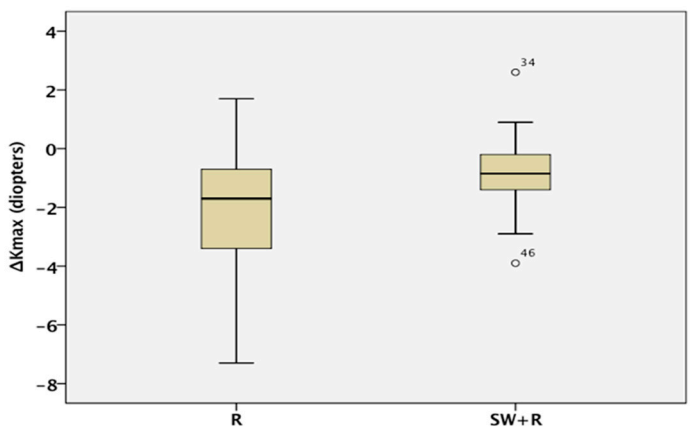


Figure 9. Change in Kmax 1 year after treatment with riboflavin only (R) or riboflavin + sterile water (SW+R).

There were two outliers in the group treated with hypoosmolar riboflavin + sterile water. Patient no. 46 showed a very distinct reduction in Kmax. Patient no. 34 showed an increase in Kmax at the 1-year follow-up, however, the measurements were highly variable between visits and progression could not later be confirmed.

DISCUSSION AND CONCLUSIONS

Should disease severity be considered when evaluating progression?

The results presented in Paper I show that there is an association between disease severity and measurement error in the parameters used for the diagnosis of progression in patients with keratoconus[33]. Previous studies have shown that measurements of keratometric parameters in cohorts of patients with more advanced keratoconus have poorer repeatability[115, 116], but no association between the two has been described *per se*. The findings of the present work suggest that different detection limits should be used to define progression in patients with different degrees of disease severity. Kmax is the most commonly used parameter in the detection of progression, and an increase of 1.0 D is commonly used[44]. However, it was found in this work that the measurement error in Kmax exhibited the strongest association with disease severity. In the group of patients with the lowest disease severity, progression could be detected by an increase in Kmax of 0.32 D (95% CI 0.26-0.37 D), while in the group with higher disease severity an increase of 1.62 D (95% CI 1.33-1.91 D) was required, in other words, a five-fold difference. Less severe disease could thus be misinterpreted as non-progressive, while more severe disease could be erroneously diagnosed as progressive. Patients with less severe disease, but with preserved vision, could be especially at risk of delayed referral for CXL, and hence at risk of visual deterioration. Patients with more severe disease would instead be at risk of undergoing CXL unnecessarily, and being exposed to the risk of treatment-associated side effects. Furthermore, these findings are clearly relevant from a scientific perspective in CXL trials to determine whether recruited patients actually have progressive keratoconus or not, as this will depend on the disease severity in the investigated cohort. It is likely that the inconsistent results in previous investigations are related, at least in part, to the effects of disease severity[33].

Another interesting finding of this study was that K1 and K2 are the most repeatable parameters, as these can also be measured with an ARK, which is commonly available. In fact, K1 and K2 could be measured with the NIDEK ARF with a precision that did not differ statistically from that obtained using the Pentacam HR (K1, $p = 0.130$) (K2, $p = 0.498$). It would therefore be of interest to further evaluate

the role of K1 and K2 in the detection of progression of keratoconus, and whether an ARK could be used for the detection of progression. Despite these findings, no suggestion was made in Paper I that they should be used in clinical practice, as measurements obtained on a single occasion might not be applicable in the clinical setting in which progression is evaluated over time.

Factors such as the lower biomechanical strength of the cornea in keratoconus could contribute to increased variation in measurements between days[119]. However, other factors could possibly reduce the variability, such as the patients gaining experience in the measurements. To the best of the author's knowledge, no prospective case-control investigations based on inter-day measurements with the Pentacam HR in patients with keratoconus have been performed. It was thus deemed important to further evaluate the findings presented in Paper I in an inter-day analysis of repeatability.

At which thresholds can progression be detected when comparing measurements made at different visits?

In the study described in Paper II it was confirmed that the standard deviation of the measurements of Kmax was indeed related to disease severity in the inter-day analysis[141]. None of the other parameters investigated showed such a relation. If subjects with more advanced keratoconus are included in future studies, disease severity should be considered according to the findings presented in Paper I. Patients with mild to moderate keratoconus were recruited in Study II, as they have the most to gain from CXL in terms of preserving vision, and should have a corneal thickness allowing for uneventful CXL.

The limits of Kmax at which progression can be detected were calculated when using single measurements on each occasion, and when using the mean of four measurements on each occasion. This aspect is seldom discussed, although the difference is of both clinical and scientific interest. When using the mean of replicates, progression could be detected at an increase in Kmax of 0.44 D (95% CI, 0.27-0.62 D) in the group with disease severity below the median, but only at an increase of 1.08 D (95% CI, 0.67-1.50 D) in the group with disease severity above the median. When single measurements were used, the corresponding limits were 0.67 D (PL, –0.49-0.67 D) and 1.42 D (PL, –1.19-1.42 D). The variability between the four measurements was included in the calculation of the prediction limits for single measurements, in order to obtain more accurate limits. The use of only one measurement on each day would thus have led to unnecessarily narrow limits, that would not represent the true inter-day repeatability of single measurements.

From these results it was concluded that the use of stratified detection limits based on inter-day measurements taking disease severity in account is an important step

towards the early detection of progressive disease in subjects with less advanced keratoconus, and for timely referral to CXL in order to preserve visual acuity. This would also help avoid overdiagnosis of progression in patients with moderate to advanced keratoconus, and subjecting them to unnecessary CXL and possible treatment-associated side effects. Although the role of ARKs in assessing progression requires further evaluation, it would be advantageous if such a widely available instrument could be used in the detection of progression of keratoconus. It is therefore recommended that the thresholds given above be used in clinical practice, although it would be valuable if they were confirmed in future studies.

Future investigations using machine-learning tools to analyse tomographic data would also be of interest. These thresholds could then be incorporated into the software in tomographic systems such as the Pentacam HR. An immediate improvement would be obtained if tomographic systems were equipped with software that allows for the comparison of means of replicate measurements. The findings presented in Paper II clearly show how the mean of replicate measurements on each occasion improves the capacity to detect progression, and is thus highly desirable.

The repeatability of measurements of the keratometric parameters was improved on Day 3 when using the Pentacam HR. This is an interesting observation, and could indicate that the improvement is due to a learning effect in the patient. It would thus be interesting to investigate whether a few "trial" measurements could improve the repeatability of the measurements.

Can the parameters A, B and C in the Belin Progression Display be used to detect progressive keratoconus?

The results of the *post hoc* analysis of the parameters A, B and C showed that the measurement error in A and C, but not that in B, was associated with disease severity[142]. As A is measured in millimetres, and not in dioptres, a lower value indicates more advanced keratoconus. Thus, the correlation is negative, in contrast to parameters measured in dioptres. Similarly, a reduction in C indicates a thinner cornea, and thus more advanced keratoconus. It has been suggested that the Belin ABCD Progression Display is superior in detecting disease progression as the parameters A and B are measured in a 3 mm zone around the thinnest point of the cornea. Thus, measurements are obtained over an area, rather than at a single point, as in the case of Kmax. This could reduce the variability, as the mean value over an area can be expected to be less prone to variation than a value at a specific point. However, the values of CV, expressed as %, for A (0.64%) and for Kmax (0.57%) were similar when obtained from the inter-day analysis of the mean of four replicates. The corresponding values for B (CV = 0.21%) and Rmin (CV = 1.49%) clearly show that B, which is obtained over an area, is the more reliable parameter.

The posterior surface is more difficult to analyse as the reflected light is also affected by the anterior surface before being recorded in the Pentacam HR, which could explain the variability in the measurements. Little difference was seen in the CV for parameter C (CV = 0.60%) and the MCT (CV = 0.53%).

The ability of Kmax and the Belin ABCD Progression Display to detect progression has been evaluated previously, and it was suggested that the latter could be used to detect progression earlier than Kmax[143]. However, such an analysis is difficult as there is no gold standard for diagnosing progression. In order to determine whether there is a difference between two values of the same parameter it is necessary to compare the accuracy of these measurements. It was thus of considerable interest to evaluate the Belin ABCD Progression Display using inter-day measurements, as the measurements on which the thresholds are based are obtained on different days[51]. The data on which the thresholds in the Belin Progression Display in the Pentacam HR are based are reproduced in Table 7[51]. In order to understand the differences, these thresholds should be compared with the magnitude of the repeatability of the inter-day measurements (mean of four replicates) and the magnitude of the prediction limits (single measurements). The prediction limits presented in Paper III for the single inter-day measurements are clearly wider than those used in the Pentacam HR software, indicating a risk of erroneous diagnosis of progression. In fact, the empirical evaluation showed false progression in 6 of 25 patients. Using the inter-day repeatability of the mean of four replicates reduced the 95% CIs, but they were still somewhat wider than those used in the Pentacam HR for the A and B parameters. Subsequent dichotomization of the values of parameters A and C increased the risk of overdiagnosis in the above-median group, while in the below-median group the limits were similar to the thresholds in the Belin ABCD Progression Display. The thresholds for the healthy controls obtained with the Belin ABCD Progression Display are of little use, as at some point there will be a clear discrepancy between the measurement accuracy for healthy controls and patients with keratoconus. A more reliable solution would be to stratify the limits according to keratoconus disease severity, and to use reference data based on inter-day measurements.

Table 7. One-sided confidence intervals for normal and keratoconic population underlying the thresholds determining progression using the parameters A, B and C in the Belin ABCD Progression Display

	Standard deviation	95% CI 1-tailed*	80% CI 1-tailed*
Keratoconus ARC (n=252)	0.062 mm	0.102 mm	0.052 mm
Normal ARC? (n=135)	0.015 mm	0.024 mm	0.012 mm
Keratoconus PRC	0.062 mm	0.102 mm	0.052 mm
Normal PRC?	0.050 mm	0.083 mm	0.042 mm
Keratoconus min pach	6.03 µm	9.92 µm	5.07 µm
Normal min pach=?	4.79 µm	7.88 µm	4.03 µm

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Can sterile water be used in CXL when the cornea is thin?

The study described in Paper IV addressed the issue of performing CXL on corneas with a thickness of less than 400 µm[144], which is considered the limit below which endothelial damage can occur[55]. No significant difference was found in the outcome between the group treated with hypoosmolar riboflavin alone (n = 14) and the group treated with hypoosmolar riboflavin with the addition of sterile water (0 mOsmol/L) (n = 28) (p = 0.065). However, as this investigation was not prospectively evaluated with a non-inferiority design no conclusions could be drawn regarding non-inferiority between the cohorts. It can be debated whether CXL with hypoosmolar riboflavin is as effective as CXL with isoosmolar riboflavin. Clinical outcomes have generally shown halting of progression[78, 145], but rare cases of failure have been reported[146]. It is possible that the hydrated state of artificially swollen corneae, both by hypoosmolar riboflavin and sterile water, could lead to a reduction in the crosslinking effect due to the lower relative concentration of collagen in the swollen cornea[77, 147]. However, crosslinking appears to be take place mainly within the anterior 200 µm, and the swelling effect could thus be relatively less important[148]. In fact, pre-clinical results have shown similar biomechanical strengthening effects when using isoosmolar or hypoosmolar riboflavin[124]. Endothelial cell measurements before and after CXL would have been useful to rule out negative effects on endothelial cell density. It is unclear whether the addition of sterile water could dilute the riboflavin to a level at which the UV shielding effect is compromised, thus leading to negative effects on the endothelial cells. In addition, the hypoosmolar riboflavin *per se* could cause a lower UV shielding effect[77].

Contact-lens-assisted CXL[122] is an alternative to the addition of sterile water. However, possible reflections of UV light from the contact lens and the risk of oxygen depletion could reduce the crosslinking effect[61, 85, 86, 88, 124]. The same reasoning can

be applied to so-called epi-on protocols. Although the corneal thickness is not reduced during treatment, the intact epithelium reduces riboflavin[58, 95] and oxygen diffusion[87, 88] and reflects some UV irradiation[85, 86], thus reducing the crosslinking effect[62, 90-92]. A recent treatment protocol for thin corneas, the "sub400 protocol", is based on an algorithm in which the intensity of the UV light is adapted according to the thickness of the cornea[149]. It would be interesting to compare the results of the riboflavin + sterile water protocol described in this work to the sub400 protocol. CXL using HPMC-riboflavin is also interesting, as pre-clinical investigations have shown an increase in the corneal thickness[81], however, clinical investigations have shown that this preserves the corneal thickness, rather than increasing it[150]. Results from a pre-clinical investigations[151] have suggested that CXL using HPMC-riboflavin induces a weaker biomechanical strengthening compared to CXL using dextran-based riboflavin and clinical investigations have shown both positive[79] as negative results[152].

Our group is currently evaluating three different CXL protocols in randomized clinical trial (ClinicalTrials.gov Identifier: NCT04427956). One of the protocols is CXL with isoosmolar riboflavin. Some of the subjects randomized to this group had corneal thicknesses less than 400 µm prior to CXL, or showed a reduction in corneal thickness during the CXL treatment, and thus sterile water was added. This will create a fourth group in the study, which will be compared with the other groups. Confocal microscopy measurements are performed pre- and post-CXL, as well as examinations using OCT and the Pentacam HR. We believe this study will provide sufficient evidence of the viability of CXL treatment with riboflavin + sterile water.

Given the lack of other scientifically validated protocols for the treatment of thin corneae with CXL, and the results of Study IV, together with 10 years' experience of good clinical outcome, we believe that the addition of sterile water is useful and reliable in the treatment of thin corneae with CXL. However, further investigations are needed.

Strengths and limitations of the studies

One of the strengths of the studies presented in Papers I-III is the attention to statistical detail. The within-subject standard deviation forms the core of the statistical calculations, and thus the subject standard deviation must be independent of the subject mean[131]. Such a dependency can probably explain some of the incongruences in previous publications. The severity of the disease varied in the cohorts studied in other investigations, and consequently also the outcomes regarding the repeatability of the measurements. In Studies II & III the variance component of the replicates was included in the calculation of the prediction limits[132] for single measurements. If only one measurement is compared to another measurement, the

prediction limits will be erroneously narrow, resulting in erroneous diagnosis of progression. This aspect was considered, and resolved, by including the variance between the four replicates on each day in the calculation of the prediction limits, thus providing more accurate results. To the best of the author's knowledge, this statistical aspect has not been considered in previous studies on the assessment of progression in keratoconus.

A possible limitation of these studies was that the optimal time interval for the assessment of inter-day repeatability is not known. Three days was chosen as it was deemed that this would be sufficiently long to allow inter-day changes, but sufficiently short to avoid true progression that could affect the repeatability calculations. Another possible limitation is that the possible effects of diurnal variation, or rather time after awakening[127, 128], were not considered. As the measurements were generally made between 09.00 and 15.00, diurnal effects were not considered relevant. In fact, no significant diurnal effects were found in a previous investigation on corneal curvature and thickness in subjects with keratoconus when measured between 09.00 and 17.00[153]. The optimal number of replicate measurements is another important factor. On the one hand, increasing the number of replicates could narrow the 95% CIs and facilitate the diagnosis of progression[141]. On the other hand, many measurements are time consuming, and exceeding the patient's attention span could negatively affect the repeatability of the measurements.

Another possible limitation of these studies is the under-representation of females. No studies have been published on the prevalence of keratoconus in Sweden. Previous studies in various parts of the world, including North America[30], China[31] and Saudi Arabia[32], have found no evidence of a gender-associated prevalence of keratoconus, while a 60%-67% male predominance was found in a Dutch study[27] and in a recently published Danish study[13]. A search was therefore carried out in the National Swedish Patient Register to identify all diagnoses of keratoconus at our hospital between the years 2014 and 2018. This showed that 1759 patients had been diagnosed with keratoconus, 1305 of whom were males (74%); the proportion of males ranging from 73 to 79% over this period. This could indicate a higher prevalence of keratoconus among Swedish males. Further studies should therefore be carried out on the gender distribution of keratoconus in Sweden.

The retrospective study described in Paper IV has several limitations; the cohorts are of different sizes and no endothelial cell analysis was carried out, which would have been important in describing safety aspects. Also, visual acuity was not considered.

Conclusions

The following conclusions were drawn based on the findings of the studies presented in this thesis.

1. The error in the measurement of parameters commonly used for the detection of progression of keratoconus is related to the disease severity. Limits for the detection of progression should therefore be stratified according to disease severity in order to avoid undertreatment or overtreatment with CXL.
2. Limits at which progression is detected should be based on inter-day repeatability, as this affects the limits at which progression is detected. Furthermore, limits should be calculated for both single measurements and for the mean of replicate measurements to suit the particular clinical setting.
3. The Belin ABCD Progression Display would benefit from using thresholds based in inter-day measurements, stratified according to disease magnitude. There is currently a clear risk of erroneously diagnosing keratoconus as progressive when using this software. The Pentacam HR software should be updated to allow the comparison of mean values in order to improve its capacity to diagnose progressive keratoconus.
4. The addition of sterile water during the treatment of thin corneae with CXL is an effective way of increasing the corneal thickness, thus allowing thin corneae to be treated. However, further studies are required to obtain more scientific evidence of the value of this treatment modality.

FUTURE PERSPECTIVES

The results of the Cochrane Review of 2015[44], the late approval of CXL by the US FDA[108] in 2016, and the more recent Cochrane Review of 2021[154] all suggest that the scientific evidence of the efficacy of CXL must be improved. Well-designed randomized clinical trials are needed, and consensus on how progressive keratoconus is defined is necessary to allow for meta-analysis. Furthermore, research is required to identify patients at risk of rapid progression, for example, by identifying genetic variants or other specific factors that increase the risk of fast progression. To properly assess this risk, the diagnosis of progression must be as correct as possible in order to draw conclusions from time-to-event analyses. The ability to compare means of replicate measurements in tomographic equipment would constitute a direct improvement.

Further validation of the results presented in this thesis, i.e. inter-day repeatability and the effects of disease severity, would be of importance. Studies should also be carried out on the ability of machine-learning tools to evaluate progression in keratoconus. Furthermore, the way in which the need for re-treatment with CXL is defined should be investigated. Relatively few investigations have been carried out on the repeatability of measurements following CXL, and factors that affect the ability to recognize the need for re-treatment. In this context, further studies should address the question of how crosslinked corneal tissue is affected by natural collagen turnover.

A further step would be to identify the best parameter for the detection of progression and assessment of treatment efficacy. A change in Kmax and other keratometric parameters is currently used for these purposes. However, changes in these parameters reflects an indirect change in the corneal shape due to a reduction in the biomechanical strength in the case of progressive keratoconus. In the follow-up after CXL, the change in Kmax (and other parameters) indirectly reflects an increase in the biomechanical strength, and consequently a flattening of the cornea. Kmax and other indirect parameters are, however, probably not the best parameters in this context.

Furthermore, it would be interesting to determine the degree of crosslinking achieved in treated subjects. One CXL protocol may not be suitable for all patients[14]. In fact, both undercrosslinking[96] and overcrosslinking[155] have been reported. It is thus important to assess the amount of crosslinking required for each patient in order

to reduce the risk of progression, while avoiding the risk of treatment-associated side effects. Brillouin microscopy can be used to measure the corneal biomechanics directly, which is the central factor in the pathophysiology and clinical management of keratoconus. This technique could therefore play an important role in the future in the assessment of progression and in quantifying the degree of crosslinking achieved[156, 157].

In order to address the issues discussed above, consensus is required on the diagnosis of keratoconus and the definition of progression of the disease. This would facilitate the meta-analysis of data, and lead to the timely implementation of evidence-based treatment protocols. Consensus would also facilitate the introduction of novel techniques such as Brillouin microscopy. The creation of keratoconus and CXL registers would also be useful for scientific studies. Such improvements would facilitate the implementation of evidence-based treatment in clinical practice.

DATA AVAILABILITY

The data underlying Paper I are available at:

https://doi.org/10.1371/journal.pone.0228992.s001 (SPSS)

The data underlying Paper II are available at:

https://data.mendeley.com/datasets/gsn2gncpcj/1 (Excel)

The data underlying Paper III are available at:

https://www.nature.com/articles/s41598-021-95503-8#Sec15 (Excel)

The data underlying Paper IV have been published as non-open access. Data are available upon request.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Keratokonus är en sjukdom som orsakar förtunning och oregelbunden form av hornhinnan och därmed försämrad syn. Oftast debuterar sjukdomen under ungdomsåren. Därefter föreligger det en risk för försämring av sjukdomen under ett antal år innan den spontant avstannar. Det är sällsynt att sjukdomen fortsätter att försämras efter 40-års ålder. Under denna tid kan dock försämringen orsaka ett allvarligt synhandikapp.

Sedan 2003 kan försämringen av keratoconus stoppas med en teknik som kallas korneal crosslinking (som förkortas CXL). Denna teknik är av samma art som används exempelvis hos tandläkaren. Har man hål i tanden fyller tandläkaren hålet med en flytande plastlösning och lyser sedan med UV-ljus för att skapa korsbindningar och därmed få plasten att stelna. På liknande sätt droppar vi riboflavin (B-vitamin) på hornhinnan och lyser sedan med UV-ljus för att skapa korsbindningar. Det finns rekommenderade gränsvärden för hur tjock hornhinnan måste vara för att behandla den med CXL. Då keratoconus är en sjukdom där hornhinnan förtunnas, så finns det en betydande del av patienterna som riskerar att inte kunna behandlas med den CXL-teknik som är mest dokumenterad som effektiv.

Indikationen för korneal crosslinking är dokumenterad försämring. Vi använder avancerad bilddiagnostik för detta ändamål men gränsvärdena för vad som tolkas som försämring kan diskuteras. Flera vetenskapliga studier har föreslagit olika gränsvärden men då resultaten varit så olika mellan studierna har man hållit sig till de vanliga gränsvärdena. Genom min forskning har jag sett att mätsäkerheten för de parametrar som oftast används är beroende av sjukdomsgraden. Jag har kunnat visa att de med lägre sjukdomsgrad behöver lägre satta gränser medan de med högre sjukdomsgrad behöver högre satta gränser. I dagsläget riskerar de med lägre sjukdomsgrad att underbehandlas och de med högre sjukdomsgrad att överbehandlas. De flesta studier har dessutom undersökt mätsäkerheten under en dag. Men då vi uppskattar tillväxt av keratoconus genom mätningar över tid, oftast månader, så är det av intresse att granska mätsäkerheten mellan olika dagar för att på så sätt inkludera de naturliga förändringar som kan ske i hornhinnans form i beräkningarna av gränsvärden. Mina resultat visar på att detta är en värdefull aspekt att ha med som påverkar gränsvärdena.

I denna avhandling har jag även utvärderat hur hornhinnans tjocklek kan ökas under CXL behandling genom att lägga till sterilt vatten som absorberas av hornhinnan och således får den att svälla. På detta sätt kan fler personer behandlas med syftet att hindra synförsämring. Jag jämförde två grupper patienter där den ena behandlats med vanlig CXL och den andra med CXL + sterilt vatten. Ett år efter behandlingarna verkade det inte föreligga några skillnader mellan grupperna. Detta resultat i kombination med en 10-årig erfarenhet av god effekt av denna behandling gör att vi kan fortsätta med den framöver. Eftersom jag endast jämförde journaluppgifter från tidigare genomförda behandlingar, så behöver denna studie upprepas inom ramen för en kontrollerad behandlingsstudie. En sådan studie pågår just nu.

RIASSUNTO IN ITALIANO

Il cheratocono è una malattia degenerativa del tessuto corneale (lo stroma) con una conseguente riduzione della stabilità biomeccanica, risultando in una protrusione della cornea, perciò il nome cheratocono. Questa protrusione causa un astigmatismo irregolare che riduce la capacità visiva. Il cheratocono debutta nei soggetti adolescenti e generalmente progredisce durante l'arco di uno o due decenni per poi arrestarsi intorno ai 40 anni di età. La severità della malattia è molto variabile ma prima che la malattia completi il suo ciclo può aver causato un handicap visivo severo con una forte riduzione della qualità della vita.

Dal 2003 esiste una terapia, crosslinking corneale, che può fermare la malattia. Questa tecnica è basata su un processo di polimerizzazione in cui crosslinks covalenti vengono creati tra le molecole proteiche. Si tratta di un intervento in anestesista locale in cui si procede alla rimozione dell'epitelio corneale ed all'istillazione di riboflavina (vitamina B) che viene assorbita dallo stroma corneale. Una volta che la cornea è stata saturata di riboflavina, si procede ad irradiarla con luce laser ultravioletta (UV-A); questo processo trasforma la riboflavina in uno stato attivo in modo che gli elettroni che vengono rilasciati inducano crosslinks covalenti tra le proteine stromali. Questo intervento aumenta la forza biomeccanica del tessuto corneale ed inibisce il progredire della malattia.

È importante da chiarire che il crosslinking arresta il continuo peggioramento, ma non cura l'attuale deformazione corneale. Per questo motivo è di fondamentale importanza la diagnosi precoce del cheratocono e il monitoraggio periodico del soggetto per verificarne un eventuale peggioramento, e pianificare un intervento di crosslinking in modo tempestivo. Attualmente il crosslinking è indicato esclusivamente per soggetti in cui il cheratocono è attivo. I rischi associati con un intervento di crosslinking sono contenuti, ma il dolore post-chirurgico è notevole ed esiste un rischio non trascurabile di infezioni corneali ed altre complicanze postoperatorie che potrebbero compromettere la vista. Dato questo trade-off fra rischio e beneficio, l'intervento di crosslinking è al momento indicato solo in pazienti con un peggioramento documentato.

Per diagnosticare il cheratocono si ricorre alla tomografia corneale che permette di ottenere grande accuratezza e può rivelare anche le forme meno evidenti di cheratocono, mentre la diagnosi del deterioramento è spesso più complicata. L'ostacolo maggiore per un efficacie diagnostica del deterioramento è dovuto alla

mancanza di guidelines internazionali e alla mancanza di parametri affidabili. Un parametro generalmente accettato a livello internazionale è rappresentato dal "maximum keratometry value". Un suo aumento di 1.0 diottrie rappresenta un peggioramento che suggerisce l'indicazione per un crosslinking corneale. Negli ultimi anni si sono succeduti numerosi studi il cui scopo è stato di identificare dei limiti oggetivi per appurare un deterioramento del cheratocono, ma i risultati di queste ricerche evidenziano chiare incongruenze nella determinazione delle soglie. Per questo motivo non sono ancora disponibili degli standard internazionali.

In questo lavoro di ricerca ho tentato di capire perché i risultati varino così tanto tra i diversi studi, al fine di indentificare dei parametri riproducibili per la diagnostica del deterioramento del cheratocono. Con il primo studio di questa tesi ho dimostrato che la precisione con cui si misura un peggioramento dipende dalla severità del cheratocono. La precisione è superiore tra soggetti con cheratocono più lieve e inferiore tra soggetti con una forma più severa. Con questa ricerca ho dimostrato che i limiti devono essere relativizzati alla severità del cheratocono. Con i limiti attuali (per esempio un aumento di 1.0 di maximum keratometry value) sotto diagnostichiamo i pazienti con le forme di cheratocono più lieve, cioè i pazienti che hanno più vantaggi se sottoposti a crosslinking in termini di preservazione della vista. Dall'altra parte sovra diagnostichiamo i pazienti con le forme più severe di cheratocono, cioè una parte di questi pazienti non hanno un deterioramento ma vengono comunque sottoposti a crosslinking. In questi casi i pazienti vengono inutilmente esposti al rischio di effetti collaterali.

Da un punto di vista metodologico, le soglie che vengono utilizzate attualmente a fini diagnostici sono ottenute da studi clinici a "singolo episodio di misura". Questo però non rispecchia la situazione clinica abituale, dato che le misurazioni in realtà vengono confrontate tra visite mediche separate nel tempo. Inoltre, è ben noto che la stabilità corneale è ridotta nei soggetti con cheratocono e si può presumere che la forma della cornea possa variare giornalmente, anche in assenza di un progredire della malattia. Perciò abbiamo condotto una ricerca in cui abbiamo ottenuto misurazioni ripetute, separate nel tempo (giorno 0 e giorno 3). Ho presunto che questo periodo di tempo (3 giorni) fosse sufficientemente lungo per permettere cambiamenti naturali della forma corneale ma, allo stesso tempo, abbastanza breve da non permettere peggioramenti nel cheratocono. I risultati hanno dimostrato che è molto importante considerare la variabilità inter-giornaliera oltre che la necessità di relativizzare i limiti con cui si diagnostica il deterioramento. Come risultato finale, propongo dei nuovi limiti da usare clinicamente, al fine di migliorare il framework decisionale per la determinazione dell'opportunità, o meno, di un intervento di crosslinking nel soggetto affetto da cheratocono.

Oltre agli studi per identificare i soggetti in cui è davvero attiva la malattia e per i quali ci si può avvalere di un intervento di crosslinking, ho studiato come si possa effettuare un crosslinking in soggetti con una cornea sottile. È ben descritto in

letteratura che uno spessore corneale al di sotto dei 400 micrometri comporta danni all'endotelio corneale con una conseguente riduzione della vista, che potrebbe necessitare un trapianto corneale. Dato che il cheratocono è una malattia degenerativa, la riduzione dello spessore corneale è patognomonica, e di conseguenza parecchi soggetti non sono eleggibili per il crosslinking. Questo è un chiaro limite che si presenta frequentemente nella clinica di tutti i giorni. Vari studi hanno suggerito modi diversi per superare questo problema, ma finora non esiste un gold standard per il trattamento di questi casi. Una tecnica per aggirare questo problema consiste nell'aumentare lo spessore corneale tramite riboflavina ipotonica. Purtroppo una notevole parte dei soggetti non raggiunge lo spessore adeguato malgrado questo trattamento. Un'alternativa ulteriore consiste nell'applicazione di una lente a contatto (UV permeabile) sulla cornea, per aumentare lo spessore in maniera artificiale. La procedura prevede di saturare il tessuto corneale di riboflavina prima dell'applicazione della lente, ma questa tecnica si presume poco efficace per indurre crosslinks a causa del fatto che il processo di crosslinking richiede ossigeno, che viene invece bloccato dalla lente a contatto. In questa tesi illustro, come soluzione migliorativa del procedimento d'inspessimento base, una tecnica che si avvale dell'aggiunta di acqua distillata. L'aggiunta di acqua distillata, assieme alla riboflavina ipotonica durante il crosslinking, apporta un efficace aumento dello spessore oltre 400 micrometri. In pratica tutti i soggetti, tranne quelli con livelli critici di spessore corneale, raggiungono uno spessore di oltre 400 micrometri, senza riduzione di ossigeno. Per conferma l'efficacia di questa tecnica, il test è stato condotto su due gruppi di pazienti: il primo con soggetti con uno spessore corneale oltre 400 micrometri e il secondo gruppo con uno spessore al di sotto dei 400 micrometri. In quest'ultimo gruppo di soggetti, l'acqua distillata è stata aggiunta alla riboflavina, evidenziando gli stessi risultati con significatività statistica. Dato che questo studio è stato condotto in passato con alcune limitazioni, deve essere sottolineata l'importanza di una sua replicazione con un design controllato.

In conclusione, i risultati riportati in questa tesi sono di grande importanza in termini di diagnosi precoce di progressione del cheratocono. Inoltre tali risultati potenzialmente permettono di selezionare quei soggetti che beneficiano di un crosslinking corneale. L' identificazione di una procedura piu' sicura per poter effettuare interventi di crosslinking rappresenta una conquista di grande importanza clinica, consentendo una piu' vasta applicazione di questa tipologia di trattamento del cheratocono.

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Paper I

PLOS ONE

RESEARCH ARTICLE

Association between keratoconus disease severity and repeatability in measurements of parameters for the assessment of progressive disease

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Abstract

Background

Progressive keratoconus can lead to severely impaired vision, but there is currently no consensus on the definition of progressive disease. Errors in the measurement of the parameters commonly used to establish progressive disease were evaluated in an attempt to determine the limits at which a true change in the values can be detected. The possible association between measurement error and disease severity was also investigated to evaluate the need for limits based on disease severity.

Methods

Sixty-one eyes were studied in 61 patients with keratoconus. Four replicate measurements were made in each patient using a Scheimpflug-based tomographic system (denoted the PC) and an auto-keratometer (denoted the AK). The repeatability coefficient, i.e., the level below which differences between two measurements are found in 95% of paired observations, was calculated. Patients were further divided into three groups based on disease severity (parameter magnitude).

Results

Increasing magnitude of all the keratometric parameters investigated was significantly associated with increasing measurement errors, and thus worse repeatability. The maximum keratometry value (Kmax) was the least repeatable parameter (1.23 D, 95% CI 1.11–1.35 D) and showed the strongest association between parameter magnitude and measurement error. The repeatability coefficient ranged between 0.32 and 1.62 D, depending on disease severity. The most repeatable parameter was the flattest central keratometry value (K1), measured with the PC (0.51 D, 95% CI 0.46–0.56 D) and the AK (0.54 D, 95% CI 0.48–0.59 D). K1 showed the weakest association between parameter magnitude and measurement error. The repeatability coefficient for K1 ranged between 0.40 and 0.54 D when using the PC, and between 0.34 and 0.70 D when using the AK in the three groups.

Conclusions

The association between the magnitude of the keratometric parameters and their measurement errors suggests that limits should be based on disease severity to ensure reliable detection of progressive keratoconus. Further studies are, however, required.

Introduction

Keratoconus is a corneal disease that can lead to severely impaired vision. It usually manifests in adolescents, and can have a significant negative impact on the quality of life [1]. In 2003 corneal crosslinking (CXL) emerged as a novel treatment for stabilizing progressive keratoconus [2]. Today, there is growing evidence that the progression of keratoconus can be halted by CXL [3] [4] [5], preventing further visual deterioration, and reducing the need for penetrating keratoplasty [6] [7]. Recent approval of CXL for the treatment of progressive keratoconus by the US FDA has confirmed the importance of this treatment.

It is important to detect keratoconus early, and to monitor it carefully for any signs of progression, so that CXL can be performed when appropriate. The instruments currently available for corneal imaging allow early detection of keratoconus [8], but there is no consensus on the definition of progressive keratoconus, which is the common indication for CXL. In 2015, it was reported that a consistent steepening of anterior or posterior corneal curvature and corneal thinning were suggestive of progressive disease [9]. It was also stated that the magnitude of the change should be greater than the measurement error. However, no specific limits were suggested for the magnitude of the change. Differences in measurements can result from a true change, or measurement error due to the intrinsic accuracy of the instrument. In order to define the level at which a true change can be suspected based on measurements, the repeatability coefficient (R) must be calculated. However, studies on the repeatability of measurements of topographic and tomographic parameters in keratoconus are often inconsistent. Some reports have suggested poorer repeatability in cohorts with more advanced disease [10] [11]. An increasing measurement error with increasing disease severity could explain some of the differences reported in repeatability, necessitating appropriate methods of calculating repeatability [12].

To the best of our knowledge, no studies have been performed to investigate the possible relation between measurement errors and the magnitude of parameters reflecting disease severity. The aim of this study was therefore to investigate this relation, and to calculate repeatability limits, based on disease severity, that could indicate a true change between measurements. Measurements of the anterior and posterior corneal curvature and corneal thickness were made using a Scheimpflug-based device, which is probably the most commonly used instrument in the management of keratoconus. Measurements of the anterior corneal curvature using auto-keratometry were also evaluated. As far as we know, auto-keratometry has not previously been evaluated in the management of keratoconus, but its repeatability is high in healthy corneas [13].

Subjects and methods

This study was conducted at the Department of Ophthalmology, Skåne University Hospital, Lund, Sweden, according to the Declaration of Helsinki. The Regional Ethics Committee in Lund approved the study (No. 2015/373).

Patients with keratoconus fulfilling the inclusion criteria were enrolled in the study after signing an informed consent form. The inclusion criteria were: keratoconus with no history of other ocular pathology or prior ocular surgery, and age > 18 years. Contact lens wear was discontinued at least 2 weeks before the measurements were made. Patients with corneal scarring were excluded. Pregnant and breastfeeding women were also excluded.

Keratoconus was diagnosed clinically, and by examination using a Scheimpflug-based device (see below). The sagittal curvature pattern, posterior and anterior elevation maps and corneal thickness pattern were assessed, in addition to information from the Belin-Ambrosio Enhanced Ectasia Display [14].

Sixty-one eyes in 61 patients were included. Only one eye was eligible for inclusion in 23 patients, due to prior CXL, penetrating keratoplasty or the presence of corneal scarring in the other eye. Computerized randomization was performed in the remaining 37 patients to select one eye for inclusion in the study (29 right eyes and 32 left eyes). Fifty-four participants were male, and 7 female, and the mean age was 29 years (range 18–49 years).

Four replicate measurements were made by the same examiner (IG) using the Pentacam HR system (Pentacam HR, version 1.20r10, Oculus Optikgeräte GmbH, Wetzlar, Germany). Patients were instructed to blink but not to lean back between measurements. Four replicate measurements were then made using auto-keratometry (NIDEK ARK-560A, NIDEK Co. Ltd., Japan) under the same conditions and by the same examiner, using auto-alignment mode. Only examinations deemed "OK" by the Pentacam HR system and error-free by the NIDEK ARK-560A instrument were accepted.

Instruments and parameters measured

The Pentacam HR (denoted PC) is a Scheimpflug-based tomographic system, the technical features of which have been described elsewhere [15]. The default setting of 25 images/s was used. The flattest central keratometry value (K1), the steepest central keratometry value (K2), the maximum keratometry value (Kmax), the posterior minimum radius (r-min) and the minimum corneal thickness (MCT) were measured with this instrument. K1 and K2 were measured in the central 3 mm zone.

The NIDEK ARK-560A (denoted AK) is a combined refractometer and keratometer. It captures a mire ring on the cornea on which analysis is based. K1 and K2 were obtained in auto-alignment mode using the standard 3.3 mm diameter zone of measurement.

Statistical methods and calculations

IBM SPSS Statistics 22 for Windows (IBM Corporation, Armonk, NY, USA) and SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc., Cary, NC, USA) were used for statistical analyses. Statistical significance was defined as a p-value of ≤ 0.05. Descriptive statistics are given as subject mean, standard deviation, median, and minimum and maximum values. Repeatability was assessed by calculating the within-subject standard deviation with 95% confidence intervals, the repeatability coefficient with 95% confidence intervals, intraclass correlation and the coefficient of variation [16] [17] [18]. Kendall's Tau-b was used to analyse correlations between the mean and standard deviation of replicate measurements. Transformed (natural logarithm) data were analysed where appropriate. K1, K2 and Kmax values were divided into three groups based on parameter magnitude to give groups of as equal size as possible. Differences between coefficients of variation were assessed using a regression test [19]. Bland-Altman plots were used to analyse the agreement between the two instruments. Limits of agreement were calculated using a linear mixed model for replicate measurements [16]. A professional medical statistician was consulted.

Definitions

- Repeatability: the variation in repeated measurements made on the same subject under identical conditions. The underlying values are assumed to be constant during the measurements [20].
- Within-subject standard deviation (S_w): the square root of the mean of subject variance [17].
- Repeatability coefficient (R) (2.77 x S_w): the difference between two measurements should be below this limit for 95% of pairs of observations [17].
- Coefficient of variation (CV): S_w divided by the overall mean [12][17].
- Intraclass correlation coefficient (ICC): (the variance between subjects) divided by (the variance between subjects plus the variance within a subject) [18].

Results

Repeatability of measurements

The value of ICC was high for all parameters, and the variability was attributed to differences between subjects, rather than within subjects. The CV was used for intra-instrument comparison of parameters. K1 showed the best repeatability (PC, CV = 0.41%) (AK = 0.43%), followed by K2 (PC, CV = 0.57%) (AK, CV = 0.50%), Kmax (CV = 0.80%), MCT (CV = 1.05%) and r-min (CV = 1.28%) (Table 1). In order to evaluate differences in repeatability between the instruments for K1 and K2 a regression test was performed to compare the CV for each parameter. No statistically significant differences were found in measurements between the PC and the AK instruments (K1, p = 0.130, K2, p = 0.498) (Table 2). The repeatability of K1 was then compared to that for K2 with both instruments, revealing a statistically significant higher repeatability for K1 than for K2 (PC, p = 0.002, AK, p<0.001) (Table 2). In parameter-specific units, the repeatability was best for K1 (PC, R = 0.51 D, 95% CI 0.46–0.56 D) (AK, R = 0.54 D, 95% CI 0.48–0.59 D), followed by K2 (PC, R = 0.76 D, 95% CI 0.68–0.83 D) (AK, R = 0.69 D, 95% CI 0.62–0.76 D), Kmax (R = 1.23 D, 95% CI 1.10–1.35 D), MCT (R = 14.2 μm, 95% CI 12.7–15.6 μm) and r-min (R = 0.18 mm, 95% CI 0.16–0.19 mm).

Stratified repeatability of measurements

The positive correlation between the magnitude of the measured parameter and its standard deviation corresponds to worsening repeatability of the measurements with increasing parameter magnitude (Table 1). Kmax showed the strongest such association (Kendall's Tau-b = 0.532, p<0.001) followed by K2 (PC)(Kendall's Tau-b = 0.305, p = 0.001)/(AK)(Kendall's Tau-b = 0.320, p = 0.001) and K1 (PC)(Kendall's Tau-b = 0.239, p<0.008)/(AK)(Kendall's Tau-b = 0.230, p = 0.016). No significant association was found for MCT (Kendall's Tau-b = 0.134, p = 0.129) or r-min (Kendall's Tau-b = -0.153, p = 0.083).

To interpret the effect of the clinical correlation between the magnitude of the keratometric parameters and their standard deviations, the repeatability coefficients were calculated. Measurements were stratified on three levels based on parameter magnitude (disease severity) and the repeatability coefficient was calculated for each group (Table 1). As can be expected from the Tau-b data, Kmax showed the greatest discrepancy in repeatability between groups, ranging from 0.32 to 1.62 D. K2 had a smaller discrepancy, ranging from 0.35 to 1.11 D using the PC, and from 0.32 to 0.95 D using the AK. K1 showed the smallest discrepancy between groups, ranging from 0.40 to 0.57 D (PC) and from 0.34 to 0.70 D (AK) (Table 1). Fig 1 shows the mean values for each parameter.

Table 1. Repeatability of the PC and AK measurements.

		n	Mean (SD)[a]	Median (Min–Max)[a]	S_w (95% CI)	Repeatability (95% CI)	ICC	CV (%)	Kendall's Tau[b]	p[b]
K1 (D)										
PC		61	44.0 (3.13)	43.5 (38.9–60.2)	0.18 (0.16–0.20)	0.51 (0.46–0.56)	0.997	0.41[c]	0.239	0.008
	<43.0	20	41.4 (1.32)	41.4 (38.9–42.9)	0.14 (0.12–0.17)	0.40 (0.33–0.47)	0.988	0.35	0.092	0.597
	≥43.0 <44.5	20	43.6 (0.38)	43.4 (43.0–44.1)	0.21 (0.17–0.24)	0.57 (0.47–0.68)	0.753	0.47[c]	0.330	0.049
	≥44.5	21	46.8 (3.49)	45.3 (44.5–60.2)	0.19 (0.16–0.23)	0.54 (0.44–0.63)	0.997	0.41	0.302	0.057
AK		61	44.8 (3.09)	44.2 (40.7–62.2)	0.19 (0.17–0.21)	0.54 (0.48–0.59)	0.996	0.43[c]	0.230	0.016
	<43.5	18	42.3 (0.72)	42.2 (40.7–43.3)	0.12 (0.10–0.15)	0.34 (0.28–0.40)	0.971	0.29	-0.145	0.444
	≥43.5 <45.2	20	44.1 (0.42)	44.1 (43.5–44.7)	0.16 (0.14–0.19)	0.46 (0.37–0.54)	0.862	0.37	0.111	0.546
	≥45.2	23	47.4 (3.55)	46.7 (45.2–62.2)	0.25 (0.21–0.29)	0.70 (0.58–0.82)	0.995	0.53	0.051	0.746
K2 (D)										
PC		61	47.0 (4.23)	46.1 (41.4–67.8)	0.27 (0.25–0.30)	0.76 (0.68–0.83)	0.996	0.57[c]	0.305	0.001
	<44.8	20	43.3 (1.04)	43.6 (41.4–44.8)	0.13 (0.10–0.15)	0.35 (0.29–0.42)	0.985	0.29	-0.154	0.373
	≥44.8 <47.8	20	46.3 (0.94)	46.1 (44.8–47.6)	0.21 (0.17–0.24)	0.57 (0.47–0.67)	0.954	0.44[c]	0.338	0.040
	≥47.8	21	51.3 (4.32)	50.2 (47.8–67.8)	0.40 (0.33–0.47)	1.11 (0.92–1.31)	0.991	0.78	0.129	0.415
AK		61	47.6 (4.55)	46.6 (41.9–73.6)	0.25 (0.22–0.27)	0.69 (0.62–0.76)	0.997	0.50[c]	0.320	0.001
	<45.3	20	44.0 (0.96)	44.4 (41.9–45.2)	0.12 (0.095–0.14)	0.32 (0.26–0.38)	0.986	0.26	0.150	0.405
	≥45.3 <48.6	20	46.9 (0.98)	46.6 (45.3–48.3)	0.23 (0.19–0.27)	0.63 (0.52–0.74)	0.949	0.49	-0.091	0.608
	≥48.6	21	51.7 (5.38)	50.1 (48.6–73.6)	0.34 (0.28–0.40)	0.95 (0.78–1.12)	0.996	0.66	0.035	0.830
Kmax (D)										
PC		61	52.0 (6.14)	50.6 (42.5–77.1)	0.44 (0.40–0.49)	1.23 (1.10–1.35)	0.995	0.80[c]	0.532	<0.001
	<48.2	20	46.2 (1.62)	46.6 (42.5–48.0)	0.11 (0.094–0.13)	0.32 (0.26–0.37)	0.995	0.25	0.281	0.090
	≥48.2 <53.9	21	51.1 (2.03)	50.6 (48.2–53.9)	0.48 (0.40–0.56)	1.33 (1.10–1.56)	0.946	0.94	0.298	0.061
	≥53.9	20	58.8 (5.27)	57.3 (53.9–77.1)	0.59 (0.48–0.69)	1.62 (1.33–1.91)	0.988	1.00	0.316	0.052
MCT (μm)										
PC		61	485.5 (40.6)	483.0 (394.5–578.0)	5.11 (4.59–5.63)	14.2 (12.7–15.6)	0.984	1.05	0.134	0.129
r-min (mm)										
PC		61	4.93 (0.71)	4.93 (2.99–6.29)	0.063 (0.057–0.070)	0.18 (0.16–0.19)	0.992	1.28	-0.153	0.083

[a] Subject Mean,

[b] Subject SD versus subject Mean,

[c] Calculated from transformed data, K1 (flattest central keratometry value), K2 (steepest central keratometry value), Kmax (maximum keratometry value), MCT (minimum corneal thickness), r-min (minimum posterior corneal radius), PC (Pentacam HR), AK (Nidek ARK 560A)

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Agreement

Fig 2 illustrates the agreement between the PC and AK instruments for K1 and K2. It can be seen that the AK system estimates higher keratometric values than the PC.

Table 2. Results of the regression test of CV for K1 and K2 within and between instruments.

	PC	AK	
K1 (D)	0.41	0.43	p = 0.130
K2 (D)	0.57	0.50	p = 0.498
	p = 0.002	p<0.001	

K1 (flattest central keratometry value), K2 (steepest central keratometry value)
PC (Pentacam HR), AK (Nidek ARK 560A)

https://doi.org/10.1371/journal.pone.0228992.t002

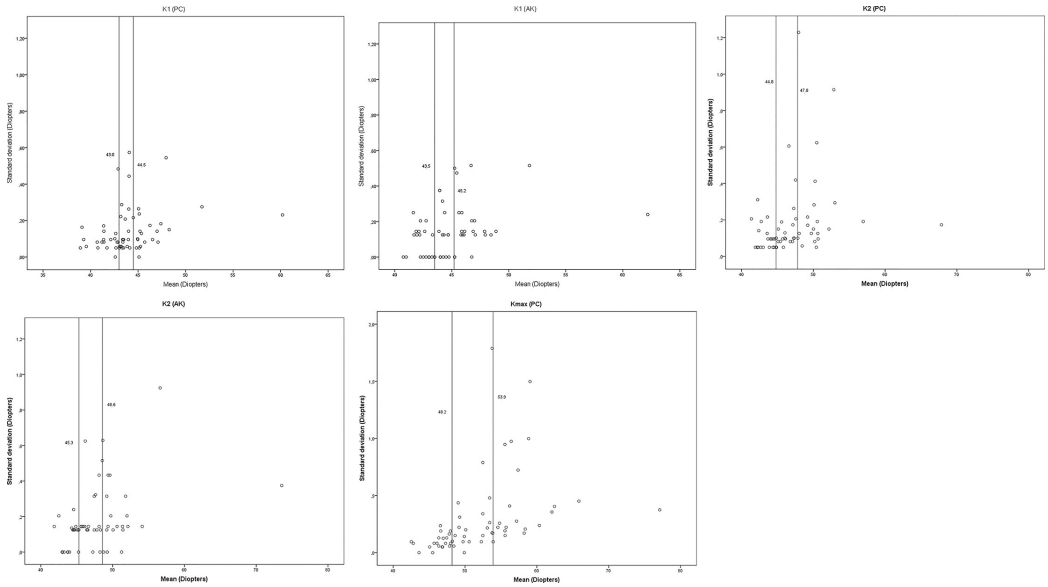

Fig 1. Mean values of the flattest central keratometry value (K1) obtained with the PC (a), and the AK (b), the steepest keratometry value (K2) obtained with the PC (c), and the AK (d), and the maximum keratometry value (Kmax), plotted against the standard deviation (e). The reference lines indicate the division into groups according to Table 1.

https://doi.org/10.1371/journal.pone.0228992.g001

Discussion

The findings of this study demonstrated a statistically significant association between measurement error and disease severity in terms of the magnitude of keratometric parameters in patients with keratoconus. These variations in measurement uncertainty for various degrees of severity of keratoconus should be considered when defining progression of the disease. To the best of our knowledge, this has not been attempted previously. Some previous studies have found poorer repeatability in cohorts of patients with more advanced disease. Flynn et al. [10] suggested such a relationship for Kmax, but not for K1, K2 or MCT, whereas Hashemi et al. [11] reported poorer repeatability in measurements of K1 and K2 in a cohort with K2 > 55 D (Kmax was not investigated). In these studies the focus was on describing the differences between cohorts, rather than on analysing the behaviour of the parameter per se. Kmax is probably the parameter most often used for the detection of progressive keratoconus [21], and is thus of particular interest. In the current study, a limit of 1.23 D for Kmax (95% CI 1.10–1.35 D) was found to indicate a true change between measurements. However, the effect of stratifying limits, based on disease severity, is clinically relevant. In patients with less severe disease (Kmax<48.2 D) a true change could be detected at a limit as low as 0.32 D (95% CI 0.26–0.37 D). However, the limit should be increased to 1.33 D (95% CI 1.10–1.56 D) in patients with Kmax ≥48.2 D<53.9 D, and to 1.62 D (95% CI 1.33–1.91 D) in patients with Kmax ≥53.9 D. There is thus a five-fold difference in the ability to detect a true change between consecutive measurement in the group with the least severe and the group with the most severe disease in this cohort. As a single limit is often used for all patients, usually an

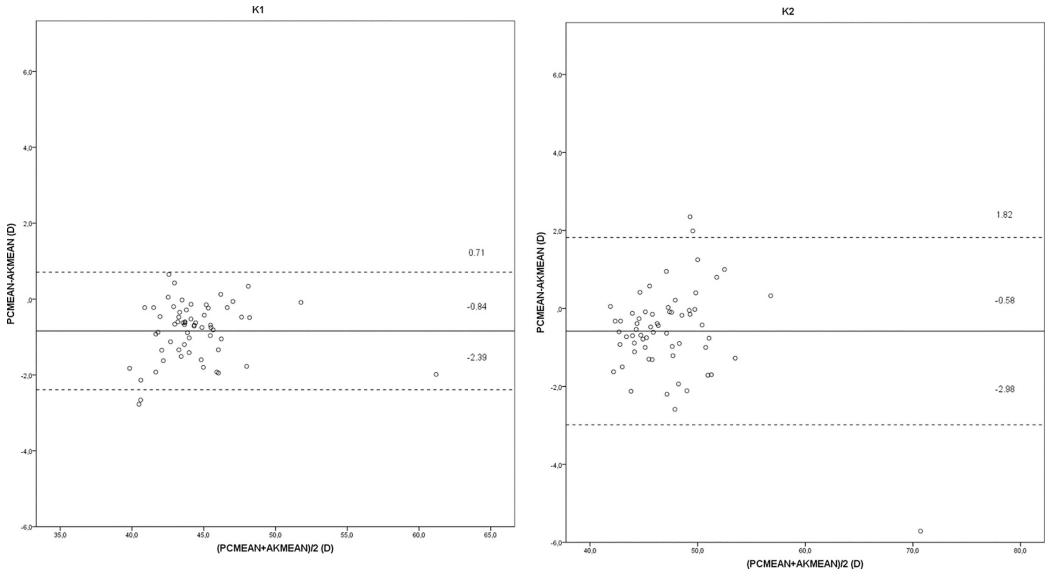

Fig 2. Mean difference, with 95% limits of agreement, between the two instruments, the PC (Pentacam HR) and the AK (Nidek ARK-560A) for: (a) the flattest central keratometry value (K1), and (b) the steepest central keratometry value (K2).

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increase of ≥ 1 D in Kmax [21], this finding is of considerable clinical importance. Less severe disease could be misinterpreted as non-progressive, while more severe disease could be erroneously diagnosed as progressive. Patients with less severe disease, but with preserved vision, could be especially at risk of delayed referral for CXL, and thus the risk of visual deterioration. Patients with more severe disease would instead be at risk of undergoing CXL unnecessarily, and being exposed to the risk of treatment-associated side effects.

The parameters associated with central keratometry, K1 and K2, showed a higher degree of repeatability and a lower association with disease severity than did Kmax. The most repeatable parameter was K1. When measuring K1 with the PC, a difference between measurements could be detected at 0.51 D (95% CI 0.46–0.56) in the cohort as a whole, and when using the AK at 0.54 D (95% CI 0.48–0.59 D), with no significant difference between the two instruments ($p = 0.130$). Due to the relatively low, but significant, association between the measurement error and the parameter magnitude, stratified limits ranged from 0.40 to 0.57 D for the PC and from 0.34 to 0.70 D for the AK.

K2 had a significantly worse repeatability than K1. When measured with the PC, the limit for the cohort as a whole was 0.76 D (95% CI 0.68–0.83 D), and the stratified limits ranged from 0.35 to 1.11 D. The corresponding values obtained with the AK were 0.69 D (95% CI 0.62–0.76 D) and a range in limits from 0.32 to 0.95 D.

To the best of our knowledge, no studies have been carried out on the repeatability of AK measurements in subjects with keratoconus. K1 and K2 are measured in the central 3 mm (PC) or 3.3 mm (AK) zone of the cornea, and may thus not cover the cone area. Kmax, on the other hand, is measured over the cone area, but has poorer repeatability. It would be

interesting to investigate whether central keratometry, and especially K1, could play a more important role in the detection of disease progression, and if such a commonly used instrument as the AK could be used. The findings of the present study show that measurements of K1 and K2 with the two instruments are not interchangeable, due to wide limits of agreement.

In contrast to the keratometric parameters, no statistically significant correlation was found between the magnitude of r-min and MCT and their associated measurement errors. This may be advantageous, since the error in these measurements does not depend on disease severity. However, the coefficient of variation in measurements of both r-min and MCT was poorer than that in the keratometric parameters.

One possible limitation of this study is the under-representation of females (11.5%). No studies have been published on the prevalence of keratoconus in Sweden. Previous investigations from various parts of the world, including North America [22], China [23] and Saudi Arabia [24], have found no evidence of a gender-associated prevalence of keratoconus. However, it was concluded in a Dutch study that there was a 60.6% male predominance [25], similar to 66.9%, in a recently published Danish study [26]. All diagnoses made at Swedish hospitals are recorded in patient registers. A search was carried out to identify all diagnoses of keratoconus at our university hospital between the years 2014 and 2018, showing that 1759 patients had been diagnosed with keratoconus, 454 of whom were female (25.8%). The proportion of females ranged from 21–27% over this period. This finding could indicate a lower prevalence of keratoconus in Swedish females, suggesting that further studies should be carried out on the gender distribution of keratoconus in Sweden.

In conclusion, we have demonstrated that measurement uncertainties increase with disease severity, i.e., the magnitude of keratometric parameters. Stratified repeatability limits were therefore calculated based on disease severity. Less severe disease could be misinterpreted as non-progressive, while more severe disease could be erroneously diagnosed as progressive if a single limit is used for all patients. However, it is important to emphasize that these findings require further evaluation before they can be applied in clinical practice. As progression in keratoconus is diagnosed over time, future investigations must be performed on inter-day repeatability, stratified according to the severity of keratoconus disease. This would be an important step towards understanding true progression, and reaching consensus on the definition of progressive keratoconus.

Supporting information

S1 File. SPSS spreadsheet. Data underlying the findings.
(SAV)

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Paper II

The Interday Repeatability of Parameters for the Assessment of Progressive Disease in Subjects With Less Advanced Keratoconus

INGEMAR GUSTAFSSON, ANDERS BERGSTRÖM, ANNA CARDIAKIDES, ANDERS IVARSEN, AND JESPER ØSTERGAARD HJORTDAL

• PURPOSE: **To evaluate the interday repeatability in the measurement of parameters used for the detection of progression of keratoconus by prediction limits (PL) for single measurements, and the repeatability coefficient (RC) for the mean of replicate measurements.**

• DESIGN: **Prospective reliability analysis for cases and control eyes.**

• METHODS: **Twenty-five eyes in 25 subjects with KC and 25 eyes in 25 healthy controls were included. Four consecutive measurements were made, 3 days apart, with a Pentacam HR tomographic instrument (denoted the Pentacam) and a Nidek ARK 560-A autokeratometer (denoted the keratometer). Main outcome measures were the intra- and interday RC of parameters used in the detection of progression of keratoconus.**

• RESULTS: **The most repeatable parameter obtained with the Pentacam was the curvature power of the central flat meridian (K1, 0.44 D [RC], -0.55 to 0.60 diopter [D] [PL]), followed by the central steep meridian (K2, 0.72 D [RC], -0.90 to 0.94 D [PL]). The interday repeatability of K1 and K2 was similar when using the keratometer (K1, 0.32 D [RC], -0.66 to 0.57 D [PL], K2, 0.93 D [RC], -1.36 to 1.08 D [PL]). The interday repeatability of the curvature power of the steepest point (Kmax, 0.84 D [RC], -0.90 to 1.11 D [PL]) would benefit from being stratified: RC = 0.44 D and PL = -0.49 to 0.67 D for Kmax < 49.0 D, and RC = 1.08 D and PL = -1.19 to 1.42 D for Kmax ≥ 49.0 D.**

• CONCLUSIONS: **The interday repeatability of measurements, single or replicate, in subjects with keratoconus should be considered when diagnosing progressive disease. K1 exhibited the best intraday repeatability. Kmax benefits from being stratified according to disease severity. (Am J Ophthalmol 2021;225:38–46. © 2021 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).)**

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KERATOCONUS IS A CORNEAL DISEASE THAT CAN cause severe visual impairment. Growing evidence suggests that corneal cross-linking (CXL) halts the progression of the disease, preventing further visual deterioration.[1,2] The general indication for CXL is progressive KC, as stated in the US FDA approval of CXL in 2016. However, there is currently no consensus on the definition of progressive disease. This matter was addressed by an international expert panel in 2015, which concluded that a consistent steepening of the anterior or posterior corneal curvature and corneal thinning were indicative of progressive disease.[3] The panel further concluded that the magnitude of the change between measurements should be greater than the measurement error of the equipment. As there is no reference instrument for these measurements, the measurement error in the parameters used in the detection of progression must be assessed. The measurement error is commonly expressed as the repeatability coefficient (RC). RC is defined as the level below which the difference between 2 measurements should be for 95% of pairs of observations. Several studies have been carried out to investigate RC in measurements of the parameters used in the detection of progressive keratoconus.[4–7] However, RC is usually calculated based on measurements on the same day, rather than on different days.[8] The evaluation of the *interday* repeatability in subjects with keratoconus is thus of the utmost importance, as progression is diagnosed over time. Day-to-day variations in the corneal shape can be expected in subjects with keratoconus owing to the lower biomechanical strength of the cornea.

The aim of this study was, therefore, to evaluate the interday repeatability of measurements in subjects with keratoconus. Subjects with keratoconus stage ≤ 2 according to the Amsler-Krumeich classification were recruited.[9] Accurate detection of the progression of keratoconus in subjects with less severe disease, followed by timely treatment with CXL, is important to preserve vision, and possibly avoid future corneal surgery.[10] Furthermore, it has been demonstrated that measurement error increases with disease severity, and should therefore be considered when evaluating progression.[7,11]

Measurements were made with a Pentacam HR (hereafter denoted the Pentacam), which is the most commonly used tomographic system in the management of

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keratoconus.[12] Central keratometry measurements were also made with an auto-keratometer, Nidek ARK 560-A (hereafter denoted the keratometer), as it has shown high repeatability in subjects with keratoconus, and auto-keratometers are widely available.[11]

METHODS

THIS STUDY WAS CONDUCTED AT THE DEPARTMENT OF Ophthalmology at Skåne University Hospital, Lund, Sweden, according to the Declaration of Helsinki. The Regional Ethics Committee in Lund, Sweden, approved the study (DNR2015/373).

• **ENROLLMENT:** Patients with keratoconus fulfilling the inclusion criteria were enrolled consecutively in the study after signing an informed consent form. The inclusion criteria were keratoconus stage ≤ 2 (Amsler-Krumeich classification) with no history of, and no current signs of, other ocular pathology. This includes ocular surface disease and external diseases such as dry eyes and atopy. Furthermore, only subjects with no prior ocular surgery and age ≥ 18 years were recruited. Contact lens wear was discontinued at least 2 weeks before the measurements were made. Pregnant and breastfeeding women were excluded. As the purpose of this study was to investigate progression in mild-to-moderate keratoconus, subjects with advanced stages (3 and 4) were excluded.

Keratoconus was diagnosed clinically, and by examination using a Scheimpflug-based device (see below). The sagittal curvature pattern, posterior and anterior elevation maps, and corneal thickness pattern were assessed, in addition to information from the Belin-Ambrosio Enhanced Ectasia Display.

Twenty-five patients were enrolled. Only 1 eye was eligible for inclusion in 8 patients owing to prior CXL (n = 3) or keratoconus stage > 2 (n = 5). If 2 eyes were eligible for inclusion, then both were examined (see "examination" section below). Computerized randomization was performed in the 17 patients where both eyes met the inclusion criteria to select 1 eye for inclusion in the study (12 right eyes and 13 left eyes). Twenty-two participants were male and 3 were female, and the mean age was 27 years (21-45 years).

Healthy controls (n = 25) were enrolled from among medical students and residents in ophthalmology after signing an informed consent form. The inclusion criteria were age ≥ 18 years and no history of any ocular pathology or prior ocular surgery. Pregnant and breastfeeding women were excluded. Ocular pathology was excluded by a clinical examination and by examination using a Scheimpflug-based device. Only 1 eye was eligible for inclusion in 3 patients, owing to scarring of the cornea. If 2 eyes were eligible for inclusion, both were examined and computerized randomization was performed as described above, resulting in 12 right eyes and 13 left eyes. Fourteen participants were male and 11 were female, and the mean age was 29 years (23-41 years).

• **INSTRUMENTS:** The Pentacam HR is a Scheimpflug-based tomographic system (Pentacam HR, version 1.20r10, Oculus Optikgeräte GmbH, Wetzlar, Germany). The technical features of this system have been described elsewhere.[5] We used the default setting of 25 pictures/s. The Nidek ARK 560-A (Nidek Co Ltd, Tokyo, Japan) is a combined refractometer and keratometer. It captures a mire ring on the cornea on which the analysis is based. The measurements were made using auto-alignment mode and the standard 3.3-mm-diameter zone of measurement.

• **EXAMINATION:** Measurements were made on 2 separate occasions, 3 days apart (denoted day 0 and day 3). Four consecutive measurements were made on each day, first with the Pentacam HR and then with the Nidek ARK 560-A, by the same examiner (I.G.). Subjects were instructed to blink between measurements, but not to lean back. Measurements were made during normal working hours without taking corneal diurnal variation into account. Only examinations deemed "OK" by the Pentacam and "error-free" by the keratometer were accepted. The right eye was examined first, then the left, if both eyes were eligible for inclusion. This represents the normal clinical scenario where both eyes of the patient are usually measured. When recruitment to the study was complete, computerized randomization was performed to select 1 participating eye per subject.

• **STATISTICAL METHODS AND CALCULATIONS:** The values obtained for the 4 replicate measurements on day 0 and day 3 were averaged for each day and were used to calculate the interday repeatability for the clinical scenario when using the mean value of measurements to assess progression. When calculating prediction limits (PL) in the clinical scenario when single measurements are used to assess progression, the variance between replicate measurements was included in the calculation to provide more accurate results.

IBM SPSS Statistics 22 Windows (IBM Corporation, Armonk, New York, USA) and SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc, Cary, North Carolina, USA) were used for statistical analyses. A P value below .05 was considered significant. Descriptive statistics are given as subject mean, standard deviation (SD), and minimum and maximum values. Repeatability was assessed by calculating the within-subject standard deviation, precision, RC, intraclass correlation coefficient, and coefficient of variation with associated confidence intervals (CI).[13–15] Kendall's tau was used to assess relationship between mean and SD and natural logarithm-transformed data were

analyzed when appropriate. The limits of agreement were calculated with the replicates by a linear mixed-effect model.[16] The sample size was calculated[17] considering the interday repeatability of Kmax as the primary outcome variable. A within-subject standard deviation of 0.36 was used based on a prior investigation.[18] The width of the 95% CI was set to 30% of the within-subject standard deviation on either side. This resulted in 22 subjects. Twenty-five subjects were recruited to secure the number. A professional medical statistician was consulted.

• **DEFINITIONS AND ABBREVIATIONS:** Measurements used are defined as follows:

- Within-subject standard deviation (S_w): The square root of the variance between subjects.
- Precision = 1.96 × S_w. The difference between a measurement and the true value should lie below this limit in 95% of the measurements.
- Repeatability coefficient (RC) = 2.77 × S_w. The difference between 2 measurements should lie below this limit for 95% pairs of observations.[13]
- Coefficient of variation (CoV): S_w divided by the total subject mean.
- Intraclass correlation coefficient: The variance between subjects divided by the variance between subjects plus the variance within subjects.
- Prediction limits (PL): 95% CI for differences between 2 future single measurements.
- K1: Curvature power of the central flat meridian.
- K2: Curvature power of the central steep meridian.
- Kmax: Curvature power of the steepest point on the anterior surface.
- Rmin: Curvature power of the steepest point on the posterior surface.

RESULTS

THE VALUE OF THE INTRACLASS CORRELATION COEFFICIENT was high for all parameters among both the healthy subjects and the keratoconus cohort. The variability was thus attributed to differences between subjects, rather than within subjects (Table 1). The CoV was used to compare parameters with different units. The interday comparison between single measurements is presented as PL, and the mean of 4 measurements is presented as RC.

As statistical models assume a constant measurement error, the SDs were plotted against the magnitude of the investigated parameters and the association analyzed using Kendall's Tau-b in the preanalysis of data.[15] Among the subjects with keratoconus in this investigation, Kmax showed the strongest association between magnitude and SD (Kendall's Tau-b = 0.483, P = .0001) and was the only parameter that could have a clinical impact. Graphically, the SD of Kmax begins to increase at approximately 50.0 diopters (D) (Figure 1). As the median value of Kmax for the subjects was 49.0 D, this value was chosen for calculations of the repeatability of measurements of Kmax below and above this value. No such correlation was seen in Kmax for the healthy controls (Kendall's Tau-b = −0.158, P = .28) (Figure 2) or for any other parameters.

• **INTRADAY REPEATABILITY OF MEASUREMENTS IN HEALTHY CONTROLS:** Data are presented in Table 1. K1 was the most repeatable parameter when measured with the Pentacam, followed by K2, also with the Pentacam. Measurements of K1 and K2 using the keratometer showed a somewhat poorer repeatability. Kmax also exhibited a high level of repeatability, but measurements of both minimum corneal thickness (MCT) and Rmin showed a poorer repeatability than K1, K2, and Kmax. The CoV expressed as % was used for this interunit comparison. No statistically significant differences in the magnitude of repeatability between measurements on day 0 and day 3 were seen in any of the parameters except K1.

• **INTRADAY REPEATABILITY OF MEASUREMENTS IN KERATOCONUS:** Data are presented in Table 1. K1 was the most repeatable parameter when measurements were made with the Pentacam and the keratometer. K2 showed a poorer repeatability than K1 with both instruments. The repeatability of measurements of Kmax was similar to the repeatability of K2. There was a tendency toward improvement from day 0 to day 3 in the repeatability of keratometric measurements using the Pentacam. This effect was most prominent for Kmax: day 0, R = 0.70 D, 95% CI 0.59-0.81 D; and day 3, R = 0.52 D, 95% CI 0.44-0.61 D. This effect was not seen in measurements of K1 and K2 using the keratometer. The repeatability in measurements of MCT remained unchanged between day 0 and day 3. In contrast, the repeatability of measurements of Rmin deteriorated between day 0 and day 3.

• **INTERDAY REPEATABILITY OF MEASUREMENTS IN THE HEALTHY CONTROLS AND THE KERATOCONUS SUBJECTS: SINGLE MEASUREMENTS AND THE MEAN OF 4 REPLICATE MEASUREMENTS:** Data are presented in Table 2 regarding single measurements, and in Table 3 regarding the mean of 4 replicates. The interday repeatability of measurements of all the parameters was better in the control group than in the keratoconus cohort, using both the Pentacam and the keratometer. However, MCT showed little difference between healthy controls and subjects with keratoconus. No clear difference could be seen in the interday repeatability of measurements of K1 and K2 between the Pentacam and the keratometer in subjects with keratoconus. Among healthy controls, measurements of K1 and K2 demonstrated a better interday repeatability when made with the Pentacam than with the keratometer. Furthermore, the interday repeatability in measurements of K1

TABLE 1. Descriptive Statistics and Repeatability of the Pentacam and Auto-keratometer Measurements on Day 0 and Day 3, in the Healthy Controls and Keratoconus Cohort

	Day	Mean (SD)[a]	Min-Max[a]	Sw (95% CI)	CV%	Repeatability (95% CI)	ICC (95% CI)
Controls							
K1 (D)							
PC	0	43.1 (1.2)	(40.9-45.6)	0.09 (0.08-0.11)	0.20[b]	0.25 (0.21-0.29)	0.99 (0.99-1.00)
	3	43.0 (1.3)	(40.8-45.7)	0.06 (0.05-0.06)	0.13	0.15 (0.13-0.18)	1.00 (1.00-1.00)
AK	0	43.3 (1.3)	(40.8-46.0)	0.15 (0.13-0.18)	0.35[b]	0.42 (0.35-0.49)	0.99 (0.97-0.99)
	3	43.3 (1.3)	(40.8-45.9)	0.12 (0.10-0.14)	0.28	0.34 (0.29-0.40)	0.99 (0.98-1.00)
K2 (D)							
PC	0	43.9 (1.4)	(41.4-46.6)	0.05 (0.05-0.06)	0.12	0.15 (0.13-0.17)	1.00 (1.00-1.00)
	3	43.9 (1.4)	(41.4-46.6)	0.07 (0.06-0.08)	0.15	0.19 (0.16-0.22)	1.00 (1.00-1.00)
AK	0	44.2 (1.4)	(41.3-46.8)	0.11 (0.09-0.13)	0.25	0.31 (0.26-0.36)	0.99 (0.99-1.00)
	3	44.1 (1.4)	(41.3-46.7)	0.12 (0.10-0.14)	0.28	0.34 (0.29-0.39)	0.99 (0.99-1.00)
Kmax (D)							
PC	0	44.3 (1.5)	(41.8-47.4)	0.10 (0.08-0.11)	0.22	0.27 (0.23-0.31)	1.00 (0.99-1.00)
	3	44.3 (1.5)	(41.6-47.4)	0.11 (0.09-0.12)	0.24	0.30 (0.25-0.34)	1.00 (0.99-1.00)
MCT (μm)							
PC	0	538.2 (22.9)	(493.0-580.3)	3.95 (3.32-4.59)	0.73	11.0 (9.21-12.7)	0.97 (0.95-0.99)
	3	539.3 (23.9)	(501.0-585.0)	3.89 (3.26-4.51)	0.72	10.8 (9.05-12.5)	0.97 (0.95-0.99)
Rmin (mm)							
PC	0	6.1 (0.24)	(5.6-6.6)	0.044 (0.037-0.051)	0.72	0.12 (0.10-0.14)	0.97 (0.94-0.98)
	3	6.1 (0.24)	(5.6-6.6)	0.038 (0.032-0.044)	0.62	0.11 (0.089-0.12)	0.98 (0.96-0.99)
Keratoconus cohort							
K1 (D)							
PC	0	43.6 (1.8)	(40.7-47.5)	0.15 (0.13-0.18)	0.35	0.42 (0.35-0.49)	0.99 (0.99-1.00)
	3	43.6 (1.8)	(40.6-47.2)	0.14 (0.12-0.16)	0.32	0.38 (0.32-0.44)	0.99 (0.99-1.00)
AK	0	44.3 (1.7)	(42.0-48.4)	0.18 (0.16-0.21)	0.42	0.51 (0.43-0.59)	0.99 (0.98-0.99)
	3	44.4 (1.6)	(42.1-48.2)	0.24 (0.20-0.27)	0.53	0.66 (0.55-0.76)	0.98 (0.96-0.99)
K2 (D)							
PC	0	46.0 (2.8)	(42.8-56.0)	0.24 (0.20-0.28)	0.54[b]	0.67 (0.56-0.78)	0.99 (0.99-1.00)
	3	46.0 (2.7)	(42.8-55.7)	0.19 (0.16-0.22)	0.41	0.52 (0.44-0.61)	1.00 (0.99-1.00)
AK	0	46.5 (2.7)	(43.5-55.6)	0.32 (0.27-0.37)	0.69	0.89 (0.75-1.03)	0.99 (0.97-0.99)
	3	46.6 (2.7)	(43.5-55.6)	0.33 (0.28-0.39)	0.67[b]	0.92 (0.77-1.07)	0.98 (0.97-0.99)
Kmax (D)							
PC	0	50.3 (4.8)	(44.5-65.4)	0.25 (0.21-0.29)	0.46[b]	0.70 (0.59-0.81)	1.00 (0.99-1.00)
	3	50.2 (4.7)	(44.4-64.7)	0.19 (0.16-0.22)	0.35[b]	0.52 (0.44-0.61)	1.00 (1.00-1.00)
MCT (μm)							
PC	0	493.0 (35.1)	(442.8-560.3)	3.76 (3.16-4.37)	0.76	10.4 (8.76-12.1)	0.99 (0.98-0.99)
	3	493.1 (35.2)	(437.5-561.3)	3.86 (3.24-4.48)	0.78	10.7 (8.99-12.4)	0.99 (0.98-0.99)
Rmin (mm)							
PC	0	5.1 (0.65)	(3.9-6.2)	0.051 (0.043-0.059)	1.00	0.14 (0.12-0.16)	0.99 (0.99-1.00)
	3	5.1 (0.63)	(3.9-6.1)	0.19 (0.16-0.22)	3.85[b]	0.52 (0.44-0.60)	0.92 (0.85-0.96)

AK = Nidek ARK 560-A auto-keratometer; K1 = flattest central keratometry value; K2 = steepest central keratometry value; Kmax = maximum keratometry value; MCT = minimum corneal thickness; PC = Pentacam HR; Rmin = minimum posterior corneal radius.

[a]Subject mean.

[b]Calculated using natural logarithm transformation.

and K2 improved when using the mean of replicates, compared to single measurements, when using both instruments.

The results suggest that progression in keratoconus can be detected by a change in magnitude between *single measurements* of 0.60 D (K1), 0.94 D (K2), 1.11 D (Kmax), 12.41 μm (MCT), or 0.38 mm (Rmin), when using the Pentacam. When measurements are made with the keratometer, progression can be detected by a change in magnitude of 0.57 D (K1) and 1.08 D (K2). These values refer to the upper prediction limit. When stratifying Kmax, progression in keratoconus subjects can be

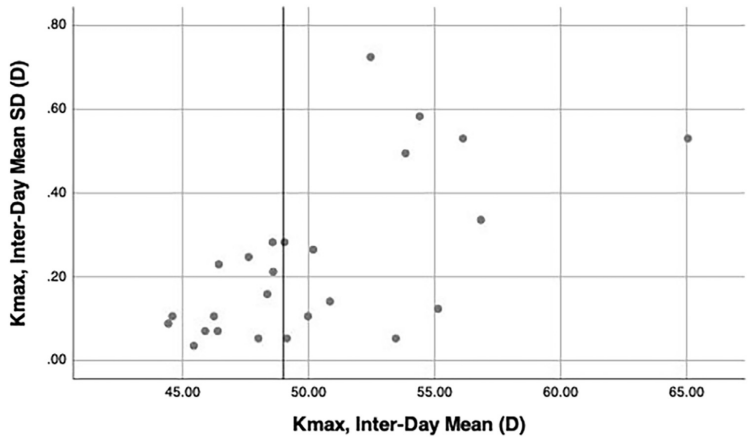

FIGURE 1. Mean standard deviation in the curvature power of the steepest point (Kmax) plotted against the mean interday values of Kmax for the keratoconus subjects. The reference line indicates the median mean value at 49.0 diopters (D).

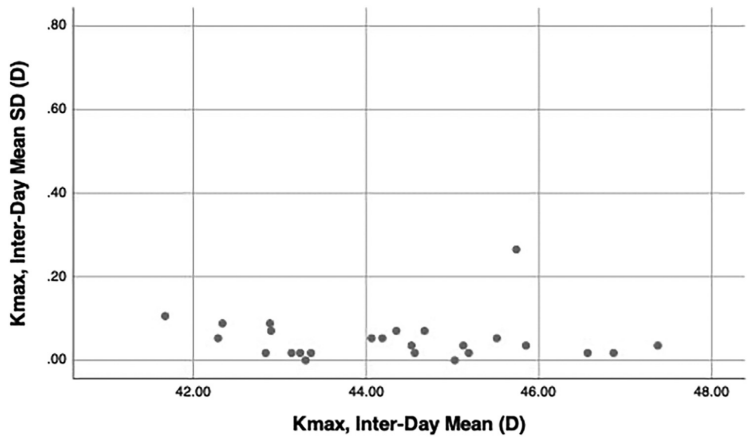

FIGURE 2. Mean standard deviation in the curvature power of the steepest point (Kmax) plotted against the mean interday values of Kmax for the healthy controls. D = diopters.

detected at a change in magnitude of 0.67 D (Kmax < 49.0 D) and 1.42 D (Kmax ≥ 49.0 D). The results suggest that progression in keratoconus can be detected by a change in magnitude between *the means of 4 replicates* of 0.44 D in K1 (95% CI 0.32-0.56 D), 0.72 D in K2 (95% CI 0.52-0.92 D), 0.84 D in Kmax (95% CI 0.61-1.07 D), 8.11 μm in MCT (95% CI 5.86-10.4 μm), or 0.21 mm in Rmin (95% CI 0.15-0.27 mm) when using the Pentacam. For central keratometry readings obtained with the keratometer, progression can be detected by a change of 0.32 D in K1 (95% CI 0.23-0.41 D) and 0.93 D in K2 (95% CI 0.67-1.18 D). When stratifying Kmax, progression in keratoconus subjects can be detected at a change in magnitude of 0.44 D (95% CI 0.27-0.62 D) for Kmax < 49.0 D, and 1.08 D (95% CI 0.67-1.50 D) for Kmax ≥ 49.0 D.

TABLE 2. Interday Differences Between Single Measurements With Prediction Limits for the Healthy Controls and the Keratoconus Cohort

	Variance Components			Mean Difference	Lower Prediction Limits	Upper Prediction Limits
	$\hat{\tau}^2$	$\hat{\sigma}_1^2$	$\hat{\sigma}_2^2$	$\hat{\alpha}_1 - \hat{\alpha}_2$	$\hat{\alpha}_1 - \hat{\alpha}_2 - 2 \times \sqrt{2\hat{\tau}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$	$\hat{\alpha}_1 - \hat{\alpha}_2 + 2 \times \sqrt{2\hat{\tau}^2 + \hat{\sigma}_1^2 + \hat{\sigma}_2^2}$
Controls						
PC						
K1 (D)	0.0002	0.0083	0.0031	0.009	−0.21	0.23
K2 (D)	0.0027	0.0029	0.0046	0.029	−0.20	0.26
Kmax (D)	0.0023	0.0096	0.0115	0.033	−0.29	0.35
MCT (μm)	1.4244	16.0578	15.0967	−1.131	−12.79	10.53
Rmin (mm)	0.0001	0.0020	0.0015	0.005	−0.11	0.13
ARK						
K1 (D)	0.0005	0.0231	0.0152	−0.010	−0.41	0.39
K2 (D)	0.0177	0.0123	0.0150	0.082	−0.41	0.58
Keratoconus cohort						
PC						
K1 (D)	0.020	0.023	0.019	0.027	−0.55	0.60
K2 (D)	0.058	0.059	0.036	0.018	−0.90	0.94
Kmax (D)	0.078	0.063	0.036	0.10	−0.90	1.11
Kmax < 49 (D)	0.017	0.035	0.015	0.092	−0.49	0.67
Kmax ≥ 49 (D)	0.14	0.089	0.055	0.12	−1.19	1.42
MCT (μm)	5.26	14.16	14.90	-0.17	−12.75	12.41
Rmin (mm)	0.0011	0.0026	0.035	−0.017	−0.42	0.38
ARK						
K1 (D)	0.0019	0.034	0.056	−0.043	−0.66	0.57
K2 (D)	0.080	0.10	0.11	−0.14	−1.36	1.08

AK = Nidek ARK 560-A auto-keratometer; D = diopters; K1 = flattest central keratometry value; K2 = steepest central keratometry value; Kmax = maximum keratometry value; MCT = minimum corneal thickness; PC = Pentacam HR; Rmin = minimum posterior corneal radius.

Definitions: $\hat{\tau}^2$ = squared between-subject mean variance day 0 and day 3; $\hat{\sigma}_1^2$ = squared within-subject mean variance on day 0; $\hat{\sigma}_2^2$ = squared within-subject mean variance on day 3; α_1-α_2 = difference between means from day 0 and day 3.

DISCUSSION

THE RESULTS OF THIS STUDY SUGGEST THE MAGNITUDE OF the change in interday measurements, single or replicates, at which a progression in keratoconus can be suspected in patients with keratoconus of stage ≤ 2. The best interday repeatability in the keratoconus cohort was seen in measurements of central keratometry parameters, in particular K1, when using the Pentacam as well as the keratometer. It would therefore be of interest to further investigate the role of K1 and K2 in the evaluation of keratoconus progression and to further investigate whether such a widely used instrument as a keratometer could be useful in the evaluation of progression. Patients with stage > 2 were excluded from this investigation, which could reduce the effects of disease severity, and thus enable the identification of a single detection limit for progression applicable to subjects with less advanced disease. We have recently published a study on the effects of disease severity on the repeatability of intraday measurements.[11] However, a statistically and clinically significant association was also found in this investigation for Kmax, which suggests the need for stratified detection limits based on disease severity. Kmax is currently the most commonly used parameter in the evaluation of progression and an increment in Kmax of 1.0 D is commonly used, as a single parameter or in combination with more parameters, to define progression regardless of disease severity.[1,2,12] The results of the present study suggest that progression could be detected at an increase in magnitude of 1.11 D in Kmax when using single measurements and at an increase of 0.82 D in Kmax when using the mean of replicates. Hence, the results are similar in magnitude to the common detection limit of 1.0 D. However, when considering disease severity, significant progression can be considered as true in increases of 0.67 D in subjects with Kmax < 49.0 D and at an increase in Kmax of 1.42 D in subjects with Kmax ≥ 49.0 D, when comparing single measurements. If the mean of 4 replicates is used, significant progression can be considered true in increases of 0.44 D in subjects with Kmax < 49.0 D and at an increase of 1.08 D in subjects with Kmax ≥ 49.0 D. Also, given the rather wide 95% CIs in the ≥49.0 D group, further stratification could be necessary in this group. The 95% CIs in the group with Kmax < 49.0 D were narrow. It can be concluded that an increase of 1.0 D in Kmax will be suboptimal in diagnosing progression in less pronounced cases of

TABLE 3. Descriptive Statistics and Interday Repeatability of Measurements for the Keratoconus Cohort and Healthy Controls

	Mean (SD)[a]	Min-Max[a]	Sw (95% CI)	CV%	Repeatability (95% CI)	ICC (95% CI)
K1 (D)						
PC						
Keratoconus	43.6 (1.8)	(40.6-47.2)	0.16 (0.11-0.20)	0.36	0.44 (0.32-0.56)	0.99 (0.98-1.00)
Controls	43.0 (1.3)	(40.9-45.6)	0.040 (0.029-0.051)	0.093	0.11 (0.080-0.14)	1.00 (1.00-1.00)
AK						
Keratoconus	44.4 (1.7)	(42.0-48.3)	0.12 (0.084-0.15)	0.26	0.32 (0.23-0.41)	1.00 (0.99-1.00)
Controls	43.3 (1.3)	(40.8-46.0)	0.072 (0.052-0.092)	0.17	0.20 (0.14-0.25)	1.00 (0.99-1.00)
K2 (D)						
PC						
Keratoconus	46.0 (2.8)	(42.8-55.8)	0.26 (0.19-0.33)	0.57	0.72 (0.52-0.92)	0.99 (0.98-1.00)
Controls	43.9 (1.4)	(41.4-46.6)	0.063 (0.045-0.080)	0.14	0.17 (0.13-0.22)	1.00 (1.00-1.00)
AK						
Keratoconus	46.6 (2.7)	(43.5-55.6)	0.33 (0.24-0.43)	0.72	0.93 (0.67-1.18)	0.98 (0.97-0.99)
Controls	44.1 (1.4)	(41.3-46.7)	0.15 (0.11-0.20)	0.35	0.43 (0.31-0.54)	0.99 (0.97-0.99)
Kmax (D)						
PC						
All						
Keratoconus	50.3 (4.8)	(44.4-65.1)	0.30 (0.22-0.39)	0.57[b]	0.84 (0.61-1.07)	1.00 (0.99-1.00)
<49						
Keratoconus	46.7 (1.5)	(44.4-48.6)	0.16 (0.10-0.22)	0.34	0.44 (0.27-0.62)	0.99 (0.96-1.00)
≥49						
Keratoconus	53.6 (4.3)	(49.1-65.1)	0.39 (0.24-0.54)	0.73	1.08 (0.67-1.50)	0.99 (0.97-1.00)
Controls	44.3 (1.5)	(41.7-47.4)	0.072 (0.052-0.092)	0.16	0.20 (0.15-0.26)	1.00 (0.99-1.00)
MCT (μm)						
PC						
Keratoconus	493.0 (35.1)	(442.3-560.8)	2.92 (2.11-3.73)	0.63[b]	8.11 (5.86-10.4)	0.99 (0.98-1.00)
Controls	538.8 (23.4)	(497.0-582.6)	2.40 (1.74-3.07)	0.45	6.66 (4.81-8.51)	0.99 (0.98-1.00)
Rmin (mm)						
PC						
Keratoconus	5.1 (0.63)	(3.9-6.1)	0.076 (0.055-0.10)	1.49	0.21 (0.15-0.27)	0.99 (0.97-0.99)
Controls	6.1 (0.24)	(5.6-6.6)	0.023 (0.016-0.029)	0.37	0.063 (0.045-0.080)	0.99 (0.98-1.00)

AK = Nidek ARK 560-A auto-keratometer; D = diopters; K1 = flattest central keratometry value; K2 = steepest central keratometry value; Kmax = maximum keratometry value; MCT = minimum corneal thickness; PC = Pentacam HR; Rmin = minimum posterior corneal radius.

[a]Subject mean.

[b]Calculated using the natural logarithm transformation.

keratoconus, leading to delayed referral for CXL. On the other hand, more severe keratoconus could be erroneously diagnosed as progressive keratoconus, and subjects could be subjected to unnecessary treatment and the associated side effects. This investigation focused on repeatability in subjects with mild-to-moderate keratoconus, who can benefit the most from CXL, as their baseline vision is still preserved. However, owing to exclusion of severe keratoconus eyes, the results of this study cannot be applied to that patient population.

Rmin showed the poorest interday repeatability of all the parameters studied. This parameter is not commonly used per se, but it is included in the *ABCD progression display* in the Pentacam for the detection of progression.[19] In the *ABCD progression display*, Rmin is obtained from a 3-mm-diameter zone under the thinnest point of the cornea, and the repeatability of measurements could thus differ from those found in this study. Further comparisons are thus inappropriate. However, given the poor interday repeatability found in this study, and the fact that the keratoconus reference data in the ABCD classification are based on intraday repeatability, it would be of interest to evaluate the interday repeatability of the *ABCD progression display* parameters.

Apart from assessing the magnitude of interday repeatability, it is interesting to consider the dynamics of the intraday repeatability on day 0 and day 3 in subjects with KC. The repeatability of the measurement of Rmin with the Pentacam deteriorated from day 0 to day 3, while the repeatability of intraday measurements of MCT remained stable, and the intraday repeatability of measurements of K1, K2, and Kmax showed some degree of improvement

between day 0 and day 3. This effect was most evident for Kmax, although the 95% CIs overlapped. An association between astigmatism and variability in the measurement of topographic parameters in keratoconus has been reported previously.[20] It could thus be interesting to investigate whether increased patient experience of such measurements improves the repeatability in subjects with keratoconus. No difference was seen between the intraday repeatability of measurements on day 0 and day 3 in healthy controls, which could support this observation.

There is no consensus regarding whether single measurements or the mean of replicates should be used when clinically assessing progressive keratoconus over time. It is also uncommon for scientific investigations to specify whether single or replicate measurements have been used. The magnitude of the difference between 2 future single measurements at which progression can be detected will be higher than when comparing the mean of replicate measurements. However, it is important to avoid erroneously narrow PL, which would result if only 2 measurements were used in the calculations. The statistical analysis of PL used in this study included the variance of the replicate measurements. In this way, the results approached the true PL for single measurements.[16]

A possible weakness of this investigation is that the optimal time interval for the assessment of interday repeatability is not known. We chose 3 days, as we deemed this would be sufficiently long to allow interday changes but short enough to avoid a true progression that would affect the calculations of repeatability. Furthermore, although progression is evaluated over time, investigations on the interday repeatability are rare, and methodological differences make comparisons difficult.[8,20] Another consideration is the optimal number of replicates. Increasing the number of replicates could narrow the 95% CIs and, as suspected in this investigation, there could be a positive learning effect. On the other hand, too many measurements will take more time, and the patient's attention span may be exceeded. It is of note that we did not adjust or control for diurnal variation in tomographic indices. Yet, we hypothesize that these variations are minimal and do not substantially affect the results presented in this study.

Males predominated in the keratoconus cohort in this investigation, as in our previous study.[11] However, the results are applicable regardless of sex. The purpose of this investigation was to evaluate single parameters and optimize their use in clinical practice. As more than 1 parameter is commonly used in the assessment of progression, it may seem rational to combine several parameters into one. However, covariation between parameters cannot be excluded, which would lead to unreliable results, so this approach was not employed in the current investigation. We believe it is advisable to analyze several parameters individually when determining progression in keratoconus.

In summary, a better definition of progression in keratoconus is important in both clinical practice and scientific investigations. We believe the results of the present study can be considered in clinical practice. Future, large-scale investigations are, however, required to further include the effects of disease severity and interday variation. The results of such investigations may also contribute to the development of machine-learning tools.

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Paper III

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An inter-day assessment of the ABC parameters in the evaluation of progressive keratoconus

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The progression of keratoconus is commonly determined by comparing the results of corneal tomographic measurements on different occasions. However, investigations on the repeatability of measurements are commonly performed within the same day, thus not taking the inter-day variation into account. The effect of keratoconus disease severity on the measurement error is also seldom considered. In this post hoc investigation, the parameters A, B and C in the Belin ABCD Progression Display were evaluated in relation to disease severity in intra-day and inter-day measurements. Four consecutive measurements were performed on 61 patients with keratoconus on the same day (intra-day). In another cohort, four consecutive measurements were obtained and then repeated 3 days later in 25 patients with keratoconus and 25 healthy controls (inter-day). The results suggest that the diagnosis of disease progression would benefit from inter-day measurements, and the stratification of the parameters A and C according to disease severity. It is also recommended that tomographic systems such as the Pentacam HR be modified to allow the comparison of both single measurements and the mean of replicate measurements of the parameters used in the assessment of progression of keratoconus.

Keratoconus is the most common form of primary ectasia, and in cases of progressive disease, timely corneal crosslinking (CXL) can prevent further progression[1–3]. Although CXL was introduced in 2003[4], the scientific evidence of its efficacy in halting continued progression was deemed to be of very low quality in a Cochrane Review in 2015[5]. The lack of robust evidence-based results also appears to have contributed to the seemingly late approval of CXL for the treatment of progressive keratoconus by the US FDA[6]. A serious drawback in scientific investigations on the effect of CXL in treating progressive keratoconus is that there is no consensus on the definition of progressive keratoconus nor adequate means of assessing treatment efficacy (i.e. treatment outcomes)[7], which would facilitate the meta-analysis of data and accelerate the implementation of evidence-based treatment protocols.

The Belin ABCD Progression Display was recently developed with the aim of improving the diagnosis of progressive keratoconus, and is incorporated in the Pentacam HR tomography system[8]. The ABCD progression display assesses the anterior corneal curvature (A), the posterior corneal curvature (B) and corneal pachymetry at the thinnest point (C) in a 3 mm zone centred on the thinnest point. The visual acuity (D) can be added manually. The change in each of these parameters can be used to detect progression by making measurements over time. The software illustrates the changes graphically and calculates whether the change in the magnitude of a parameter (apart from D) exceeds the 80% or 95% one-tailed confidence interval, based on a reference population of subjects with keratoconus or healthy individuals. The latter population is suggested to be more representative of milder cases of keratoconus. It has been proposed that the ABCD progression display could be used to detect progression earlier than the commonly used maximum keratometry reading, K_{max}[9].

We have previously demonstrated an association between measurement error and the severity of keratoconus in measurements made with the Pentacam HR[10]. In that study, we suggested the stratification of detection limits for different parameters according to disease severity. This has also recently been proposed by other authors[11]. In a more recent study, we presented the inter-day repeatability of measurements in subjects with keratoconus and in healthy controls, using the Pentacam HR[12]. Apart from considering the effects on inter-day repeatability, the significant effects of disease severity were also elucidated. Furthermore, the difference between using single measurements and the mean of replicate measurements when assessing progression between visits was

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	Mean (SD)[a]	(Min–Max)[a]	S_w (95% CI)	RC (95% CI)	ICC (95% CI)	CoV (%)	Kendall's tau-b[b]	P[b] (2-tailed)	Spearman's rho[b]	P[b] (1-tailed)
(n = 61)										
A (mm)	7.02 (0.56)	(5.14–8.10)	0.067 (0.060–0.074)	0.18 (0.17–0.20)	0.99 (0.98–0.99)	0.95	−0.162	0.07	−0.246	0.03
B (mm)	5.37 (0.58)	(3.56–6.49)	0.087 (0.078–0.095)	0.24 (0.22–0.26)	0.98 (0.97–0.99)	1.61	−0.031	0.73	−0.035	0.39
C (µm)	485.2 (40.5)	(394.5–574.5)	5.47 (4.91–6.03)	15.2 (13.6–16.7)	0.98 (0.97–0.99)	1.13	0.174	0.05	0.265	0.02

Table 1. Descriptive statistics and repeatability of Pentacam measurements made on a single day in subjects with keratoconus. A = Anterior curvature of the 3 mm zone over the thinnest point (mm). B = Posterior curvature of the 3 mm zone under the thinnest point (mm). C = Thickness of the thinnest point on the cornea (µm). [a]Subject mean. [b]Subject SD versus subject mean.

investigated. As the ABCD Progression Display is integrated into the Pentacam HR, which is the most commonly used tomographic instrument in the management of keratoconus[5], it can be assumed that this software is widely used in both clinical practice and scientific investigations. It is therefore important to evaluate whether our previous findings are also relevant for the ABC parameters. It is of particular interest to analyse the inter-day effect on the ABC parameters, as these are based on intra-day measurements. Values of the ABC parameters obtained in our previous investigations were therefore analysed.

Definitions and abbreviations.

- Within-subject standard deviation (S_w). The square root of the variance between subjects.
- Precision = $1.96 \times S_w$. The difference between a measurement and the true value should lie below this limit in 95% of the measurements.
- Repeatability coefficient (RC) = $2.77 \times S_w$. The difference between two measurements should lie below this limit in 95% of the pairs of observations.
- Coefficient of variation (CoV). S_w divided by the total subject mean.
- Intra-class correlation coefficient (ICC). The variance between subjects divided by [the variance between subjects + the variance within subjects].
- Prediction limit (PL) = 95% CI for differences between two future single measurements.
- A: Anterior curvature of the 3 mm zone over the thinnest point of the cornea.
- B: Posterior curvature of the 3 mm zone under the thinnest point of the cornea.
- C: Thickness at the thinnest point of the cornea (µm).

Results

The ICC showed high values for all the measured parameters in all intra and inter-day measurements in all the groups. Therefore, variability could be interpreted as resulting from differences between subjects rather than within subjects (Tables 1, 2 and 3).

Repeatability and disease severity. A correlation between the magnitude of a measured parameter and its SD indicates a worsening of the repeatability of the measurements with increasing parameter magnitude. Disease severity was found to be significantly associated with measurement error for the parameters A and C, but not for B (the correlation for the parameter A was not significant in the group n = 61 for two-tailed CIs but was significant for 1-tailed CIs). This correlation was more pronounced in inter-day measurements. One-tailed 95% CIs showed a stronger association than two-tailed 95% CIs (Table 3). The strongest association was seen in inter-day measurements of A in subjects with keratoconus (Spearman's rho = −0.481, p = 0.007, Kendall's Tau-b = −0.377, p = 0.009), followed by measurements of C in the same group (Spearman's rho = −0.480, p = 0.008, Kendall's Tau-b = −0.350, p = 0.02), and in C in the intra-day measurements in subjects with keratoconus (Spearman's rho = −0.265, p = 0.02, Kendall's Tau-b = 0.174, p = 0.05) and in C in the same group (Spearman's rho = −0.265, p = 0.02, Kendall's Tau-b = 0.174, p = 0.05). Nevertheless, in intra-day measurements of A in subjects with keratoconus the correlation was close to being significant (Spearman's rho = −0.246, p = 0.03, Kendall's Tau-b = −0.162, p = 0.07) (Tables 1 and 3). No significant association was found in measurements of B in subjects with keratoconus (Tables 1, 2 and 3). Neither was any significant association found between the repeatability and magnitude in the inter-day measurements of any of the parameters in the control group (Table 3). No significant association was found in intra-day measurements for all the parameters in both subjects with keratoconus and the control group with the exception of parameter A in day 0 and 3 in subjects with keratoconus (Spearman's rho = −0.396, p = 0.025, Kendall's Tau-b = −0.284, p = 0.047 and Spearman's rho = −0.387, p = 0.028, Kendall's Tau-b = −0.264, p = 0.065) and parameter B in day 0 in the control group (Spearman's rho = 0.34, p = 0.048, Kendall's Tau-b = 0.230, p = 0.107) (Table 2). Figure 1 illustrates the mean values for each parameter for inter-day and intra-day measurements in subjects with keratoconus and the healthy control group.

Intra-day repeatability of measurements. In intra-day measurements in subjects with keratoconus, the best repeatability was found for parameter A, followed by C and B (Table 1) and the same happened when

	Mean (SD)[a]	(Min–Max)[a]	S_w (95% CI)	RC (95% CI)	ICC (95% CI)	CoV (%)	Kendall's Tau-b[b]	pb (2-tailed)	Spearman's rhob	pb (1-tailed)
Intra-day measurements										
Keratoconus patients (n = 25)										
A (mm)										
Day 0	7.18 (0.48)	(6.34–7.99)	0.054 (0.045–0.063)	0.15 (0.13–0.17)	0.99 (0.98–0.99)	0.78	−0.284	0.047	−0.396	0.025
Day 3	7.17 (0.49)	(6.34–7.90)	0.045 (0.037–0.052)	0.12 (0.10–0.14)	0.99 (0.98–1.00)	0.62	−0.264	0.065	−0.387	0.028
B (mm)										
Day 0	5.53 (0.51)	(4.68–6.45)	0.049 (0.041–0.056)	0.13 (0.11–0.16)	0.99 (0.98–1.00)	0.88	−0.030	0.833	−0.070	0.370
Day 3	5.52 (0.52)	(4.58–6.43)	0.054 (0.046–0.063)	0.15 (0.13–0.18)	0.99 (0.98–0.99)	0.99	−0.193	0.176	−0.312	0.065
C (µm)										
Day 0	492.6 (35.0)	(442.8–560.3)	3.85 (3.23–4.46)	10.7 (8.96–12.4)	0.99 (0.98–0.99)	0.78	−0.087	0.543	−0.116	0.290
Day 3	492.8 (35.3)	(437.5–561.3)	3.84 (3.23–4.46)	10.6 (8.94–12.4)	0.99 (0.98–0.99)	0.78	−0.044	0.761	−0.029	0.446
Healthy controls (n = 25)										
A (mm)										
Day 0	7.77 (0.23)	(7.34–8.20)	0.012 (0.010–0.013)	0.032 (0.027–0.037)	1.00 (1.00–1.00)	0.15	0.024	0.864	0.18	0.466
Day 3	7.77 (0.23)	(7.34–8.23)	0.013 (0.011–0.015)	0.035 (0.030–0.041)	1.00 (0.99–1.00)	0.16	−0.78	0.590	−0.076	0.358
B (mm)										
Day 0	6.31 (0.19)	(5.83–6.64)	0.044 (0.037–0.051)	0.12 (0.10–0.14)	0.95 (0.91–0.98)	0.69	0.230	0.107	0.34	0.048
Day 3	6.31 (0.20)	(5.83–6.66)	0.026 (0.022–0.030)	0.073 (0.061–0.084)	0.98 (0.97–0.99)	0.42	−0.114	0.427	−0.141	0.251
C (µm)										
Day 0	538.2 (23.0)	(493.0–580.3)	3.94 (3.31–4.57)	10.9 (9.17–12.7)	0.97 (0.95–0.99)	0.73	−0.179	0.215	−0.243	0.120
Day 3	539.4 (23.8)	(501.0–585.0)	4.13 (3.46–4.79)	11.4 (9.60–13.3)	0.97 (0.95–0.99)	0.76	−0.120	0.400	−0.212	0.154

Table 2. Descriptive statistics and repeatability of intra-day Pentacam measurements in subjects with keratoconus and healthy controls. A = Anterior curvature of the 3 mm zone over the thinnest point (mm). B = Posterior curvature of the 3 mm zone under the thinnest point (mm). C = Thickness of the thinnest point on the cornea (µm). [a]Subject mean. [b]Subject SD versus subject mean.

the repeatability in the intra-day measurements in day 0 and day 3 were evaluated in subjects with keratoconus (Table 2). In the control group, the repeatability in the intra-day measurements in day 0 and day 3 was clearly superior to the measurements in subjects with keratoconus (Table 2).

Inter-day repeatability of measurements using a mean of replicates. The repeatability of inter-day measurements of the parameters A, B and C was better in the control group than in subjects with keratoconus (Table 3). It was a factor of 4 worse for A, a factor of 2 worse for B, and a factor or 1.2 worse for C in subjects with keratoconus.

The best repeatability in the inter-day measurements was seen in the control group for parameter A (RC = 0.033 mm, 95% CI 0.024–0.042 mm, CoV 0.15%), followed by B (RC = 0.056 mm, 95% CI 0.041–0.072 mm, CoV 0.32%) and C (RC = 6.47 µm, 95% CI 4.68–8.27 µm, CoV 0.43%). In subjects with keratoconus, the repeatability in the inter-day measurements was best for parameter C (RC = 8.17 µm, 95% CI 5.91–10.4 µm, CoV 0.60%), followed by A (RC = 0.13 mm, 95% CI 0.092–0.16 mm, CoV 0.64%) and B (RC = 0.12 mm, 95% CI 0.088–0.16 mm, CoV 0.79%). When stratifying parameter A, subjects with keratoconus with a value below the median value for that parameter (7.33 mm) showed a repeatability about 2 times better that those with a value above the median (RC = 0.017 mm, 95% CI 0.10–0.23 mm vs. RC = 0.007 mm, 95% CI 0.040–0.0943 mm). Repeatability was also approximately two times better when stratifying parameter C for subjects with keratoconus with a value of that parameter above the median value (482.5 µm) than for those with a value below the median (RC = 5.75 mm, 95% CI 3.54–7.96 mm vs. RC = 10.2 mm, 95% CI 6.10–14.2 mm) (Table 3).

Inter-day repeatability of measurements using single measurements (PLs). The PLs for single inter-day measurements in subjects with keratoconus were − 0.19 to 0.17 mm for parameter A, − 0.19 to 0.16 mm for B and − 12.5 to 12.9 µm for C. In the control group, the PLs for single inter-day measurements were − 0.04 to 0.05 mm for A, − 0.10 to 0.11 mm for B and − 10.6 to 12.9 µm for C (Table 4). When stratifying the parameters A and C according to the median value, the PLs for single inter-day measurements in subjects with keratoconus were − 0.25 to 0.22 mm for values of A below the median value, and − 0.11 to 0.081 mm for values above the

	Mean (SD)[a]	(Min–Max)[a]	S_w (95% CI)	RC (95% CI)	ICC (95% CI)	CoV (%)	Kendall's Tau-b[b]	pb (2-tailed)	Spearman's rhob	pb (1-tailed)
Inter-day measurements										
Keratoconus patients (n = 25)										
A (mm)										
All	7.17 (0.48)	(6.34–7.95)	0.046 (0.033–0.059)	0.13 (0.092–0.16)	0.99 (0.98–1.00)	0.64	− 0.377	0.009	− 0.481	0.007
< 7.33	6.81 (0.36)	(6.34–7.33)	0.060 (0.037–0.082)	0.17 (0.10–0.23)	0.97 (0.92–0.99)					
≥ 7.33	7.57 (0.20)	(7.34–7.95)	0.024 (0.015–0.034)	0.07 (0.040–0.094)	0.98 (0.95–1.00)					
B (mm)	5.52 (0.51)	(4.63–6.44)	0.044 (0.032–0.056)	0.12 (0.088–0.16)	0.99 (0.98–1.00)	0.79	0.113	0.427	0.132	0.265
C (µm)										
All	492.7 (35.1)	(442.3–560.8)	2.95 (2.13–3.77)	8.17 (5.91–10.4)	0.99 (0.98–1.00)	0.60	− 0.350	0.016	− 0.480	0.008
< 482.5	463.9 (12.9)	(442.3–482.4)	3.67 (2.20–5.13)	10.2 (6.10–14.2)	0.92 (0.76–0.98)					
≥ 482.5	519.3 (26.8)	(482.5–560.8)	2.07 (1.28–2.87)	5.75 (3.54–7.96)	0.99 (0.98–1.00)					
Healthy controls (n = 25)										
A (mm)	7.77 (0.23)	(7.34–8.21)	0.012 (0.009–0.015)	0.033 (0.024–0.042)	1.00 (0.99–1.00)	0.15	− 0.069	0.638	− 0.104	0.311
B (mm)	6.31 (0.20)	(5.83–6.65)	0.020 (0.015–0.026)	0.056 (0.041–0.072)	0.99 (0.98–1.00)	0.32	0.189	0.190	0.260	0.105
C (µm)	538.8 (23.4)	(497.0–582.6)	2.34 (1.69–2.98)	6.47 (4.68–8.27)	0.99 (0.98–1.00)	0.43	0.171	0.240	0.209	0.158

Table 3. Descriptive statistics and repeatability of inter-day Pentacam measurements in subjects with keratoconus and healthy controls (mean of replicates). A = Anterior curvature of the 3 mm zone over the thinnest point (mm). B = Posterior curvature of the 3 mm zone under the thinnest point (mm). C = Thickness of the thinnest point on the cornea (µm). [a]Subject mean. [b]Subject SD versus subject mean.

median value, − 15.4 to 14.0 µm for values of C below the median value and − 9.51 to 11.5 µm for values above the median value (Table 4).

Inter-day progression. In a randomized comparison between two measurements in each subject with keratoconus, six subjects (24%) showed progression according to one parameter (in three of these subjects the parameter A indicated progression, while in two subjects the parameter B suggested progression), and in one subject both parameters A and B indicated progression. In a second randomized comparison among the subjects with keratoconus, progression was indicated by one parameter in two of the subjects (8%). In one of these subjects parameter A indicated progression, while in the other B suggested progression. Two parameters (A and B) indicated progression in three of the subjects (12%), and all three parameters suggested progression in one of the subjects (4.0%).

Discussion

The results of this study demonstrate the statistically significant association between disease severity and measurement error in the parameters A and C, but not B, in the Belin ABCD progression display. This association was more pronounced in inter-day measurements than in intra-day measurements. One-tailed 95% CIs also showed a stronger association with disease severity than two-tailed 95% CIs. These findings suggest progression should be diagnosed based on limits stratified according to disease severity for the parameters A and C. There appears to be a threshold at 7.0 mm for A, i.e. approximately 48 D, at which the measurement error begins to increase. This threshold appears to be equivalent to that for K_{max}, which is not surprising as they are based on the same measurements[12]. The association between measurement error and disease severity was statistically significant for both A (Kendall's Tau-b = − 0.377, p = 0.009) and K_{max} (Kendall's Tau-b = 0.483, p = 0.0001), although the association for A was somewhat weaker. As a lower value of A indicates greater disease severity, Kendall's Tau-b is negative, whereas a lower value of K_{max} indicates less severe disease. The threshold for C is at approximately 500 µm, below which measurements are more prone to error. It was also reported in a recent study that the repeatability of measurements of A, B and C deteriorated with increasing disease severity[11]. However, those calculations were based on intra-day measurements, and the association between deteriorating repeatability and disease severity was not investigated per se. An inter-day scenario is more appropriate as this reflects the clinical situation. Factors such as changes in the shape of the cornea due to diurnal variation or the natural biomechanical weakness of corneas affected by keratoconus could lead also to deterioration in the repeatability of inter-day measurements. However, other factors may improve the repeatability of measurements, such as learning effects among the patients. No association was seen between the measurement error and the magnitude of the measured parameters among healthy controls, and the repeatability of these measurements was clearly superior to those obtained in patients with keratoconus, in particular regarding the parameters A and B.

Progression can be assessed in the ABCD progression display by comparing the results with the 80% or 95% CIs obtained from a reference cohort of patients with keratoconus, or from a reference cohort of healthy

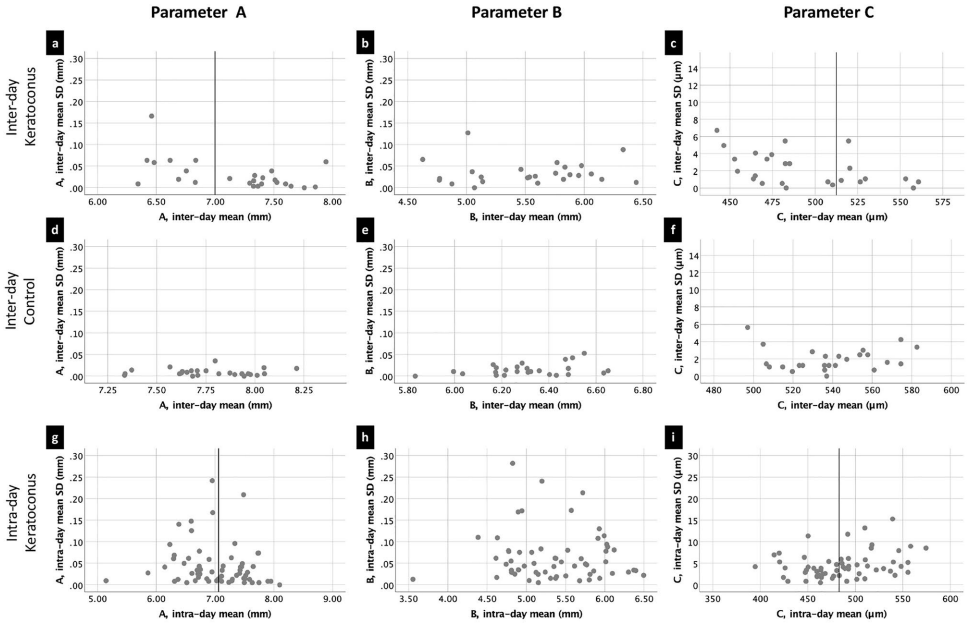

Figure 1. Mean values of inter-day and intra-day measurements of the parameters A, B and C, plotted against the standard deviation. (**a**) Inter-day measurements of parameter A in subjects with keratoconus, (**d**) inter-day measurements of A in control patients, (**b**) inter-day measurements of B in subjects with keratoconus, (**e**) inter-day measurements of B in the control group, (**c**) inter-day measurements of C in subjects with keratoconus and (**f**) inter-day measurements of C in the control group. Lower values of parameters A and B indicate more severe keratoconus, while lower values of C indicate less severe disease. The vertical lines indicate the median.

	Variance components			Mean difference	Lower prediction limit	Upper prediction limit
	$\widehat{\tau}^2$	$\widehat{\sigma}_1^2$	$\widehat{\sigma}_2^2$	$\widehat{\alpha}_1 - \widehat{\alpha}_2$	$\widehat{\alpha}_1 - \widehat{\alpha}_2 - 2 \times \sqrt{2\widehat{\tau}^2 + \widehat{\sigma}_1^2 + \widehat{\sigma}_2^2}$	$\widehat{\alpha}_1 - \widehat{\alpha}_2 + 2 \times \sqrt{2\widehat{\tau}^2 + \widehat{\sigma}_1^2 + \widehat{\sigma}_2^2}$
Subjects with keratoconus						
A (mm)	0.0015	0.0020	0.0029	−0.013	−0.19	0.17
<7.33	0.0027	0.0035	0.0046	−0.013	−0.25	0.22
≥7.33	0.00039	0.00035	0.0011	−0.012	−0.11	0.081
B (mm)	0.0011	0.0030	0.0024	−0.019	−0.19	0.16
C (µm)	5.34	14.8	14.8	0.18	−12.5	12.9
<482.5	10.2	17.7	16.1	−0.69	−15.4	14.0
≥482.5	0.94	12.0	13.6	0.98	−9.51	11.5
Healthy controls						
A (mm)	0.0001	0.0002	0.0001	0.003	−0.04	0.05
B (mm)	0.0001	0.0007	0.0019	0.003	−0.10	0.11
C (µm)	0.90	17.02	15.51	1.17	−10.6	12.9

Table 4. Inter-day differences between single measurements of the parameters A, B and C with prediction limits for subjects with keratoconus and healthy controls (single measurements). A = Anterior curvature of the 3 mm zone over the thinnest point (mm). B = Posterior curvature of the 3 mm zone under the thinnest point (mm). C = Thickness at the thinnest point on the cornea (µm). $\widehat{\tau}^2$ = squared between-subject mean variance between Day 0 and Day 3; $\widehat{\sigma}_1^2$ = squared within-subject mean variance on Day 0; $\widehat{\sigma}_2^2$ = squared within-subject mean variance on Day 3;$\widehat{\alpha}_1 - \widehat{\alpha}_2$ = difference between means on Day 0 and Day 3.

subjects. The latter could be appropriate in subjects with less severe keratoconus, as the repeatability of these measurements will probably be more similar to those in a healthy cohort than a general cohort of patients with all stages of disease. In fact, in the abovementioned study[11] the repeatability of measurements of A, B and C was reported to be identical in healthy subjects and in subjects with subclinical keratoconus. However, there will be a threshold at which some subjects with keratoconus will be over-diagnosed as progressive if compared to a healthy cohort. If stratified limits were implemented in the detection of progression in keratoconus, there would be no need for a comparison with a healthy cohort.

As well as considering the effects of disease severity, the thresholds at which progression could be detected were evaluated assuming two clinical scenarios: using one measurement on each occasion, and using the mean of replicate measurements (in this case the mean of four). This has been addressed in a few studies[12–14] but it is seldom considered in the enrolment of subjects in clinical studies on CXL, and there is no software in the Pentacam HR allowing for the comparison of mean values. In order to avoid unnecessarily narrow and erroneous prediction limits for single measurements, the variance between the four replicates was included in the statistical analysis[15]. This provided more accurate results and reduced the risk of over-interpreting the results as indicating progression. However, and as expected, it can be concluded that comparing the mean values obtained on each occasion further improves the ability to detect progression, and it is therefore recommended that appropriate software be developed for this purpose.

The ABCD progression display is based on one-tailed 80% and 95% CIs. On the one hand, one-tailed intervals seem logical, as only a decrease in the magnitude of the parameter indicates progression; but on the other, the parameters can increase or decrease, which suggests that two-tailed intervals are more appropriate. Two-tailed 95% CIs were used in this study, and 80% CIs were avoided. The 95% CIs of the non-stratified repeatability of measurements of the parameters A and B in this study were wider than those used in the ABCD progression display, suggesting that there is a risk of over-interpreting the results as indicating progression. Empirically, the proportion of false positive results in the inter-day scenario was 24% (n = 6), for one or more parameters. When this analysis was repeated the same results were obtained. This empirical analysis describes a one-to-one measurement scenario and the false positive results are explained by the fact that the 95% prediction limits (reflecting a one-to-one measurement scenario) are wider than the 95% CIs in the Belin ABCD progression display, in particular for parameters A and B. It is important to note that only subjects with Stage 1–2 AKC (Amsler Krumeich Classification System) were included in this inter-day analysis. If subjects with Stage 3 disease had also been included, this would most likely have increased the proportion of false positive results due to the association between measurement error and disease magnitude. However, if the means of replicates were compared between days this would, as expected, reduce the number of false positive progressions. Unfortunately, this feature is not available in the Pentacam HR and could thus not be tested empirically.

When stratifying the parameters A and C above/below the median value, those with more advanced disease showed an approximately two times poorer repeatability for both the single measurements and the mean of replicates than those with less advanced disease. If comparing the limit in the ABCD Progression Display with the results for subjects with more advanced disease (bearing in mind that the whole cohort consisted of subjects with less advanced keratoconus) there would have been a further shift towards false positive results. However, in the group with the lower disease severity, the repeatability was close to the limit in the ABCD Progression Display for the scenario involving single measurements. If, on the other hand, the mean of replicate measurements is used, the 95% CIs of the repeatability of measurements of parameters A and C are below the limit in the ABCD Progression Display, leading to the risk of false negative results. In this case, it appears reasonable to compare this group with the suggested limits for a normal population in the Belin ABCD Progression Display. While the limits for parameter C are rather similar, the repeatability of the measurements of parameter A is still three times higher in the below-median group of keratoconus than in the normal population in the ABCD Progression Display, highlighting the difference in the repeatability between healthy subjects and subjects with keratoconus. The subjects included in the below-median group had K_{max} values ranging from 44.8 to 48.6 D. The repeatability of the measurements in the healthy controls in this investigation was similar to that presented in the Belin ABCD Progression Display.

A possible weakness of this study is that the optimal time frame for comparing inter-day repeatability is unclear. We chose three days as we deemed this to be sufficient to allow for inter-day changes in corneal shape, but sufficiently short to avoid true disease progression. Males were overrepresented in the keratoconus groups, reflecting the gender difference in patients with keratoconus at our clinic[10], and the healthy controls were not matched for sex or age. We believe that diurnal variation would not affect the measurements significantly. The measurements were in general obtained between 09.00 a.m. and 15.00 p.m. It has been suggested previously that the corneal thickness is significantly reduced within the first 1–2 h after awakening but then remains relatively unchanged during the daytime[16,17]. In fact, the diurnal variation of keratometric and corneal thickness measurements in subjects with keratoconus has been suggested to be clinically insignificant[18] if obtained between 09.00 a.m. and 17.00 p.m. We therefore believe that the results in this investigation are applicable in a daytime setting.

There is no gold standard for measuring progress in keratoconus, and thus measurement accuracy is of paramount importance, in both clinical practice and scientific investigations. As mentioned in the introduction, there is no consensus on the definition of progression. However, a consensus on which parameters should be used may be less important than understanding the repeatability and the dynamics of the parameters used and designing the investigation accordingly. This would be an important step towards facilitating the meta-analysis of data. More specifically, the use of reference data in the Belin ABCD Progression Display based on inter-day measurements should be considered. The association between measurement error and disease severity should also be considered for parameters A and C as this would allow progression to be diagnosed earlier in patients with less severe disease, and help avoid erroneous diagnosis of progression in those with more advanced disease.

Furthermore, it is desirable that tomographic systems such as the Pentacam HR allow for the comparison of both single measurements and the mean of replicates for parameters used in the assessment of progression of keratoconus.

The findings of this investigation could be of interest for developers of software for the detection of progression in keratoconus, but may also be useful in clinical practice. The results of measurements of the A, B and C parameters are presented in the Progression Display and changes in the magnitude of the parameters between visits can be evaluated by comparing with the results of this investigation. However, clinicians would probably find it more practical to compare single measurements between visits as the mean of replicates would have to be calculated manually, as the current system does not allow for the comparison of mean values.

Subjects and methods

The studies were conducted at the Department of Ophthalmology at Skåne University Hospital, Lund, Sweden, according to the declaration of Helsinki. The Regional Ethics Committee in Lund, Sweden, approved the studies (No. 2015/373).

Enrolment. Patients with keratoconus fulfilling the inclusion criteria described below were enrolled consecutively after signing an informed consent form. The inclusion criteria were: keratoconus Stage ≤ 3 (Investigation 1)[10] and keratoconus Stage ≤ 2 (Investigation 2)[12] with no history of, and no current signs of, other ocular pathology, including ocular surface disease and external diseases such as dry eyes and atopy. Only subjects who had not undergone prior ocular surgery and who were aged ≥ 18 years were recruited and pregnant and breastfeeding women were also excluded[10,12]. Contact lens wear was discontinued at least 2 weeks before the measurements were made[10,12]. Subjects with advanced keratoconus (Stage 4) were excluded from Investigation 1[10] due to the presence of corneal scarring. In Investigation 2[12], patients with Stage 3–4 keratoconus were excluded as the purpose was to study subjects with less advanced disease. In both investigation 1[10] and 2[12] keratoconus was diagnosed clinically and by examination using The Pentacam HR. More specifically, the sagittal curvature pattern, posterior and anterior elevation maps, and corneal thickness pattern were assessed, in addition to information from the Belin-Ambrosio Enhanced Ectasia Display.

Sixty-one patients (Investigation 1)[10] and 25 patients (Investigation 2)[12] were enrolled. Only one eye was eligible for inclusion in 31 subjects in these investigations due to previous CXL, previous penetrating keratoplasty or too advanced stage of keratoconus. If two eyes were eligible for inclusion, both were examined (see "Examination" below). Computerised randomisation was performed in subjects where both eyes met the inclusion criteria to select one eye for inclusion in the study (41 right eyes and 45 left eyes). Seventy-six participants were males, and 10 females, and the mean age of all participants was 28 years (18–45 years).

Healthy controls (Investigation 2)[12] (n = 25) were enrolled from among medical students and residents in ophthalmology after signing an informed consent form. The inclusion criteria were: age ≥ 18 years, no history of any ocular pathology or previous ocular surgery. Pregnant and breastfeeding women were excluded. Ocular pathology was excluded by clinical examination and by examination using the Pentacam HR. Only one eye was eligible for inclusion in three patients, due to scarring of the cornea. If two eyes were eligible for inclusion, both were examined and computerized randomization was performed, as described above, resulting in 12 right eyes and 13 left eyes. Fourteen participants were males, and 11 females, and their mean age was 29 years (23–41 years).

Instruments. The Pentacam HR is a Scheimpflug-based tomographic system (Pentacam HR, version 1.20r10, Oculus Optikgeräte GmbH, Wetzlar, Germany). The technical features of this system have been described elsewhere[19]. The default setting of 25 pictures/s was used.

Examination. Measurements were made on a single day (Investigation 1)[10] and on two separate occasions (Investigation 2)[12] by the same examiner (IG). In the latter study, 4 consecutive measurements were made on Day 0, and four on Day 3. Subjects were instructed to blink between measurements, but not to lean back. Measurements were made during normal working hours without taking diurnal corneal variation into account. Only examinations deemed "OK" by the Pentacam were accepted. The right eye was examined first, then the left, if both eyes were eligible for inclusion. This represents normal clinical practice where both the patient's eyes are usually examined. When recruitment to the study was complete, computerised randomisation was performed to select one participating eye per subject.

Statistical methods and calculations. The values obtained from the four replicate measurements were used to calculate the repeatability in Investigation 1. The measurements obtained on Day 0 and Day 3 in Investigation 2 were averaged for each day, and used to calculate the inter-day repeatability in the clinical situation where the mean value of several measurements is used to assess progression. When calculating prediction limits in the clinical scenario where single measurements are used to assess progression, the variance between replicate measurements was included in the calculation to provide more accurate results.

IBM SPSS Statistics 22 for Windows (IBM Corporation, Armonk, NY, USA) and SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc., Cary, NC, USA) were used for statistical analyses. Results were considered statistically significant when the p-value was ≤ 0.05. Descriptive statistics are given as subject mean, standard deviation (SD), and minimum and maximum values. Repeatability was assessed by calculating the within-subject SD, precision, repeatability coefficient, intra-class correlation and coefficient of variation with associated confidence intervals (CIs)[20–22]. Kendall's Tau-b was used to assess the relationship between the mean and SD, and natural logarithm transformed data were analysed when appropriate. The limits of agreement (denoted prediction limits) were calculated including the variance of the replicates using a linear mixed-effect model[15].

In the empirical analysis of progression, the four measurements in the inter-day data were randomised to define one measurement as the baseline (at Day 0), and the other as the follow-up measurement (at Day 3), for each subject. The procedure was repeated to confirm the results.

Data availability

All data are available as "Supplementary information".

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Author contributions

I.G., A.V., J.H., A.I. proposed the idea. I.G., A.V. and T.F. analysed data and drafted the manuscript. I.G., T.F., A.V. J.H., A.I., A.B. analysed data and revised the manuscript. I.G. made the measurements with the Pentacam HR. T.F. collected the data. J.H., A.I., A.B. supervised the project. All authors have read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Paper IV

ERRATUM

Paper IV

The sentence below contains an error.

“In the sterile water C riboflavin group (Figure 1), an increase in maximum K of 2.6 D was seen in 1 patient (identified as “46” in the figure).”

(identified as “46” in the figure) should read (identified as “34” in the figure).

However, this does not affect the outcome or interpretation of the results.

Retrospective analysis of the effects of using sterile water in addition to hypoosmolar riboflavin during corneal collagen crosslinking for keratoconus

The efficacy in halting progressive keratoconus by epithelium-off (epi-off) corneal collagen crosslinking (CXL) using isoosmolar riboflavin 0.1% and dextran 20.0% in combination with ultraviolet-A (UVA) irradiation (3 mW/cm2) for 30 minutes is well documented.[1,2] To avoid endothelial toxicity, the cornea should have a minimal deepithelialized thickness of 400 μm.[3,4] Unfortunately, because keratoconus is a thinning disorder, a group of patients that could benefit from CXL is thus excluded.

Hypoosmolar riboflavin, which swells the cornea, permits the treatment of thinner corneas. Positive indications of its clinical efficacy and safety have been reported,[5] but 1 failure in an extremely thin cornea has also been reported.[6] Despite the use of a hypoosmolar solution of riboflavin, the corneas of some patients are too thin. In these cases, we have used sterile water to swell the cornea.

PATIENTS AND METHODS

A retrospective study was performed at Skåne University Hospital. Patients with progressive keratoconus were treated with hypoosmolar riboflavin and sterile water or with hypoosmolar riboflavin alone during CXL. Progression was defined as an increase in the maximum keratometry (K) value equal to or greater than 1.0 diopter (D) in 12 months or equal to or greater than 0.5 D in 6 months.

Contact lens use was discontinued 2 weeks prior to topographic measurements or CXL. Scheimpflug corneal topography (Pentacam HR, Oculus Optikgeräte GmbH) was analyzed immediately before CXL and again at 1 year. The CXL was performed with the epi-off technique. Hypoosmolar riboflavin 0.1% (Mediocross H, Medio-Haus Medizinprodukte GmbH) was instilled every 3 minutes for 30 minutes, followed by UVA irradiation (3 mW/cm^2 UV-X 1000, IROC Innocross AG) and continuous riboflavin application. Corneal thickness was measured immediately before irradiation and repeatedly during irradiation using a pachymeter (SP-100, Tomey Corp.). Sterile water (0 Osmol/L) (Braun Melsungen AG) was added (1 drop/s for at least 1 minute) when treating corneas with a thickness equal to or less than 400 μm immediately prior to irradiation. The addition of sterile water was repeated if corneal thickness decreased to equal to or less than 400 μm during irradiation.

Statistical analysis and graphic presentation were obtained using SPSS software (version 23, International Business Machines Corp.). A P value less than .05 was considered significant. Median values are presented with interquartile range (IQR).

RESULTS

Twenty-eight patients (32 eyes) were in the group treated with sterile water and riboflavin (sterile water + riboflavin) (6 women, 22 men; mean age 25.8 years ± 5.3 [SD]). The baseline median maximum K was 54.9 D (IQR = 9.4 D), and the median thinnest point on the cornea was 451 μm (IQR = 42 μm) (range 388 to 537 μm).

Fourteen patients (17 eyes) were in the group treated with riboflavin alone (2 women, 12 men; mean age 24.9 ± 6.1 years). The baseline median maximum K was 55.7 D (IQR = 11.5 D), and the median thinnest point on the cornea was 465 μm (IQR = 59 μm) (range 374 to 516 μm). No significant differences were observed in the baseline characteristics of the patients.

At 1 year, the median maximum K was statistically significantly reduced in both groups. The change in maximum K was −0.85 D, IQR = 1.35 D (P < .001) in the sterile water + riboflavin group and −1.7 D, IQR = 3.25 D (P = .006) in the riboflavin alone group. No statistically significant difference was seen between the change in maximum K in the 2 groups (P = .065) (Figure 1). These data do not suggest noninferiority.

In the sterile water + riboflavin group (Figure 1), an increase in maximum K of 2.6 D was seen in 1 patient (identified as "46" in the figure). The patient had highly variable keratometric measurements at follow-up visits, and progression could not be confirmed later.

DISCUSSION

The strategy of using hypoosmolar riboflavin with sterile water appears to be clinically efficacious. Because of the prominent swelling effect of sterile water, we have rarely, if at all, had to exclude a patient from CXL because of a thin cornea.

Regarding safety, it has been suggested that the effect of UVA irradiation is increased at the endothelial level when hypoosmolar riboflavin is used because of the poor shielding effect of the hypoosmolar riboflavin solution.[7] One limitation of this study is the lack of endothelial cell analysis before and after CXL.

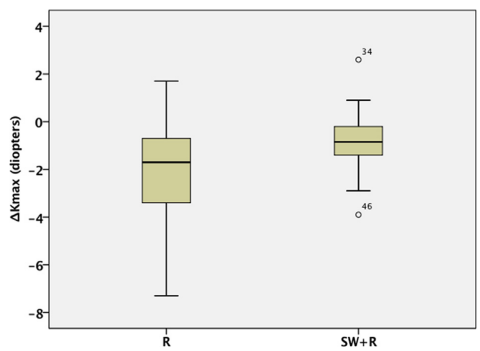

Figure 1. Change in maximum K 1 year after treatment in the 2 groups (sterile water + riboflavin and riboflavin alone) (ΔKmax = change in maximum K; R = riboflavin; SW = sterile water).

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Further prospective studies are needed to evaluate the safety and efficacy of this treatment modality. However, we conclude that the application of sterile water together with hypoosmolar riboflavin during CXL may allow the treatment of thin corneas with progressive keratoconus.

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