Diplomarbeit

Does corneal collagen cross-linking promote the new appearance of iris freckles and iris nevi?

eingereicht von

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zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitäts-Augenklinik

unter der Anleitung von

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Graz, 31.08.2020

Eidesstattliche Erklärung

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Acknowledgments

First, I would like to give special thanks to my first thesis advisor Priv.-Doz. DDr. Schwab for coming up with the idea for the study, for carefully supervising me all along the way, for conducting ophthalmologic study examinations (and explaining a multitude of aspects of what has to be looked out for), for instructing me on how to accurately identify iris freckles and nevi in the slit-lamp examination and in images, for supervising me during statistical analysis and for valuable advice during the writing process.

Likewise, I wish to express my gratitude to my second thesis advisor Dr. Glatz for carrying out study examinations providing important tips, for demonstrating and explaining the CXL procedure to me and for careful advice during the writing process.

Additionally, I thank Priv.-Doz. DDr. Steinwender for carrying out study examinations.

Also, big thanks to the Clinical Photography Team for creating high quality iris images for our analysis, and to the nurses and secretaries at the Department of Ophthalmology, with whom I have had the honor to work with during the study.

Furthermore, I would like to express my thanks to all participants who volunteered for our study.

Last, but certainly not least, huge thanks to my family, who tremendously supported me in my life and without whom my medical studies would have been impossible to achieve for all intents and purposes; and to my dear friends, who always stayed with me through thick and thin, showing me compassion and good sportsmanship.

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Abbreviations

a	annus, year
CCT	central corneal thickness
CXL	corneal collagen cross-linking
D	Diopter
Δt	time difference
LASIK	laser-assisted in situ keratomileusis
n	number of observations
PACK-CXL	Photo Activated Chromophore for Keratitis-CXL
PIPH	primary iris pigment epithelial hyperplasia
PMD	pellucid marginal corneal degeneration
UV	ultraviolet

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Abstract

Purpose

We aim to investigate, if corneal collagen cross-linking (CXL) is related to an increase in the number of iris freckles and nevi in treated eyes in comparison with untreated eyes.

Patients and Methods

We conducted a prospective observational, cross-sectional, single-center study. Volunteers with history of unilateral CXL had bilateral iris images taken. We determined iris pigmented lesion counts for each iris quadrant by image inspection and compared the results of post-CXL eyes versus non-CXL eyes. In additional analyses, we examined the possible effects of post-CXL time difference and participant age.

Results

Data of 20 participants were evaluated. No significant increase in iris pigmented lesion count was found between post-CXL eye to the non-CXL eye, except for the inferior nasal quadrant, though small in magnitude. No significant correlation between iris pigmented lesion count and CXL-to-examination time difference was detected. The number of iris pigmented lesions also did not significantly correlate with age.

Conclusions

In our study population, we detect no clear indication of CXL leading to an increase in the number of iris freckles and nevi.

Keywords

Iris freckle, iris nevus, corneal collagen cross-linking

Zusammenfassung

Ziele

Ziel unserer Studie ist es zu untersuchen, ob es einen Zusammenhang zwischen kornealem Kollagen-Cross-Linking (CXL) und der Entstehung von Iris Freckles und Iris Nävi gibt.

Patientinnen und Patienten und Methoden

In unserer prospektiven monozentrischen Querschnitt-Beobachtungsstudie haben wir bilaterale Irisfotos von freiwilligen Personen, die in der Vergangenheit ein unilaterales CXL erhalten hatten, angefertigt und auf die Anzahl ihrer Iris Freckles und Iris Nävi, unter Berücksichtigung ihrer topographischen Lage, untersucht. Wir haben die Ergebnisse von post-CXL-Auge und non-CXL-Auge miteinander verglichen. In weiteren Analysen haben wir einen möglichen Einfluss der post-CXL-Zeitdifferenz und des Alters der Studienteilnehmerinnen und Studienteilnehmer auf die Anzahl der Iris Freckles und Iris Nävi untersucht.

Ergebnisse

20 Personen wurden untersucht. Es konnte kein signifikanter Unterschied in der Anzahl der pigmentierten Irisläsionen zwischen post-CXL-Auge und non-CXL-Auge festgestellt werden, außer einer minimalen Erhöhung im inferior nasalen Quadranten. Weiters zeigte sich keine signifikante Korrelation zwischen CXL-zu-Untersuchung-Zeitdifferenz und Anzahl der pigmentierten Irisläsionen. Auch das Alter der Studienteilnehmerinnen und Studienteilnehmer korrelierte nicht signifikant mit der Anzahl der pigmentierten Irisläsionen.

Fazit

Wir können in unserer Studienpopulation keinen klaren Hinweis dafür finden, dass CXL mit der Entstehung von Iris Freckles und Iris Nävi zusammenhängt.

Schlüsselwörter

Iris Freckle, Iris Nävus, korneales Kollagen Cross-Linking

Prior publications

No part of the present manuscript has been published previously.

Funding

Our study was funded by the Medical University of Graz.

No author has any financial or proprietary interests in the mentioned materials or methods.

1 Introduction

1.1 Background (summary)

Benign iris pigmented lesions

Iris freckles are small flecks of brown pigment on the anterior iris surface, consisting of colonies of atypical melanocytes with large melanin granules. [1] Iris nevi are benign nodular melanocytic lesions, which replace the iris stroma. [1,2] The term '(benign) iris pigmented lesions' encompasses both iris freckles and nevi. [3]

Recent studies have explored the potential clinical significance of these lesions: The presence of iris freckles and nevi has been linked to an increased risk for cutaneous melanoma and an association between iris nevi and uveal melanoma has also been reported. [3,4]

Furthermore, measures of ultraviolet light exposure have been linked to the presence of iris pigmented lesions. [2,5,6] However, this relationship has not been consistently shown in all study populations [3]; and iris nevi alone do not seem to be significantly associated with sunlight exposure. [7]

Iris pigmented lesions do seem to occur more frequently with increasing age, possibly suggesting an accumulation of UV-related effects over time, or a potential threshold UV-level required for their formation. [2,3,5]

Additionally, genes which influence the formation of iris pigmented lesions have been identified (e.g. variants of HERC2/OCA2 and IRF4). [3]

However, more generally speaking, the factors influencing the formation of iris freckles and nevi are not fully clear yet and subject for ongoing research. [2,3,5]

Corneal collagen cross-linking

Corneal collagen cross-linking (CXL) is a procedure used in the management of corneal thinning disorders, such as keratoconus, pellucid marginal degeneration and keratectasia after laser in situ keratomileusis (LASIK). [8]

During CXL, UVA radiation and riboflavin are utilized to create additional covalent bonds in the corneal stroma's collagen, in order to increase its mechanical stability. [8,9] The light's wavelength used during CXL (370 nm) has been designed to correspond with an absorption maximum of riboflavin, which leads to a high proportion of the emitted light being absorbed within the corneal stroma. [8,9] Residual radiant exposure has been reported at 0.32 J/cm² at the corneal endothelium level and even lower for deeper structures. [8] This level of radiant exposure has been shown to cause no damage to the corneal endothelium, lens and retina. [8] To ensure this, safety precautions of a central corneal thickness (CCT) of at least 400 µm and adequately homogenous UVA irradiation are required during CXL. [8]

Though, it is currently not clearly known, if the UV-light applied during CXL could have any effects on the formation of iris freckles and nevi.

1.2 Background (extended)

Benign iris pigmented lesions

Definitions

The term ‘benign uveal pigmented lesions’ may include choroidal nevi, iris nevi, iris freckles and primary iris pigment epithelial hyperplasia (PIPH). [2,6,10] In our study, we examine benign iris pigmented lesions (i.e. iris freckles and iris nevi) only, due to the irradiation area used during CXL (i.e. a circle with a diameter of 9 mm aimed at the corneal center). [8]

Origin

The formation of benign iris pigmented lesions is thought to be influenced by a number of endogenous and exogenous factors, some of which will be mentioned in the following: [2,5]

Ultraviolet and blue light

UV irradiation is known to induce melanocyte proliferation and having immunosuppressive and immunomodulatory effects, which, in turn, may increase the risk of both nevus and melanoma development. [6]

Sunlight

Measurement of sunlight exposure has been noted to be extremely complex, because no clear definitions exist for types and levels of exposure. [11] In recent studies, questionnaires have been used to elucidate this topic. [2,5,6] Self-reported measures, which have been observed to be associated with presence of iris pigmented lesions are: higher number of lifetime sunburns, a history of severe sunburns with blisters, frequency of sunbathing and time spent outdoors during adulthood. [2,5,6] Consequently, it has been proposed, that iris freckles could indicate a high cumulative dose of lifetime sun exposure. [5]

Neonatal blue light phototherapy

Prevalence of benign pigmented ocular lesions has also been reported to be significantly associated with neonatal blue light phototherapy, which is used in the management of neonatal jaundice in order to prevent kernicterus. [2,6]

During neonatal blue light phototherapy, light with a wavelength of approximately 370-600 nm is used, with 0.3 % being UVA radiation. [6] The biologic effects of blue light and ultraviolet light have been noted to possibly be partly similar. [6]

Iris color

Iris color has been shown to influence the number of iris pigmented lesions, with fewer iris freckles found in dark irises compared to medium and light colored ones. [2,3,5,12] Possible explanations could be, either, that dark irises might be less photosensitive, or, that in very dark colored irises, iris freckles are more difficult to discern. [5]

Age

Higher age has been reported to be related to a higher number of iris pigmented lesions, which has been discussed to possibly indicate an accumulation of UV-related effects. [2,3,5]

Genetics

Genetic factors appear to have a major influence on melanocytic nevus development, and thus are an important focus of current research. [6] The genes, which affect development of iris pigmented lesions, have been noted to be distinct from the genes, which affect cutaneous pigmented lesions. [3] For example, variations of the HERC2/OCA2 gene and IRF4 gene (which are also known to affect eye color) have been shown to be associated with iris pigmented lesions. [3] On the other hand, variants of the MC1R gene (which influences skin color) and of the HAL gene (which plays a role in UV-induced immunosuppression) have been shown to have no clear association with uveal pigmented lesions. [6]

Cutaneous melanocytic lesions

Associations of presence of ocular pigmented lesions with total cutaneous nevus count, cutaneous atypical melanocytic nevi, and a family history of high number of cutaneous melanocytic nevi have been observed. [2,5,6]

Therefore, it has been suggested, that the pathways for formation of iris and cutaneous pigmented lesions might be related; and that the presence of iris

pigmented lesions may possibly serve as an indication for an inherent propensity for melanocyte proliferation (and also transformation into melanoma). [3,5,13]

Topography

Iris freckles have been observed to occur more frequently in the inferior iris quadrants (approx. 70 %) compared to the superior iris quadrants (approx. 30 %). [5] For iris nevi, that difference in distribution has been found to be about 77 % in the inferior quadrants versus 23 % in the superior quadrants. [7]

One possible explanation for this could be, that the superior iris half is more shielded from solar UV-rays by the eyelids and the superior orbital rim. [7,14]

Iris freckles

Definition

Iris freckles appear as small flecks of pigment on the anterior iris surface, which range in color from yellow-tan to deep chocolate-brown, and feature characteristic sharp feathery margins and a granular surface. [1]

Ultra-morphologically, iris freckles are discrete colonies of atypical melanocytes with varying capability to synthesize pigment. [1] Cells of iris freckles have been found to exhibit raspberry-like structures, which could be pigment-filled processes of the cells underneath. [1]

It has been hypothesized, that iris freckles might be a small, nonprogressive variant of iris nevi. [1]

Epidemiology

Iris freckles are the most common pigmented lesion of the iris [1]; their prevalence has been reported to vary from 25 % to 76 % in different study populations. [2,3,5]

Clinical significance

Biomarker for chronic sun damage

According to current hypotheses on their formation, iris freckles have been explored by Schwab et al. as a potential biomarker for cumulative sunlight exposure, with possible implications on new insights on UV-related ophthalmologic diseases. [5]

Biomarker for cutaneous melanoma risk

An Australian study by Laino et al. shows, that the presence of iris pigmented lesions is associated with an increase cutaneous melanoma risk, especially in individuals aged under 40 years. [3] This confirms similar findings of a 1985 study by Nordlund et al. from the USA, who counted iris nevi (defined as tan or brown, round or oval minimally raised spots) without the use of a slit lamp and found at least one in 51 % of melanoma patients versus in 39 % of controls. [3,15]

Laino et al. have put their findings into context with the divergent pathway model, which differentiates between two melanoma risk groups: on the one hand persons with long history of sun exposure (and usually older age of melanoma onset), and on the other hand persons with propensity to develop nevi (mostly) regardless of sun exposure (and usually younger age of melanoma onset). [3,13] Iris freckles are thought to be able to be used to identify persons in the nevus-prone pathway - but possibly not necessarily in the UV-induced pathway. [3]

Iris nevi

Definition

Iris nevi appear as raised, discrete pigmented lesions of tan to dark-brown color, replacing the iris stroma and obscuring the normal iris architecture. [2] Iris nevi are benign neoplasms composed of atypical melanocytes with growth potential, which could originate from the neoplastic transformation of normal iris stromal melanocytes. [1]

When compared to iris freckles, iris nevi have been noted to contain an increased number of cytologically benign cells, are much larger and tend to form a definite nodule. [1] Cells of iris nevi have slender, fusiform, or dendritic processes, which are similar to the normal iris stroma. [1]

Forms

Three forms of iris nevi may be distinguished according to their appearance: solitary (small and roundish), incomplete sectoral (elongated and triangular) and sectoral. [7]

Epidemiology

Prevalence of iris nevi is about 4 – 6 %. [2,7] Presence of iris nevi has been reported to be associated with higher age. [7]

Clinical significance

It has been proposed that iris nevi could be used as a risk indicator for uveal melanoma and for cutaneous melanoma. [2,7]

Uveal melanoma

Definition, Prognosis

Uveal melanoma is the most common primary malignant tumor of the eye in adults, often with poor long term outcome due to metastasis. [16]

Forms

Uveal melanoma may be categorized by anatomical location: iris, ciliary body, or choroid. [16] The term 'posterior uveal melanoma' refers to melanoma in the ciliary body and choroid. [4,12] Iris melanoma typically has a better prognosis than the other two forms. [16]

Epidemiology

In absolute numbers, uveal melanoma is quite uncommon (cited at approximately 5-8 cases per million individuals per year for Caucasians), and usually affects older Caucasian individuals, but may also rarely be found in younger patients (i.e. around puberty). [17] The disease affects both male and female individuals. [17]

Risk factors

The risk factors for uveal melanoma are not fully clear yet, though, a combination of genetic predisposition and environmental factors (e.g. UV light) seems likely. [2,4]

Host

Host factors for uveal melanoma are: light-colored eyes, light skin, self-reported susceptibility to sunburn. [4] Furthermore, increased choroidal pigmentation in white persons with light iris color has been linked to a higher risk for posterior uveal melanoma. [18]

Environmental

The role of UV radiation in the pathogenesis of uveal melanoma is currently not fully understood, as no relationship with sunlight exposure has been convincingly proven, though, arc welding exposure has been identified as a possible environmental influencing factor. [4,18]

Combined

Known combined host and environmental associations are: atypical and common cutaneous nevi, cutaneous freckles and iris nevi. [4]

Corneal thinning disorders

Keratoconus

Definitions

Keratoconus is a noninflammatory progressive thinning disorder affecting the central or paracentral cornea, leading to cone-like ectasia, which causes irregular astigmatism. [19–21] The disorder occurs bilaterally, though, asymmetry between the eyes is common. [21,22]

Subclinical keratoconus is characterized by either a topographically normal eye that has frank keratoconus in the fellow eye, or subtle topographic changes without clinical signs of keratoconus or a change in visual acuity. [23] The mildest and earliest form of the disease in fellow eyes in patients with unilateral keratoconus, is also known under the term ‘forme fruste keratoconus’. [22] However, use of these definitions in literature has been noted to be inconsistent. [22,23] Early diagnosis and corneal collagen cross-linking (CXL) of these forms may prevent disease progression and preserve visual function. [22]

Epidemiology

Prevalence of keratoconus is often reported at 1 in 2000, though, this number apparently widely varies in different study populations from 0.2 - 0.4 per 100,000 (in Russia) to 2340 per 100,000 (in Israel), possibly due to genetic variations and also differences in diagnostic criteria. [19,21,24] Recent studies indicate a preponderance of men over women with keratoconus. [25] The disease is usually sporadic, but familial cases have been observed. [21]

Onset of keratoconus usually occurs in puberty or early adulthood and the disease then commonly progresses until the third or fourth decade of life. [19,21,24] Late onset keratoconus may be related to a history of current or recent pregnancy. [24]

Etiology

The disease's exact etiology is unknown, though some contributing environmental factors (e.g. contact lens wear, chronic eye rubbing and allergic eye disease) as well as associations with genetic factors (e.g. Leber congenital amaurosis, Down syndrome, Ehler-Danlos syndrome) have been identified. [21]

Pathogenesis

The cornea affected by keratoconus has been shown to feature altered proteomics, as well as a reduced number of collagen lamellae within the affected region (possibly due to proteolysis, inter-lamellar slippage or lamellar unraveling), leading to stromal thinning. [21]

Symptoms and diagnosis

Patients may present with blurred vision from irregular astigmatism. [21] Patient history may reveal eye rubbing, atopic eye disease, frequent change of glasses, pregnancy, Down Syndrome, connective tissue disorders, retinitis pigmentosa or Leber congenital amaurosis. [24]

Clinical signs of keratoconus include stromal thinning, Fleischer ring, Vogt striae and apical stromal scar. [19] Using Scheimpflug imaging systems, such as Pentacam, various indices can be determined, by which keratoconus may be differentiated from normal eyes. [23]

Combining clinical data with patient demographics and Pentacam indices in the diagnostic process has been recommended. [23]

Pentacam

Pentacam ® (Oculus, Wetzlar, Germany) is a corneal tomography system, which creates a three-dimensional map of the inner and outer corneal surfaces. [23,26] In order to achieve this, a Scheimpflug camera is used to capture multiple cross-sectional images through the cornea and anterior chamber, rotating around a

central axis for each picture. [26] From this, ocular indices, such as corneal topography and tomography, corneal thickness, chamber angle, chamber volume and height, lens densitometry and radius of anterior lens surface curvature can be derived. [23,27]

Pentacam is applied in screening for corneal changes, for example prior to refractive surgery (including LASIK) and cataract surgery. [23]

Similar devices are available from different manufacturers. [23]

Management

Vision correction may be achieved with spectacles, rigid gas-permeable contact lenses, or other specialized lenses. [24] Progressive keratoconus can be treated with corneal collagen cross-linking under the standard Dresden protocol. [24,28] Other treatment methods include intra-corneal ring segments and topography-guided ablation treatment. [24] Lamellar or penetrating keratoplasty is used in advanced keratoconus with corneal scarring. [24] Keratoconus is the most common reason for keratoplasty in the developed world. [21]

Pellucid marginal degeneration

Definition

Pellucid marginal degeneration (PMD) is a progressive noninflammatory form of corneal ectasia characterized by bilateral, clear (“pellucid”), inferior corneal thinning, which also leads to irregular astigmatism. [29,30]

Epidemiology

PMD is the second most common noninflammatory corneal thinning disorder after keratoconus. [30] PMD occurs in both men and women. [29] Typical age of onset of PMD is reported as the second to fifth decade of life. [30] The disease then slowly progresses over many years. [30]

Etiology and pathogenesis

The exact etiology and pathogenesis of PMD is unknown. [29]

Diagnosis

Patients are asymptomatic, except for progressive visual deterioration. [30]

Differential diagnosis

The feature, which differentiates PMD from keratoconus and keratoglobus, is the thinning location at 1 – 3 mm from the limbus in the 4 – 8 o'clock position. [29,30] Age of onset could be considered another differentiating feature between PMD and keratoconus: keratoconus typically presents in puberty or early adulthood, while PMD's commonly makes its first appearance up to the fifth decade of life. [21,30] Inflammatory peripheral corneal disorders, such as Terrien's marginal degeneration and Mooren's ulcer cause vascularization, and pain, while PMD does not. [29,31,32]

Management

CXL can be used to delay or prevent the need of corneal transplantation; and the procedure may improve visual acuity. [29]

Other possible treatment methods for PMD have been reported to include intrastromal ring insertion, lamellar keratoplasty and penetrating keratoplasty. [29] Cyanoacrylates may be used in managements of severe cases with corneal perforation. [33,34]

Refractive surgery in patients with PMD can lead to severe complications, thus, prior screening is necessary. [30]

Keratoglobus

Keratoglobus is a rare, non-inflammatory form of corneal ectasia, characterized by progressive, bilateral, generalized thinning and globular protrusion of the cornea. [35,36] Congenital and acquired forms of keratoglobus have been reported. [36] Management of keratoglobus is very challenging; new surgical procedures have been developed recently. [36]

Keratectasia after laser in situ keratomileusis (LASIK)

Post-LASIK corneal ectasia is a serious complication of refractive surgery. [8,37] This disease can be prevented by screening patients prior to refractive surgery using corneal imaging. [37] If already present, its progression can be effectively stopped by CXL. [37]

Corneal collagen cross-linking

Synonyms

Corneal collagen cross-linking has also been referred to as corneal X-linking, or CXL in current literature. [8,29]

Definition

CXL is a treatment method, which utilizes riboflavin and ultraviolet A light to induce the formation of collagen cross-links within the cornea, which has been shown to be effective for stopping the progression of keratectasia in patients with keratoconus. [9,19]

Indications

A keratoconus progression, which indicates CXL, may be defined as a > 1 Diopter (D) increase in maximum keratometry and/or > 1 D increase in average keratometry and/or refractive astigmatism of > 1 D and/or decrease in pachymetry of more than 10 % in the preceding 12-18 months. [28] Patients with signs of high-risk for progression could also be considered for CXL without waiting for documented progression. [24]

Additionally, corneal collagen cross-linking has been used in the management of keratectasia after laser in situ keratomileusis (LASIK), pellucid marginal degeneration (PMD); and it has also been explored for use in cases of infectious keratitis. [8,9,19,28,29] However, the latter, also known as ‘Photo Activated Chromophore for Keratitis’-CXL (PACK-CXL), used in conjunction with antimicrobial treatment, has not been shown to lead to improved outcomes over antimicrobial treatment alone. [28]

Contraindications

CXL should be avoided in eyes with previous herpes simplex infections (to prevent reactivation) and in current atopic eye disease (to prevent corneal melting). [28]

Procedure

Dresden protocol (epithelium-off)

In the following, the standard CXL method is described step-by-step. [8,28]

- The procedure is conducted under sterile conditions in the operating room. [19]
- Preoperative pachymetry should measure a central corneal thickness of at least 400 µm, otherwise CXL should be avoided. [19]
- preoperative local anesthesia with eyedrops (e.g. proxymetacainhydrochloride 0.5 %; or tetracaine 1 %) [19,28]
- abrasion of the corneal epithelium in a 9 mm diameter [8]
- instillation of drops of 0.1 % riboflavin solution in 20 % dextran (or instead, containing hydroxypropyl methylcellulose) onto the cornea every 2-3 minutes for a total of 30 minutes. [8,28,29]
- ultrasound pachymetry, to verify central corneal thickness (CCT) of at least 400 µm; otherwise, hypotonic riboflavin eyedrops are applied until the required CCT is reached [28]
- verification of riboflavin in the anterior chamber by blue light slit-lamp examination. [8]
- UV irradiation of the cornea (wavelength = 370 nm, irradiance = 3 mW/cm², cropped beam diameter = 9 mm; duration = 30 minutes), and rinsing with riboflavin/dextran and topical anaesthetic every 2-5 minutes [8,28]
- application of antibiotic ointment (e.g. oxofloxacine, tobramycin), steroid eyedrops (e.g. fluorometholone) and a bandage lens until reepithelialization [8,19,29]

Epithelium-on protocols

The corneal epithelium essentially acts as a diffusion barrier; so, in the original CXL method it is recommended to be removed, in order to improve and accelerate the diffusion of riboflavin into the corneal stroma, and to avoid eliciting photokeratitis. [8] However, the epithelium-off technique is associated with significant postoperative

pain and also risk of infection and scarring. [28] To address this, in epithelium-on techniques, the corneal epithelium is not removed, though, these procedures have been shown to be less efficacious than the original epithelium-off technique. [28]

Accelerated CXL

In accelerated CXL, a higher UV-irradiance is applied for a shorter time, when compared to the standard procedure, arriving at the same energy dose delivered. [28] Accelerated CXL has not consistently been shown to be as effective as the standard protocol. [28]

Effects of CXL

Photochemical effects

Production of oxygen radicals by riboflavin, ambient oxygen and UVA light induces changes at the end of amino groups of collagen chains, which in turn may form new covalent bonds (cross-links), leading to the intended biomechanical effects. [8]

Biomechanical effects

It has been shown, that in CXL, the keratoconic cornea's anterior 200 µm are stiffened to a greater degree when compared to the posterior layer, in total arriving at mechanical properties, which are similar to a normal cornea. [9]

Effects on keratocytes

CXL leads to keratocyte apoptosis approximately in the anterior 300 µm of the cornea, but repopulation has been shown 6 months after the procedure. [8]

Clinical effects

CXL can stop the progression of keratoconus, PMD and post-LASIK-keratectasia, potentially leading to improved best-corrected visual acuity and corneal topography measurements. [19,28]

Possible complications

Complications of CXL include: corneal pseudo-haze, permanent stromal scarring, bacterial infection, sterile infiltrates, melting, delayed epithelial healing, perforation of the cornea, or failure of treatment. [28,29]

The most common complication of these is anterior corneal haze, usually appearing 1 - 2 months after CXL and disappearing by 6 - 12 months, though, it should be noted, that permanent scarring, affecting patients' vision, may also occur. [28]

Corneal endothelial damage may occur due to failure to meet the corneal thickness requirement or UV device misalignment or irradiation inhomogeneity. [28]

Radiation safety

A possible safety concern of CXL was the potentially resulting ocular damage caused directly by UVA radiation, as well as photochemical damage caused by the induced radicals. [8] Vulnerable structures include the corneal endothelium, lens and retina. [8] However, it has been shown, that the irradiance during CXL is below the damage thresholds, provided, that the required minimum corneal thickness of 400 µm is met and that the light source provides homogenous irradiance. [8] The residual UV radiant exposure of the corneal endothelium has been reported at 0.32 J/cm², and even less for deeper structures. [8]

1.3 Objectives

Rationale

In summary of the above, CXL appears to be a rather safe procedure. However, it is not fully clear yet, to which extent UVA light penetrates the eye in vivo and if this might cause effects which possibly have not been recognized previously. To elucidate this subject further, we intend to explore iris freckles as a potential marker for such UV-related effects.

Research questions

Our central research question is, if there is an increase in the number iris freckles and nevi in eyes, which have received CXL, when compared to eyes, that have not.

Additionally, we intend to evaluate possible differences between the iris quadrants, as well as the influence of elapsed time since CXL and participant age, with regards to iris pigmented lesion count.

Thereby, we aim to add further knowledge on the origin of iris freckles and nevi and examine the possible role of CXL and UV radiation in this process.

2 Patients and Methods

2.1 Ethical considerations

Our study was approved by the local ethics committee (30-302 ex 17/18) and was conducted in accordance with the WMA Declaration of Helsinki (amendment of 2013).

2.2 Study design and population

In our prospective observational cross-sectional single-center study, we examined non-hospitalized volunteers who in the past had undergone unilateral corneal collagen cross-linking (CXL). All individuals were examined by slit-lamp (Haag-Streit), followed by capture of bilateral iris images for each participant. In turn, we arrived at two matched samples (i.e. post-CXL eyes and non-CXL eyes), which we then analyzed and compared with regards to iris pigmented lesion count. By means of this design, we could control for variables, which are established influencing factors for iris pigmented lesion count, such as age and iris color. [3,5]

Both female and male persons over the age of 14 years, who were able to give informed consent, were eligible to be included in the study. The age limit was chosen to ensure optimal compliance during study assessments; other than this, no additional exclusion criteria had to be defined.

All participants were recruited from the Medical University of Graz Department of Ophthalmology and all study examinations were conducted there as well. Recruitment, including confirmation of eligibility and providing initial information on the study, was carried out by telephone and took place from July to August 2018. Examinations were performed from July to September 2018. Data collection, image analysis and statistical analysis were completed in December 2018.

Our aim was to invite as many eligible persons as possible to participate in our study examinations. Preliminary assessment of our database revealed that 130 individuals met our inclusion criteria and were available to be contacted. Our final study population size (n = 20) was determined by the number of volunteers who accepted our invitation.

2.3 Iris imaging

First, informed consent was obtained from the participants. Next, every volunteer received a bilateral ophthalmologic examination, including Pentacam® (Oculus Optikgeräte GmbH, Wetzlar, Germany), visual acuity testing and slit lamp (Haag-Streit) examination. Then, images of both irises were captured by our department's professional clinical photographers.

2.4 Diagnostic criteria

Primarily, we aimed to identify and count all iris freckles in the images. However, we also included solitary iris nevi (iris pigmented lesions with disturbance of the iris stroma) in our count, because in photographs no certain distinction can be made between freckles and nevi - analysis during slit lamp examination would be required for this. [3,7]

Consequently, an iris pigmented lesion to be included was defined as any visually discernible small, roundish pigmented spot of yellow-to-brown color within the area of the iris. [1,5] These are considered to be acquired during the course of an individual's life, with UV-radiation having a possible role in the process. [3,5,7]

Sectoral and incomplete-sectoral iris nevi, which could be clearly identified as such by their triangular shape, were excluded from our count, since these are deemed likely to be of congenital origin as opposed to the aforementioned acquired types. [7]

2.5 Image analysis

Once all images had been collected, they were visually inspected to identify all iris pigmented lesions which met our diagnostic criteria and to determine their number by quadrant. The quadrants were defined by approximately placing a horizontal line and a vertical line at the center of the pupil, dividing its area into four parts of equal size (temporal inferior, nasal inferior, temporal superior and nasal superior). [7]

The resulting counts of iris pigmented lesions were recorded in Microsoft Excel, organized within a pseudonymized table by participant identification number and by CXL-side (i.e. number of iris pigmented lesions in post-CXL eye, number of iris pigmented lesions in non-CXL eye).

During image analysis, we additionally documented iris color (categorized into light, medium and dark). These categories were chosen to be approximately equivalent to other studies on the subject. [5,7]

2.6 Additional variables

The following additional parameters were taken note of for each person: examination date, age at examination date, sex, indication for CXL, as well as CXL date and side (i.e. left eye or right eye).

2.7 Statistical analysis

Statistical software

Statistical analysis was carried out using IBM SPSS Version 25. Microsoft Excel was utilized for spreadsheet calculations and Microsoft Word was employed for graph creation. Our level for accepting significance was set at < 0.05.

Comparison between post-CXL eye and non-CXL eye

One-sample Kolmogorov-Smirnov tests were used to check for presence of a normal distribution of the number of iris pigmented lesions in the post-CXL eye and the non-CXL eye.

Because our data were not normally distributed, a non-parametric test, in our case a Wilcoxon signed-rank test for paired samples, was performed in order to compare the post-CXL eye's undivided iris freckle count with the non-CXL eye's undivided iris freckle count. [38] This test was repeated for comparing the corresponding quadrants' iris freckle counts of the post-CXL eye to the ones of the non-CXL eye.

Additionally, we performed a Wilcoxon signed-rank test for all datasets where the individual's CXL-to-examination time difference exceeded 12 complete months, in order to address the hypothesis that iris pigmented lesions might only appear after some time has elapsed since UV-exposure.

Post-CXL time-difference

Furthermore, we calculated Spearman's rank correlation coefficient between the total number of iris freckles in the post-CXL eye and the time difference between CXL and examination date. We tested for one-tailed significance as to whether there was an increase in iris freckle count relative to time difference.

Age

We also compared the ages of persons with and without iris pigmented lesions. [5] Since our sample sizes were too small for a t-test for independent samples, we chose to conduct a Mann-Whitney U-test instead. [5,38] The tested variable was age at examination in years.

Spearman's rank correlation coefficient was calculated between age at examination in years and the number of iris pigmented lesions, for both eyes, and for the post-CXL eye and the non-CXL eye separately. [5] Again, we calculated one-tailed significance to test for an increase in the number of iris pigmented lesions relative to age.

2.8 Reporting

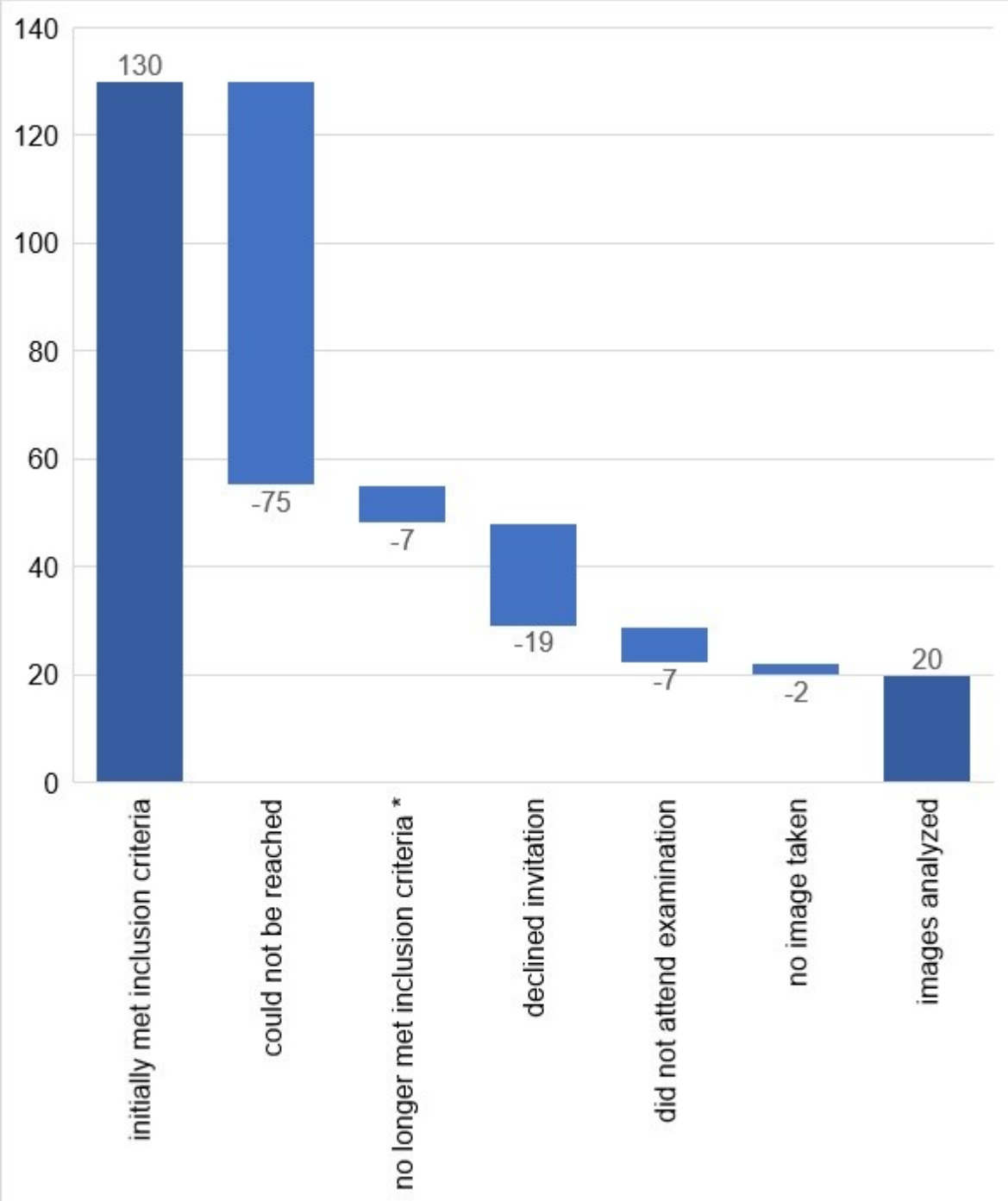
For general guidance on composing and structuring this document, the STROBE-Checklist was followed, as well as the Medical University of Graz's most current diploma thesis guidelines. [39]

3 Results

3.1 Participants

The reasons for participant dropout at the different stages of our study are broken down in Figure 1.

Figure 1: *dropout*



** All persons who no longer met our inclusion criteria stated that they had in the meantime received bilateral corneal collagen cross-linking.*

After image analysis, no additional exclusions had to be made, therefore our final study population for statistical analysis consisted of 20 individuals, whose key features are summarized in Table 1.

Data with regards to CXL-to-examination time difference are covered in their own chapter.

Table 1: *study population data (n = 20)*

	total number	**percentages**
sex		
female	2	10 %
male	18	90 %
age at examination date		
mean	35.15 a	
standard deviation	11.15 a	
median	33 a	
range (minimum - maximum)	18 a – 54 a	
iris color		
dark	2	10 %
medium	2	10 %
light	16	80 %
CXL-side		
left eye	11	55 %
right eye	9	45 %
indication for CXL		
keratoconus	19	95 %
pellucid marginal corneal degeneration	1	5 %

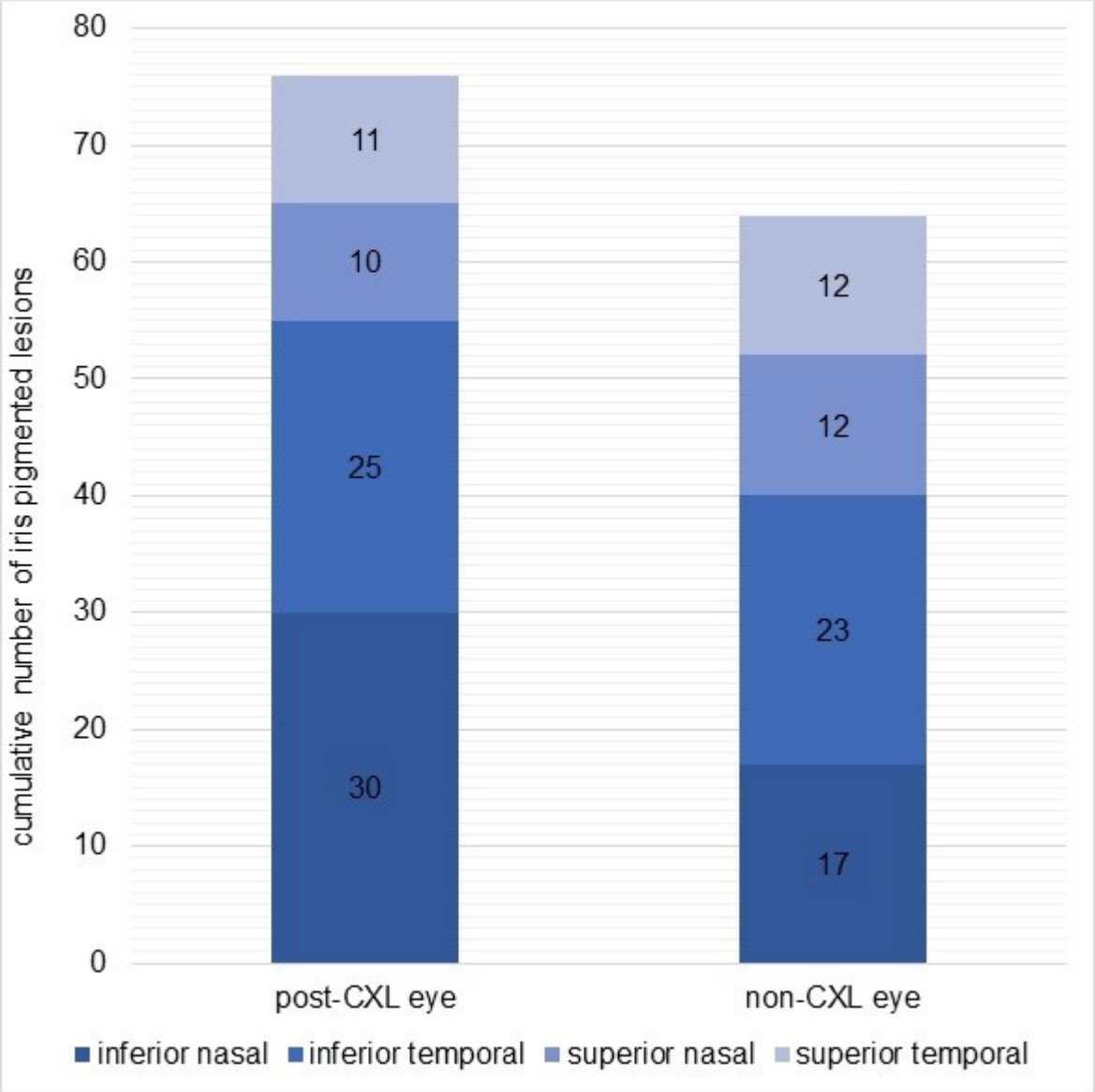
3.2 Iris pigmented lesion count

In 16 participants (80 %) at least one iris pigmented lesion could be identified. Approximately 67.9 % (95/140) of all iris pigmented lesions were located in the inferior quadrants.

Cumulative

The cumulative counts of iris pigmented lesions are summarized in Figure 2, organized by side and by quadrant. In all post-CXL eye's irises, 76 iris pigmented lesions were found, while in all non-CXL eye's irises that total was 64.

Figure 2: *distribution of iris pigmented lesions in absolute numbers (n = 20 irises per side)*



Frequency distribution

The maximum number of iris pigmented lesions observed in a single quadrant was 8. The bar charts in Figure 3 illustrate the frequency with which each pigmented lesion count (from 0 to 8) was observed. This data is also presented in Table 2 in tabular form.

Figure 3: *frequency of iris pigmented lesion counts by quadrant and by CXL-side (n = 20 irises per side)*

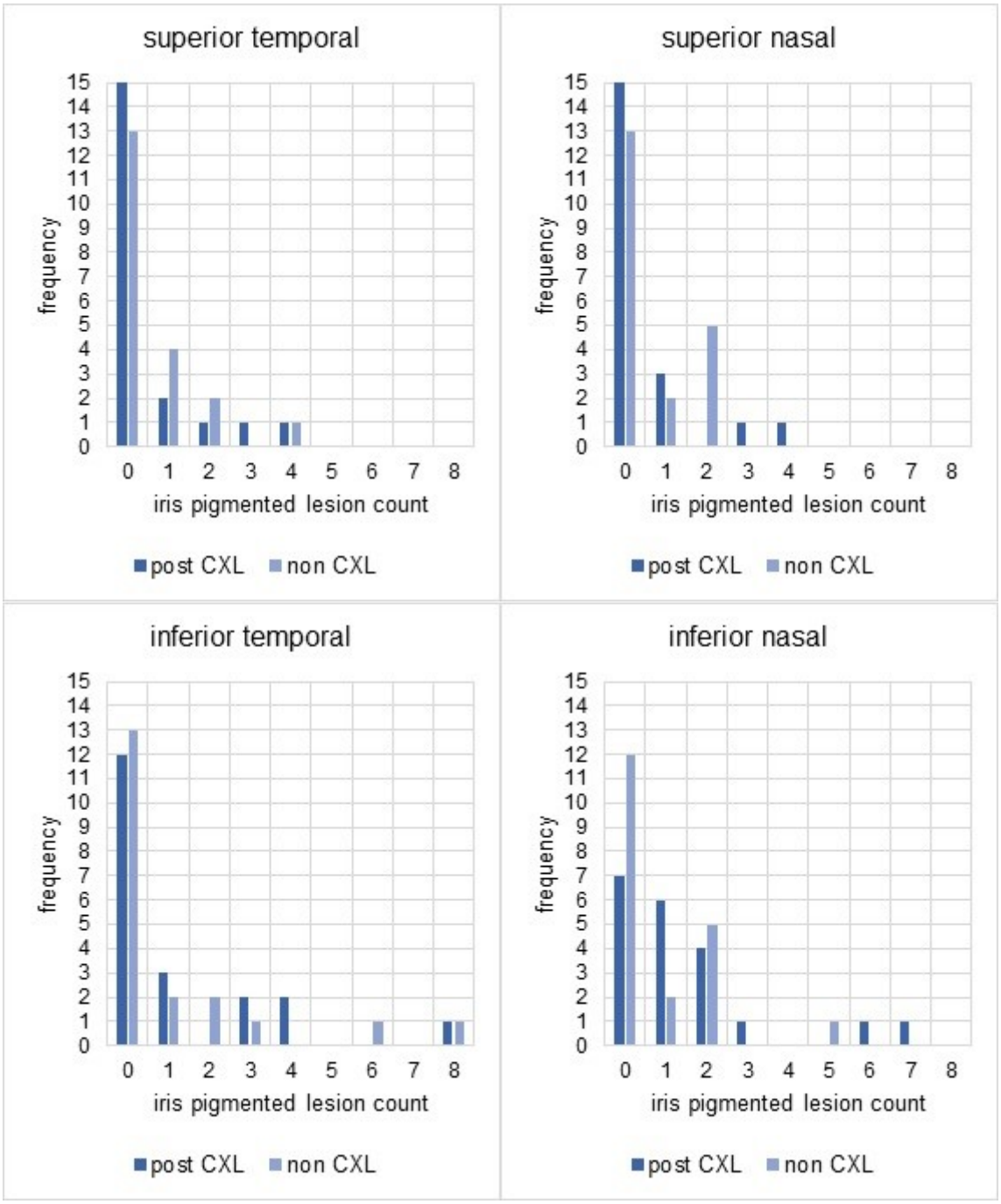


Table 2: *frequency of iris pigmented lesion counts by quadrant and by CXL-side*

iris pigmented lesion count	**superior temporal**		**superior nasal**		**inferior temporal**		**inferior nasal**	
	post CXL	non CXL	post CXL	non CXL	post CXL	non CXL	post CXL	non CXL
0	15	13	15	13	12	13	7	12
1	2	4	3	2	3	2	6	2
2	1	2	0	5	0	2	4	5
3	1	0	1	0	2	1	1	0
4	1	1	1	0	2	0	0	0
5	0	0	0	0	0	0	0	1
6	0	0	0	0	0	1	1	0
7	0	0	0	0	0	0	1	0
8	0	0	0	0	1	1	0	0

Boxplot summary

Some additional descriptive statistical results, including medians, are displayed in the graphs in Figure 4 and Figure 5, respectively. Note that in the post-CXL eyes' inferior nasal quadrant the median iris pigmented lesion count is 1, whereas all other examined quadrants exhibited a median of 0.

Figure 4: *boxplot diagram by quadrant (n = 20 irises per side)*

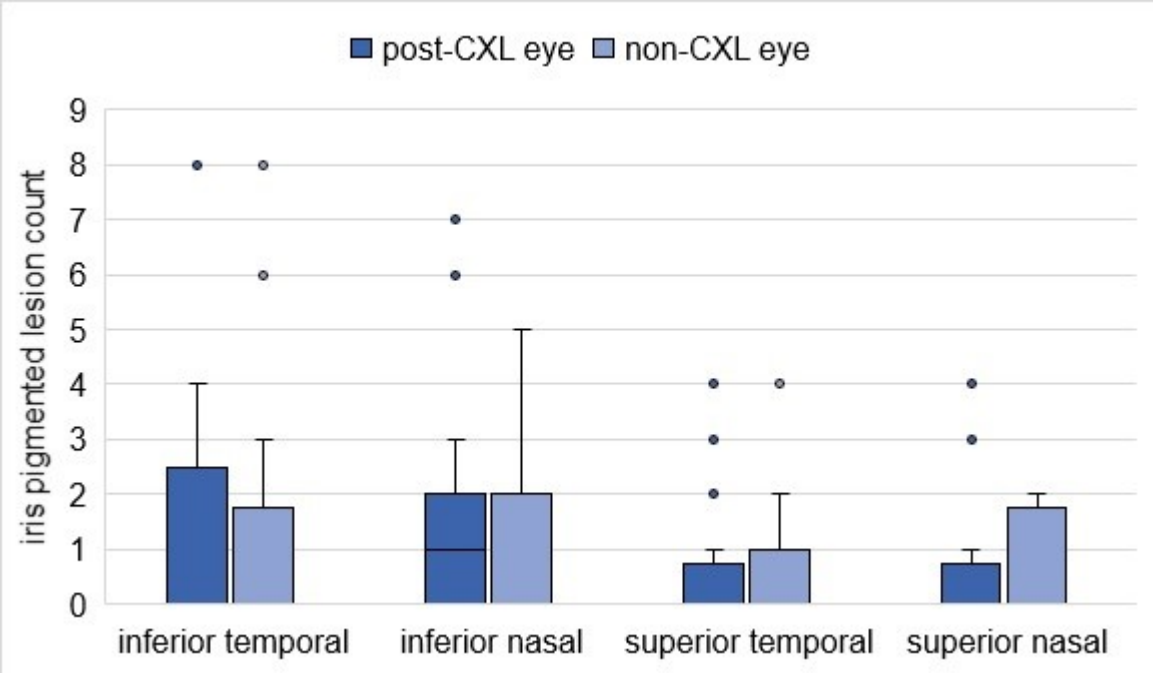
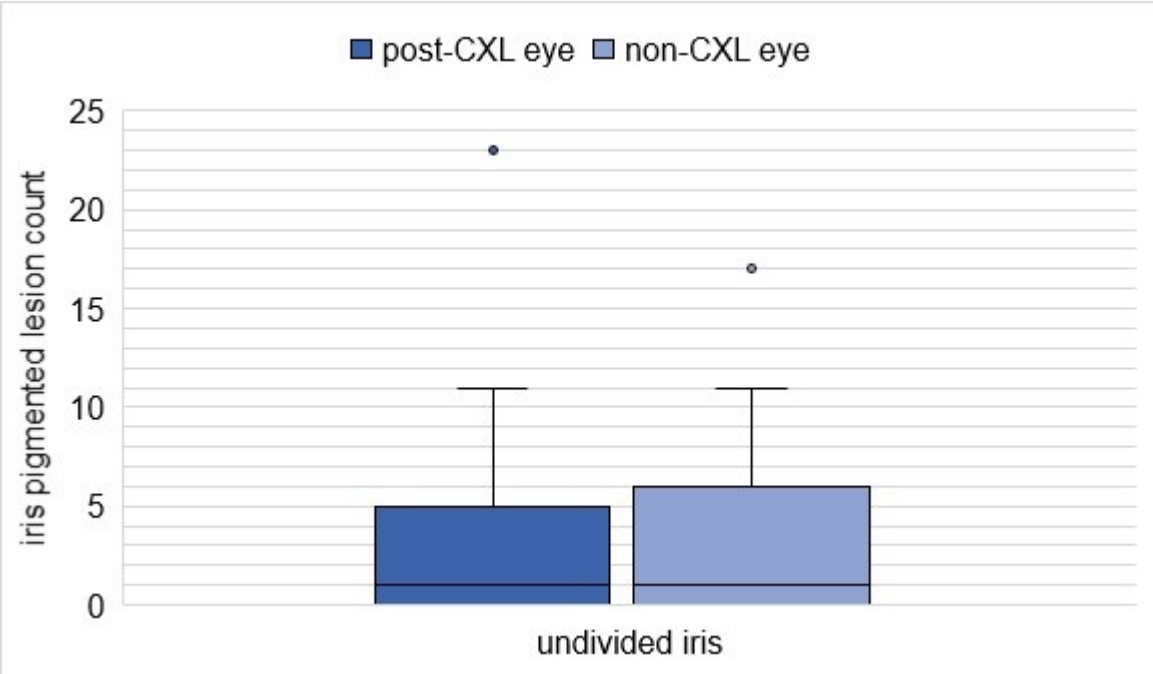


Figure 5: *boxplot diagram for the undivided iris (n = 20 irises per side)*



3.3 Comparison between post-CXL eye and non-CXL eye

Entire study population

The results of the Wilcoxon signed-rank tests comparing the freckle counts of the corresponding iris areas of the post-CXL eye and the non-CXL eye are displayed in Table 3. The null hypothesis H_0 in this test is defined as the median of differences between the compared samples being equal to zero. [38] The threshold for accepting significance was $p < 0.05$.

Table 3: *Wilcoxon signed-rank test for related samples (post-CXL vs. non-CXL; n = 20 pairs of data)*

tested iris area	result	p-value
inferior temporal	accept H_0	0.681
inferior nasal	reject H_0	0.032
superior temporal	accept H_0	0.792
superior nasal	accept H_0	0.732
undivided iris	accept H_0	0.305

Subgroup, where CXL-to-examination time difference > 12 months

The reduced sample size of our study population with a CXL-to-examination time difference of greater than 12 complete months was 15 pairs of data. The Wilcoxon signed-rank test comparing this subset's undivided iris pigmented lesion counts, again between post-CXL eye and non-CXL eye result was to accept H_0 ($p = 0.215$).

3.4 Post-CXL time difference and iris pigmented lesion count

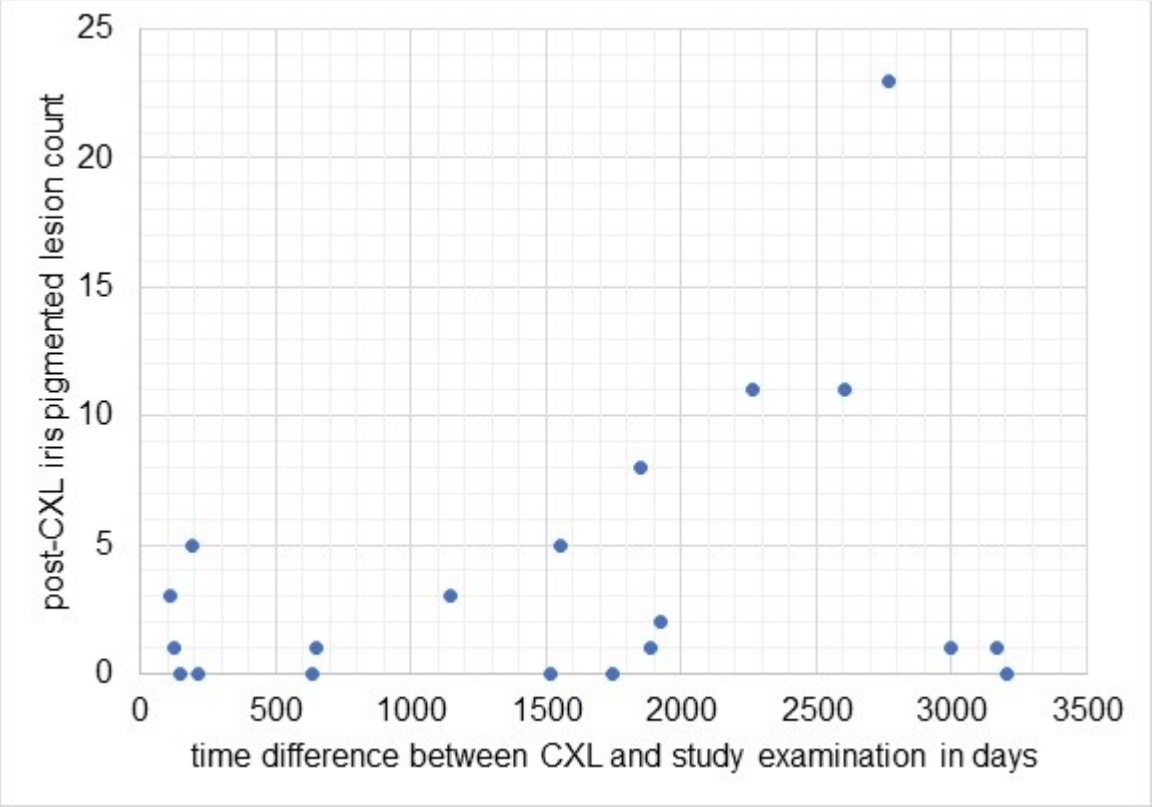
Key parameters regarding time difference between CXL and the examination are presented in Table 4 in precise number of days as well as rounded down to complete months. A full data overview is available in Figure 6.

Our calculations with regards to correlation between this CXL-to-examination time difference (in days) and iris freckle count in the post-CXL eye yielded a Spearman's rank correlation coefficient of ρ = 0.214 with one-tailed significance of 0.183.

Table 4: *Time difference (Δt) from CXL to examination (n = 20)*

	Δt in days	**Δt in years and complete months**
median	1650	4 years 6 months
shortest	113	3 months
longest	3202	8 years 9 months

Figure 6: *comparison between time difference (CXL-examination) and post-CXL iris pigmented lesion count (n = 20)*



3.5 Age and iris pigmented lesions

Age profiles

When dividing our study population in two subgroups (examining both eyes): one with 0 iris pigmented lesions, and another with 1 or more iris pigmented lesions, we arrived at the age profile statistics listed in Table 5:

Table 5: *absence/presence of iris pigmented lesions and age (n = 20 in total)*

	persons with 0 iris pigmented lesions	**persons with ≥ 1 iris pigmented lesions**	**total study population**
sample size	4	16	20
age arithmetic mean	30.5 years	36.3 years	35.2 years
age standard deviation	10.2 years	11.1 years	11.1 years
age median	33.5 years	29.0 years	33 years
age range	18 – 54 years	18 – 46 years	18 – 54 years

The Mann-Whitney U test's result was to accept the null hypothesis H_0 of there being no significant difference in the age distributions of these samples (U = 19.500; exact significance = 0.249).

Correlation

Spearman's correlation coefficients between age at examination and the number of iris pigmented lesions, including one-tailed significance are listed in Table 6.

Table 6: *correlation between age at examination and iris pigmented lesion count (n = 20 for each test)*

tested area	**Spearman's ρ (comparing to age)**	**one-tailed significance**
both eyes	0.061	0.400
post-CXL eye	-0.015	0.474
non-CXL eye	0.034	0.443

Full data

Full data comparing age at examination versus number of iris freckles are disclosed in Figure 7 for both eyes of each individual, and in Figure 8 for post-CXL eye and non-CXL eye separately.

Figure 7: *scatterplot comparison between age and number of iris pigmented lesions (n = 20) trendline is second degree polynomial*

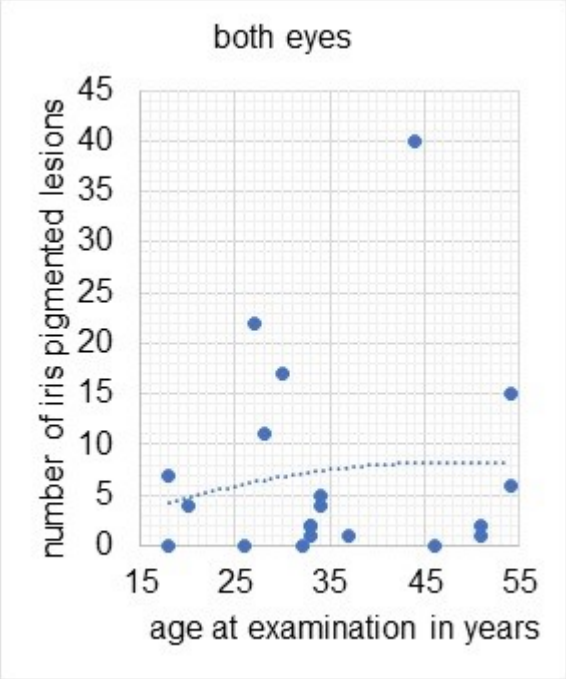
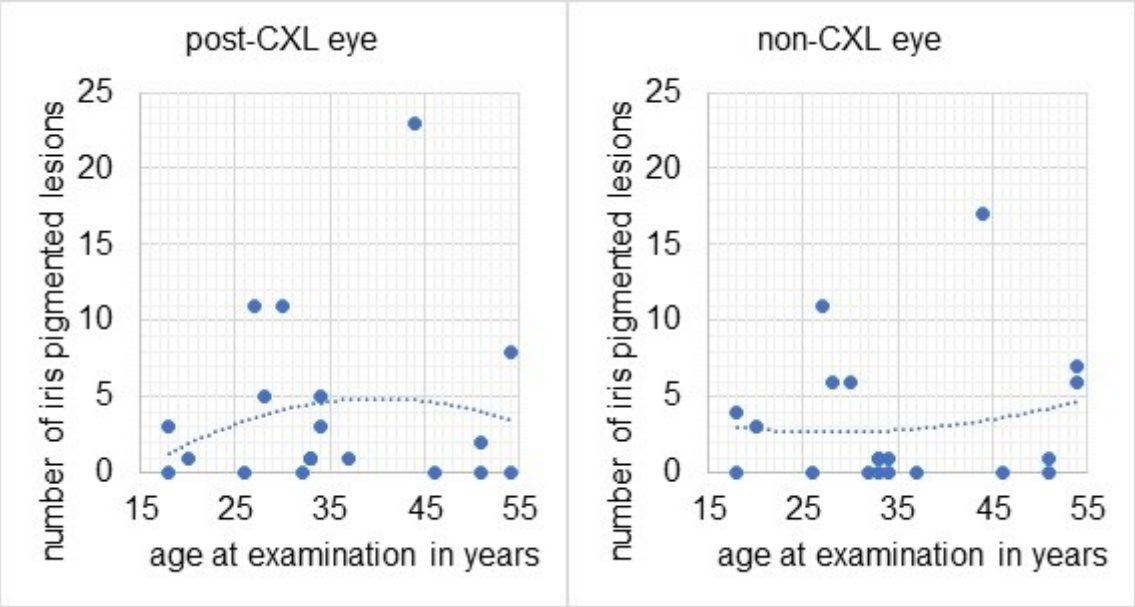


Figure 8 *the influence of age on the number of iris pigmented lesions for post-CXL side and non-CXL side (n = 20 for each side) trendlines are second degree polynomial*



4 Discussion

4.1 Summary of key results

In our study, we compared the number of iris freckles and nevi in eyes, which have received CXL, to eyes, that have not, in order to gain additional knowledge on the origin of these types of melanocytic lesions:

- We found no significant increase in iris pigmented lesion count in the post-CXL eye, when compared to the non-CXL eye. The same was true when comparing each side's corresponding quadrants, except for the inferior nasal quadrants (see Table 3 and Figure 4) (n = 20).
- For the subset of data, where CXL-to-examination time difference was greater than 12 complete months (n = 15), we also detected no significant difference in iris pigmented lesion count between post-CXL eye and non-CXL eye.
- No significant correlation between post-CXL time difference and iris pigmented lesion count in the post-CXL eye was identified. (n = 20)
- The age profile of persons with at least 1 iris pigmented lesion (n = 16) was not significantly different to the age profile of persons with 0 iris pigmented lesions (n = 4).

4.2 Iris freckle and nevus count

Prevalence

The proportion of individuals with one or more iris pigmented lesions in our evaluation (80 %) is comparable to other recent studies with larger study populations and similar phenotypes, who found percentages of about 76 %. [3,5] However, in other study populations, a much lower prevalence has been noted; for example, Csoma et al. have reported a prevalence of 25.35 % for persons with at least one iris freckle and 5.98 % for iris nevi. [2]

Topography

The topographic distribution of iris freckles and nevi in our study resembles previous findings: We found approximately 67.9 % of all iris pigmented lesions in the inferior quadrants, while former studies observed that proportion to be at slightly more than 70 % for iris freckles and about 77 % for iris nevi. [5,7] As a possible explanation for this, current literature mentions the inferior quadrants' higher exposure to solar UV radiation, when compared to the superior quadrants, because the latter might be more protected from sunlight by the superior orbital rim and the superior eyelid. [5,7,14] The same theory has been applied to iris melanoma, which also appears more frequently on the inferior iris half. [40]

In our study, we would have expected possible UV-induced changes in iris freckle and nevus count to occur predominantly in the superior quadrants because of this probable lower UV light exposure during the course of an individual's life, whereas during CXL all quadrants should be equally irradiated. [5] However, this hypothesis could not be verified by our results, since the superior quadrants did not exhibit any signs of significant change in pigmented lesion count.

4.3 Possible explanations and implications

High UVA absorption in the riboflavin treated cornea

The absence of a significant increase of iris freckle and nevus count in the post-CXL eye, when compared to the non-CXL eye, could be explained by considering the following previous findings:

- Laino et al. suggest, that there may be a threshold UV level required for the formation of iris pigmented lesions, which may also explain the development of iris pigmented lesions with age. [3]
- Kohlhaas et al. describe, that during the corneal collagen cross-linking procedure, riboflavin treatment causes high absorption of UVA radiation within the corneal stroma, (i.e. 65 % - 70 % in the anterior 200 µm and 20 % in the posterior 200 µm), which is intended in order to prevent damage to the corneal endothelium. [9]

With regards to the present study, this would mean, that the iris might only be exposed to a low level of UVA irradiation during the CXL-procedure, one that is possibly too low to reach the threshold for the formation of iris freckles and nevi as a biological response. However, it remains unclear, what quantity of UV irradiation would be required for this; and our results neither support, nor reject the existence of such a threshold.

Light spectrum

In our study, we aimed to examine the possible effects of the single-wavelength UVA light applied during CXL. In contrast, other publications on the topic of iris freckles and nevi oftentimes analyze the effects of solar radiation, where exposure to sunlight (and use of adequate protection) is oftentimes assessed by questionnaire. [3,5,7] Especially UVB radiation has been mentioned as a possible cause for the formation of iris freckles. [5]

Consequently, an additional possible explanation for our current findings could be, that radiation in the UVA spectrum alone could be insufficient to elicit the formation of iris freckles and nevi.

Observations in the inferior nasal quadrant

In the inferior nasal quadrant, Wilcoxon's signed rank test did show a statistically significant difference ($p = 0.032$; see Table 3), and when examining that quadrant's absolute iris pigmented lesion counts, the median was 1 in the post-CXL eye versus 0 in the non-CXL eye (see Figure 4).

However, because this inconsistency is quite small in magnitude, we suspect that it may possibly be attributable to random experimental error and that it is rather unlikely to serve as an indication for any true biological effect. Nevertheless, it might still be interesting to see, if similar results could be replicated in future studies.

Formation time

The analysis taking into account a possible iris pigmented lesion formation period by only including datasets with > 12 complete months post-CXL time, and also the correlation analysis between post-CXL time difference and iris pigmented lesion count are in line with our other results, in that there is no measurable change, possibly due to the low total influence of UVA light on the iris during CXL.

Age

When comparing the subgroups with and without iris freckles, Schwab et al. found a significant difference in the age distributions. [5] In our current study, we could not replicate this finding. One possible explanation for this may be, that the current study had a much smaller population size (20 versus 632), with only 4 persons in the zero iris freckles and nevi group, and also an overall narrower age profile (see Table 7). [5] Laino et al. also report an increase of iris pigmented lesion count with age (comparing age profiles of persons with < 3 versus ≥ 3 iris pigmented lesions). [3]

Table 7: *comparison of study population age profiles*

	Schwab et al. 2017 (n = 632) [5]	**present study (n = 20)**
age arithmetic mean	38.4 a	35.2 a
age standard deviation	18.4 a	11.1 a
age range	4 a – 84 a	18 a – 54 a

4.4 Limitations

Study design

The generalizability of our results may be affected by our recruitment method, which could be prone to sampling bias, as well as the single-center character of our examination. [38]

Study population

Furthermore, our final study population (n = 20) was quite small, which might impact the strength of some of our conclusions. For example, only 2 persons each were categorized as having dark and medium colored irises, respectively. Consequently, no extensive statistical analyses of these subgroups were possible. We faced a similar problem when conducting our age profile analysis.

Even when considering keratoconus' higher prevalence in male individuals, it still seemed somewhat surprising that only two women (10 % of the total study population) volunteered for our study. [25] However, in current literature no major sex-related differences in iris freckle count have been reported, hence, this factor might not have greatly influenced our results. [5]

Time from exposure to examination

The short time intervals between CXL and our study examination could be another limitation. Studies on the origin of iris freckles commonly mention lifetime accumulation of UV-light as a potentially causal factor. [3,5] Comparatively, CXL has only been employed in clinical practice for approximately 15 years, and typical time after exposure in our study was even shorter (see Table 4). [19] Future research on longer term effects of CXL might therefore come to different conclusions.

Image analysis

The missing distinction between iris freckles and iris nevi, which could have been achieved by slit-lamp examination, could also have influenced our results slightly. [3] However, both iris freckles and solitary iris nevi are thought of as being acquired lesions, which is why we considered our image analysis method adequately effective for the outcomes we aimed to examine. [3,5,7]

In our study, no blinding was used when determining iris pigmented lesion counts in the post-CXL eye and the non-CXL eye. In future studies, it may be possible to eliminate this potential source of observer bias by implementing appropriate measures in the analysis method.

Lastly, a possible source of imprecision in our observations may be, that only one person identified iris freckles and nevi, while other studies controlled for inter-observer variability by employing two to four experienced observers. [3,7]

4.5 Strengths

A possible advantage of our study could be, that we did not have to rely on self-reported questionnaires to assess the differences in UV exposure, possibly increasing our results' accuracy.

Another strength of our examination might be, that each patient (being unilaterally cross-linked) contributed to post-CXL group and non-CXL group equally, essentially arriving at two matched samples. This allowed us to control for known influencing variables for iris freckle and nevus count, such as age. [3]

5 Conclusion

Overall, we conclude that CXL is not significantly associated with the formation of iris freckles and nevi in our study population.

It might be an additional reassurance to affected patients, that CXL may be viewed as quite safe in that regard.

Further research on the origins of iris freckles and nevi, and, more specifically, the role of UV light in the process could be required because the precise underlying principles are not fully understood yet.

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