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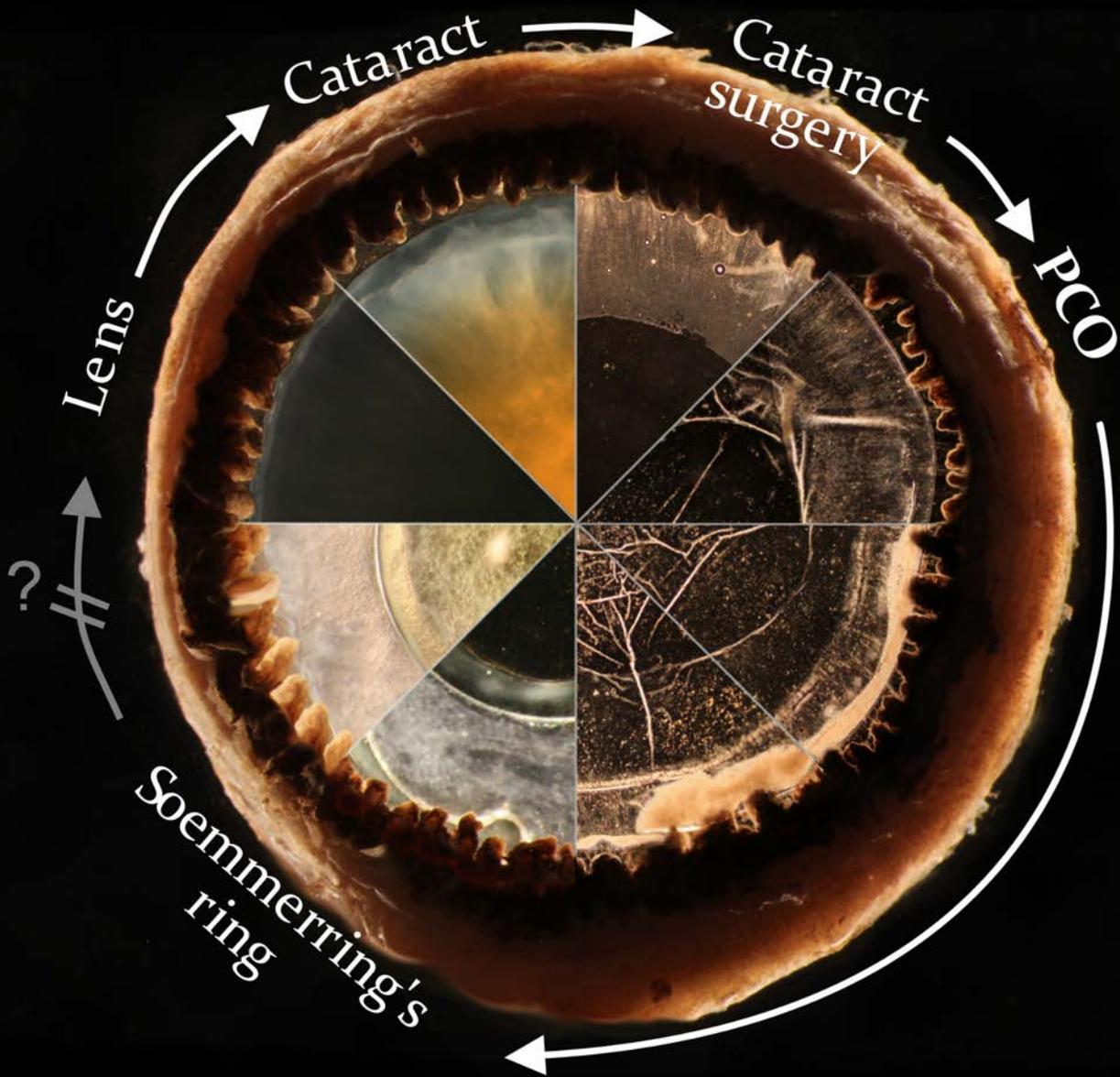
DOCTORAL THESIS

Lens capsule tissue culture for the investigation and prevention of Posterior Capsule Opacification.

by

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To my wife,

for always believing in me and making me feel that I can do anything.

Abbreviations list

αSMA	Alpha smooth muscle actin
A-cells	Lens epithelial cells lining the anterior capsule
ALE	Anterior lens epithelium
AQP0	Aquaporin 0
bFGF	Basic fibroblast growth factor
BSS	Balanced salt solution
BUTC	Banc d´Ulls per a Tractaments de Ceguesa
CCC	Continuous curvilinear capsulorhexis
CE	Corneal epithelium
DAB	3,3'-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
ECCE	Extracapsular cataract extraction
E-cells	Lens epithelial cells lining the equator of the capsular bag
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
FHL124	Spontaneously immortalized embryonic lens explant cell line
FOXE3	Forkhead box protein E3
GBD	Global Burden of Disease
H&E	Hematoxilin and Eosin
H2O2	Hydrogen peroxide
H2Od	Distilled water
HBSS	Hanks balanced salt solution

HGF	Hepatocyte growth factor
HLEB3	Immortalized human lens epithelial cell line
ICCE	Intracapsular cataract extraction
IGF-II	Insulin-like growth factor
IOL	Intraocular lens
Ki-67	Nuclear protein marker of proliferation Ki-67
LEC	lens epithelial cell
Nd:YAG	Nodymium:yttrium-aluminum-garnet
Pax6	Paired box protein 6
PBS	Phosphate-buffered saline
PCO	Posterior capsule opacification
PDGFb	Platelet derived growth factor subunit B
PMMA	Polymethylmethacrylate
PSR	Picro Sirius Red
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
SRA01/04	Immortalized human lens epithelial cell line
TGF-β	Transforming growth factor beta
US	United States
USD	United States dollar
WGA	Wheat germ agglutinin
WHO	World Health Organization

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1. Introduction

1.1. Lens development

To understand the pathology of Posterior Capsule Opacification (PCO), we will first describe the development of the embryological and adult lenses.

1.1.1. Embryology of the lens

The development of the eye serves to remind us that there are many instances in normal embryology in which development continues after birth and can be significantly altered by events in postnatal life.[1]

The embryological development of the eye is very similar among mammals, only with slight variations in the onset of the different stages.[2, 3] In humans, the developing eye (optic primordia) is first distinguishable at about day 22 (Carnegie stage 10) as bilateral evaginations of the neuroectoderm of the forebrain (optic pits) (Figure 1.1). These evaginations, at about day 27 become large, single-layered vesicles of neuroectoderm, known as the primary optic vesicles (Figure 1.1 & Figure 1.2 A). As these reach the surface ectoderm, they induce the formation of the lens placodes which also invaginate. (Figure 1.1 & Figure 1.2 B)
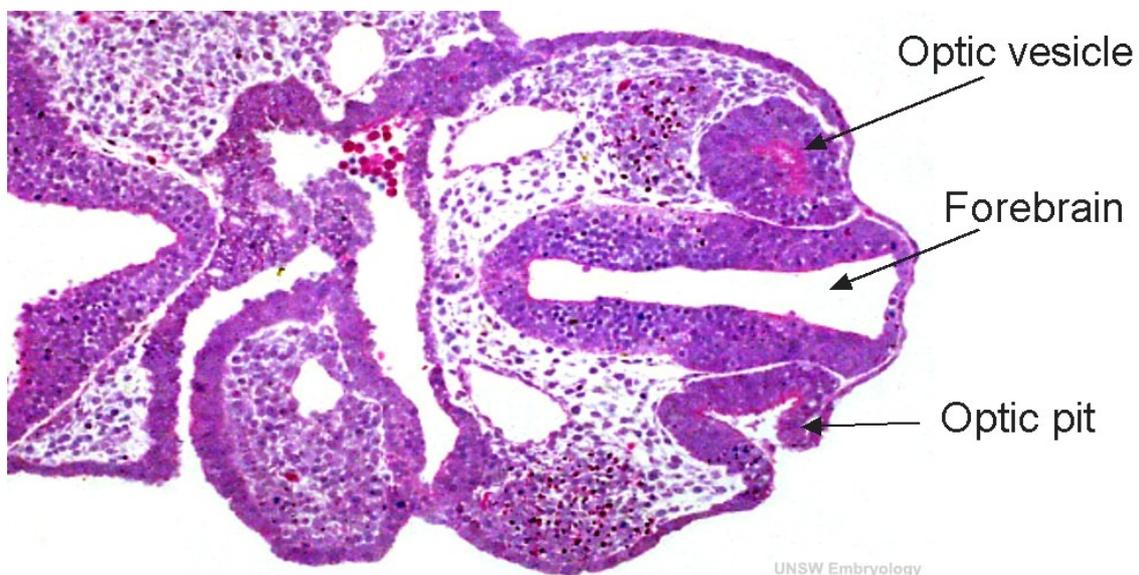
Figure 1.1 This is a ventral histological section from the human embryo at Carnegie stage 11 (25 days) showing: an optical vesicle, the forebrain and an optic pit.[4]

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After 4 weeks, as the optic vesicle doubles back on itself (invaginates) forming the optic cup (Figure 1.2 C & D), the external retinal layer becomes the pigmented layer of the retina and the internal layer differentiates into the neural layer. The point where these two layers meet anteriorly will extend over the developing lens to form the pupillary opening and the iris.[1]
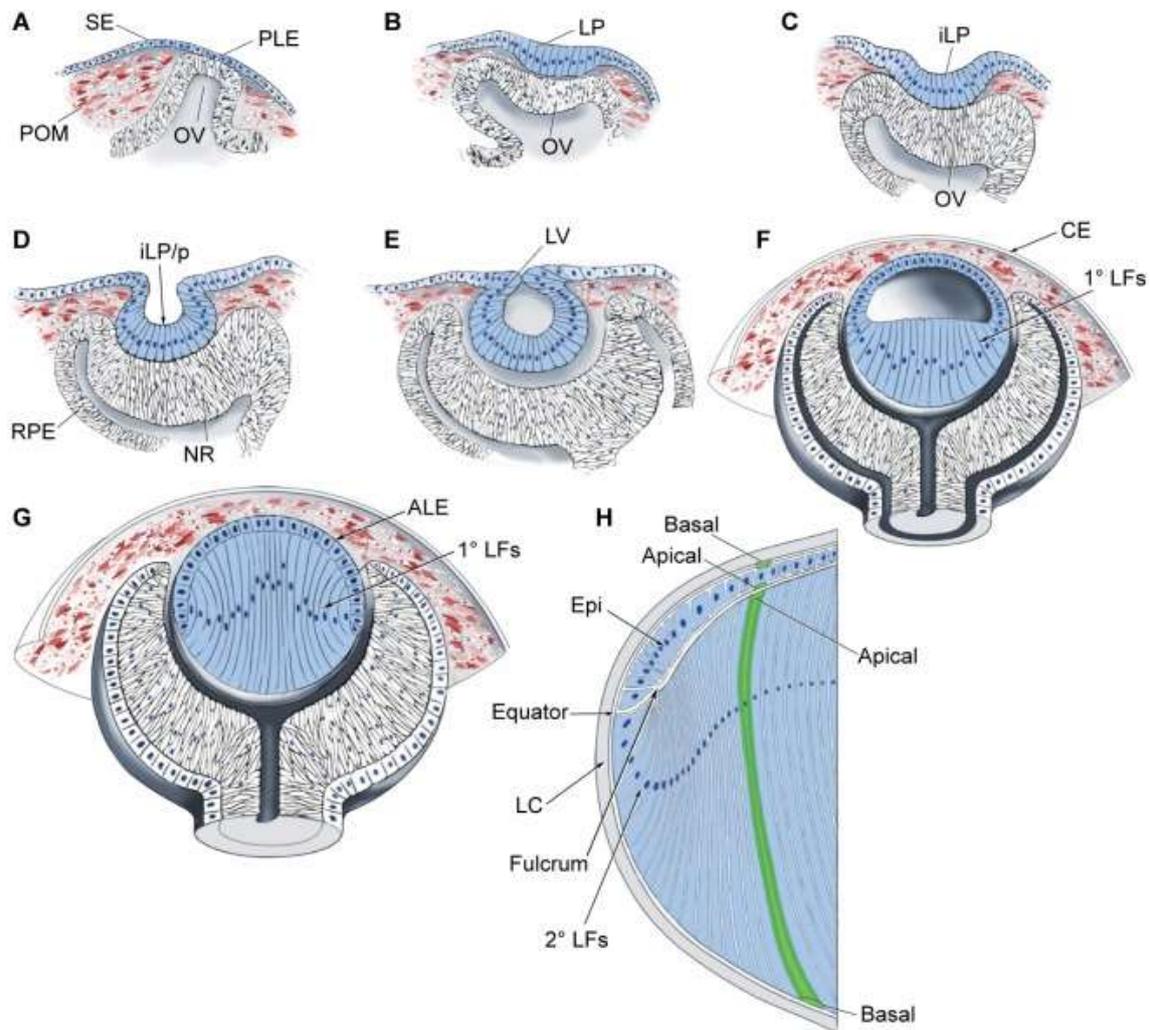
During the sixth week, the invaginating lens placode loses its connection to the surface ectoderm, forming the lens vesicle (Figure 1.2 E). The inner cells of the lens vesicle multiply and extend primary lens fibers towards the opposite external cells, gradually filling the lens vesicle (Figure 1.2 F). By the seventh week, these fibers extend between the walls of the vesicle, filling it; forming the embryonic lens nucleus (Figure 1.2 G).[1]

Lens epithelial cells (LECs) are polarized with an apical surface that faces the lens nucleus and a basal surface that attaches to a thick basement membrane, the lens capsule (Figure 1.2 H). LECs form a monolayer lining the inner surface of the anterior lens capsule up to the equator. As LECs differentiate into lens fibers, the apical tips of the cells constrict to form an anchor point at the equator, known as the fulcrum. Lens fiber cells elongate, maintaining contact to LECs at their apical end and to the lens capsule at their basal end, until both ends terminate at opposite poles of the lens (Figure 1.2 H). The precise alignment and orientation of these fibers generate the characteristic lens suture lines.[5,6]

Figure 1.2 Stages of lens formation in mouse embryos. Schematics showing the stages of lens development at various points during mouse embryogenesis. (A) E9.0, prospective lens ectoderm. (B) E9.5, lens placode. (C) E10, invaginating lens placode. (D) E10.5, invaginating lens placode to lens pit. (E) E11, open lens vesicle. (F) E12.5, primary lens fiber cell differentiation. (G) E13.5-E14.5, completion of primary lens fiber cell elongation to secondary lens fiber cell formation. (H) Lens growth and secondary lens fiber cell differentiation in adult ocular lens. The apical-basal polarity of lens epithelial and fiber cells is indicated. The area where the apical tips of elongating epithelial cells at the equator constrict to form an anchor point before fiber cell differentiation and elongation at the equator was recently named the 'lens fulcrum' (Sugiyama et al., 2009). ALE, anterior lens epithelium; CE, corneal epithelium; iLP, invaginating lens placode; iLP/p, invaginating lens placode/lens pit; LC, lens capsule; Epi, lens epithelium; LP, lens placode; NR, neuroretina; OV, optic vesicle; POM, periocular mesenchyme; 1° and 2° LFs, primary and secondary lens fibers; PLE, prospective lens ectoderm; RPE, retinal pigmented epithelium; SE, surface ectoderm. **(Cvekl A. and Ashery-Padan R. 2014)[5]**

After synthesis of the full complement of lens specific proteins: α, β and γ crystallins, the cell organelles and nuclei of the primary lens fibers are broken down in a process similar to apoptosis, while leaving the fibers intact.[7] Following organelle degradation, lens fiber

cell membranes form ball and socket structures in the deep cortex, minimizing intercellular space and preventing fibers from sliding along each other. This is important for a homogenous refractive index over the whole lens and for the flexing of the lens, as a whole, during accommodation.[5] Furthermore, once secondary lens fiber cells reach a crystalline content around 35–40 %, they also start to lose cell organelles and nuclei in the same manner as described for primary lens fibers. This absence of organelles minimizes light scattering within the pupillary space.[7]

The anterior lens capsule thickens with collagen type IV secreted by anterior LECs, but due to the different ability to synthesize collagen of LECs and the posterior endings of fibers, the lens capsule is thicker anteriorly and thinner in the posterior pole.[8]

The lens keeps adding layers of fibers (secondary lens fibers) to its circumference throughout life, at a pace which decreases with age but continuously increases its size.[1]

It is accepted that the transparency of the crystalline lens can be attributed in large part to the complex and organized arrangement of its components at both microscopic and molecular levels. At the microscopic level, the regular array of flattened, hexagonal cross-sections of fiber cells epitomizes lens tissue to most researchers and clinicians. At the molecular level, the homogeneous packaging of proteins close together in the cytoplasmic network, minimizes scatter by white light. Hence, the light scattering of the concentrated protein solution is significantly low, leading to transparency.[9]

1.1.2. Structure of the normal adult lens

The human lens is a uniquely transparent, biconvex, avascular structure that, together with the cornea, refracts light in order to focus it on the retina. The refractive power of the lens in its natural environment is approximately 18 diopters, roughly one-third of the eye's total power while the cornea accounts for the other two-thirds, approximately 43 diopters, of the eye's total optical power. The anterior surface of the lens is less curved than the posterior. In adults, the lens is typically around 10 mm in diameter and has an axial length of about 4 mm.

The lens and cornea are part of the anterior segment of the human eye. Behind the cornea is the anterior chamber, filled with the aqueous humor. In front of the lens is the iris, which regulates the amount of light entering the eye. Posterior to the lens is the vitreous body, which, along with the aqueous humor on the anterior surface, bathes the lens. The lens, especially the outer cortex with its proliferating and differentiating LECs, is a metabolically active organ and its transparency strongly depends on proper

nourishment. Because of its avascularity the lens depends on the surrounding aqueous and vitreous for its supply of nutrients, oxygen and for the removal of waste products.[10]

The lens is enclosed by a thick capsular basement membrane, which is a secretory product of the lens epithelium, and suspended in place by the suspensory ligament of the lens (zonules), a ring of fibrous tissue that attaches to the lens at its equator and connects it to the ciliary body. The lens capsule is the thickest basement membrane in the body (3–10 μm).[11]

The lens has the unique ability to change its shape, in order to change the focal distance of the eye so that it can focus on objects at various distances, thus allowing a sharp real image of the object of interest to be formed on the retina. This adjustment of the lens is known as accommodation. Accommodation is similar to the focusing of a photographic camera via movement of its lenses.[12]

The accommodative process in the lens is dependent upon the inherent elasticity of the lens capsule, the contractile property of the lens cells and the forces applied by the ciliary muscles through the zonular fibers.[13] Within the eye, the lens is supported by the axially-oriented zonular fibers which connect to the ciliary body (a sphincter muscle). The ciliary body acts as a unit and contains a mixture of meridional, radial and circular muscle fibers, just behind the root of the iris. When accommodation is 'relaxed' for distance vision, the ciliary ring increases its diameter and the zonular fibers are stretched. The tension in the zonular fibers exerts strong radial forces on the capsule, stretching it, causing the lens to flatten and attain the lower power required for distance vision (Figure 1.3, Left).[14] Contraction of the ciliary body during accommodation, when the main muscle mass moves towards the axis, reduces the diameter of the ciliary ring. This allows the tension in the zonules to relax and reduces the stretching forces acting on the lens capsule. The crystalline lens can then move towards its natural, rounder and more powerful form for near vision (Figure 1.3, Right).[13, 14]
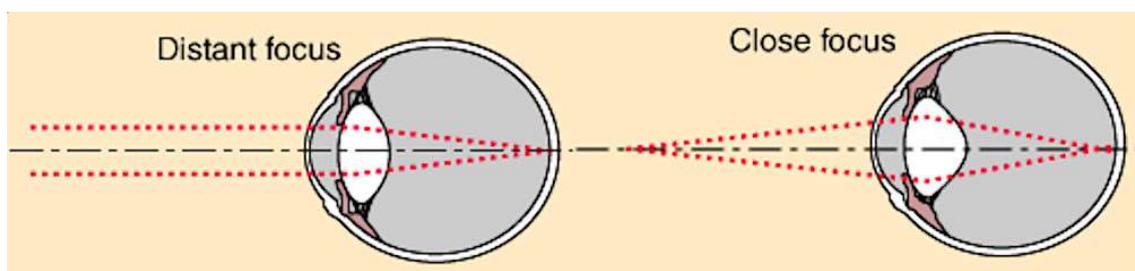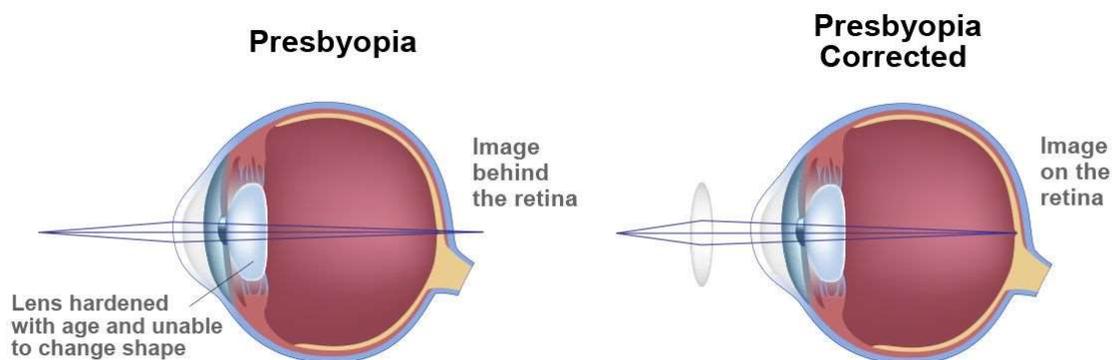

Figure 1.3 Graphic representation of visual accommodation. To shift the eye's focus from a distant target (Left) to a close target (Right), the eye muscles controlling lens thickness receive different signals. Relaxing in order to stretch and flatten the lens or contracting in order to let the lens become rounder. **(Sweet, B.T. and Kaiser M.K. 2011)**[14]

However, as we age, there is a progressive decline in the ability to accommodate the lens, once the subjective amplitude of accommodation falls below three diopters, this limiting condition is known as presbyopia. The ability to accommodate falls almost linearly with age, starting at around the age of 40 and reaching zero at around the age of 50.[15, 16] Everyone eventually develops presbyopia, but symptoms may vary. The major risk factor for presbyopia is age although the condition may be affected by other factors including disease, trauma and medications.[17]

A variety of factors are considered to play a role in the development of presbyopia, such as, changes in the internal gradients of refractive index, a reduction in the elasticity of the capsule, an increase in capsular thickness, a reduction in ciliary body activity and an increase in lens stiffness.[13, 17]

While many of these factors may play a part, the most important and widely accepted seems to be that the lens loses viscoelasticity and becomes harder to deform with age. It has been suggested that the changes in the mechanical characteristics of the lens occur as a result of increased adhesion and compaction of the nuclear fibers, in part due to oxidized protein sulfhydryl groups within lens fiber cells that form intraprotein cross-links. Also, continuous lens growth increases the inelastic mass that must change shape for accommodation to occur.[13, 17-19]

Current treatments for presbyopia are corrective in nature either by optical (bifocals, trifocals or contact lenses) (Figure 1.4) or surgical refractive modifications (accommodative intra-ocular lenses, laser or conventional corrective surgical techniques).[17]

Figure 1.4 Graphic representation of the visual effects of presbyopia. The stiffened lens cannot accommodate to focus light from near objects on the retina, instead, it focuses light behind the retina, thus blurring the image (Left). In order to adjust the focal point, corrective lenses can be used in front of the eye in order to focus near images on the retina. **https://www.fishmansheridan.com/conditions-we-treat/presbyopia**

However, none of these treatments help to regain the ability to accommodate.[16] Recently, some promising new pharmaceutical approaches have been suggested to reduce the effects of presbyopia, but no clear results have been obtained.[20] Currently, presbyopia is still a condition that will affect every adult, in both the developed and developing world, reducing quality of life.[17]

1.2. Cataract development and treatments

Further ageing of the lens can lead to the formation of cataracts. Cataracts are the world's leading cause of blindness and the second cause of moderate and severe vision impairment according to the Global Burden of Disease, Injuries and Risk Factors Study (GBD) especially due to its prevalence in underdeveloped countries. A large number of etiological factors for cataracts have been identified, of which ageing is the most common.[21]

According to the GBD and the World Health Organization (WHO) data, cataracts account for 33% and 48% of global visual impairment respectively. The GBD showed that there were 10.8 million cataract blind people (1/3 of blind people worldwide) in 2010. The WHO has estimated that this number will increase to 40 million in 2025 as populations grow and age, with greater life expectancies.[22]

From a clinical point of view, cataracts can be defined as a clouding of the lens in the eye that impairs vision, while biochemically, cataracts are due to the accumulation of denatured proteins, yellow pigments and oxidative stress in the normally transparent lens fiber cells[23], this can lead to a slight clouding or distortion of vision, to total blindness (Figure 1.5).
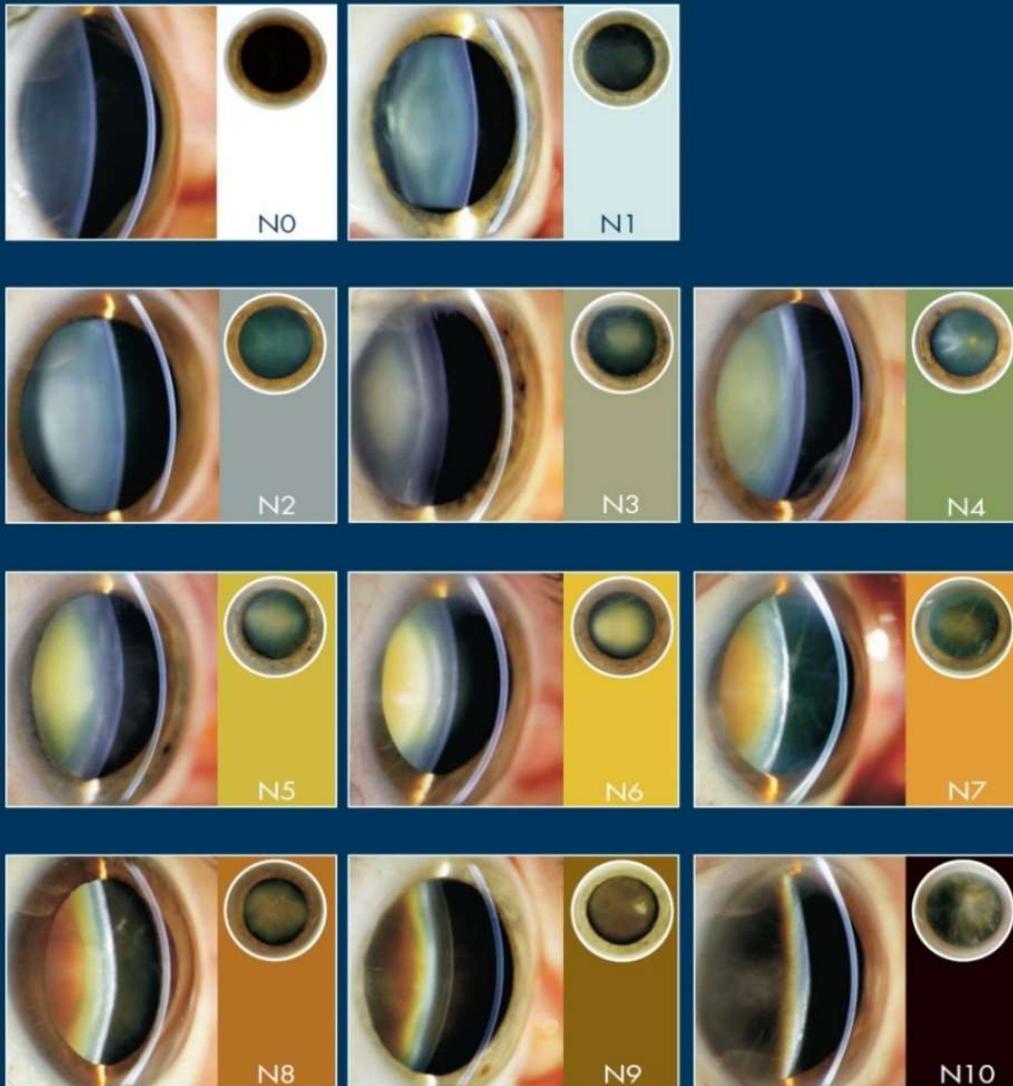
Figure 1.5 BCN 10 nuclear grading system chart showing a slit-lamp cross-sectional image, a smaller frontal view image, and the relative color for each stage of cataract development (N0 to N10). **(Barraquer, R.I. et al. 2017)**[24]

There are various known factors that can lead to the formation of cataracts, such as, genetics, metabolic, chemical or mechanical insults occurring to the lens, irradiation, trauma, disease or just ageing.[25]

Inherited or congenital cataracts can be due to genetic mutations and present all types of inheritance patterns in a syndromic and nonsyndromic form with more than 100 causative genes. However, congenital cataracts are defined as any unilateral or bilateral lens opacification present at birth or early childhood. Thus, congenital cataracts can also be due to conditions such as, maternal nutrition, infections and a deficiency of oxygenation due to placental hemorrhage.[26, 27]

On the other hand, the most common type of cataract is Age-related or Senile Cataract, which as its name implies, is simply due to aging and usually start around the age of 50-60.

Traumatic Injuries can also lead to cataracts and are the most common cause of unilateral cataract in young adults. The causes of these injuries can be perforating or blunt trauma[28], electric shock, ultraviolet radiation[29], ionizing radiation[29] or chemical injuries.[30]

Different types of diseases can produce cataracts as a direct or indirect side effect. This is the case in primary ocular diseases such as, chronic anterior uveitis, acute congestive angle closure, high myopia or hereditary fundus dystrophies like retinitis pigmentosa.[31] This can also happen in systemic diseases, such as, myotonic dystrophy, atopic dermatitis or neurofibromatosis type 2 [32] and in endocrine diseases, such as, diabetes mellitus, hypoparathyroidism or cretinism.[33]

External environmental factors such as drugs like corticosteroids or anticholinesterase, alcohol abuse, smoking or poor nutrition (specifically a diet deficient in anti-oxidants and vitamins)[34] can all accelerate the development and formation of cataracts.[35, 36]

Not only are there various possible causes for cataracts, but there are different types of cataracts, depending on the region of the lens that becomes opaque(Figure 1.6A).[37, 38] The most common form of cataracts is nuclear cataracts (Figure 1.6B) which are usually due to deposition of pigments or denatured proteins in the lens nucleus. Another form is cortical cataracts (Figure 1.6C), which are characterized by white, wedge-like opacities that start in the periphery of the lens, in the cortex, and work their way to the center in a spoke-like fashion. Cortical cataracts are associated with the local disruption of the structure of mature fiber cells, this can be due to the sheer stress between a stiff older lens nucleus and soft lens cortex.[39] Finally, there are subcapsular cataracts (Figure

1.6D), which can either occur on the anterior capsule due to aberrations in the LEC monolayer, such as fibrous metaplasia, or on the posterior capsule, appearing as granulose clouding.
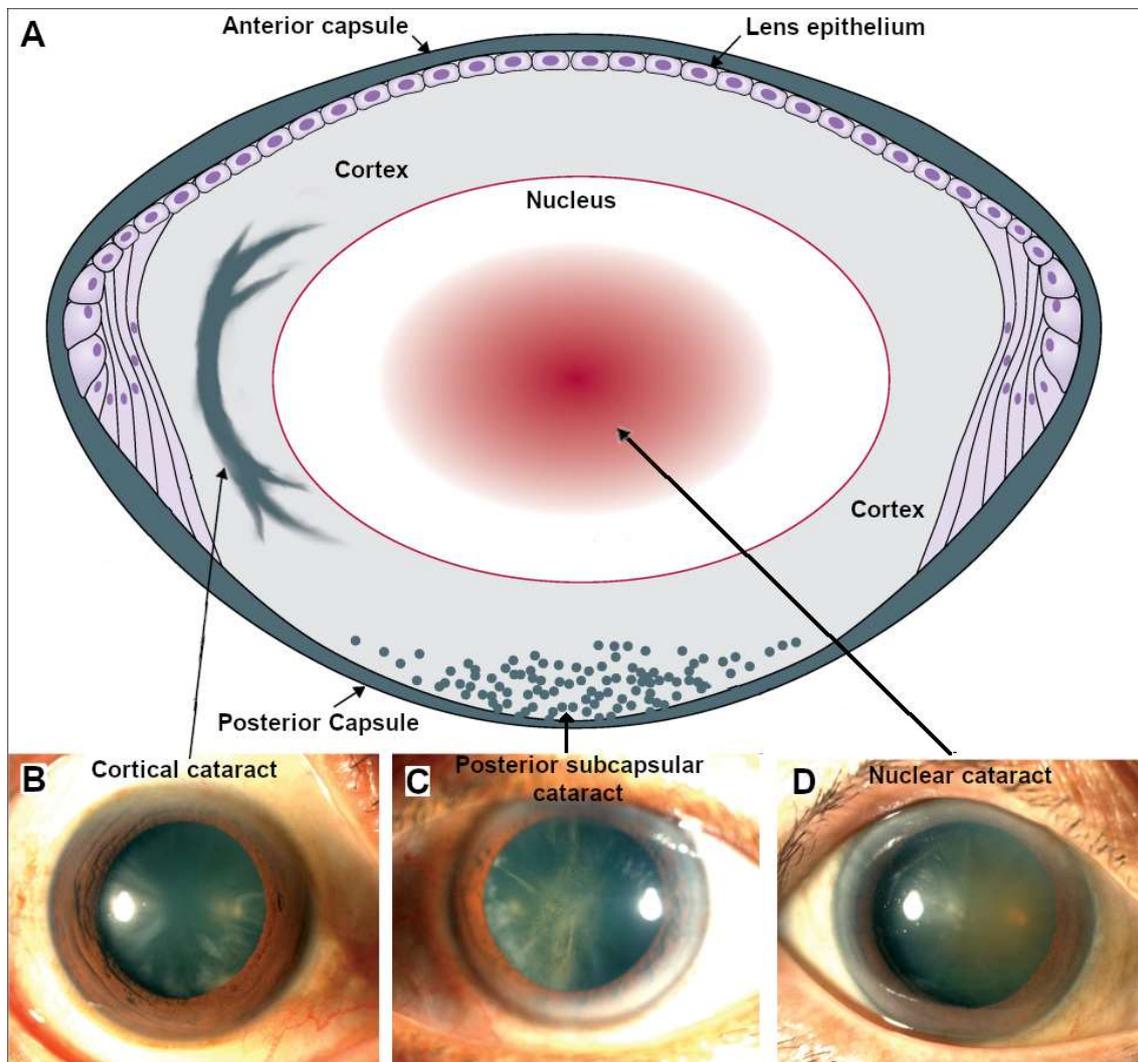
Figure 1.6 Characteristics of lens structures and different types of cataracts (A) A schematic view of lens structures and corresponding types of cataracts. Slit lamp biomicroscopy photos showing (B) nuclear cataract, (C) wedge-shaped cortical cataract, and (D) subcapsular posterior cataract that has plaque opacity in the axial posterior cortical layer. Most patients have more than one type of cataract. Figure adapted from (Liu Y.C. et al. 2017)[38]

Cataract surgery, which was first documented in the fifth century BC, is one of the oldest and most frequently performed surgical procedures in the world. The first technique implemented to treat cataracts was called "couching", which consisted in dislodging the mature cataract out of the visual axis and into the vitreous cavity. This could be accomplished either by striking the eye with a blunt object with sufficient force to break the zonules or by inserting a sharp fine instrument into the eye to break the zonules to

cause the dislocation.[40] These methods could only be performed when the lens had become completely opaque, rigid, and heavy to the point that the supporting zonules had become fragile.[41] The cataract remained in the eye but no longer blocked light, producing a limited yet instantaneous improvement in vision (Figure 1.7). Certainly, in the very immediate postoperative period, couching was considered a success, but the fact that the cataractous lens remained in the eye and the lack of aseptic conditions soon had deleterious effects on the eye, often resulting in blindness shortly after the procedure. Unfortunately, couching is still in practice in some developing countries[42].
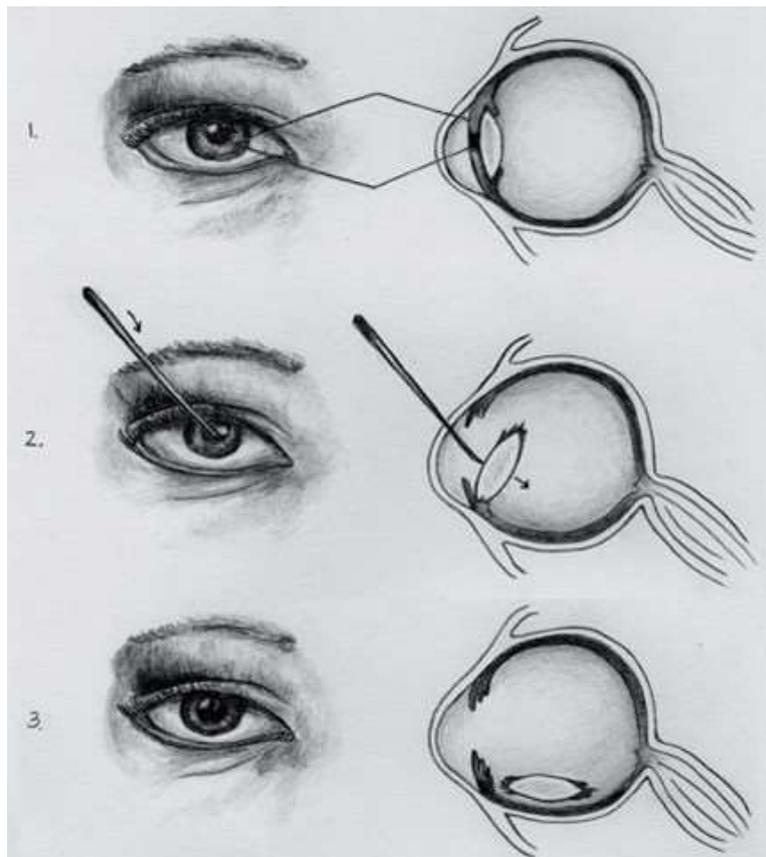
Figure 1.7 Schematic representation of the eye (1) and the couching process, a sharp fine instrument is inserted into the eye to break the zonules (2) and cause the dislocation of the lens in to the posterior chamber of the eye (3). http://www.uniteforsight.org/traditional-eye-practices/module3

In the 18th century a new surgical technique, intracapsular cataract extraction (ICCE), was developed and became the norm.[43, 44] ICCE is a procedure in which the opacified lens within its capsule is removed in one piece through a limbal incision using traction and pressure from the thumb.[45] Since after the surgery, the patient's eye is left aphakic (without an artificial or natural lens), vision was further corrected with extremely thick eyeglasses or by contact lenses. As was to be expected, the procedure had high rates

of complications due to the large incision (8-10mm), the traction on the zonules and pressure placed on the vitreous body.[46]

Some of these problems were mitigated by the development of innovative new tools. For example, the need to apply pressure to the vitreous body was avoided through the development of a suction cup–like devices to remove the lens by Prof. Ignacio Barraquer in 1917.[47]

Another innovation to reduce zonular traction, was the use of the enzyme alpha Chymotrypsin during surgery in order to selectively dissolve or weaken only the lens zonules in the eye (zonulolysis), vastly facilitating the removal of the lens after only 2-3min of the treatment, this technique was pioneered by Joaquín Barraquer in 1958[48].

In the early 1950s, the advance of microsurgical instrumentation allowed for the development of extracapsular cataract extraction (ECCE). This procedure consists of the surgical removal of the lens through an anterior opening in the lens capsule, leaving the rest of the capsule intact. The anterior capsule is opened by means of an anterior circular continuous capsulorhexis, a circular opening, usually of 5-6mm, that prevents further tearing of the elastic capsule. This allows for the implantation of an artificial intraocular lens (IOL), of appropriate refractive power (Figure 1.8)[49], within the remaining capsule. Originally the lens content was removed through a large 12 mm incision in the sclera or cornea, however, further technological advancements led to the development of sutureless ECCE surgery.[50]

Currently, surgeons either use ultrasonic fragmentation (phacoemulsification)[51-53], or manual fragmentation [54] of the lens in order to remove it from the capsule. With phacoemulsification, the lens nucleus is emulsified by ultrasonic waves and is aspirated together with the cortex (Figure 1.8). These changes in extraction technique, allow incisions to be between 2.4 to 2.8mm in width, although they can be even smaller for certain IOLs and larger for others.
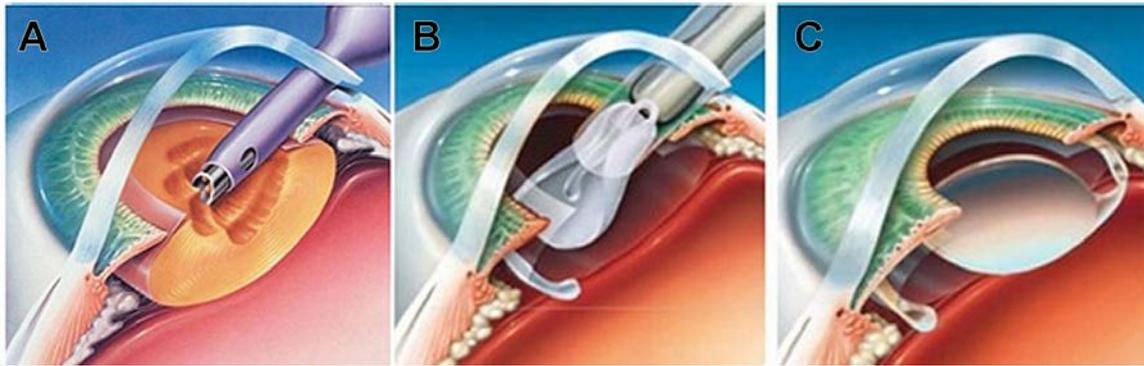
Figure 1.8 Schematic representation of modern cataract phacoemulsification surgery with implantation of a foldable IOL. First the lens material is broken up with ultrasonic vibrations, emulsifying it, and aspirated simultaneously (A). Once the capsule is empty the folded IOL is injected in to the capsule (B). Finally, the lens unfolds and sits wedged in the capsular bag (C). **https://nurseslabs.com/cataract/**

Since ECCE leaves the posterior capsule in place, the anatomical barrier between the posterior and anterior segments of the eye remains and reduces the risk of posterior segment complications after surgery. However, this also has a disadvantage, the posterior lens capsule can become cloudy.[54, 55]

1.2.1. *Impact of cataract surgery on lens epithelial cells*

The current norm for operating cataracts is phacoemulsification, while it is currently the best treatment for cataracts, LECs can be negatively affected by the surgery. During surgery the contents of the lens capsule are extracted through a combination of irrigation and aspiration. Almost all the LECs lining the remaining part of the anterior capsule can be aspirated. However, realistically not all the LECs can be removed, especially those at the equator of the bag. These will remain in the bag together with the newly implanted IOL. They will not only be injured mechanically by the instruments but also by the circulating nuclear fragments, the irrigation solutions and their osmotic effect. Many *in vitro* studies have shown that these injured LECs react to this surgical trauma within 24 hours by proliferating and can cover the posterior and anterior capsule completely within as little as one week.[56] This outgrowth of LECs can be interpreted as a wound healing response and will lead to PCO[55, 57].

1.3. Posterior Capsule Opacification

While ECCE is a highly effective procedure with a low initial complication rate, the medium and long-term outcomes are less promising due to the occurrence of PCO some two to five years after surgery in 20-40 percent of patients depending on numerous factors including patient age, surgical method, type of IOL implanted and post-surgical follow-up time.[58, 59]

PCO, also referred to as secondary or after-cataract, can clinically be defined as changes on the posterior capsule leading to loss of vision and necessitating a second surgical intervention to restore vision.[60-62] Despite its name, it is not exactly the posterior capsule itself that becomes opaque, rather, the opacification is caused by the proliferation, migration and transdifferentiation of residual lens epithelial cells (LECs) on the internal face of the capsular bag[55, 63, 64] (Figure 1.9).
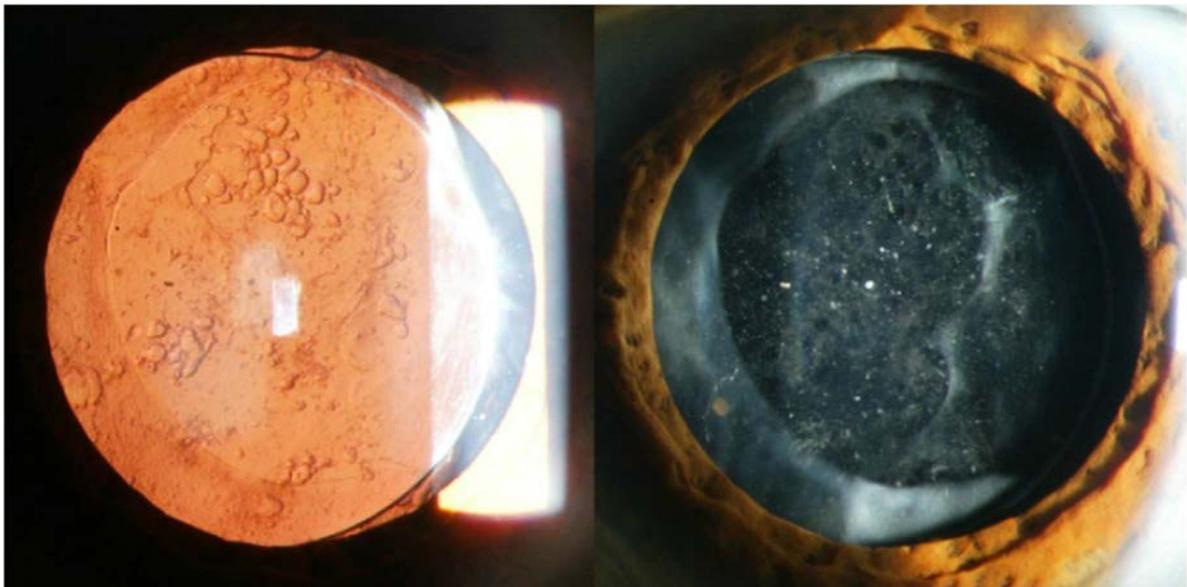
Figure 1.9 Retroillumination (Left) and slit-lamp-derived, reflected-light (Right) images of PCO in the same eye. Different aspects of PCO morphology are observed in each image. **(Montenegro G.A. et al. 2010)** [65]

Residual LECs are the epithelial cells left in the capsular bag after cataract surgery, when these cells migrate and proliferate on the posterior capsule, they can impair vision by blocking and forward scattering incoming light. Furthermore, the contraction of cells as they migrate, can cause wrinkling of the posterior capsule and even, traction-induced IOL displacement (tilting or dislocation), resulting in further visual distortions and glare.[66]

PCO has been observed and documented as early as when the first extracapsular cataract extractions (ECCE) had been introduced to ophthalmology. Ridley already

reported on the occurrence of PCO after having performed his first IOL implantations in the 1950s [67, 68]

1.3.1. Types of posterior capsule opacification

Clinically two different types of PCO can be distinguished, depending on how the residual LECs develop.

PCO is considered fibrotic if the LECs undergo epithelial to mesenchymal transition (EMT), changing their morphology, deposit mesenchymal type extracellular matrix proteins (such as proteoglycans and collagen fibrils) and wrinkle the posterior capsule. (Figure 1.10)

Alternatively, PCO is regenerative if the LECs undergo fiber cell differentiation, presumably in an attempt to regenerate the lens[69], giving rise to Elsching's plates, pearls and Soemmerring's rings[70] (Figure 1.10).

Although fibrotic PCO tends to appear somewhat earlier (around 6 months) than regenerative PCO (after several years), they can still appear together.
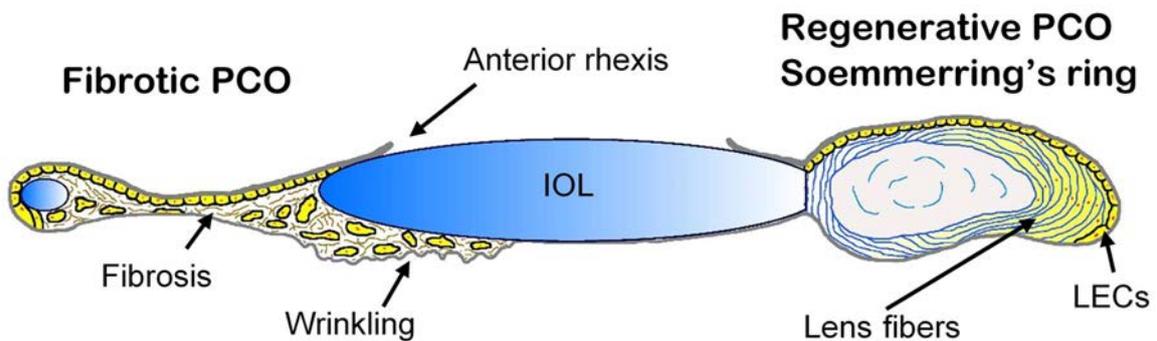
Figure 1.10 Schematic representation of different types of PCO developing around an IOL (Intraocular lens). On the left is represented typical fibrotic PCO where cells have deposited extracellular matrix proteins and contracted the posterior capsule. On the right is represented an advanced type of regenerative PCO known as a Soemmerring's ring, where LECs have reorganized into a monolayer and fiber cell differentiation is taking place.

Furthermore, the two types of PCO seem to be related to the two types of LECs, the anterior epithelial cells (A-cells) and the equatorial lens bow cells (E-cells). Although all

LECs originate from a single cell line, these can be divided into two different functional groups, which is useful for better understanding their role in the pathological process[71].

A-cells while under normal physiological conditions are relatively quiescent, with minimal mitotic activity, and form only a monolayer of cells on the inner face of the anterior capsule.[72, 73] However, after surgery, A-cells that are not removed undergo changes. First, they become almost immediately opaque, probably as a result of the swelling induced by the surgical trauma. Then they proliferate and undergo fibrous metaplasia (EMT) gaining motility, allowing them to migrate over the capsule. This causes anterior and posterior capsular opacification, posterior capsule wrinkling and in some cases contraction of the anterior capsule, all of this promotes fibrotic PCO.[71, 74] However, some studies have shown the tendency of A-cells to maintain or regain their original shape and mitotic properties after analyzing post-mortem human capsular bag samples, years after cataract surgery, which showed a stable monolayer of A-cells on the inner surface of the anterior capsule.[75]

E-cells are found in the equatorial region of the capsule, all around the lens periphery. These cells are moderately mitotically active throughout life and can be considered as stem cells.[76] After mitosis, one of the daughter cells remains in the equatorial region and starts a new cycle of mitosis, and the other differentiates in to a lens fiber cell. As a result of cataract surgery, these cells can lead to both fibrotic and regenerative PCO.[77] However, E-cells contribute more to the formation of pearls, plates and Soemmerring's rings through fiber cell differentiation.[72] This behavior can be considered as a wound healing response after surgical trauma. Furthermore, E-cells can reform a lens bow-like structure at the capsular equator, where they can differentiate to lens fibers. This will lead to the formation of the Soemmerring's ring, a donut like growth composed of newly formed fibers[78].

To a certain extent, a Soemmerring's ring is formed in virtually all lens capsules after extracapsular cataract extraction.[74, 79] Interestingly, it has been shown that the presence of newly formed lens fibers can decrease the mitotic activity of E-cells, thus reducing the risk for PCO progression.[72] This inhibition could explain why LEC migration slows down or even stops in 60 to 80 % of patients after cataract surgery. Although it has also been suggested that given enough time, PCO will develop in all post-surgical cases. For example, even when a Soemmerring's ring is formed, over time some cells will migrate into the space behind the IOL where they can form Elsching's pearls.[63]

1.3.2. Lens epithelial cell responses leading to PCO

As previously implied, PCO is initiated by the inflammatory response of the body to the trauma caused by cataract surgery. However, it is not yet very clear which factors trigger the LECs to proliferate, migrate and differentiate after ECCE. Currently the major stimulating factor seems to be the disruption of the blood aqueous barrier during surgery, this would activate the release of inflammatory mediators such as cytokines and local growth factors.

Cytokines are peptides secreted from cells which can then act in either a paracrine or autocrine way on their target cells. Various studies have identified cytokines as key elements in the development of PCO. Transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) are considered the main cytokines involved in PCO.[80-84]

TGF-β is known to affect LECs in various ways, promoting tissue fibrosis, EMT, myofibroblast formation, fibrosis, cell proliferation, and apoptosis. It does this by up-regulating genes such as α-SMA and tropomyosins, which are implicated in a variety of pathogenic processes, including cataractogenesis.[85]

TGF-β inhibits epithelial cell growth but induces mitosis in mesenchymal cells such as fibroblasts. It is known to control the differentiation of a variety of cell types, such as metaplastic transformation of LECs. It is important in embryogenesis, where it is expressed at high levels in areas undergoing rapid morphogenesis. It has even been shown to induce cataractous changes in rat lenses.[86] TGF-β has also been shown to be important in modulating the remodeling of the extracellular matrix through the production of components like collagen and it also controls cell-cell and cell-matrix interactions. Human LECs produce TGF-β *in vitro*, and both TGF-β1 and β2 have been immunolocalized to equatorial and anterior LECs, with relatively stronger labelling at the equator.[87, 88] The equatorial cells also expressed the receptors for β1 and β2, indicating that there is autocrine stimulation.[80]

Several mechanisms for TGF-β activation have been reported, including proteases, trauma, integrin, and Reactive oxygen species (ROS).[89] In addition, TGF-β1 has been shown to increase ROS generation through the down-regulation of antioxidant-related genes.[85]

bFGF is a polypeptide which influences the proliferation and differentiation of a variety of cells, in the lens it stimulates both LEC mitosis and differentiation in to fiber cells. High levels of bFGF have been observed in the vitreous humor after cataract surgery.[81]

The concentration of TGF-β is lowest immediately after cataract surgery, which is when bFGF seems to be most active. After TGF-β returns to normal levels, about 2 weeks after surgery, it can induce further PCO changes like myo-fibroblastic differentiation, extracellular matrix formation, and attachment of LECs to the posterior capsule through α-SMA expression.[80] Furthermore, bFGF has been shown to exacerbate the effects of TGF-β.[90] (Figure 1.11)
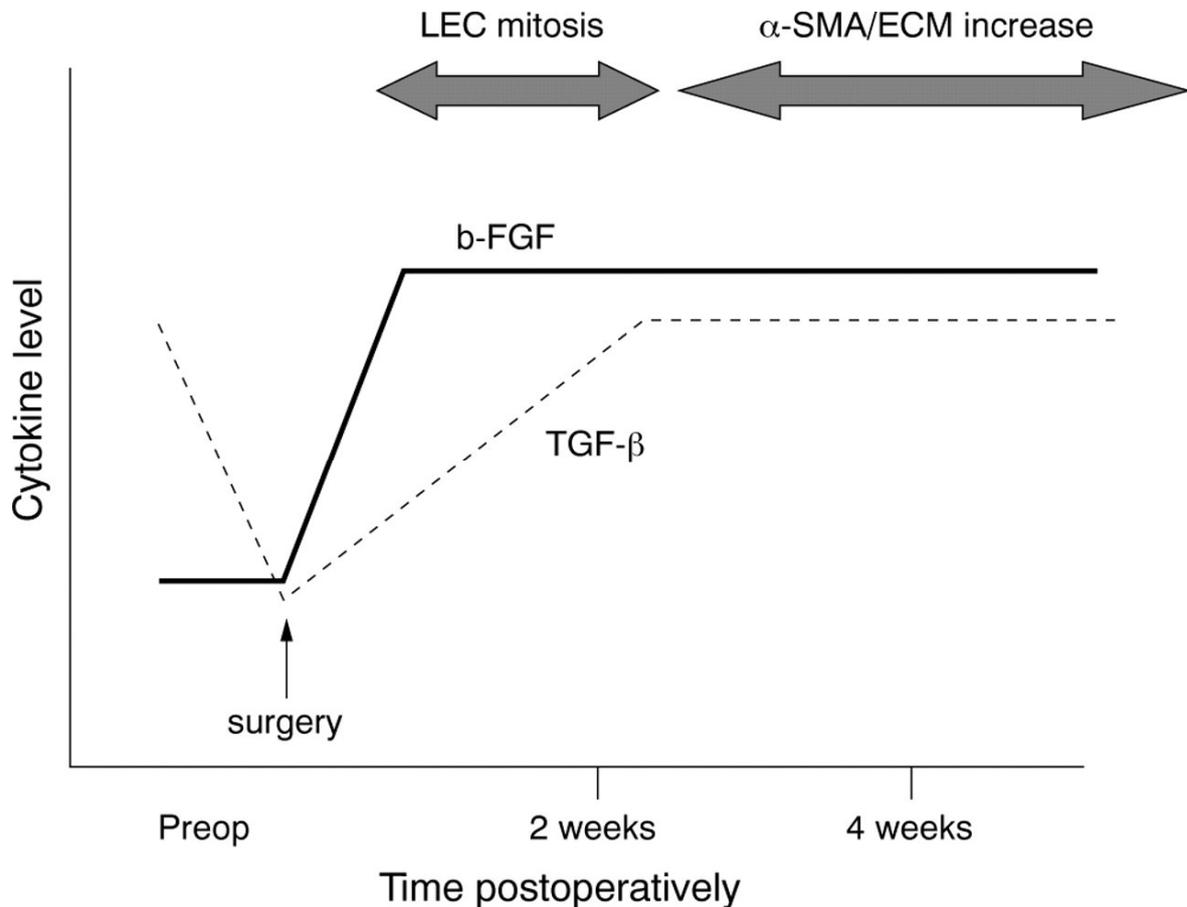
Figure 1.11 Graph expressing the concentration and actions of TGF-β and b-FGF following cataract surgery in rabbits. LEC (Lens epithelial cells); α-SMA (Alpha smooth muscle actin) b-FGF (Basic fibroblast growth factor) TGF-β (Transforming growth factor beta) **(Meacock, W.R. et al. 2000)[91]**

Various other cytokines, such as epidermal growth factor (EGF), insulin-like growth factor (IGF-II), hepatocyte growth factor (HGF), and Notch can also trigger EMT of LECs through aberrant signaling.[92] These changes in signaling patterns can be due to the changes in the blood-aqueous barrier as a result of cataract surgery.[80]

However, despite the obvious effects of the exogenous growth factors that are introduced through disruptions in the blood aqueous barrier, the endogenous growth factors

produced by the LECs are enough to promote proliferation, migration and differentiation of residual LECs.[93]

Furthermore, the removal of the lens content itself might be enough to induce PCO without the participation of cytokines or local growth factors, due to cell-cell inhibition of mitosis.[72] Most LECs stop proliferating as soon as the available space is filled up, and this might be another reason why LECs start to proliferate after lens extraction.[94, 95]

1.3.3. Treatment of PCO

Regardless of the type of PCO or the triggers that led to its development, currently there are only 2 effective methods for treating PCO, surgical or laser assisted capsulotomy. These treatments consist in making an opening in the central posterior lens capsule in order to remove the central opacification, thus providing a clear visual axis and restoring vision (Figure 1.12). However, they are invasive procedures with additional risks for patients, such as: damage or luxation of the IOL, elevation of intraocular pressure, cystoid macular edema, and increased incidences of retinal detachment. This risk further increases in patients with certain preexisting conditions, such as high myopathy which directly increases the risk for retinal detachment after treatment.[55, 96-99]

The most common treatment method used today is neodymium:yttrium-aluminium-garnet (Nd:YAG) laser capsulotomy. Beside the complications, the considerable high procurement costs of a Nd:YAG laser makes laser capsulotomy not universally available for all patients suffering from PCO.

Another factor to take into account is capsulotomy size (Figure 1.12).[100] Smaller capsulotomies benefit from lower risks of retinal detachment, intra ocular pressure rises and IOL dislocation. However, reduced visual acuity due to contrast and glare with small capsulotomies are significantly worse than those with larger capsulotomies.[101]

Furthermore, in the case of children and infants, which are the groups most prone to developing PCO, Nd:YAG laser capsulotomy can usually only be performed under general anesthesia, which presents its own risks.[102, 103]

All these drawbacks indicate that there is a strong need to prevent PCO instead of treating it with laser capsulotomy[59].
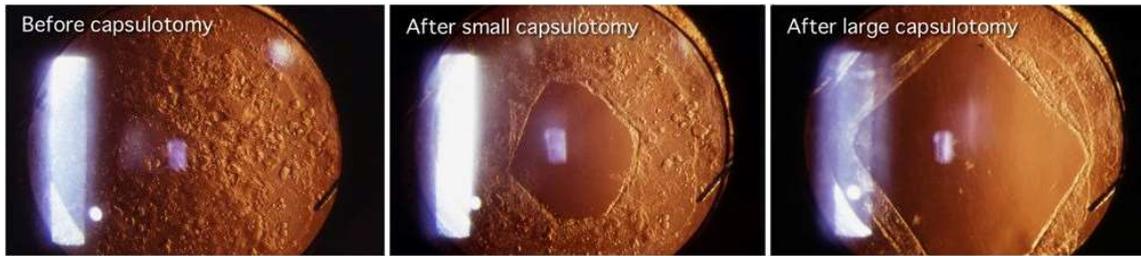
Figure 1.12 Retro-illumination photographs of a representative eye that underwent two-step Nd:YAG laser capsulotomy. The Elschnig pearls type of posterior capsule opacification is seen before Nd:YAG laser capsulotomy (top). An Nd:YAG laser posterior capsulotomy of smaller than the pupillary size was first made (middle). At 2 weeks after this small capsulotomy, the capsulotomy opening was enlarged to greater than pupillary size (bottom). **(Hayashi, K. et al. 2010)** [100]

1.3.4. *Epidemiology of PCO*

Although PCO is still the most common complication after ECCE, with or without IOL implantation, its incidence has decreased over time thanks to improvements in surgical techniques and IOL designs.

Until the early 1990s the prevalence of PCO after cataract surgery was as high as 50 % after just one year.[55] This was most probably due to the lack of cortical and cellular clean up during surgery and that the IOLs used were manufactured of a rigid biomaterial (polymethylmethacrylate = PMMA).

As research in to PCO and cataract surgery progressed, different factors were taken into account during surgery in order to reduce incidences of PCO further. In the early 2000s, postmortem studies highlighted the importance of better cortical clean up, IOL fixation, IOL material and smaller continuous curvilinear capsulorhexis that covered the IOL optic in reducing the incidences of PCO.[104] A study in the early 2000s with over 16,500 postmortem IOL specimens showed that the implementation of these new techniques and types of IOLs had reduced PCO rates to between 3.8 % and 21.7 %.[105]

The three most important recent improvements in IOL design were the development of foldable IOLs, the use of square edged optics and hydrophobic materials, which in the best cases can reduce PCO rates in adults to under 3% at 3 years.[106, 107]

However, despite these improvements, the prevalence of PCO in infants and children is still very high, ranging from 60 to 90%.[108, 109] This highlights the fact that the most important factor in PCO rates is still the patients age.

Not only is the prevalence of PCO a health and quality of life problem, it is a serious financial burden for the health care budgets of developed countries. For example,

according to the Centre for Medicare Services data for the year 2010, they estimated costs for Nd:YAG laser capsulotomy accounted for 187 million USD. Furthermore, the costs of PCO are not limited to the cost of performing Nd:YAG capsulotomy but should also include the cost of managing its complications, which further increases the financial burden.[110] With this, the annual expenses for the treatment of PCO in the United States, are estimated at 250 million USD, which supports the conclusion that "PCO remains the second most expensive surgical cost of the US Medicare System after cataract surgery itself".[111]

Furthermore, in the case of the developing world, due to a lack of adequate equipment or surgical expertise, there is an increased prevalence of PCO. This is commonly left untreated due to insufficient finances and access to proper equipment such as Nd:YAG lasers. Due to these drawbacks, older techniques without risk of PCO but with higher complications for the eye are often preferred such as ICCE often without IOL implantation.

All these problems further highlight the need for PCO prevention.

1.3.5. Prevention of PCO

PCO prevention will not only improve post-surgical outcomes of cataract treatment but can also benefit new techniques that are being developed to treat other lens problems such as refractive errors and presbyopia. Recently, ECCE is also being used to correct visual acuity for both distant and near vision using new IOLs that have the ability to accommodate. Due to the design of some of these new IOLs, PCO instances increase, affecting ocular transparency and IOL stability which seriously impairs the visual outcome of these accommodative IOLs.[112, 113]

Two major strategies have been used to prevent PCO: surgical techniques to minimize the number LECs left in the capsular bag after surgery and non-surgical techniques to stop the remaining LECs from proliferating and migrating into the visual axis.

1.3.5.1. Surgical methods to prevent PCO

Every aspect of cataract surgery is important for the prevention of PCO development and many improvements have been made over the years that have reduced PCO rates, of these, the most important aspects are:

The size and technique used to perform the anterior capsulorhexis, which are known to directly affect visual outcome and PCO. Originally the anterior capsule was simply torn

in order to remove the lens nucleus, but this promoted PCO, glare and even IOL extrusion. Currently the best technique is laser assisted continuous curvilinear capsulorhexis (CCC), which allows for very precise and regular openings. Furthermore, using a diameter slightly smaller than the diameter of the IOL optic is known to better support the IOL and reduce PCO by reducing contact with the aqueous humor.[114-116]

Another aspect is lens content removal, which has been improved with the use of two relatively simple techniques, hydrodissection and anterior lens capsule polishing. Hydrodissection separates the nucleus from the capsule utilizing low hydrostatic pressure and precise kinetic movement of a balanced salt solution. This facilitates the phases of phacoemulsification and irrigation/aspiration, reducing residual lens fragments and the risk of capsule perforation or rupture.[117-119] Capsular polishing consists of manually "scraping" the anterior capsule with a blunt or rough cannula to further remove and aspirate the monolayer of LECs. However, this is complicated due to the fact that even when dilated, the iris still covers part of the equator of the capsular bag.[66, 120]

Another important aspect is the fixation of the IOL within the capsular bag. This improves optic centration and reduces PCO by enhancing the IOL-optic barrier effect, which prevents LEC migration to the posterior capsule. This is accomplished by complete contact of the IOL optic with the posterior capsule. If contact is not complete, a potential space is created, allowing LECs to migrate and grow on the visual axis. In cases where the capsular bag or zonules are damaged, the IOL can be fixed to the sclera or the iris. However, these techniques have their own complications aside from increased PCO.[121]

This barrier effect might be the aspect that has most reduced the prevalence of PCO. Thus, many components of the IOL itself have been improved to further increase the barrier effect. The IOL haptics have been elongated and angulated upwards to increase the contact and pressure between the back of the IOL optic and the posterior capsule. Even changes in the IOL material have been implemented to improve its adhesiveness to the capsule.[122] Furthermore, most modern IOLs have sharp square-edge optic designs in order to form stronger mechanical barrier for the LECs.[123, 124]

Although, not all modifications of the IOL are mechanical. Since the IOL can cause specific foreign body responses, the biocompatibility of IOL has also been studied in order to further reduce PCO. The chemical composition of the IOL has been noted to play a role in the proliferation, migration, differentiation and fibrotic transition of LECs and thus PCO. Normally, biocompatibility is defined as the capability of a prosthesis implanted in the body to exist in harmony with tissue, without causing deleterious changes. However, in ophthalmic literature biocompatibility is defined as the reaction of

LECs and the capsule to IOL material, leading to the least amount of PCO. For example, hydrophobic materials induce more fibrous transformation of the LECs and hydrophilic materials support LEC proliferation better but with less metaplasia.[125-128] The ideal biomaterial should be biocompatible and not induce a foreign-body reaction, but should inhibit LECs' proliferation, and therefore be bio-incompatible for the LECs. In view of this, the "ideal" IOL biomaterial is intrinsically confronted with a conflicting demand since most cells in the body will react the same.

Even a change to the IOL implantation technique itself has been proposed to prevent PCO. The "bag-in-the-lens" technique consists of creating a CCC in both the anterior and posterior capsules, and then inserting the residual capsule "ring" in to the modified IOL.[129, 130] This IOL has an equatorial groove where the capsules are inserted and in turn support the IOL, thus the term bag-in-the-lens as opposed to the currently used lens-in-the-bag technique. (Figure 1.13) While this technique has very good PCO prevention, it brings with it its own side effects, such as vitreous prolapse, hypertension and glaucoma.[131, 132]
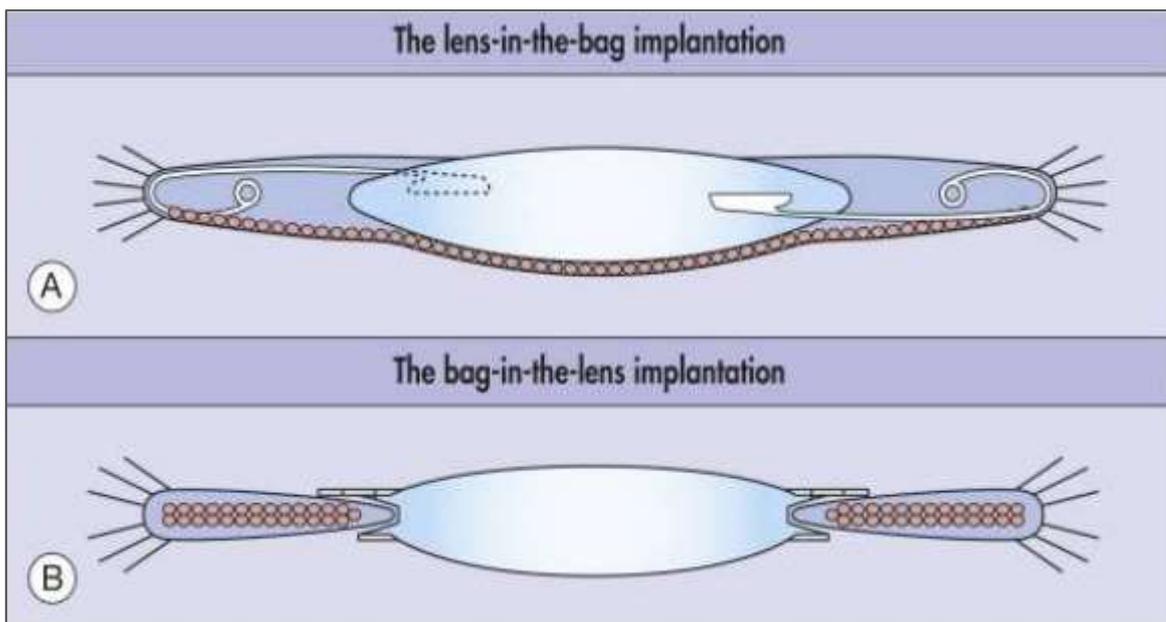
Figure 1.13. Schematic drawing of a side view of the lens in the bag vs the bag in the lens techniques. In the lens in the bag, cells can migrate behind the IOL (A) while in the bag in the lens, cells are trapped in the periphery (B). **(Verbruggen, K.H. et al. 2007)[133]**

1.3.5.2. Non-surgical strategies to prevent PCO

For more than 20 years researchers have tried many different substances to interfere with the biological processes underlying PCO formation in order to inhibit its development.[59] The three main approaches are: inducing LEC death, preventing cell proliferation/migration and interfering with the immune response. (Table 1.1)

Antimetabolites have been shown to effectively inhibit LEC proliferation and differentiation *in vitro*, but they have been abandoned since the therapeutic doses required to significantly affect LECs reached the toxic dose for other ocular tissues in humans.[134-138]

Other cytostatic substances have focused more on inhibiting specific growth factors, like TGF-β or EGF. However, these usually only showed transient inhibition, probably due to the short time which the substances had to act.[139-142]

Like most cytostatic substances, anti-inflammatory substances were effective in reducing PCO, but only in the early postoperative period.[143-147]

Finally, in the case of apoptosis inducing substances, while most are effective in eliminating LECs, they all have the drawback of possibly effecting the surrounding tissue as well. In order to more specifically effect the LECs, gene therapies and specific antibodies have also been studied. Gene therapies have focused on cell death or growth inhibition. However, for this to be viable, retro and adenoviruses must be used as vehicles. Beside the fact that there is some controversy in using altered viruses for human treatments, current tests have still not been successful in significantly preventing PCO.[148-151] In the case of antibodies, while their use is promising, more research is required in order to find an effective target.[139, 152, 153]

Target cell cycle regulation

1. Anti-proliferation (author, experimental model)

Cytostatic drugs

Anti-metabolites

5-fluorouracil	(Duncan, 2007 (EVH); Fernandez, 2004 (IVA); Huang, 2013 (CL/IVA))
Methotrexate	(Koopmans, 2014 (IVA); Sternberg, 2010 (EVH/IVA))

Cytotoxic antibiotics

Actinomycin D	(Koopmans, 2011-2014 (IVA); van Kooten, 2006 (CPA/EVA); Sternberg, 2010 (EVH/IVA)) (Geissler, 2001 (CL/EVA/EVH))
Calcimycin	
Cycloheximide	(Koopmans, 2011 (IVA); van Kooten, 2006 (CPA/EVA))
Doxorubicin	(Guha, 2013 (IVA))
Mitomycin C	(Fernandez, 2004 (IVA); Inan, 2001a-2001b (IVA); Kim, 2007 (IVA))

Other cytostatics

Colchicine	(Legler, 1993 (IVA))
Paclitaxel	(Koopmans, 2014 (IVA))

TGF-β inhibitors

Anti-TGF- β antibody	(Sun, 2013 (CL))
CAT-152	(Wormstone, 2004 (CL))
Pirfenidone	(Yang, 2013 (CL))
Zebularine	(Zhou, 2011-2012 (CL))

Rho inhibitors

H-7	(Sureshkumar, 2012 (EVH))
Lovastatin	(Urakami, 2012 (CPA))

EGF inhibitors

Erlotinib	(Wertheimer, 2013-2014 (CL))

Histone deacetylase inhibitors

Trichostatin A	(Chen, 2013 (CL); Xie, 2014 (CPA/CPH))
Vorinostat	(Xie, 2014 (CPA/CPH))

Metabolites

Retinoic acid	(Inan, 2001a (IVA))
Tripan blue	(**Sharma, 2013 (IVH)**)

Tyrosine kinase inhibitor

Genistein	(Zhang, 2013 (CL))

Local anesthetics

Lidocaine	(Vargas, 2003 (EVA))

2. Anti-inflammatory

NSAIDs

Celecoxib	(Chandler, 2007 (CPA/EVA); Davis, 2012 (EVA))
Diclofenac sodium	(Inan, 2001b (IVA))
Indometacin	(Nishi, 1995 (IVA))
Rofecoxib	(Chandler, 2007 (CPA/EVA))

Steriods

Dexamethasone	(Inan, 2001b (IVA); Kugelberg, 2010 (IVA))

Immunosuppressivants

Cyclosporin A	(Cortina, 1997 (CPH); Totan, 2008 (IVA); Pei, 2013 (IVA))
Rapamycin	(Liu, 2009-2010 (CPA/IVA))

Anticoagulants

Heparin	(**Maedel, 2013 (IVH)** ; **Ronbeck, 2009 (IVH)** ; **Wejde, 2003 (IVH)** ; Xie, 2003 (IVA))

3. Apoptosis inducing

Cytotoxins

Immontoxins

FGF2-saporin	(Behar-Cohen, 1995 (IVA))
MDX-RA (Ricin A)	(**Clark, 1998 (IVH)** ; **Meacock, 2000 (IVH)**)
Polylysin-saporin	(Bretton, 1999 (IVA))

Sponge-derived

Latrunculin B	(Sureshkumar, 2012 (EVH))

Gene therapy

Transfer β -galactosidase gene	(Coudrec, 1999 (CPA/IVA); Malecaze, 1999 (CPA/IVA))
Transfer BMP-7	(Saika, 2006 (IVA))
Transfer HSVtk gene	(Coudrec, 1999 (CPA/IVA); Malecaze, 1999 (CPA/IVA); Yang, 2012 (CL))
Target snail	(Li, 2013a (CL))
Target TGF-β receptor	(Zheng, 2012 (CL))

Osmotic effective solutions

Distilled (deionized) water	(Crowston, 2004 (EVH); Duncan, 2007 (EVH); Fernandez, 2004 (IVA); Kim, 2007 (IVA); **Rabsilber, 2007 (IVH)** ; **Rekas, 2013 (IVH)**)
NaCl	(Duncan, 2007 (EVH))

Detergents

Triton X-100	(Maloof, 2005 (IVA))

Irradation β-irradation isotope

P-32	(Joussen, 2001 (CPA/IVA))
Ultraviolet B	(Wang, 2013a (CL))

Iontophoresis

Acetic acid	(Fernandez, 2004 (IVA))

Photodynamic therapy

Bacteriochlorin A	(van Tenten, 2002 (IVA))
Indocyanin green	(Melendez, 2005 (CL))
Tripan blue	(Melendez, 2005 (CL))

SERCA inhibitors (Duncan, 1997-2007 (EVH))

Thapsigargin	

Anti-migration and anti-adherence

Matrix metalloproteinase inhibitors

Caffeic acid phenethyl ester (CAPE)	(Hepsen, 1997 (IVA); Koopmans, 2014 (IVA))
Ethylenediamine-tetraacetic acid (EDTA)	(Fernandez, 2004 (IVA); Hazra, 2012a (IVA); Inan, 2001b (IVA); Nishi, 1996-1997 (IVA))
Ilomastat / GM 6001	(Awasthi, 2008 (CL/CPH/EVH))
Nobiletin	(Miyata, 2013 (CL))
Secreted protein acidic and rich in cysteine (SPARC)	(Gotoh, 2007 (CPA))

Proteasome inhibition

Disulfiram	(Sternberg, 2010 (EVH/IVA))
MG132	(Awasthi, 2006-2008 (CL/CPH/EVH))

Integrin anagonists/disintegrins

RGD peptide	(Inan, 2001b (IVA); Nishi, 1997 (IVA))
Salmosin	(Kim, 2002 (CPA/IVA))

Calcium antagonists

Mibefradil	(Weidmann, 2008 (CL/EVH))

Experimental models:

CL	Cell line (human)
CPA	Cell primary animal
CPH	Cell primary human
EVA	Ex vivo animal
EVH	Ex vivo human
IVA	In vivo animal
IVH	In vivo human

Table 1.1 List of agents examined in studies for PCO prevention. The agents are first divided by their point of action in the process of PCO formation and subsequently by their working mechanism. The experimental models are indicated by using abbreviations which are explained in the box in the table. The *in vivo* human (IVH) model is highlighted in bold to emphasize that only a few treatments have been applied in patients. All references can be found in **Nibourg, M.L. et al. 2015** [59]

Other groups have studied more direct approaches such as hyperthermia, in order to kill LECs through heat exposure, or photodynamic therapy which elicits cell death through molecular oxygen exposure. Both treatments are successful in preventing PCO *in vitro* and almost completely reduce LEC proliferation, but hyperthermia would destroy all neighboring tissues *in vivo* and photodynamic therapies have been shown to affect corneal integrity.[154]

The main difficulty for all these approaches is restricting the effects of the substances only to the LECs without damaging the surrounding tissue. Furthermore, since these substances have to be applied during routine cataract surgery, they must be effective after a single short treatment. Due to these concerns, very few approaches have ever been studied in *in vivo* human patients, and none of these have been very successful.

However, there are techniques that attempt to address these problems in order to make these treatments more viable. One approach is to seal the capsular bag during treatment in order to prevent collateral damage due to substance exposure. For this, a silicone device, the "Perfect Capsule™", had been designed which used suction to seal the lens capsule, allowing selective irrigation of the capsular bag following phaco-emulsification.[134] However, this device is no longer available despite promising results. Another approach is to use IOLs made of specific materials that can be coated or soaked in the desired substance in order to prolong exposure time of the residual LECs. These drug carrying IOLs are promising but still need to be studied further.[155, 156]

Even with these new techniques, to the best of our knowledge, to date there is only one studied substance, Thapsigargin, which has proven total LEC destruction.[134, 157] However, despite the promising results, Spalton and Wormestone's group have noted that the complete elimination of LECs has negative effects on IOL centration and fixation.[158] They have also brought up the concern of whether the lens capsule can even survive without LECs. Furthermore, thapsigargin is a tumor promoter in mammalian cells.

1.4. Peripheral posterior capsule opacification – Soemmerring's ring

As previously mentioned, a Soemmerring's ring is a type of PCO that occurs in the periphery of the lens capsule. It consists of an annular swelling in the periphery of the lens capsule, composed of lens epithelial and fiber cells, that develops after cataract lens extraction, between the posterior capsule and the edges of the remaining anterior capsule.

The Soemmerring's ring was first described in 1828, by D.W. Soemmerring.[159] He had examined several postmortem eye globes, from donors, who in life had undergone cataract surgery. He dissected the anterior segment of the eyes, and in eight cases saw more or less transparent, ring-like substances behind the iris (Figure 1.14). When the dissected eyes were immersed in alcohol, these almost transparent rings became opaque. His observations were followed by those of Werneck (1834)[160] and Textor (1842)[161].

The formation of these rings had already been shown in animal experiments by Dieterich (1824)[162] and Cocteau and Leroy d'Aille (1827)[163]. Gonin (1896)[164] and Wessely (1910)[165] showed that the ring formations arise more easily in younger animals than in older ones. This is most likely due to the fact that the epithelial cells in young animals have a greater capacity for growth than those of old animals.
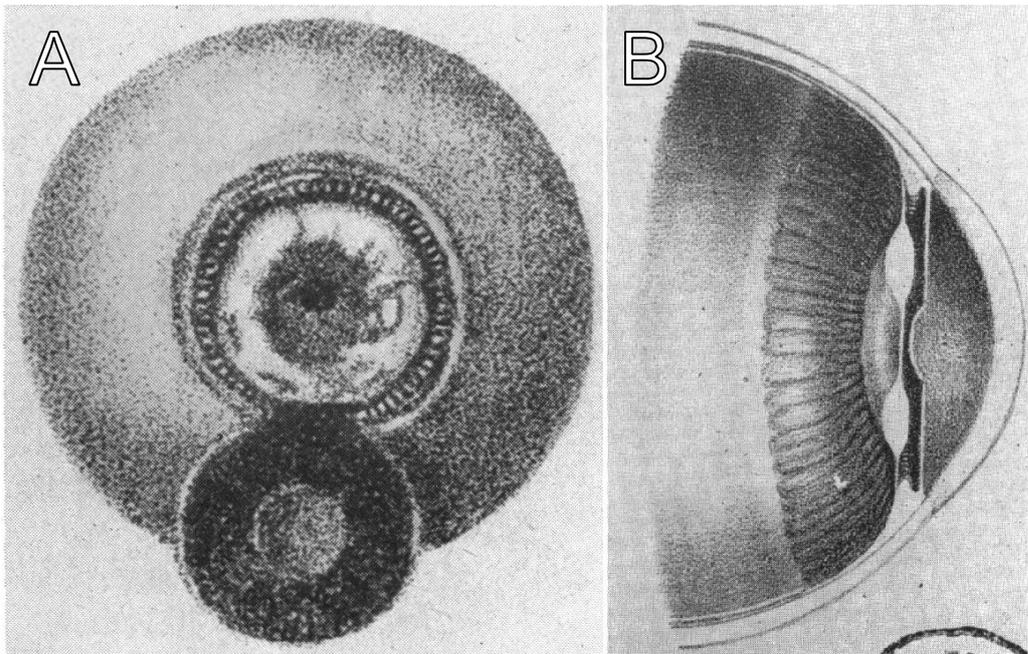
Figure 1.14 Hand drawn figures by D.W. Soemmerring in 1828[159] A) The ring of Soemmerring, showing the annular post cataract growth in a post mortem eye with the cornea and iris removed. B) The Soemmerring's ring in a cross-section.

Soemmerring's rings are common but usually pass unnoticed, since, they are only diagnosed clinically when they dislocate, when a coloboma of the iris has been made, or when the pupil has been widely dilated. By 1957, more than 100 years after Soemmerring's rings were first described, only fourteen cases of dislocation had been published[166].

Furthermore, when unobstructed, Soemmerring's rings develop alongside cells that migrate to the posterior capsule. These cells negatively affecting vision, leading to clinically relevant PCO. However, over the years, these incidences of PCO have been reduced, in large part due to the advance of square edge IOLs. These IOLs form a mechanical barrier between the IOL and the posterior capsule, blocking the migration of the residual LECs on to the posterior capsule. However, this same improvement, further favors the development of Soemmerring's rings.[167] Since they are left to develop over longer periods of time. (Figure 1.15)
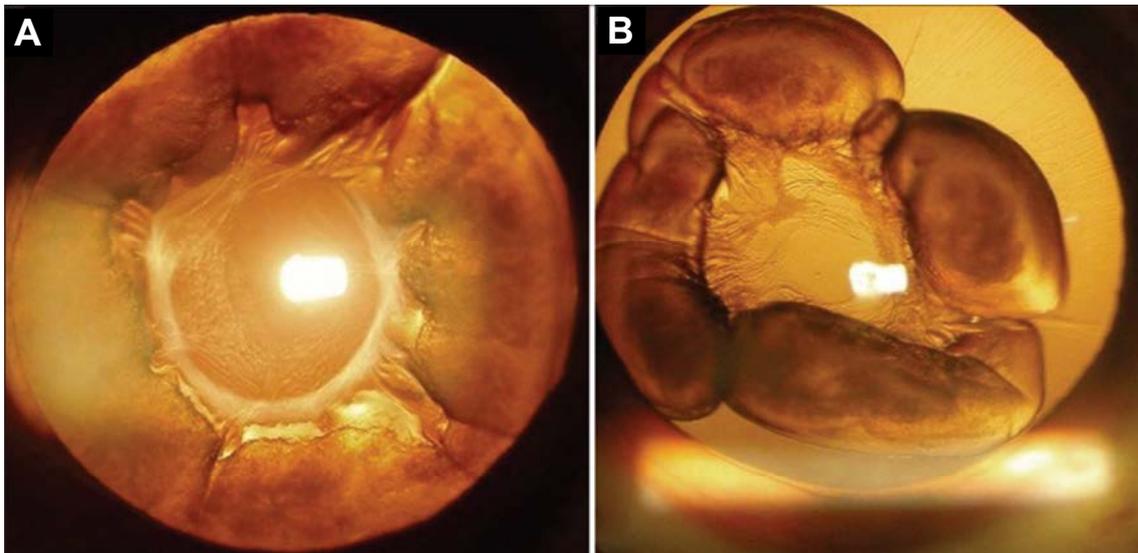
Figure 1.15 Slit lamp retroillumination photograph of (a) left eye showing dense 360° Soemmering's ring with annular fibrosis of the capsule with clear optical axis and (b) right eye showing after cataract in the form of complete 360° Soemmering's Ring with stretched zonules and clear visual axis.[78]

Moreover, even in cases where cells from the Soemmerring's ring breach the barrier and migrate on to the posterior capsule, treatment does not affect the Soemmerring's ring, since the treatment in these cases is Nd:YAG laser posterior capsulotomy, which only ever creates a hole in the posterior capsule smaller than the IOL optic (Figure 1.12).

1.5. Models used to study posterior capsule opacification

Despite the vast amount of studies on PCO prevention and although some very promising results have been obtained, no perfect technique has been found. Thus, further studies are necessary and one of the most important aspects in designing a new study or further validating past results, is to choose the correct model. There are three main models for studying surgical and pharmacological techniques: animal models, cell culture models and tissue culture models.[168] The final objective of all these models is to be transferable to *in vivo* human clinical studies. Fortunately, PCO research is well served by a variety of experimental systems (Table 1.2)

Experimental Model	Observable PCO characteristic					Possibility of IOL implantation
	Proliferation	Migration	Matrix contraction	EMT	Fiber differentiation	
In vivo Animal	Yes	Yes	Yes	Yes	Yes	Yes*
Whole Capsular bags	Yes	Yes	Yes	Yes	No	Yes*
Capsular bags fragments	Yes	Yes	Yes	Yes	Yes	No
Cell cultures	Yes	Yes	Yes	Yes	Yes	No
Human lens cell lines	Yes	Yes	Yes	Yes	No	No

Table 1.2 Experimental models and their applications for the study of PCO *Limited by lens size. EMT (Epithelial to mesenchymal transition) IOL (Intraocular lens) Table adapted from **Wormstone, I.M. and Eldred, J.A. 2016**[168]

1.5.1. Animal models

Several animal models have been used to investigate PCO development and some even permit the implantation of IOLs. The main advantage of these models is that they can develop a complete inflammatory response. However, since the inflammatory response in species can differ greatly, results are not always representative of the *in vivo* human response.[169] Furthermore, different species can have different receptor expressions and signaling profiles. For instance, adrenalin in the rat lens, leads to a release of calcium, while no such response is observed in the human lens. On the other hand, EGF mobilizes the calcium store in human lens cells, but does not affect the rat lens.[170]

The rabbit is the oldest *in vivo* model for PCO, having been used since the early 1990's[171-173], although it had been used over a century earlier for the study of lens regeneration.[174] This model allows for the study of cell regrowth, matrix modification,

EMT, differentiation and IOL displacement. However, this model undergoes a sever and rapid inflammatory response following surgery, suggested to be over 20 times higher than in humans.[175] Therefore, a drug that could be effective at inhibiting or suppressing PCO in humans may be disregarded or missed if exclusively tested using the rabbit model, where it may not have a significant impact on PCO prevention.[168] This elevated inflammatory response may also explain the rabbit lenses ability to regenerate.

The first rat model for PCO was published in 2003 by Lois et al.[176], they were able to observe LEC migration within 24h, spindle shaped cells and capsular wrinkling by 3 days and Lens fibers and Soemmerring's rings by 14 days. They related this to the low-grade inflammatory response in these animals, consisting mostly of mononuclear macrophages. Recently rat models have been used to study the roles of different growth factors in PCO development.[85, 177]

The first mouse model for PCO was also published by Lois et al. in 2005[178], and allowed for the observation of similar PCO traits, such as, migrations, wrinkling and differentiation. These changes were also associated with low-grade but significant macrophage response. However, this model had the advantage of being genetically modifiable in order to better evaluate the pathogenic mechanisms of PCO.[179] But it had the disadvantage of not allowing IOL implantation during surgery, due to the reduced size of the mouse lens.

1.5.2. Cell culture models

Cell cultures are the simplest model for studying PCO characteristics and have been used to study LEC development since 1965.[180] Generally, primary cultures derived from lenses or cell lines are used. This model has the advantage of rapidly and easily identifying factors that positively or negatively affect LEC behavior and development. Such as stimulating or inhibiting proliferation, migration, differentiation, transdifferentiation, matrix component synthesis and apoptosis.[141, 181, 182]

Primary lens cell cultures, derived from lens explants, have limited longevity in culture, surviving only for a few passages before they start to differentiate or senesce. Despite this, mouse and chick primary lens cultures have been used to study the effects of alpha-crystallins and fibronectin on LEC fate. The alpha-crystallins were shown to be important for optimal lens epithelial growth and lens transparency.[183] While fibronectin was shown to stimulate both myofibroblast and lens fiber differentiation by upregulating TGF-β signaling.[184]

In order to overcome the limited longevity of primary lens cells, three human LEC lines were created. HLEB3 and SRA01/04 were derived from virus immortalized infant lens cells.[185, 186] While, FHL124 is a spontaneously immortalized cell line derived from embryonic lens explants.[139] These cell lines allow for continuous and reproducible analytical studies, due to their reduced variability.

HLEB3 cells have been shown to express normal human crystallins and receptors for growth factors such as HGF, EGF, PDGFb and TGF-β.[187, 188] Thus, HLEB3 has been used to study the role of these factors in cell proliferation, migration, matrix contraction and cell spreading during PCO.[142]

SRA01/04 cells expressed most of the same growth factors as HLEB3 with the addition of the bFGF receptor, which when activated induces cell morphology changes, focal adhesion complexes and formation of the cortical actin stress fibers. This suggests that bFGF plays a role in cell shape and cytoskeletal changes that are often associated with myofibroblast formation and EMT.[189, 190]

FHL124 cells express genes for αA-crystallin, Pax6 and FOXE3, which all play important roles in proper lens development.[191]

These cells also possess many growth factors that are associated with cell migration, proliferation and survival.[192] Furthermore, they secrete extracellular matrix components which has helped study cell induced matrix contraction *in vitro*.

Each of these LEC lines have been used to test experimental therapies for preventing PCO formation, by attacking one of the specific PCO inducing factors they have helped elucidate.[141, 181, 182]

Despite these valuable results, this model has the big disadvantage of not taking in to account certain key factors that heavily influence LEC and PCO development. Such as, the surface on which LECs grow, extra cellular matrix components, spatial distribution of cells and media composition over time. All these factors affect LEC growth rates, molecular expression patterns and cell type. Most of these factors can be correctly represented in the *in vivo* animal and tissue culture models.

1.5.3. Capsular bag tissue culture models

As mentioned, in order to better study PCO development, lens capsule explants with attached LECs have been used as study models. Originally these samples simply consisted of lens capsular fragments obtained from routine cataract surgery or animal

explants that were placed in culture media. This system has an advantage over cell cultures in that the cells are retained on their natural basal membrane.

This explant system was improved by maintaining the entire capsular bag in order to better simulate the spatial arrangement of cells and tissue, after *in vivo* cataract surgery. Capsular bag models have been derived from many species including human[93, 193-195], porcine[196, 197], bovine[198, 199], canine[200, 201], rabbit[134] and chick[202].

To our knowledge, the first *in vitro* human lens capsular bag culture model was designed in 1996 by Liu et al.[193]. In this model, a sham cataract surgery was performed on a donor eye globe, which included anterior capsulorhexis, nucleus hydroexpression, aspiration of lens fibers and, in some cases, IOL implantation into the capsular bag. The remaining lens capsule was excised and pinned directly to a plastic petri dish and submerged in serum supplemented culture media. It could be observed by phase-contrast and dark-field microscopy for as long as 100 days. This model, showed many of the changes seen *in vivo*, including rapid LEC growth, wrinkling, tensioning, and light scatter on the posterior capsule. However, since pinning directly through the capsular bag can damage it, thus adding variables, other groups developed modifications to improve this model. Such as, using a holder to support the lens capsule [203] or a capsular tension ring to maintain the shape of the capsule[204].

In 2010, Cleary et al.[194] designed the base model for most current *in vitro* studies. This model retains the zonular fibers and the ciliary body, which could be pinned to a silicon ring, effectively suspending the capsular bag in a more physiological manner. Also, with this, IOLs can sit within the capsular bag and interact with it in manner more representative of the *in vivo* situation.

Recently variations to the culture media, such as serum concentrations, have been studied, in both cell and tissue culture models, in order to better mimic clinical events following cataract surgery [205].

In summary, animal and human tissue models allow us to mimic and establish the in vivo development of PCO, and cell cultures allow us to elucidate the principal factors and mechanisms that drive the initiation of PCO. However, human tissue is limited, and *in vivo* animal experiments provide us mainly with endpoint PCO results. All phases of PCO development, from initiation to end stage fibrosis through cellular changes over time, are critical for the establishment of new therapeutic treatments. Thus, a combination of the models would need to be used in order to obtain the most reliable results.[168]

1.6. Lens regeneration

The idea of regenerating the lens isn't a new concept. The first report of ocular tissue regeneration was recorded in 1781 by Bonnet C., where he described how after partial dismemberment of a salamander's eye, a complete eye would regenerate within several months.[206] Later scholars discovered that the new lens originated from the iris pigmented epithelial cells.

Over the years, three different types of lens regeneration processes have been reported, Wolffian lens regeneration, cornea-lens regeneration and LEC regeneration. (Figure 1.16)[207]
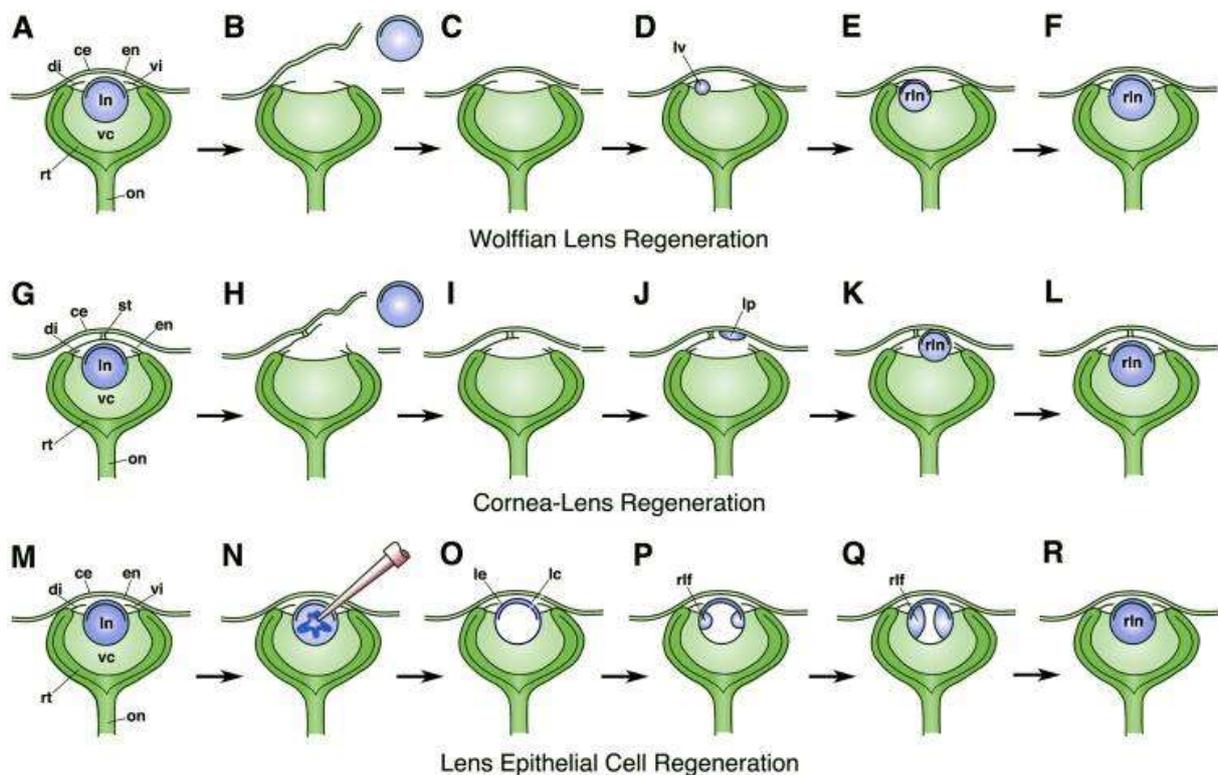
Figure 1.16 Diagrams illustrating the process of Wolffian lens regeneration (A–F), cornea-lens regeneration (G–L), and lens epithelial cell regeneration (M–R). In (B) and (H), simple lensectomy is performed to remove the intact lens along with its lens capsule. (N) Shows the process of phacoemulsification to remove the lens fiber cells while mainly leaving the lens epithelium and lens capsule intact (as seen in O). (A–F) and (M–R) show adult eyes. Unlike the case in the adult eye, notice that the Xenopus larval cornea epithelium is initially attached to the deeper cornea endothelium by only a small central stalk (as shown in G). This connection enlarges, and the collagenous stroma is deposited during later stages when the larva approaches the time of metamorphosis. Eye structures are labeled as: ce, cornea epithelium; di, dorsal iris; en, cornea endothelium; lc, lens capsule; le, lens epithelium; ln, lens; lp, lens placode; lv, lens vesicle; on, optic nerve; rlf, regenerated lens fiber cells; rln, regenerated lens; rt, retina; st, central stalk; vc, vitreous chamber; vi, ventral iris. **(Henry, J.J. and Hamilton, P.W. 2018)[207]**

Wolffian lens regeneration, occurs in newts and salamanders and consists of the transdifferentiation of iris cells into lens cells.[208] (Figure 1.16A-F)

Cornea-lens regeneration was first described by Freeman in 1963 in frogs.[209] In these cases, the new lens arises from the basal layer of the corneal epithelium, and it is stimulated by factors secreted by the retina. However, this only occurs during the larval stages as regenerative ability decreases as the larvae approach metamorphosis (Figure 1.16G-L).

Finally, there is LEC regeneration, sometimes referred to as mammalian lens regeneration. Contrary to the previous models, in this case, regeneration can only occur when the lens capsule is left behind after surgery with residual LECs adhered (Figure 1.16M-R). Therefore, removal of the capsular bag will result in the absence of regeneration.

This form of lens regeneration was first described in albino rabbits in 1824 by Cocteau.[174] Since then, it has been reported for several other mammals[207, 210, 211] such as, rats,[176] cats, dogs[212], pigs[213], sheep, cows, Guinea pigs, and primates[212], with variable degrees of success. This process has even, very recently, been described in human infants.[212] In general, this process seems to be age-dependent, achieving a higher degree of regeneration and at a faster rate in younger animals.[214] As these models (including humans) age, these processes become abnormal and lead to the development of PCO.

There seems to be a relation between this form of lens regeneration and Soemmerring's rings. LEC regeneration starts at the equator of the lens capsule, where the residual LECs accumulate and starts forming new lens fiber cells. This leads to the formation and growth of a donut shaped mass of organized fiber cells that eventually grow together in the center, reforming the lens.[212] The formation of the Soemmerring's ring seems to be a failed attempt at this process, as it is also a doughnut shaped growth that contains fibers cells but they are unorganized and opaque.[78, 215]

2. Aims of the study

The general aim of this thesis was:

> to study PCO development, from both a clinical and experimental perspective, in order to find a new approach to avoid the problems associated with it.

The specific aims of the study were:

1. To simulate the development of normal clinical PCO in a lens capsular bag tissue culture model.
2. To prevent the development of PCO in our tissue culture model, using novel substances and compare them to previously validated substances.
3. To compare the morphology of the PCO developed in our tissue culture model, PCO developed *in vivo* and adult lenses.
4. To analyze the morphology of Soemmerring's rings and how they are formed.
5. To analyze if any part of the Soemmerring's rings is or could be transparent.
6. To modify the tissue culture model in order to promote lens fiber cell formation and regeneration.

3. Methods

In the following sections, we will explain in more detail some of the materials and methods later summarized in our articles and list all off the tissue used in those studies. We will also explain the design process of our irrigation device for sample treatment along with some unused design concepts, which were not mentioned in our articles.

In order to study the development of PCO, we decided to use human tissue cultures. This choice was made due to a couple of factors. First, when compared to cell cultures, tissue cultures mimic the biomechanics of PCO better and are more representative of *in vivo* progression. Second, while animal models develop an immune response to the surgery, day to day progression of PCO development is harder to follow up. Finally, due to our eye banc, we have the unique privilege of having ready access to human donor globes.

3.1. Establishment of mock cataract surgery protocol

In order to simulate the progression of PCO, it was first necessary to perform cataract surgery on the donated ex-vivo eye globes. We based our technique on the Cleary G. 2010 model[194] of preforming the cataract surgery on the lens after having affixed it to a silicone ring. Here we will explain the procedure in extended detail.

After the globe is obtained from the eye Bank, the conjunctive is removed so that the globe can be decontaminated with a 5% Povidone-Iodine wash.

After that the corneoscleral disk is removed with a circular trephine and then the anterior segment is removed from the rest of the globe (Figure 3.1A). Next, using an operating microscope; the iris–ciliary body–lens complex is dissected from the scleral tissue in a single piece, carefully avoiding excessive traction on the ciliary body to prevent zonular dehiscence (Figure 3.1B).

Afterwards, the specimen is transferred to a sterile petri dish and placed with the posterior side up. Any residual adherent vitreous is removed from the posterior surface of the lens, being careful not to breach the posterior capsule. The iris is removed with forceps. In some cases, iridectomy may liberate iris pigment; if this occurs, the specimen is irrigated and transferred to a fresh Petri dish of HBBS. Then the specimen is placed on a silicone ring, anterior surface facing upward, and attached by passing 8 stainless-steel needles through the ciliary body, (Figure 3.1C) the ends of the needles are cut with pliers.

Once affixed, a 6mm continuous curvilinear anterior capsulorhexis was performed using capsulorhexis forceps (Figure 3.1D). After which the lens was hydroexpressed using an angled irrigation cannula and balanced salt solution (BSS). In order to do this the tip of the canula was introduced through the rhexis and placed between the lens and the anterior capsule, then the BSS was slowly injected in order to create space. This was done along the rhexis until the BSS could be seen separating the lens from the posterior capsule. In some cases, at this point the whole lens nucleus would be extruded through the rhexis (Figure 3.1E). If not, the lens was removed whole or in pieces using forceps and the canula tip. Finally the remaining lens fibers still affixed to the lens capsule would be aspirated using the canula in order to clean the capsule and only leave the monolayer of LECs behind (Figure 3.1F).
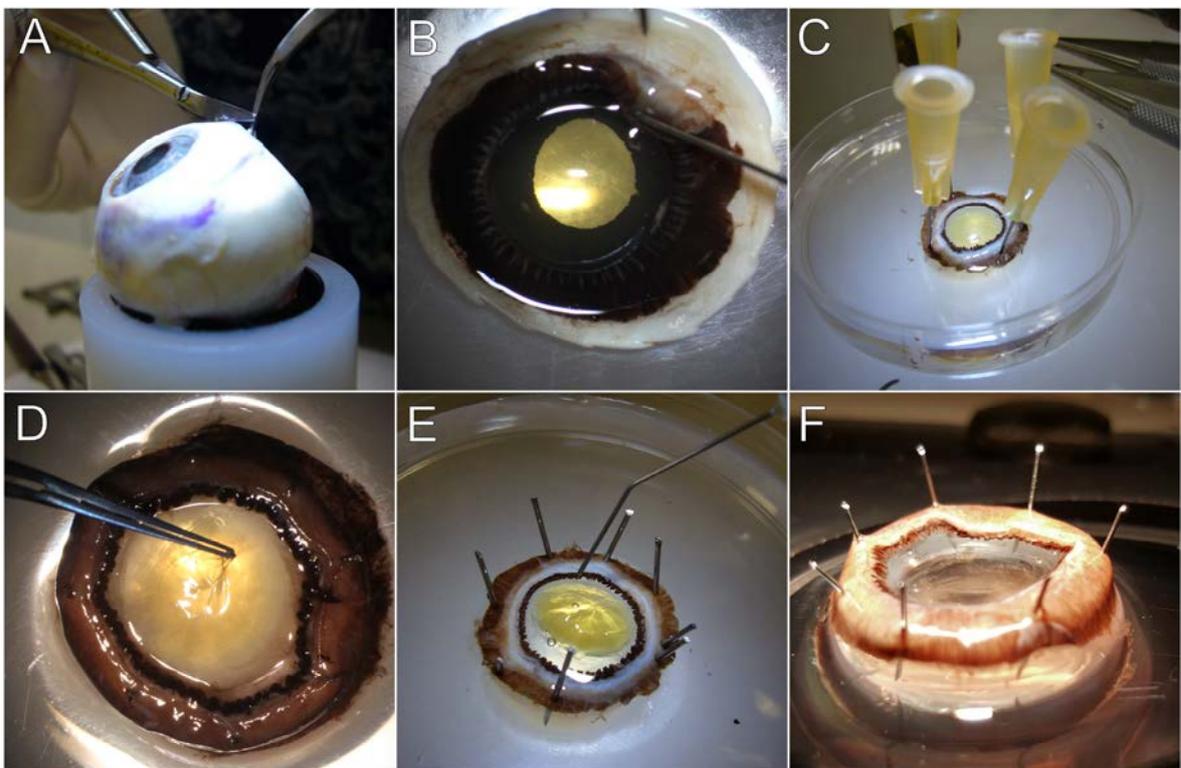
Figure 3.1 Photographs of the main steps of mock cataract surgery in order to obtain the lens capsule-ciliary body complexes. (A) Separation of the anterior segment of the eye globe, after having removed the cornea. (B) Separation of the Lens-iris-ciliary body complex from the sclera. (C) Affixing the Lens-iris-ciliary body complex to the silicone ring with 30G needles. (D) Performing the 6mm anterior capsulorhexis. (E) Hydroexpression of the lens from the capsule. (F) Finished sample.

3.2. Sample treatment protocol

In order to prevent the development of PCO using new or previously validated substances, it was first necessary to find an instrument that would allow us to apply the selected substance to all the cells within the capsule. Here we will explain the different approaches we attempted and the design process of our own treatment device, not explained in our published article.

Simply attempting to fill the capsule with the substance was not a viable option as the capsule tended to collapse on itself creating pockets where the substance would not reach. In order to overcome this problem, we needed an instrument that would maintain the capsule open during treatment.

During the time of our first experiment, there was only one commercial instrument that was designed for the irrigation of the capsule, the PerfectCapsule® by Milvella (Figure 3.2).
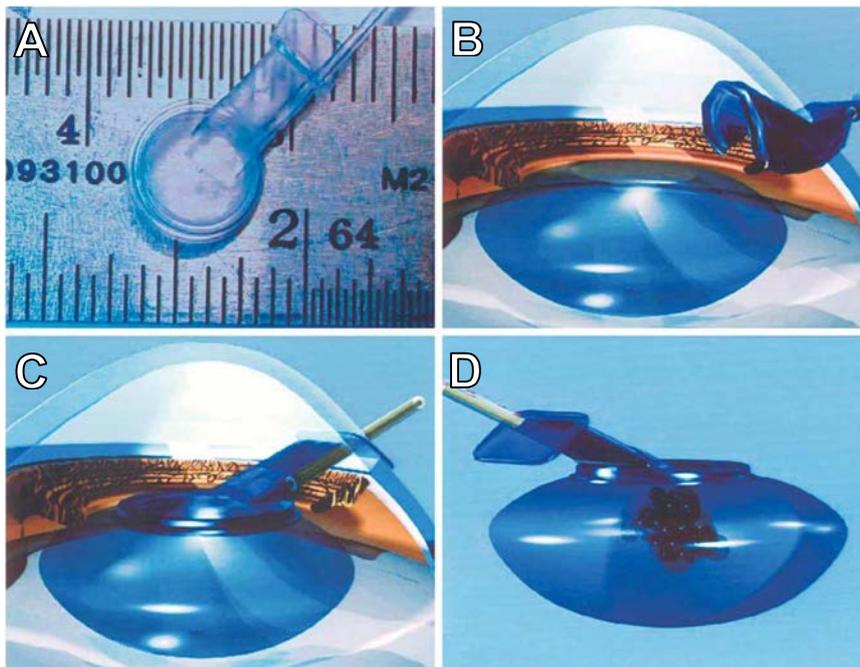
Figure 3.2 Schematic diagrams illustrating the concept of the sealed capsule irrigating device (PerfectCapsule; Milvella Pty Ltd, Sydney, Australia). This device is designed to hold the capsular bag by means of a toroidal suction ring connected to a locking suction syringe. An irrigation/aspiration port allows fluids to be injected through the device into the empty capsule, significantly reducing the possibility of potentially toxic irrigation fluid coming into contact with other ocular structures. A, Sealed capsule irrigation device viewed from the top. It consists of a round plate that seals against the capsule and an extension arm that passes outside the wound. Ruler is in millimeters. B, Sealed capsule irrigation device is folded and inserted through a 3-mm incision. C, Sealed capsule irrigation device is placed onto the capsular bag and vacuum attached by a syringe. D, Internal irrigation of the capsular bag using the sealed capsule irrigation device. **(Maloof, A.J. et al. 2005)[216]**

However, this instrument was no longer available for purchase. Due to this and a lack of alternatives, we decided to design our own irrigation device.

We conceptualize several different instruments (Figure 3.3), until we developed our own intracapsular irrigation device. All our designs were only intended for use in *ex-vivo* culture samples and not for *in vivo* surgical use.
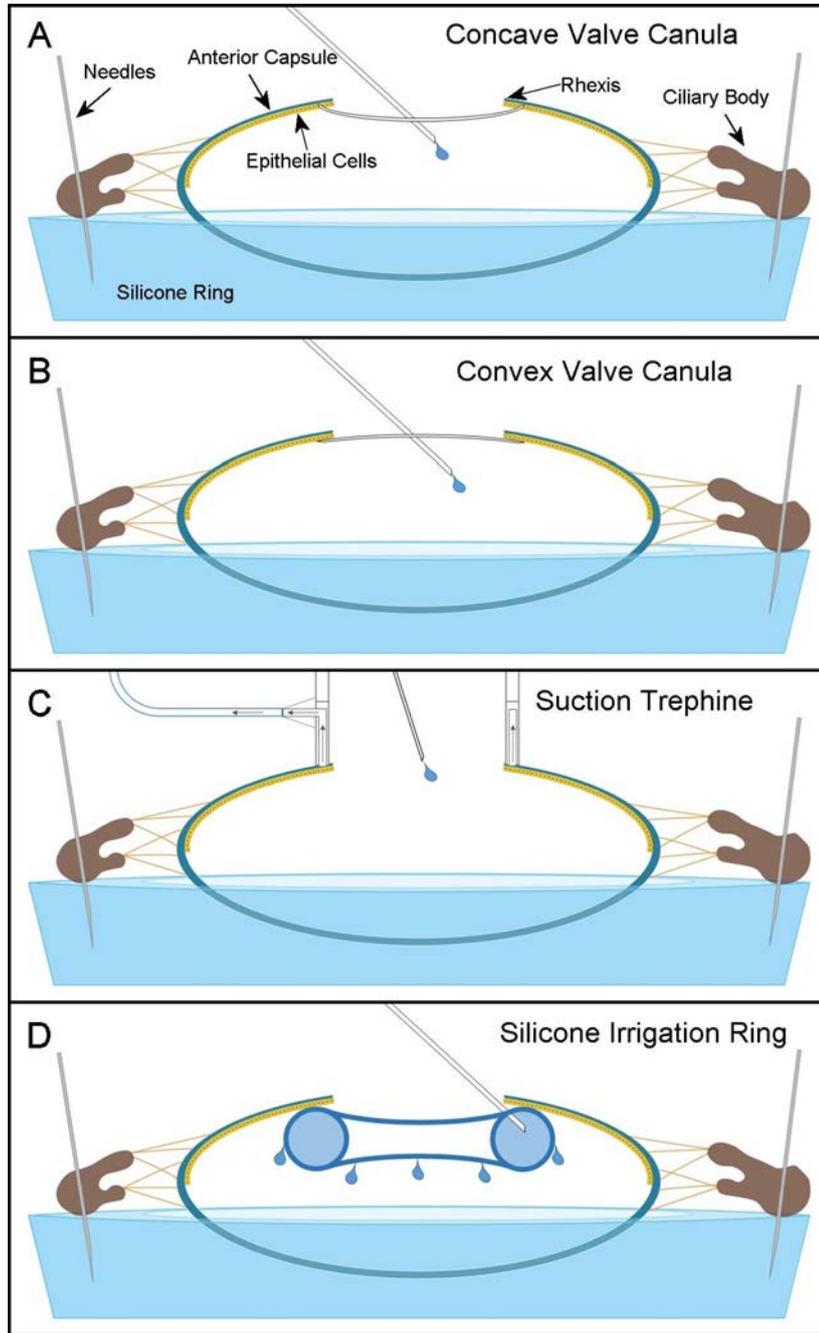
Figure 3.3 Schematic representation of the different irrigation devices we conceptualized, placed inside emptied lens capsules. **A**) A canula with a valve at the end is placed through the anterior rhexis and lifts the anterior capsule. **B**) Same concept as (A) but the valve is convex in order to damage the LECs less. **C**) The anterior capsule is affixed to a bladeless suction trephine with a vacuum and then the capsule is filled. **D**) A hollow silicone ring, with puncture holes, connected to a canula is placed inside the lens capsule.

Our first two designs (Figure 3.3A & B) of using semi-rigid valves to both seal and lift the anterior capsule were discarded. This was due to difficulty of uniformly lifting the capsule without creating unnecessary traction. This traction would damage the anterior LECs and could potentially tear the capsule. The suction trephine (Figure 3.3C), theoretically, had the advantage of not damaging the LECs and creating uniform tension around the anterior rhexis. However, in practice the pressure needed to correctly place the trephine around the rhexis, would damage the capsule or the zonules.

Our final design (Figure 3.3D) was both simple and less traumatic to the tissue due to being made from soft medical grade silicone. It consisted of a ring of silicone tubing, connected to a cannula, with 8 evenly spaced holes through which the treatment substance could be released (Figure 3.4).
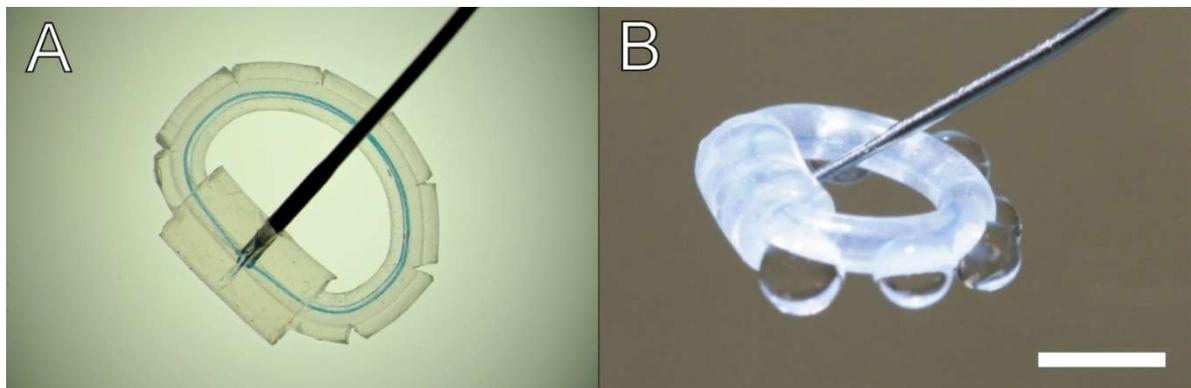
Figure 3.4 Top (A) and side (B) photos of our silicone irrigation ring. In photo (A) you can see the inner prolene suture and larger outer locking tube that maintains the ring coiled. In photo (B) you can observe how the treatment substance is released through all the holes. Scale bar = 2 mm.

3.2.1. Irrigation ring construction

The following material is needed: Silicone tubes with 1 and 2mm of exterior diameter, prolene sutures, 21G needle, forceps and Westcott scissors.

First you need to cut the larger silicone tube to a length of 4mm and the thinner tube to a length of 17mm. Next you pass the thinner tube through the larger tube. Once the tubes are together, you pass the prolene thread through the thinner tube until about 10mm extend from the end (Figure 3.5A). Then you tie both ends of the thread together in order to create a ring with the silicone tubing, and you slide the larger tube over the area where both ends of the thinner tube connect (Figure 3.5B). Finally, with the help of a 21G needle, you make 8 evenly spaced punctures around the equator of the ring (Figure 3.4A).
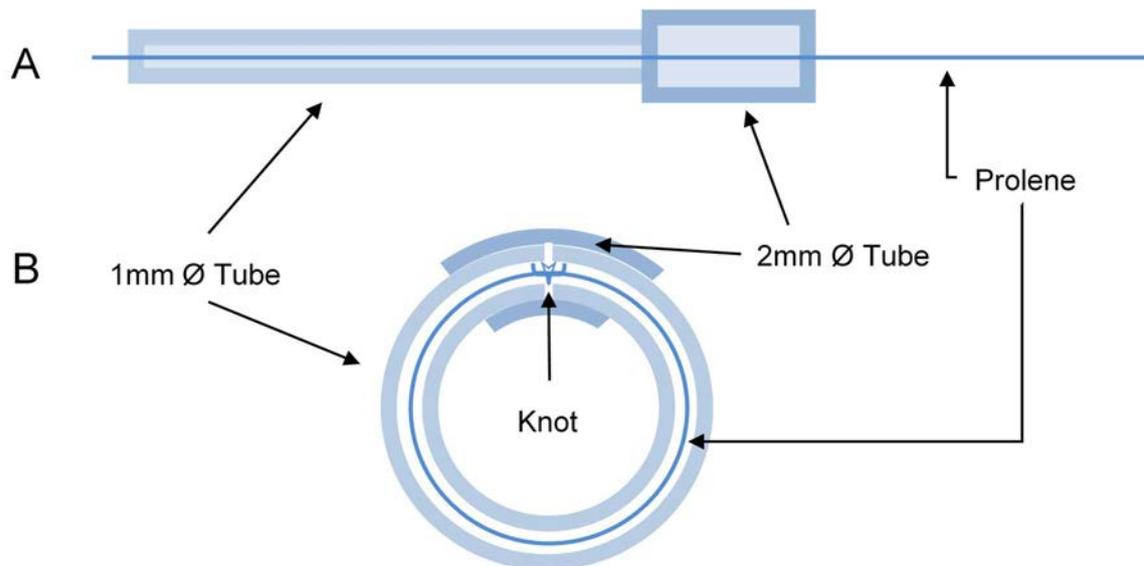
Figure 3.5 Schematic representation of the parts and construction of our silicone irrigation ring.

Our treatment protocol was as follows: Once the capsule sample is prepared, any residual BSS is removed and the silicone irrigation ring is placed within the capsule through the anterior rhexis. Once placed, the plunger of the syringe is slowly depressed while slightly rotating the ring left and right to assure complete contact of the treatment substance with the residual LECs. After the desired treatment time, the irrigation ring is removed and the capsule sample is washed with BSS. Control samples were only irrigated with BSS. Afterwards samples were taken to the culture room.

3.3. Tissue culture protocol

The completed specimens (Figure 3.1F) after treatment were transferred to a laminar flow hood and washed 3 times with Hanks balanced salt solution (HBSS) (H9269 Sigma-Aldrich) supplemented with 2% antibiotic/antimycotic solution (10000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL) (A5955 Sigma-Aldrich) for 3 minutes.

After the third wash, the specimen is placed in a petri dish with culture medium consisting of: RPMI-1640 media (R8758 Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS) (F7524 Sigma-Aldrich) and 1% antibiotic/antimycotic solution.

Finally, the petri dish is placed in a humidified CO_2-incubator at 37 °C and 5% CO_2. The culture medium is refreshed every 2 to 3 days, making sure to completely cover each sample with media.

3.4. Ocular tissue samples

This thesis consists of 4 experimental studies, for each, specific tissue types were needed. All of the tissues used in these studies are listed in the tables below (Table 3.1, Table 3.2, Table 3.3 and Table 3.4).

In total, sixty-eight human donor eye globes from the "Banc d'Ulls per a Tractaments de Ceguesa", Barcelona, and ten porcine eye globes from "Patel, S.A.U." Barcelona, were obtained. The human eye globes had been classified as non-suitable for transplantation. Written informed consent for the removal and use of the eye globes for diagnostic and research purposes was obtained from patients and/or relatives. All experimental studies were approved by the Ethical Committee for Clinical Research of the Centro de Oftalmología Barraquer and followed the tenets of the Declaration of Helsinki.

	Samples	Tissue type	Age (Years)	Sex	Postmortem Time (h)
PCO H2O2 culture (Scientific reports)	C030	Human lens capsule	66	F	120
	C031	Human lens capsule	73	M	12
	C033	Human lens capsule	43	F	24
	C035	Human lens capsule	88	M	96
	C039	Human lens capsule	86	M	72
	C040	Human lens capsule	69	M	72
	C008	Human lens capsule	80	F	48
	C018	Human lens capsule	54	F	48
	C022	Human lens capsule	63	M	120
	C026	Human lens capsule	64	F	72
	C027	Human lens capsule	70	M	120
	C023	Human lens capsule	82	F	144
	C024	Human lens capsule	89	F	120
	C025	Human lens capsule	89	F	120
	C029	Human lens capsule	79	F	96
	C032	Human lens capsule	73	M	12
	C034	Human lens capsule	43	F	24
	C036	Human lens capsule	88	M	96
	C038	Human lens capsule	59	F	24
	619B	Human lens capsule	92	M	50
	622B	Human lens capsule	54	M	120
	629A	Human lens capsule	89	M	148

Table 3.1 Relevant Information associated with all samples used in the study "**4.1 Prevention of posterior capsule opacification (Article 1)"**

	Samples	Tissue type	Age (Years)	Sex	Postmortem Time (h)
PCO Histology (Experimental Eye Research)	603a	Soemmerring's ring	73	M	168
	605a	Soemmerring's ring	96	F	143
	611a	Soemmerring's ring	88	M	43
	612a	Soemmerring's ring	92	F	44
	613a	Soemmerring's ring	92	F	40
	614a	Soemmerring's ring	91	F	69
	616a	Soemmerring's ring	91	F	100
	617a	Soemmerring's ring	89	M	32
	618a	Soemmerring's ring	94	F	83
	621b	Soemmerring's ring	92	F	121
	622a	Soemmerring's ring	54	M	127
	625a	Soemmerring's ring	74	M	98
	C030	Cultured lens capsule	66	F	111
	C031	Cultured lens capsule	73	M	12
	C033	Cultured lens capsule	43	F	25
	C035	Cultured lens capsule	88	M	90
	C039	Cultured lens capsule	86	M	61
	C040	Cultured lens capsule	69	M	80
	610a	Cataract lens	69	F	140
	615a	Cataract lens	85	M	34
	620b	Cataract lens	87	M	76
	307a	Normal lens	48	F	22
	M61	Normal lens	42	M	97
	M84b	Normal lens	35	M	49
	619b	Empty lens capsule	92	M	51
	622b	Empty lens capsule	54	M	127
	629a	Empty lens capsule	89	M	146
	624a	Normal cornea	69	M	17
	1612134A1	Corneal dystrophy	81	F	48

Table 3.2 Relevant Information associated with all samples used in the study "**4.2 Comparison of *in vivo* and *in vitro* posterior capsule opacification (Article 2)**"

	Samples	Tissue type	Age (Years)	Sex	Postmortem Time (h)
Transparency Study	705b	Transparent lens	85	M	20
	706a	Transparent lens	70	F	72
	561a	Soemmerring's ring	83	F	120
	563a	Soemmerring's ring	91	F	120
	577b	Soemmerring's ring	92	F	144
	590a	Soemmerring's ring	102	F	24
	600a	Soemmerring's ring	80	M	144
	605a	Soemmerring's ring	96	F	144
	607b	Soemmerring's ring	79	M	96
	608b	Soemmerring's ring	80	F	96
	612b	Soemmerring's ring	92	F	36
	613b	Soemmerring's ring	92	F	48
	616a	Soemmerring's ring	91	F	96
	617a	Soemmerring's ring	89	M	26
	618a	Soemmerring's ring	94	F	72
	621a	Soemmerring's ring	92	F	120
	621b	Soemmerring's ring	92	F	120
	622a	Soemmerring's ring	54	M	120
	625a	Soemmerring's ring	74	M	96

Table 3.3 Relevant Information associated with all samples used in the study "**4.3.1 Soemmerring's ring transparency analysis"**

	Samples	Tissue type	Age (Years)	Sex	Postmortem Time (h)
Regeneration culture test	C041	Test lens capsule	44	M	29
	C042	Test lens capsule	44	M	29
	C043	Test lens capsule	47	F	27
	C044	Test lens capsule	70	M	47
	C045	Test lens capsule	70	M	47
	C046	Test lens capsule	75	M	12
	C047	Test lens capsule	75	M	12
	700a	Human control capsule	54	M	17
	701a	Human control capsule	59	M	34
	LC01	Human lens capsule	89	F	7
	LC02a	Human lens capsule	61	M	18
	LC02b	Human lens capsule	61	M	18
	LC03a	Human lens capsule	74	M	22
	LC03b	Human lens capsule	74	M	22
	P93	Pig control capsule	0,5	-	9
	P94	Pig control capsule	0,5	-	9
	PLC01	Pig lens capsule	0,5	-	8
	PLC02	Pig lens capsule	0,5	-	8
	PLC03	Pig lens capsule	0,5	-	8
	PLC04	Pig lens capsule	0,5	-	8
	PLC05	Pig lens capsule	0,5	-	26
	PLC06	Pig lens capsule	0,5	-	26
	PLC07	Pig lens capsule	0,5	-	26
	PLC08	Pig lens capsule	0,5	-	26

Table 3.4 Relevant Information associated with all samples used in the study "**4.3.2 In vitro lens regeneration experiment"**

4. Results

4.1. Prevention of posterior capsule opacification (Article 1)

SCIENTIFIC REPORTS

OPEN

Prevention of posterior capsule opacification through intracapsular hydrogen peroxide or distilled water treatment in human donor tissue

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1

Prevention of posterior capsule opacification through intracapsular hydrogen peroxide or distilled water treatment in human donor tissue

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In order to determine whether posterior capsule opacification after cataract surgery, could be delayed or inhibited through the application of hydrogen peroxide (H_2O_2) or distilled water (H_2Od), we extracted lens capsules from 25 human donor eye globes. Samples were treated for 5min with either 30mM H_2O_2 or H_2Od or used as controls, and cultured for one month, during which dark field and tilt illumination photos were taken. These were used to observe and quantify, time until cellular growth and confluence on the posterior capsule. After culture, histological sections were stained for H&E, α-SMA, Ki-67 and vimentin and evaluated. We prevented cellular growth in 50% of H_2Od and 58% H_2O_2 of treated samples. The overall prevention of cell growth compared to cultured controls was significant for both treatments while there was no significant difference between them. In the cases where cellular growth was not prevented, both treatments significantly delay cellular growth. Until day 28 none of the treated samples of either type that had shown growth reached total confluence. All cultured controls reached total confluence before treated samples (median = day 11.5). Also, histologically, there was a clear morphological difference between cultured controls and treated samples.

Introduction

The development of posterior capsule opacification (PCO) is due to a combination of the processes of proliferation, migration, and transdifferentiation of residual LECs on the lens capsule, after cataract surgery, resulting in light scatter on the visual axis[65].

In the past decades, many approaches for PCO prevention have been studied, including adjustments of surgical techniques, IOL materials and design, pharmacological treatments, and prevention by interfering with biological processes (such as TGF-β signaling, proliferation, migration or membrane stability) in LECs.[55, 57, 59, 62, 217-219] So far the most effective method seems to be the implantation of an IOL with sharp edged optics to mechanically prevent PCO formation.[219]

The use of mechanical methods for the prevention of PCO might not be the best option since it limits the design of Intra ocular lenses (IOLs) and does not prevent PCO in all cases. If PCO could be prevented by chemical or pharmacological treatments, new IOLs or lens refilling techniques could be used or designed that would allow visual accommodation, eliminating the need for corrective multifocal glasses or lenses.[220, 221]

Different substances have been studied for the prevention of PCO, such as 5-Fluorouracil [134, 135], Mitomycin-C [222], Pirfenidone [140], Rapamycin [223], Ricin [152], etc., all with varying degrees of success in the prevention or postponement of PCO. Duncan G. and Wormstone I.M. studied thapsigargin [134, 158] which seems to be the most effective substance to date, leading to total lens cell destruction; however thapsigargin is cytotoxic and can damage the cornea [224].

To safely and effectively search for preventative substances, we decided to use tissue cultures. Although animal models and LEC cell line cultures are useful, animal models are more difficult to use and LEC cell line cultures are missing the anatomical conditions of the human eye. In this respect, tissue cultures of human capsular bags have advantages, mimicking surgical conditions *in vivo* and allowing growth of LECs on their preferred natural substrate, the lens capsule. The normal anatomical relationships of the capsule and LECs need to be preserved as closely as possible, if the findings of tissue culture models are to be applicable clinically [194].

We decided to test two basic substances for PCO prevention, distilled water (H_2Od) and hydrogen peroxide (H_2O_2). H_2Od since it has been tested many times before [134, 136, 158, 222, 225, 226] and gives us a point of reference with other similar studies, although its effectiveness has been inconsistent. H_2O_2 was selected due to its known presence in the

lens [227] and its dichotomous role in mammalian cells, being harmful or beneficial depending on its location and concentration. For example, rabbit LECs exposed to different concentrations of H_2O_2, result in cell death or proliferation depending on concentration.[228] Low levels of H_2O_2 have also been shown to promote faster wound healing in cornea both *in vitro* and *in vivo*.[229]

Material and Methods

The research adhered to the tenets of the Declaration of Helsinki on research involving human subjects. The experimental protocol was approved by the Ethical Committee for Clinical Research of the Centro de Oftalmología Barraquer (Study code: CultivoOCP2015-2018)

Donor eye globes were obtained from the "Banc d´Ulls per a Tractaments de Ceguesa" (BUTC). Written informed consent for the removal and use of the eye globes for diagnostic and research purposes was obtained from patients and/or relatives. In the cases that, after BUTC analysis, the eye (cornea) was classified as non-suitable for transplantation, and as long as the reason did not affect our tissue culture, we proceeded with its use.

A total of 25 donor globes were used, consisting of 3 uncultured & 7 cultured controls, 7 treated with distilled water (H_2Od) and 8 treated with hydrogen peroxide (H_2O_2).

Sample preparation

The capsular bag–ciliary body complex was dissected in our laboratory under standard biosecurity conditions based on the model proposed by Cleary G, et al.[194] The corneoscleral disk was removed from the globe with a circular trephine, and then the iris–ciliary body–lens complex was dissected from the globe in a single piece.

Specimens were transferred to sterile Petri dishes and placed posterior side up. Any residual adherent vitreous was removed from the posterior surface of the lens, being careful not to breach the posterior capsule. In some cases the vitreous membrane (hyaloid) was torn, leaving remnants on the posterior capsule.

For each sample a silicone ring mount was placed in a deep Petri dish containing balanced salt solution (BSS). The specimen were placed on the silicone ring, anterior surface facing upward, and attached by passing 8 G30 needles through the ciliary body. Afterwards the iris was removed with forceps (Figure 4.1).
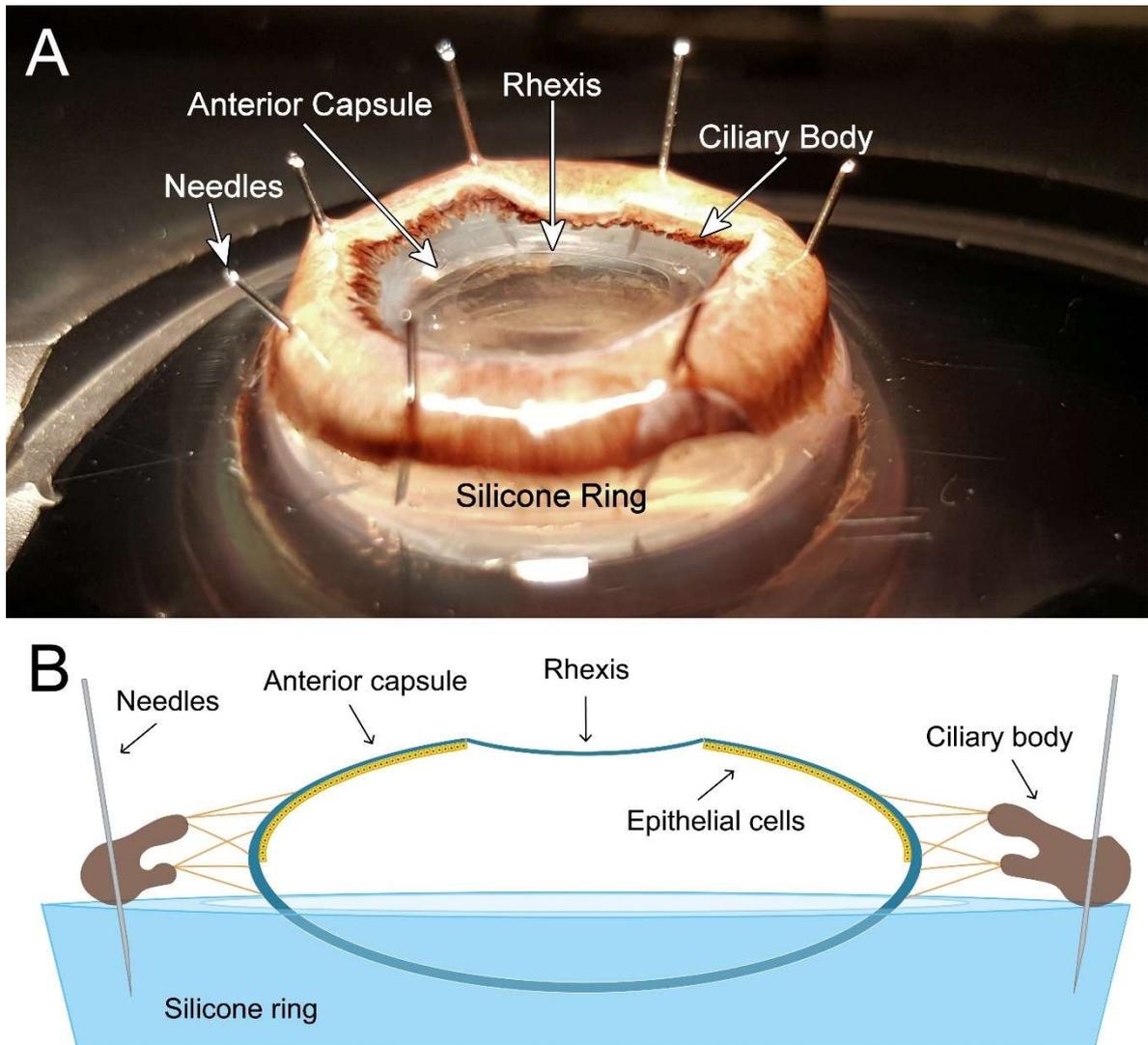
Figure 4.1 (A) Image of a prepared capsule-ciliary body complex fixed to a silicone ring before treatment or being placement in culture media. (B) A schematic diagram illustrating a prepared sample and the distribution of lens epithelial cells.

Finally the anterior capsule was breached with capsulorhexis forceps and a 6 mm continuous circular capsulorhexis was performed. Hydrodissection and hydroexpression were employed and residual lens fibers removed manually with a non-toothed forceps or aspirated with a Simcoe cannula. No IOLs were implanted in the capsule to prevent variations in the results due to the mechanical prevention of cell progression. At this point, the 3 uncultured controls were fixed in 4% paraformaldehyde, for histological analysis. We did this in order to observe the starting point of our cultures and to assure that we started from a LECs monolayer. (Supplementary Figure 4.1 & Supplementary Table 4.1)

Sample Treatment

Treatments were performed using an irrigation device designed by our group (Figure 4.2). This device is not intended for clinical use. A sealed capsule irrigation device such as the PerfectCapsule™ (Milvella Pty. Ltd.) would have been preferable, but it is no longer on the market. Such a device would avoid contact of the treatment substance with the surrounding tissue.[225] Our device consists of a 16 mm silicone tube (1 mm outer diameter), tied in to a ring with an inner suture and covered with a second 3 mm silicone tube (2 mm outer diameter) where the 2 ends of the first tube meet. Eight evenly spaced cuts are made around the tube to permit uniform liquid distribution. Afterwards it is attached to a syringe, with a blunt needle, containing the treatment substance. Finally, the device is introduced in to the capsular bag through the rhexis. This device assures a uniform distribution of the treatment substance within the capsular bag.
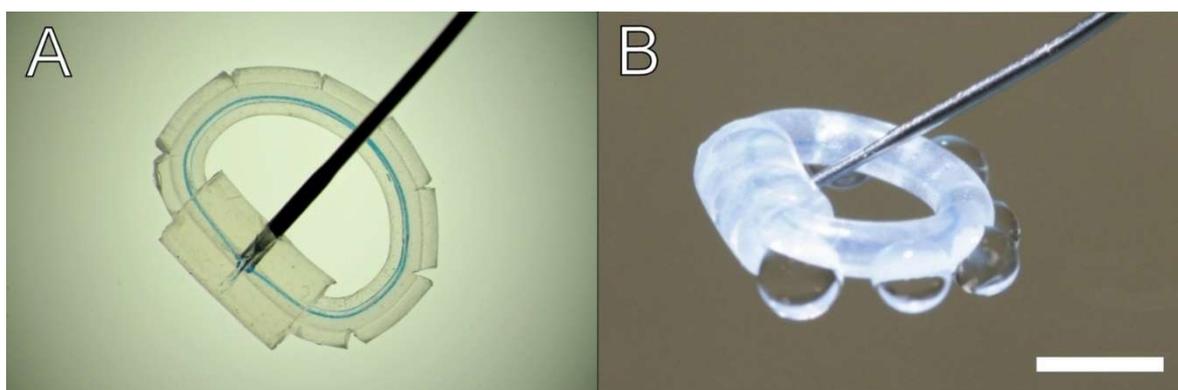
Figure 4.2 (A) The silicone irrigation ring we designed to treat our samples. (B) Visualization of treatment solution distribution from ring. Scale bar = 2 mm.

Treatments of samples were performed in the absence of BSS to avoid dilution. Samples were treated with 30 mM H_2O_2 or H_2Od during 5 min, after which, samples were thoroughly washed with BSS to remove the treatment. Cultured control samples were only irrigated with BSS.

Tissue culture

All samples once prepared and treated were transferred to a biosafety cabinet (Bio-II-A, Telstar®) and washed 3 times with a BSS and antibiotic/antimicotic solution for 3 minutes. After the third wash, samples were placed in RPMI-1640 culture medium (R8758 Sigma-

Aldrich) supplemented with 5% fetal bovine serum (F7524 Sigma-Aldrich) and 1% antibiotic-antimycotic (A5955 Sigma-Aldrich).

The culture medium was exchanged every 2 to 3 days, and LEC growth and migration across the posterior capsule was monitored and documented using an inverted phase contrast microscope (Axiovert 100, Zeiss). Samples were to be cultured for one month.

Culture monitoring

Photographs were taken with the microscope, using phase contrast (Ph2) illumination with a normal 2.5x Plan-objective, giving dark field images (Figure 4.3 A, C & E). This allowed for the evaluation of relative transparency of the capsule and migration patterns of LEC.
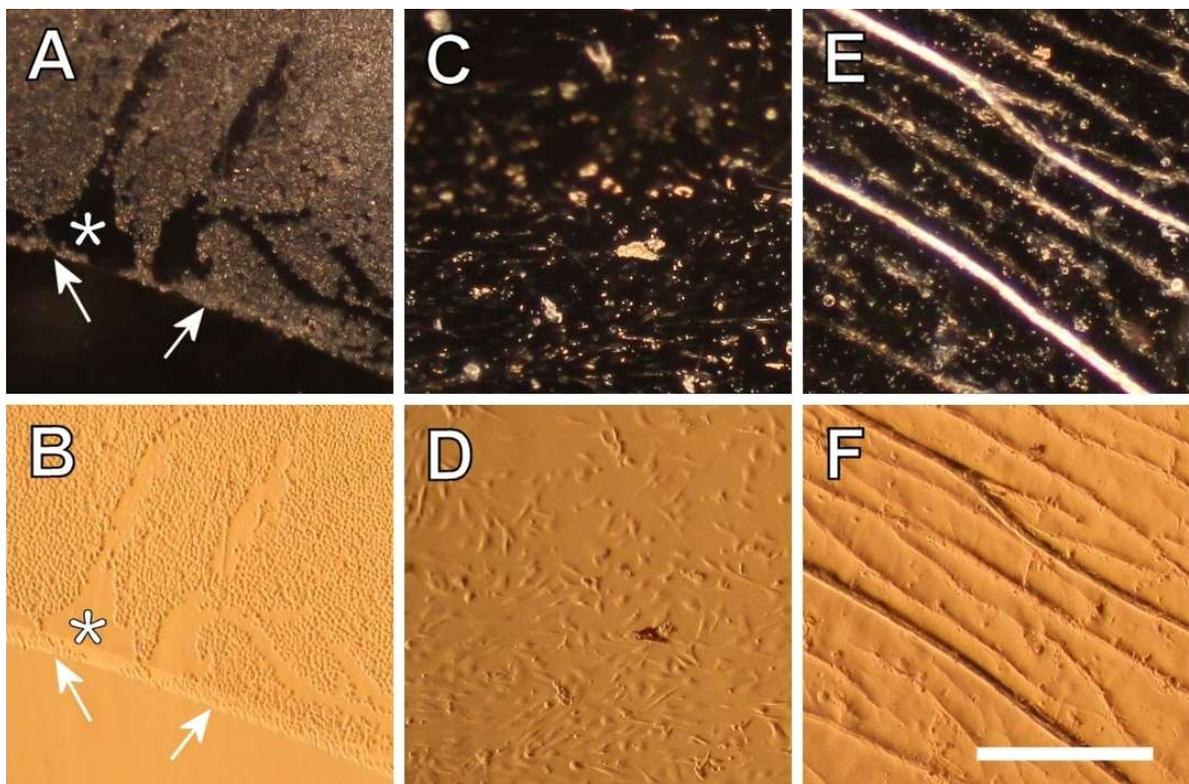
Figure 4.3 (A,C & E) Dark field and (B,D & F) tilt illumination images of cultured control samples, showing different types and stages of PCO. (A,B) Initial organized distribution of LECs on the anterior capsule and clear posterior capsule. Arrows indicate rhexis border. Asterisk shows area where LECs where accidentally scraped off during hydrodissection. (C,D) Migrated cells on the posterior capsule of a sample after 36 days in culture. (E,F) Wrinkles produced by cells on the posterior capsule of a sample after 33 days in culture. Scale bar = 400 µm.

Photographs of the same regions were also taken with the phase contrast illumination ring slightly misaligned (Technique previously described by "Eldred et al. 2014" and

named modified dark field) [230]; we termed these "tilt illumination images" (Figure 4.3 B, D & F). This technique gives better images of the individual cells, facilitating the visualization of cellular migration and morphology on the posterior capsule (Figure 4.4).
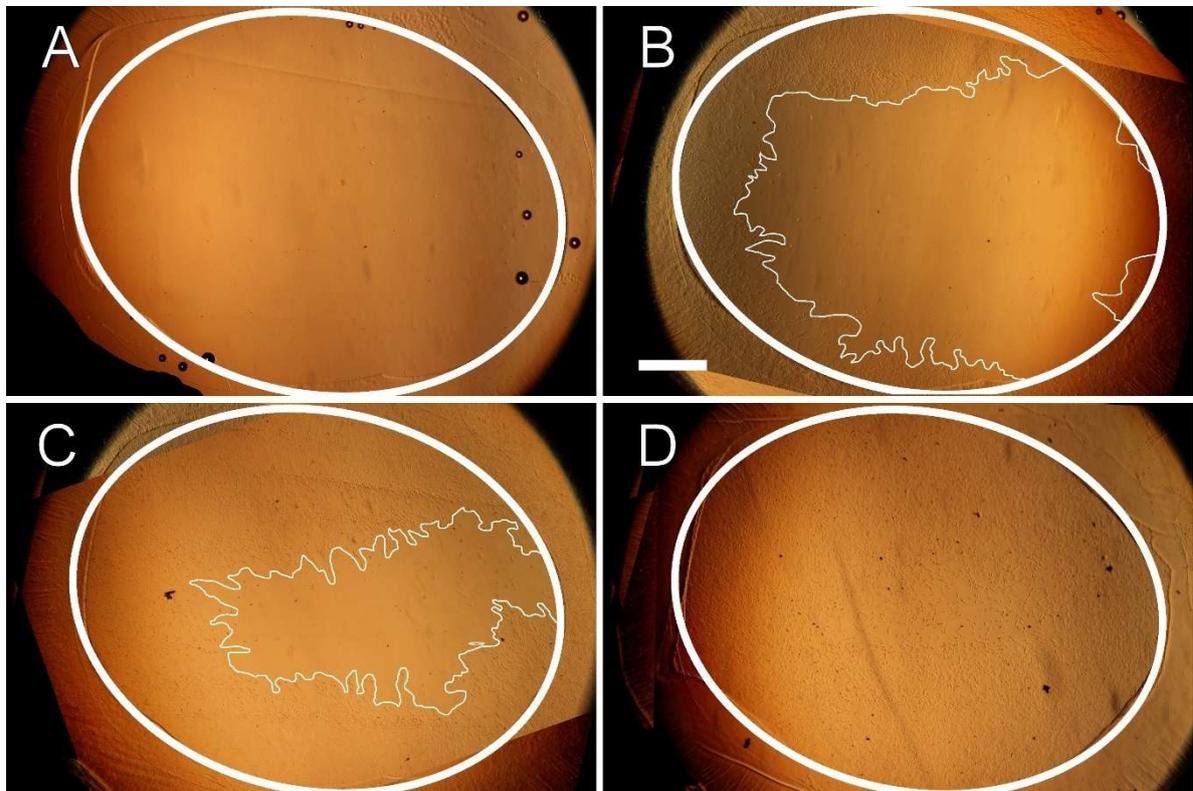
Figure 4.4 Tilt illumination images of cellular growth progression on the posterior capsule of cultured control sample C031 on (A) day 1, (B) day 8, (C) day 10 and (D) day 17 of culture. The thicker white line indicates the rhexis border and the thinner white line indicates the border of progression used to calculate total area coverage on the posterior capsule and speed of growth. Scale bar = 1 mm.

With these imaging techniques, we could distinguish different aspects of PCO, such as cellular distortions; small clusters of disorganized lens cells on the posterior capsule that scattered light in dark field images (Figure 4.3, C & D) or wrinkles; folds on the posterior capsule caused by lens cell migration and myofibroblastic differentiation resulting in visual distortions [57] (Figure 4.3, E & F).

We subjectively quantified the degrees of these distortions in order to asses PCO severity. Four degrees of wrinkles and cellular distortions were defined: transparent (-), low (X), medium (XX) and high (XXX) degree of either wrinkles or cellular distortions (Figure 4.5). We based this scale on the lowest and highest degrees of distortions observed in our samples.

We defined cellular growth as the presence of cells on the posterior capsule within the visual limits of the anterior rhexis, termed rhexis area. Once the rhexis area was totally covered in cells it was considered that total confluence was reached.

The area between the rhexis and the border of cell progression was considered the area of cell coverage. This area was calculated by outlining the rhexis and the border of migrating cells on the posterior capsule in all sample images as seen in Figure 4.4. These images were then processed using ImageJ [231], giving the area in pixels which we passed to mm^2.

Confluence speed was calculated as increase of area of cell coverage over the respective time interval and expressed in mm^2/day.

Histological staining

Immediately after samples were removed from culture, they were placed in 4% paraformaldehyde for fixation for at least one week and then embedded in paraffin. Once all samples were in paraffin, they were sectioned along the sagittal plane and stained on the same day. Tissue were stained with hematoxylin and eosin for morphological analysis, α-smooth muscle actin α-SMA (clone 14A, Cell Marque) for myofibroblast formation and intercapsular adhesion [232-234], Ki-67 (clone 30-9, Ventana) for proliferation [235] and vimentin (clone V9, Ventana) a marker of both undifferentiated lens epithelium as well as differentiating fiber cells[236]. All immunostainings were done with the BenchMark® ULTRA device (Ventana Medical Systems, Inc.). We also included negative immunostain controls to support the validity of our stains and identify possible experimental artefacts or background noise. These controls were processed in the same manner with the automated staining device but without the primary antibodies. Capsular adhesion, cellular morphology and the degree of staining of α-SMA, Ki-67 and vimentin were studied at the equatorial region of the capsule and at the center of the posterior capsule.

Statistics

For the statistical analysis of treatment efficacy, the Kaplan-Meier method with Log rank test was used. We assigned cellular growth or total confluence as end point events. A p-value of ≤ 0.05 was considered as significant.

Results

|	Samples	Pairs	Age (Years)	Sex	Postmortem Time (h)	Day 1	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	Day 35	Wrinkles (~Day 15)	Wrinkles (~Day 30)	Cellular distortion (~Day 15)	Cellular distortion (~Day 30)
Control	C030		66	F	120									XX		XXX	
	C031	a	73	M	12									-	-	XXX	XXX
	C033	b	43	F	24									XXX	XXX	XX	XXX
	C035	c	88	M	96									XX	XXX	X	XX
	C039		86	M	72									-	XXX	X	XX
	C040		69	M	72									XXX	XXX	XX	XXX
H2Od	C008		80	F	48									-		-	
	C018		54	F	48									-	-	-	-
	C022		63	M	120									-	-	-	-
	C026		64	F	72									-	-	X	XX
	C027		70	M	120									-	X	X	XX
H2O2	C023		82	F	144									-	-	-	-
	C024	d	89	F	120									-	X	-	XX
	C025	d	89	F	120									-	-	-	-
	C029		79	F	96									-	-	-	-
	C032	a	73	M	12									-	-	-	XXX
	C034	b	43	F	24									-	-	-	-
	C036	c	88	M	96									-	-	-	X
	C038		59	F	24									-	-	-	XX

No cellular growth ▢ Cellular Growth ▢ Total confluence ▢

Figure 4.5 Relevant Information associated with samples used in this study and summarized results of culture. Samples with matching letters are pairs. F = Female, M = Male. The amount of wrinkles and cellular distortion on the posterior capsule were based on tilt illumination and dark field images, on a scale from 0 (−) to 3 (XXX).

Tissue sample information and a general overview can be seen in Figure 4.5. Samples were cultured for an average of 33 days, excluding samples C030, C008 and C029. These didn't reach the planned 30 days of culture but still gave us valuable data. All cultured control samples showed signs of cellular growth within the first week of cell culture. Some H_2Od samples started showing signs 2 weeks in and H_2O_2 samples only started showing some signs of cellular growth 3 to 4 weeks in. The degree of wrinkles and cellular distortions was much higher and more severe in cultured controls than in treated samples (Figure 4.5). Visual examples of these degrees can be seen in Figure 4.6, at day 30: C034 is transparent (-) and shows no wrinkles or cellular distortions, C026 shows no wrinkles (-) but a medium degree (XX) of cellular distortions and C033 shows a high degree (XXX) of both wrinkles and cellular distortions. One cultured control and two H_2Od samples were contaminated before day 5 and no useful results could be obtained so they were discarded.
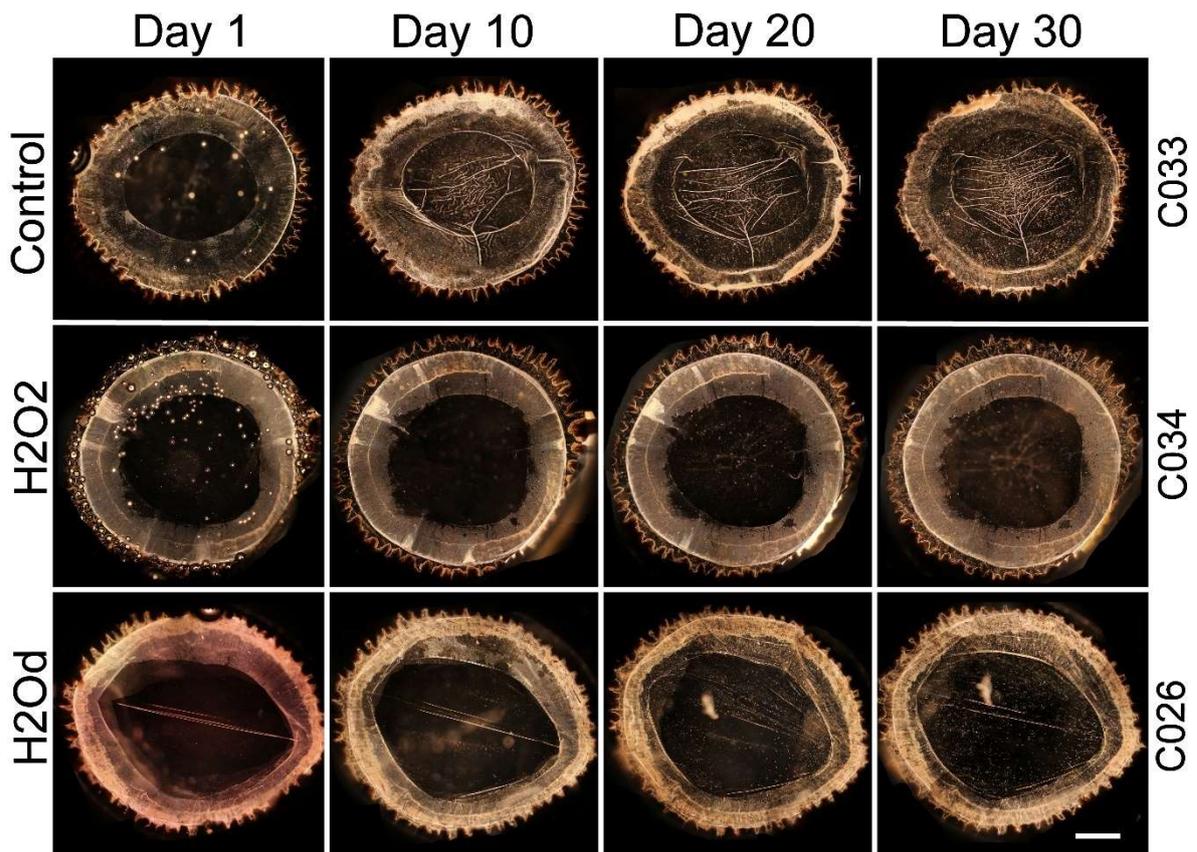
Figure 4.6 Dark field images of three samples on days 1, 10, 20 and 30 of culture. Cells clusters at the equator and wrinkles can be seen on the cultured control sample. Transparent posterior capsule can be seen throughout the whole H2O2 sample. Scattered cellular growth can be seen on the H2Od sample day 30. The wrinkles that can be seen in H2Od sample is due to the uneven tension of the support needles, not cell migration. Scale bar = 2 mm.

Most cultured control samples showed medium amounts of wrinkles on the posterior capsule by day 10 while all showed signs of cellular distortions. As culture time progressed the amount of wrinkles and cellular distortions increased. As cells start to migrate; they first concentrate on the equator of the capsule (Figure 4.6). Sometimes these cells agglomerate in to clusters of cells that seem to be the first stages of Soemmerring ring like formations.

Only one sample from each of the treatment types showed signs of wrinkles and these in low amounts. Varying amounts of cellular distortions were visible in all treated samples that showed signs of cellular growth. An example of cellular distortion can be seen in Figure 4.6, H_2Od days 20 and 30. The H_2O_2 treatment kept the posterior capsule transparent in half of the samples, including the sample shown in Figure 4.6.

All cultured control samples showed signs of cellular growth on the posterior capsule on average by day 6, 2 of 5 H_2Od samples showed growth on day 14 and 4 of 8 H_2O_2 samples showed growth on average on day 29 (Range: day 22-32) (Figure 4.5).

Cellular growth was prevented in all treated samples on day 10. On day 20, prevention was successful in half of H_2Od and all H_2O_2 samples. On day 30, prevention was still successful in half of H_2Od samples while it had failed in 2 H_2O_2 samples. The overall prevention of cellular growth compared to cultured controls was significant for both treatments according to Kaplan Meier statistics (H2Od p=0.001 and H2O2 p≤0.001) while there was no significant difference between treatments.

Total confluence occurred in all cultured controls on median by day 8 (Range: day 7 to 26), one H_2Od sample reached total confluence on day 28 and 2 H_2O_2 samples reached total confluence on days 29 and 36 (Figure 4.5). The overall prevention of confluence compared to cultured controls was significant for both treatments according to Kaplan Meier statistics (H2Od p=0.002 and H2O2 p≤0.001) while there was no significant difference between treatments.

The average rhexis area was 31.4 mm^2 (Range: 23.0 to 35.5mm^2), due to this variation, in order to compare confluence progression, we interpreted the area of cell coverage as a percentage of rhexis area. A graph expressing the increase in rhexis area coverage over time in all samples can be seen in Supplementary Figure 4.2. The average speed at which samples reached total confluence was similar among cultured controls (3.2 mm^2/day ± 0.9) and H_2O_2 treated samples (3.0 mm^2/day ± 2.2) (95% confidence interval). Confluence speed in H_2Od treated samples was slower (2.0 mm^2/day ± 0.3).

All cultured controls (except C031), H2Od sample C027 and H2O2 sample C024 presented intercapsular adhesion. The cellular morphology of uncultured control samples consisted of a single continuous LEC monolayer, while cultured control samples along the equator consisted of stacked layers of cells in all samples except C031 which only presented a monolayer. Cells were also present on the central posterior capsule of all cultured controls. Cellular morphology in treated samples was similar for both treatments; showing some dispersed cells and nuclei at the equator but manly residual cell fragments. These fragments were also present on the central posterior capsule of 4 of the 6 treated samples that had shown cell growth (Figure 4.7 and Figure 4.8).

	Samples	Capsule Adhesion	Morphology E	Morphology C	α-SMA E	α-SMA C	Ki-67 E	Ki-67 C	Vimentin E	Vimentin C
Control	C030	Yes	+++	+	XX	XX	X	X	XXX	XX
Control	C031	-	+	+	-	X	-	-	XXX	XXX
Control	C033	Yes	+++	+	X	XXX	-	X	XXX	XXX
Control	C035	Yes	+++	++	XX	XXX	-	X	XXX	XXX
Control	C039	Yes	+++	++	XX	XXX	-	-	XXX	XXX
Control	C040	Yes	+++	++	XX	XXX	-	X	XXX	XXX
H2Od	C008									
H2Od	C018		+		X		-		XX	
H2Od	C022		++		XX		X		XX	
H2Od	C026	-	o	-	X	-	-	-	XX	-
H2Od	C027	Yes	+	o	XX	XX	-	-	XXX	XXX
H2O2	C023	-	-	-	-	-	-	-	-	-
H2O2	C024	Yes	+	o	XX	XX	-	-	XX	X
H2O2	C025	-	o	-	X	-	-	-	XX	-
H2O2	C029	-	o	-	XX	-	-	-	XX	-
H2O2	C032	-	+	o	X	-	-	-	XX	XX
H2O2	C034	-	o	-	X	-	-	-	XXX	-
H2O2	C036	-	++	o	XX	-	-	-	XXX	XXX
H2O2	C038	-	o	-	-	-	-	-	X	-

Figure 4.7 Overview containing all relevant information obtained from histological stains. Intercapsular adhesion between anterior and posterior capsule was indicated as present (yes) or not (−). Observations were made at the equator (E) and the central posterior capsule (C). Cellular Morphology was classified as "clusters or stacked layers of apparently normal cells" (+++); "dispersed, but apparently intact cells with nuclei and cytoplasm" (++); "nuclei without visible cytoplasm" (+); "only residual cell fragments" (o) or "absence of cells and fragments" (−). The fractions of cell content stained with α-SMA, Ki-67 or Vimentin were classified as "all or large majority" (xxx), "about half" (xx); "few" (x) and "none" (−). Samples C008, C018 and C022 are lacking the majority of their histological results due to an error during their sectioning (positions blank).
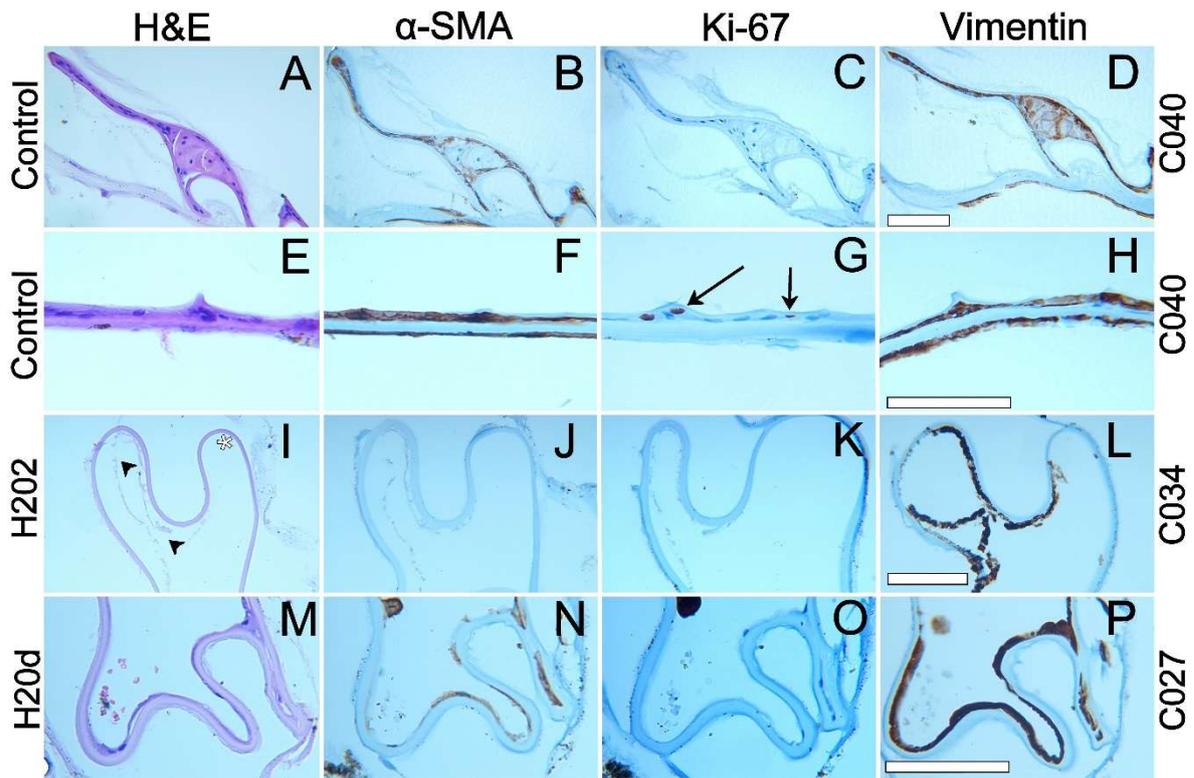
Figure 4.8 Microscopic images of the histological sections of some of our samples, showing staining for hematoxylin and eosin (H,E), alpha smooth muscle actin (α-SMA), ki-67 and Vimentin. Images (A–D) show the equator of cultured control sample C040. Images (E–H) show the central posterior capsule of sample C040. Images (I–L) show the equator of H2O2 sample C034. Images (M–P) show the equator of H2Od sample C027. Arrows highlight Ki-67 staining in image (G). Arrowheads show residual cell fragments (I). Asterisks highlight an area free of cells (I). Scale bars = 100 μm.

Histologically all cultured controls stained for α-SMA at the equator and more intensely on the center of the posterior capsule, except one (C031). This cultured control sample was the only one that does not show capsular adhesion or wrinkling (Figure 4.5 and Figure 4.7). Uncultured controls were negative for α-SMA (Supplementary Figure 4.1). Most treated samples showed α-SMA staining but to a lesser degree and even on cellular fragments (Figure 4.7). Ki-67 was present on the posterior capsule of 4 of the 6 cultured controls, showing that cells still proliferate after confluence on the posterior capsule. One uncultured control showed Ki-67 staining at the equator (Supplementary Figure 4.1). Only one treated sample (C022) showed signs of Ki-67, indicating that proliferation had been delayed but not totally prevented and given time this sample would probably have developed PCO. Vimentin was present on all samples with either cells or cell fragments (Figure 4.7, Figure 4.8 and Supplementary Figure 4.1). All negative immunostain controls confirmed the absence of background noise (Supplementary Figure 4.3).

Discussion

Overall both distilled water and hydrogen peroxide had a clear effect on PCO, delaying it or in about half of the samples preventing it for 30 days or more.

The variations between samples due to sample preparation, such as rhexis size and removal of lens fibers from within the lens capsule, could have had effects on the initial number or concentration of cells. We tried to mitigate these variables through the random distribution of samples in the different groups, and the use of paired samples in different groups. The three uncultured control samples also only showed a monolayer of LECs without lens fibers, indicating that lens fiber cell removal was complete and consistent. Some contralateral samples were used as cultured controls and their pairs were treated with H_2O_2. The significant difference between their cellular growth times supports our hypothesis that H_2O_2 can help prevent PCO. Donor age, sex or the postmortem time of the samples had no apparent effects on the results.

A previous study [228] used rabbit LEC lines to evaluate the effects of hydrogen peroxide exposure. Showing that high levels (1 mM) of hydrogen peroxide killed cells and sub lethal levels (100 µM) suppressed their proliferation. While unexpectedly from 1 nM to 1 µM of hydrogen peroxide, there was a dose dependent increase in the cell numbers. Our 30 mM H_2O_2 treatment concentration was based on this previous study [228], however due to the large difference in treatment time (5 min instead of 48 h) our final concentration of 30 mM was an approximately proportional increase to the 100 µM used to suppress LEC line cultures. Furthermore, a study by Tholozan F. et al.[237] indicated that the lens capsule is a source of essential survival factors for LECs, making them more resistant to substance induced apoptosis then LECs cultured in the absence of the capsule, such as in the rabbit study[228], in which case, a higher concentration of H2O2 is needed in order to obtain the same results.

H_2O_2 has the benefit of not only being an endogenously produced substance [227]; it has also been shown to have beneficial effects on the cornea in a similar study [229], reducing the risks of post-operative corneal complications. Low levels (10–50 µM) of H_2O_2 in culture for 48 hours, stimulated adhesion, migration, and faster wound healing on rabbit and pig corneal epithelial cells both *in vitro* and *in vivo*.

However, corneal endothelium is more delicate than corneal epithelium and lacks regenerative capabilities. There is a normal physiological concentration of around 0.5 mM H2O2 in the aqueous humor.[238] Fluctuations of up to 3 fold concentration of H2O2 can be regulated by endogenous catalase.[239] At higher concentrations, the corneal

endothelium is in danger of being damaged. This is why our treatment is meant to be applied with a sealed capsule irrigation device, thus avoiding contact of the treatment with the surrounding tissues.

These studies together with our results seem to indicate that it is possible that an ideal concentration of H_2O_2 could be found, that suppresses proliferation of LECs while not putting corneal cells in danger or even helping speed up corneal wound healing due to cataract surgery.

Our results can be considered representative of naturally occurring PCO, since the cellular transformations expected in cultured control samples due to epithelial–mesenchymal transition are appreciable in Figure 4.3. Where well organized, adhered and uniform LECs can be seen at the start of culture in A and B. While larger, elongated and unadhered mesenchymal cells can be observed on the posterior capsule in C and D after 36 days in culture, having migrated there from the anterior capsule.

It is interesting to note that even with our relatively high concentration of H_2O_2, in the cases where it did not prevent growth, but only significantly delayed it, the average speed at which samples reached total confluence, was similar to cultured controls. This shows the resilience and high proliferative capabilities of the surviving LECs. These results highlight the fine line between an effective and ineffective treatment and how difficult it is to find a 100% effective treatment.

Our results of 50% PCO prevention with H_2Od treatment after one month were comparable or slightly better than earlier studies. One study [134] with human tissue culture obtained 20% PCO prevention, while another *in vivo* study [222] using rabbits, obtained 40% prevention. To our knowledge, the only study to obtain 100% PCO prevention with LEC death was with the use of 100µM of Thapsigargin [134], however this substance is highly cytotoxic.

The instances of cellular growth and PCO that were observed in treated samples, did appear significantly later than in cultured controls and after the end point (day 28) of other similar studies.[134, 135] Based on this, future studies should aim to culture for longer than a month to assure results.

Histologically, there was a clear difference in the morphological aspect of cultured controls compared to treated samples (Figure 4.7). These controls showed cells with well-defined nuclei and tended to form unorganized agglomerations of cells near the equator (Figure 4.8A). In general, treated samples presented fewer cells which tended to be smaller and dispersed. In most H2O2 samples, only residual cell fragments with no

visible nuclei were found. These fragments can still stain positively for both α-SMA and Vimentin (Figure 4.8J and L). This was to be expected as both are found in the cytoskeleton of LECs and Vimentin also in early lens fiber cells [236].

It is known that both treatments severely damage cells, H2Od through hypotonic cell lysis [240] and H2O2 through apoptosis or necrosis.[228, 241] These processes are probably the origin of the residual cell fragments we see in our samples. Neither treatment seemed to prevent epithelial to mesenchymal transition since most samples expressed α-SMA, a known marker of this transition.[233, 234] α-SMA also plays a role in capsular contraction during cell migration.[29] Its expression in our samples correlated with these functions, since it was present in all cases of wrinkling and intercapsular adhesion and staining was highest in areas of close intercapsular adhesion (Figure 4.8B). Also, the only sample that did not present α-SMAs (C031) did not show capsular adhesion or wrinkling (Figure 4.4).

In conclusion, hydrogen peroxide is a possible treatment for the delay of posterior capsule opacification, but the resilient nature of LECs makes this a difficult task.
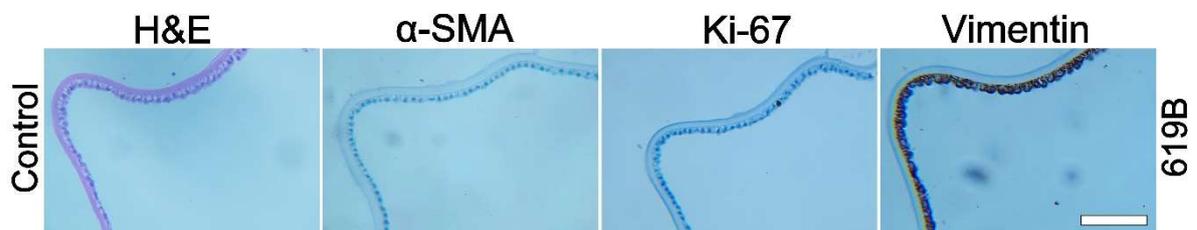
Supplementary materials

Supplementary Table S1.

|	Samples	Age (Years)	Sex	Postmortem Time (h)
	619B	92	M	50
Uncultured Control	**622B**	54	M	120
	629A	89	M	148

Supplementary Table 4.1 Table with the basic information of the three uncultured control samples.

Supplementary Figure S1.

Supplementary Figure 4.1 Microscopic images of the histological sections of one of our uncultured control sample 619B, showing staining for hematoxylin and eosin (H&E), alpha smooth muscle actin (α-SMA), ki-67 and Vimentin. Scale bars = 100 μm.

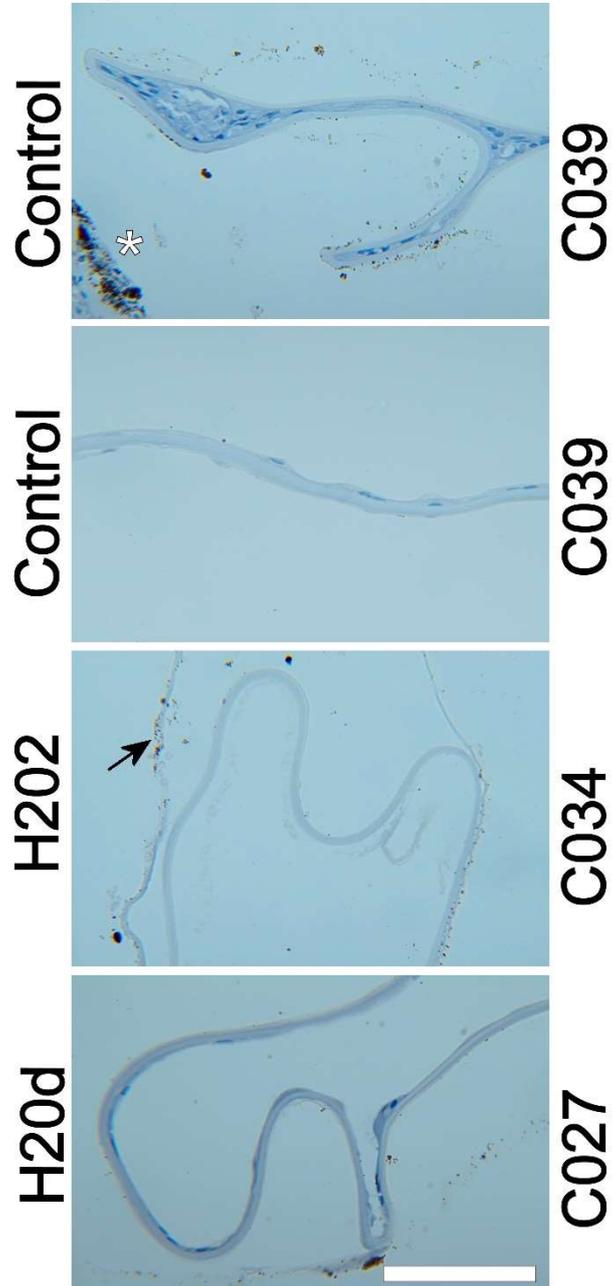## Supplementary Figure S2.
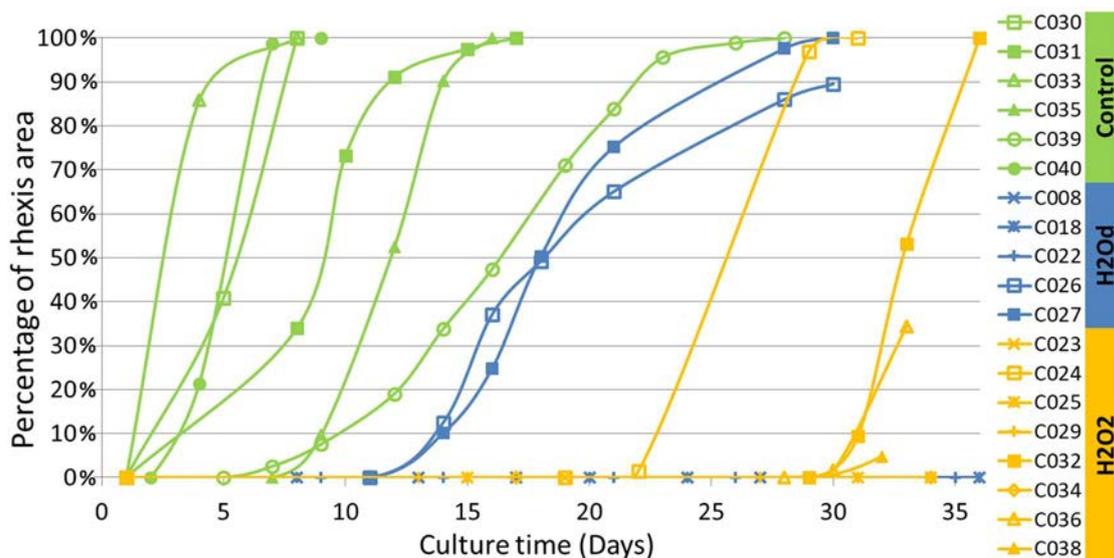
Supplementary Figure 4.2 Diagram of cellular confluence progression during cell culture. Showing percentage of confluence on posterior capsule against culture time. 0% means no signs of cell growth on posterior capsule within visual limits of the rhexis and 100% means total confluence. Samples C008, C018, C022, C023, C025, C029 and C034 showed no signs of growth.

Supplementary Figure S3.


Supplementary Figure 4.3 Histological sections of the negative immunostain controls of our samples. Immunostains processed with the BenchMark® ULTRA autostainer omitting the primary antibody. Within our capsule samples, no staining is observed. Asterisk show pigmented ciliary body next to control sample C039. Arrow points at zonule with adhered pigment Scale bars = 100µm.

4.2. Comparison of *in vivo* and *in vitro* posterior capsule opacification (Article 2)
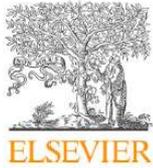
Histological comparison of in vitro and in vivo development of peripheral posterior capsule opacification in human donor tissue

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Histological comparison of *in vitro* and *in vivo* development of peripheral posterior capsule opacification in human donor tissue

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Abstract

In order to study the mechanisms involved in the development of posterior capsule opacification (PCO) we compared *in vivo* developed PCO with PCO formed in tissue culture with focus on the periphery of the lens capsule to evaluate lens regeneration potential. We studied 3 human tissue groups: Cultured lens capsules after mock cataract surgery (n=6, 30 days), lens capsules from donors that had previously undergone cataract surgery (IOL capsules) (n=12) and intact lenses (n=6). All samples were stained with Vimentin, alpha Smooth Muscle Actin, Picro Sirius Red (for collagen) and Paired box protein (Pax6). We found that cultured capsules and less developed IOL capsules consisted mainly of monolayers of mesenchymal cells, while more developed IOL capsules, contained LECs, globular cells and lens fiber cells. Many IOL capsule samples expressed collagen I and III in areas where cells were in contact with the IOL. Pax6 had a similar dispersed distribution in less developed IOL capsules and cultured capsules, while more developed IOL capsules and intact lenses, concentrated Pax6 in LECs at the equatorial lens bow. The similarities between cultured capsules and less developed IOL capsules indicate that our *in vitro* developed PCO is comparable to early *in vivo* developed PCO. The similar morphology of more developed IOL capsules and intact lenses seems to indicate an attempt at lens regeneration.

Introduction

After cataract surgery, residual LECs remaining in the lens capsule can proliferate, migrate and differentiate on the posterior capsule where they develop in an apparently unorganized manner, leading to posterior capsule opacification (PCO).[60-62, 74, 168, 242] PCO is still the most common long-term complication following cataract surgery. Clinically, PCO can be distinguished on the posterior capsule, behind the implanted intra ocular lens (IOL), as fibrotic PCO consisting of micro structures, wrinkles and fibrotic cells or as regenerative PCO consisting of plates and Elsching's pearls.[70] However, in many cases, cells do not migrate behind the IOL, rather they agglomerate at the periphery of the capsule (at the lens equator) around the implanted IOL forming a donut-like structure, known as a Soemmerring's ring.[166]

The *in vivo* developed Soemmerring's ring has previously been studied in post mortem human and rabbit eyes, using light and scanning electron microscopy.[79, 215, 243, 244] These studies showed monolayers of LECs, normal fiber cells, globular differentiating fiber cells (Elsching's pearls) and amorphous structures. Other histological studies showed that an important factor in PCO and Soemmerring's ring development is the epithelial to mesenchymal transition of LECs. Mesenchymal cells are positive for alpha smooth muscle actin (α-SMA), Vimentin and fibronectin and are fibroblastic in morphology [64, 75, 245].

To our knowledge, the first *in vitro* lens capsular bag culture model was designed in 1996 [193]. After a mock cataract surgery on a donor eye globe, the lens capsule is excised and pinned directly to a petri dish. This model, showed many of the changes seen *in vivo*, including rapid LEC growth, wrinkling, tensioning, and light scatter on the posterior capsule. However, since pinning directly through the capsular bag can damage it, other groups developed modifications to improve this model. Such as, using a holder to support the lens capsule [203] or a capsular tension ring to maintain the shape of the capsule[204]. In 2010, Cleary et al.[194] designed the base model for most current *in vitro* studies. This model retains the ciliary body, which is pinned to a silicon ring, effectively suspending the capsular bag in a more physiological manner. Recently variations in the culture media, such as serum concentrations, have been studied in order to better mimic clinical events following cataract surgery.[205]

The exact mechanisms that modulate residual LECs in the lens capsule after cataract surgery are still under study, but there are some known factors that lead to the

proliferation of LECs and finally to PCO.[246] The inflammatory response after surgery elevates the concentration of the growth factor (TGF-β) in the aqueous humor and induces epithelial to mesenchymal transition [247-249] At the same time, there is a synthesis of α-SMA, which due to its retractile properties enables cells to migrate, which in turn can produce wrinkles on the posterior capsule.[59, 250-252] However it has also been shown that LEC can migrate without the need for α-SMA [242], showing that some mechanisms still need to be studied further.

Fibrosis is another important factor in PCO development [242], leading to fibroblastic transformation and elongation of cells and to extracellular deposits such as collagen, fibronectin and myofibroblasts.[92, 188, 253] These biological processes lead to increased light scattering in the eye, reducing contrast sensitivity and visual acuity.

Animal models [69, 249, 254], cell culture models [255-257] and tissue models [194, 205, 258] have been used to study PCO development and prevention. However, to our knowledge, no in-depth histological comparisons of these models with naturally occurring PCO in humans has been performed. Furthermore, most *in vitro* models focus on the development of PCO on the central posterior capsule, which is more relevant for the vision of patients. However peripheral PCO is important, for instance, for the design of new accommodative IOLs, the study of long term PCO development or the study of crystalline lens regeneration. Therefore, the aim of our research is to describe and compare the processes that residual LECs undergo *in vivo* and *in vitro,* focusing mainly on the development of PCO at the periphery of the capsular bag.

Methods

Twenty-four human donor eye globes from the "Banc d'Ulls per a Tractaments de Ceguesa", Barcelona, were obtained. These eye globes had been classified as non-suitable for transplantation. Written informed consent for the removal and use of the eye globes for diagnostic and research purposes was obtained from patients and/or relatives. This experimental study was approved by the Ethical Committee for Clinical Research of the Centro de Oftalmología Barraquer and followed the tenets of the Declaration of Helsinki. Three different types of tissue samples extracted from these donor globes were studied.

Cultured capsules: Lens capsules from human donors, cultured for 30 days after mock cataract surgery (n = 6, median age 71, age range 43-88 years). Lens-capsule-ciliary

body complexes were affixed to a silicone ring using eight 30G needles. An anterior capsulorhexis was performed through which the lens fibers were extracted by hydrodissection and aspiration.

We did not implant IOLs since our focus was not on the effects of IOL characteristics on cell growth. The prepared lens capsules were cultured in a medium with 5% fetal bovine serum. This sustained serum concentration model was used, since it is well established and accelerates PCO development more than the serum free or graded serum models.

IOL capsules: Lens capsules from human donors that had previously undergone cataract surgery with IOL implantation (n = 12, median age 91, range 54-96 years). These samples had varying degrees of PCO and Soemmerring's ring. They were separated in to two groups, based on the size and opaqueness of the space between the IOL border and the internal part of the ciliary body (Soemmerring's ring). Less developed IOL capsules were samples where the Soemmerring's ring was apparently thinner, less opaque and did not completely surround the IOL (Figure 4.9B) and more developed IOL capsules were samples in which the Soemmerring's ring was apparently thicker, more opaque and completely surrounded the IOL (Figure 4.9C).

Intact lenses: Lenses from younger donors without cataract (n = 3, median age 42 years) (Figure 4.9D) and lenses from older donors with cataracts (n = 3, median age 85 years). Older intact lenses had different types of cataracts, two presented cortical cataracts (mild and severe) (see Appendix Figure 4.2) and the third had advanced nuclear cataract.
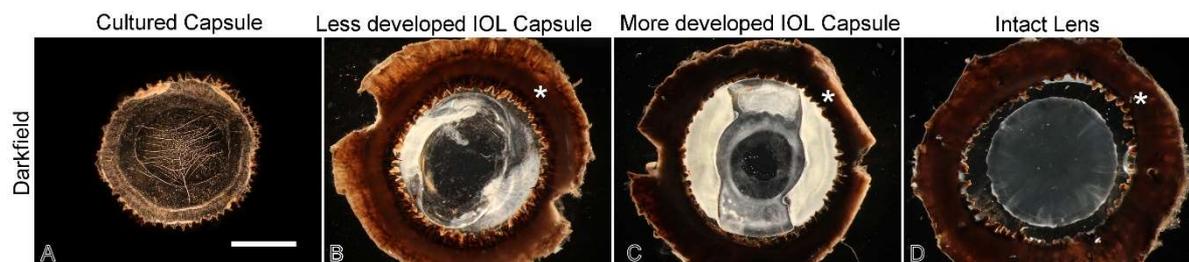
Figure 4.9 Frontal dark field images of our sample types before histological sectioning. (A) Cultured capsule sample (C033) after 30 days of culture, showing cell clusters at the equator and wrinkling on the posterior capsule. (B) Less developed IOL capsule (621b), showing a less extensive and opaque Soemmerring's ring. (C) More developed IOL capsule (618a), showing a large opaque Soemmerring's ring surrounding the IOL. (D) A transparent intact lens (M84b). Asterisk mark the ciliary body. "V" shaped notches in the ciliary body indicate the line along which histological sections were to be made. Scale bar = 4 mm.

IOL capsule and intact lens samples were frontally imaged with dark field illumination. Photographs were taken with a digital camera attached to a surgical microscope OPMI

(Carl Zeiss, Oberkochen, Germany). In the case of cultured capsule samples, photographs were taken during and after culture with an inverted microscope (Axiovert 100, Zeiss); using a phase contrast (Ph2) illumination ring with a normal 2.5x Plan-objective.

After imaging, all samples were fixed in 4% buffered formaldehyde for one week. Once fixed, IOL and cultured capsule samples where marked for sectioning along the area of apparently the most tissue growth. This was done by cutting two "V" shaped cuts in to the ciliary body (Figure 4.9B and C). All tissue samples were paraffin embedded and sectioned sagittally for histological staining.

All samples were stained with Hematoxylin and Eosin for general morphology. We used Vimentin to mark LECs and early lens fiber cells. In most other tissue Vimentin is expressed in mesenchymal cells, but in the crystalline lens it is a known marker of LECs and newly formed lens fiber cells.[259] In order to study adherence between the capsule and cells that have undergone epithelial to mesenchymal transition, we used α-SMA.[260] Vimentin and α-SMA stains were done with the BenchMark® ULTRA device (Ventana Medical Systems, Inc.), treated with methanol with 3% hydrogen peroxide for 10 minutes, subsequently incubated in 98-100% formic acid for 3 minutes, and washed in distilled water. Antigen retrieval was performed with ethylenediaminetetraacetic acid at pH 9 for 20 min for Vimentin (clone V9, Ventana) and 36 minutes for α-SMA (clone 14A, Cell Marque). Sections were then counterstained with Hematoxylin.

To identify cells with the potential to differentiate to lens fibers cells we used Paired box protein 6 (Pax6).[261, 262] Pax6 is expressed during early development in the eye, olfactory epithelium and the nervous system; it controls a series of critical steps during the embryonic formation of the eye.[263] The Pax6 staining was performed by HistoWiz Inc. (histowiz.com) using standard operating procedures and fully automated workflow. Immunohistochemistry was performed on a Bond Rx autostainer (Leica Biosystems) with heat mediated enzyme treatment (Citrate Buffer, pH6 from Leica Biosystems) using standard protocols. Mouse monoclonal to Pax6 (Abcam 78545, 1:50) was the primary antibody and were detected by polymer system (anti-rabbit Poly-HRP-IgG) from Bond Polymer Refine Detection kit (Leica Biosystems). Sections were then counterstained with Hematoxylin, dehydrated and film cover slipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). Human retina tissue was used as a control for Pax6. For all

previously mentioned immuno stains, DAB (3,3′-Diaminobenzidine) was used as a chromogen.

Picro Sirius Red (PSR) staining was used as a birefringent marker for collagen fibers type I, III and IV (Velidandla et al., 2014) It was prepared according to a procedure described previously.[264, 265] Samples were stained for 30 minutes with a solution of 0.1% Sirius red F3BA (Direct Red 80, catalog No. 365548, Sigma) in a saturated aqueous solution of picric acid. The samples were counterstained with Meyer's Hematoxylin. Details for the interpretation of PSR are given in the Appendix.

Results

Macroscopically, all cultured capsules had wrinkles on their posterior capsules and opacifications at the capsular equator (Figure 4.9A), except for one, which only presented opacifications on the posterior capsule. IOL capsules had varying degrees of opacifications at the equator (Figure 4.9B and C). Also in this location in some samples, there are transparent areas alternating with opaque areas (Figure 4.9B). We attempted to identify the IOL models, but were uncertain in many cases, therefore we did not include this ambiguous data. Of the 12 samples, 4 had undergone laser posterior capsulotomy. Capsular rhexis size was very similar in all IOL capsules with a mean diameter of 4 mm (range 3.5 to 4.5 mm) in our post-mortem tissue; it was always smaller than the IOL optics. Less developed IOL capsules had smaller Soemmerring's ring cross-sections, on average 1.2 mm in width and 0.5 mm in thickness, while more developed IOL capsules were thicker, 1.6 mm in width and 1.0 mm in thickness on average. Younger intact lenses were thinner (average 2.5 mm) than older cataractous lenses (average 3.8 mm), equatorial diameter was similar in both groups (average 8.6 mm).

Histologically, cultured capsules consisted mainly of a monolayer of cells in which wrinkles could be observed (Figure 4.10A and F) and in some cases showed some volumetric growth of cells at the equator, up to 0.3 mm in size (Figure 4.10B and G). In most cases, anterior and posterior capsules adhered to each other. Cellular growths in IOL capsules, were observed as monolayers with some clusters of small cells (Figure 4.10C and H) or as clusters of larger, irregular cells (Figure 4.10D and I). In these types of cellular growth, a clear distinction between the outer layers, which consisted of globular and fiber like cells, and the central mass, which was more an amorphous cluster, could be made. Globular cells had typical diameters between 50 and 100 with a maximum of up to 200 µm (Figure 4.10D). Some less developed IOL capsules were very

similar to our cultured capsules (Figure 4.10A and C). Some more developed IOL capsule samples presented morphologies surprisingly similar to intact lenses (Figure 4.11B and C) with a monolayer of LECs below the anterior capsule, with a lens bow of cells at the equator elongating into newly formed lens fiber cells.
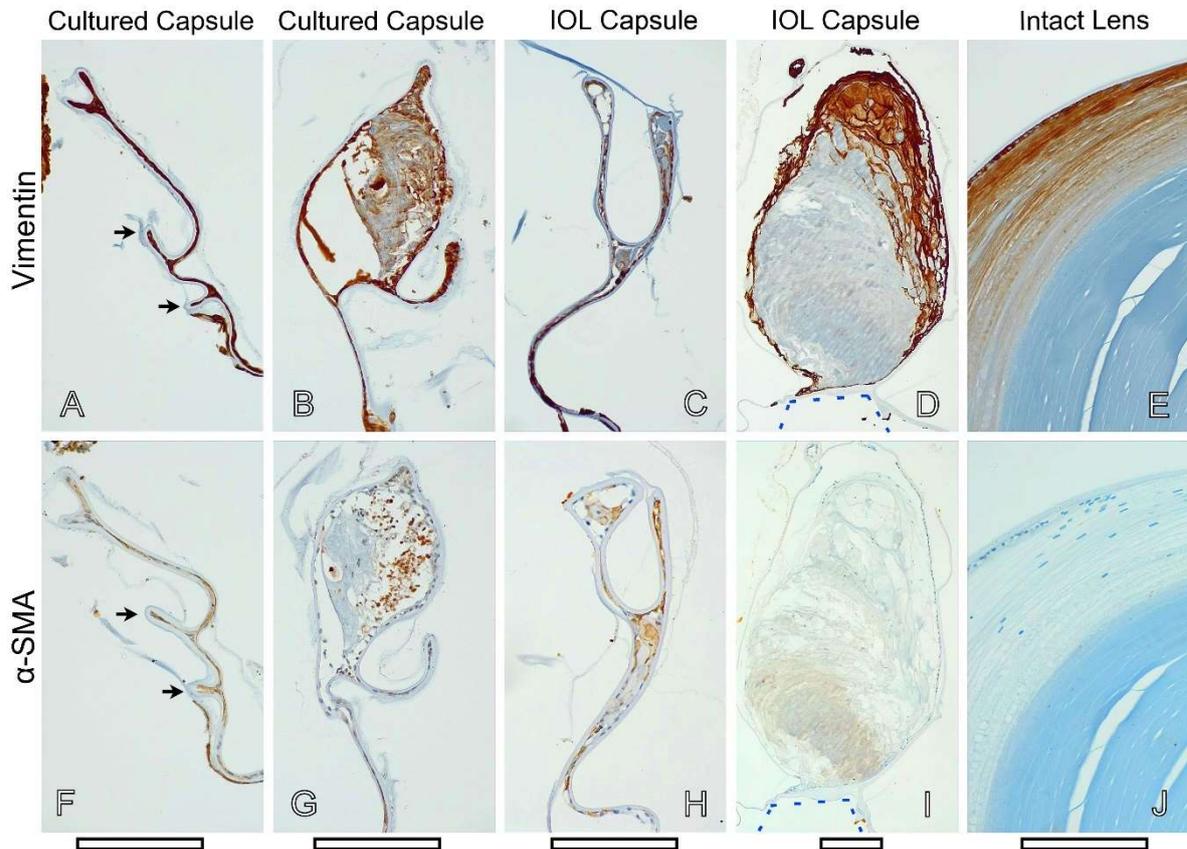
Figure 4.10 Microscopic images of the histological sections of the equatorial region of some of our samples, showing staining for Vimentin (A-E) and alpha smooth muscle actin (α-SMA) (F-J). Images (A & F) show cultured capsule sample C039, with arrows marking capsular wrinkles. Images (B & G) show cultured capsule sample C040. Images (C & H) show less developed IOL capsule 603a. Images (D & I) show more developed IOL capsule 621b, with a dashed outline of where the IOL edge was. Images (E & J) show young intact lens M84b. Scale bars = 200 μm

Vimentin and α-SMA expression was positive in most cultured capsule samples, indicating that the LECs had undergone epithelial-mesenchymal transition. In one sample, cells on the capsule were negative for α-SMA and positive for Vimentin indicating that these were LECs (Figure 4.10B and G). IOL capsules only expressed α-SMA in areas where capsules adhered to each other or cells were in contact with the IOL (Figure 4.10H and I, Figure 4.12B and C). Vimentin expression in IOL capsules varied depending on cellular morphology, if cells where irregular and large, Vimentin expression was uneven throughout (Figure 4.10D), however if lens fiber cells were

distinguishable, Vimentin expression was uniform and concentrated in LECs and at the bow region, similar to intact lens samples (Figure 4.10 and Figure 4.11). The average depth of the Vimentin stain below the capsule was 200 µm, but ranged from 20 to 700 µm. In all intact lenses, Vimentin stained epithelial cells and showed a degrading gradient at the bow region, marking the transition zone and newly formed lens fiber cells (Figure 4.10E), staining on average cells up to 160 µm below the capsule (range from 95 to 320 µm). Intact lenses did not expressed α-SMA (Figure 4.10J).
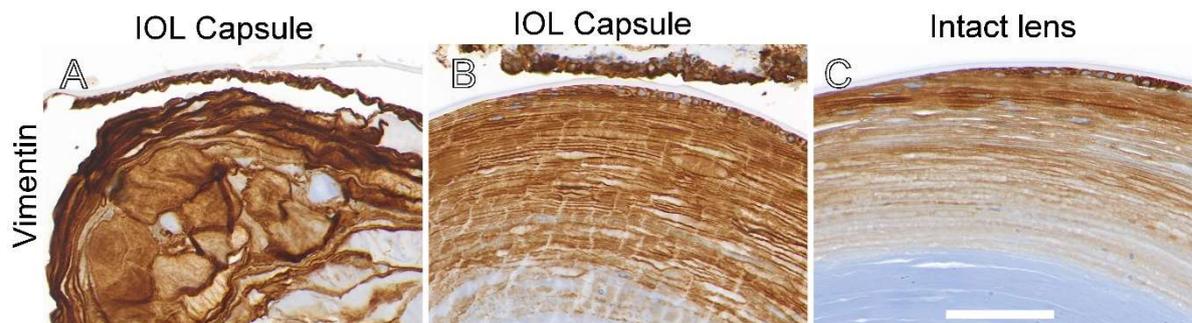
Figure 4.11 Higher magnification images of the histological sections of the equatorial region of (A) a less (621b) and (B) more (618a) organized IOL sample and (C) an intact lens (M84b), stained with Vimentin. Scale bar = 100 µm.

The lens capsule in all samples stained red with bright field illumination, but did not show any color under polarized light (indicating collagen IV)(Figure 4.12E to L). None of the cultured capsules or intact lenses expressed either collagen I or III (Figure 4.12 and Appendix Figure 4.2). However, many IOL capsule samples expressed collagen I and III in areas where cells were in contact with the IOL (Figure 4.12F, G, J, K). In two IOL capsule samples, collagen types I and III were seen around the anterior capsulorhexis. The lens cortex of intact lenses showed a light pink stain which we consider to be a background stain of PSR. Deeper lens areas showed a yellow stain of PSR, typical for cytoplasm (Figure 4.12H and Appendix Figure 4.2D). This staining pattern correlated with Vimentin and α-SMA (Figure 4.10E & J, Appendix Figure 4.2 B & C).
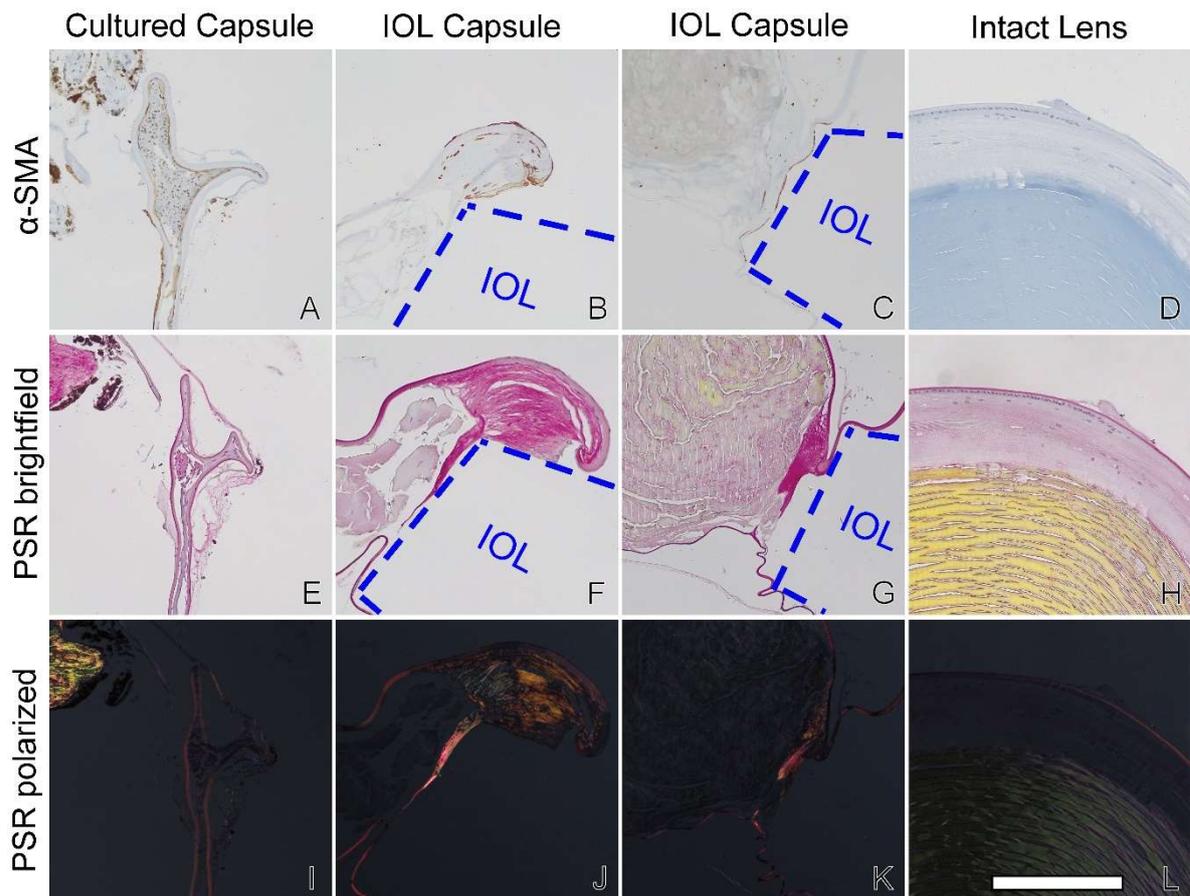
Figure 4.12 Microscopic images of the histological sections of some of our samples, showing staining for alpha smooth muscle action (α-SMA) (A-D) and Picro Sirius Red, seen in both, brightfield (E-H) and with polarized light (I-L) (highlighting collagens I & III). Images (A, E & I) show the equatorial region of cultured capsule sample C033. Images (B, F & J) show the area of contact with the IOL of less developed IOL capsule 614a, with a dashed outline of where the IOL edge was. Images (C, G & K) show the area of contact with the IOL of more developed IOL capsule 612a, with a dashed outline of where the IOL edge was. Images (D, H & L) show the equatorial bow region of young intact lens M61. Scale bar = 300 μm.

Most cell nuclei in cultured capsules and less developed IOL capsules were positive for Pax6 with a scattered distribution (Figure 4.13). In intact lenses, all LECs near the lens equator were Pax6 positive, compared to about 1 in 3 LECs at the anterior lens pole (Figure 4.14). In some more developed IOL capsules, with organized cell morphology, Pax6 expression was very similar to intact lenses (Figure 4.14).
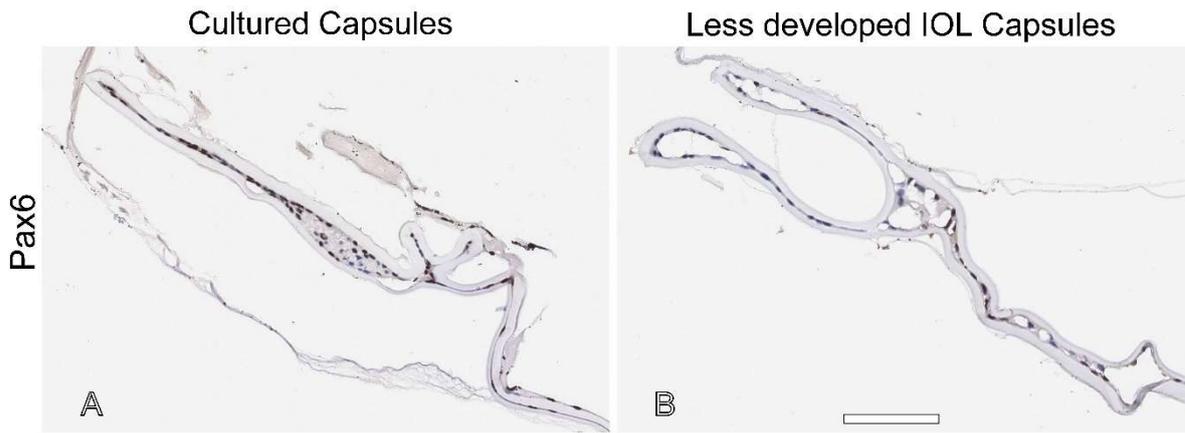
Figure 4.13 Microscopic images of the histological sections of two of our samples stained for Paired box protein 6 (Pax6). The left image shows cultured capsule sample C039 and the right image shows less developed IOL capsule 603a. Scale bar = 100 μm.
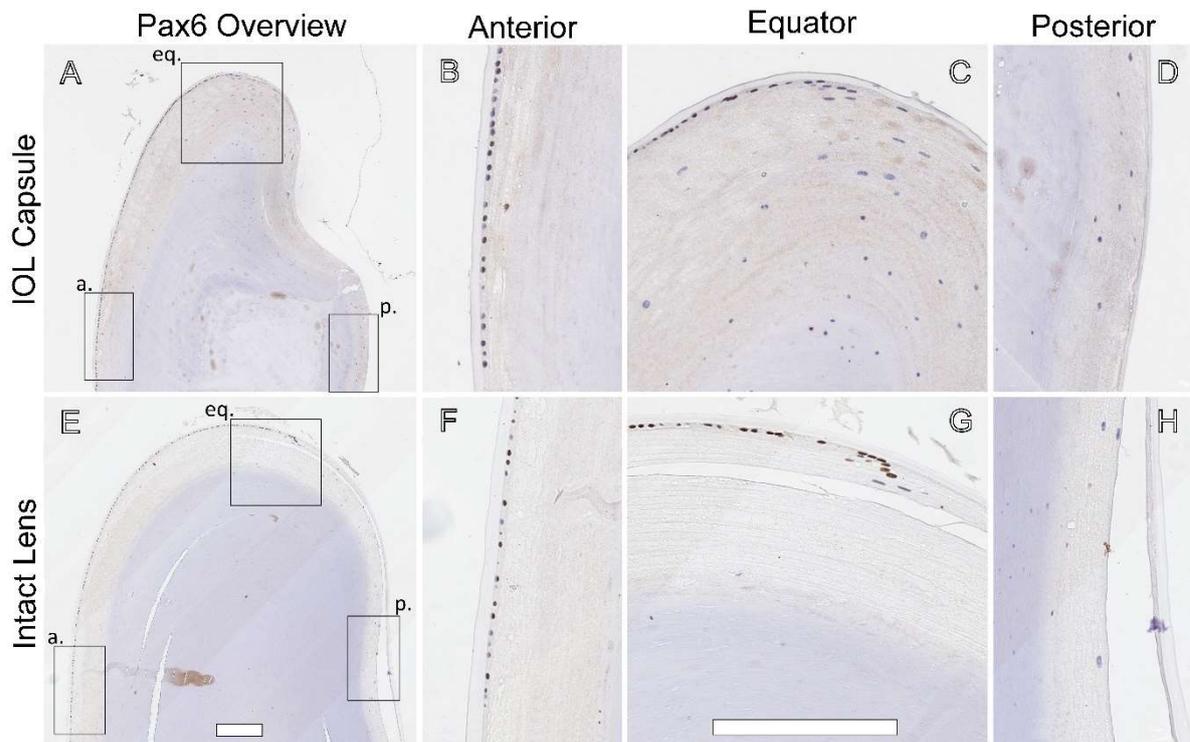
Figure 4.14 Microscopic images of the histological sections of two of our samples stained for Paired box protein 6 (Pax6). The top images are from more developed IOL capsule 618a and the bottom images shows young intact lens M84a. Both samples show an overview on the left, with boxes indicating the magnified anterior, equator and posterior regions. Scale bars = 200 μm.

Discussion

In our cultured capsules, we had previously observed the proliferation, differentiation and migration of the residual LECs and the formation of wrinkles.[258] These processes have been shown to be due to epithelial-mesenchymal transition.[252] We could see that in most samples, capsules adhered to each other and the majority of cells were mesenchymal cells, since they were stained with α-SMA. This was very similar to what we could see in the sections of our less developed IOL capsules which also showed mainly monolayers of mesenchymal cells with adhered capsules. Interestingly, a published case of an IOL capsule, just 32 days post cataract surgery showed similar results regarding mesenchymal cells.[242]

As volumetric growth in cultured capsules and IOL capsules increased, separating the capsules apart, α-SMA expression decreased. This loss of expression of α-SMA might be due to mesenchymal cell apoptosis, which has previously been reported *in vitro*.[266] In some IOL capsules and in one cultured capsule with volumetric growth (Figure 4.10B and G), we could observe small cells on the capsule that didn't express α-SMA but did express Vimentin, indicating that these were most likely LECs. Furthermore, in these samples we could also see larger globular cells that were positive for Vimentin and negative for α-SMA (Figure 4.10D and I), therefore, we hypothesize that these were developing lens fiber cells. This hypothesis is corroborated by other studies, such as one explaining that in order for fiber cells to elongate, first differentiating fiber cells must dramatically increase their volume.[267] Within the residual lens capsule, with less space to elongate and without a lens nucleus around which to grow, it makes sense that differentiating lens fiber cells would increase their volume in all directions forming globules. Another possibility is that these globular cells are swollen fiber cells, a phenomenon that has been described before in cataractous lenses.[63, 268] Yet other studies have suggested that like our globular cells, globular Elschnig's pearls are LECs differentiating into lens fiber cells.[269, 270]

While in these cases more specific immunofluorescent stains would have been easier to interpret, it wasn't an option for us during this study. If cells were both α-SMA and Vimentin positive they were myofibroblastic. According to the literature, Vimentin, as in the rest of the body, is most strongly positive in mesenchymal or myofibroblastic cells however in the lens it is also notably positive in LECs and young fiber cells.[234, 271, 272] When looking at our results, there does seem to be a slightly more intense staining in the cells we consider to be mesenchymal cells (Figure 4.10A) when compared to

epithelial or fiber cells (Figure 4.10E) and this distinction is confirmed by the presence of α-SMA in the former (Figure 4.10F).

Collagen expression has been associated with the presence of myofibroblast cells.[273] Fibrosis in PCO has also been related to collagens types I and III in the extracellular matrix[274, 275] and type II in congenital cataracts[276]. Although the differentiation between collagens type I and III with the PSR staining was somewhat subjective due to the color distinction (see Appendix Figure 4.1), we do not think that this had an impact on our results, since we always observed collagens type I and III together in our collagen positive samples. We did not observe collagen in any of our cultured capsules (Figure 4.12E and I). This indicates that in early fibrosis, α-SMA is expressed alone and later on fibrosis is characterized by collagen I and III expression. In IOL capsules, collagen was only observed in areas where cells were in contact with the IOL and in these areas, it co-expressed with α-SMA (Figure 4.12). Others have also observed collagen deposits on the haptics and optics of IOLs extracted from donor globes.[277, 278] We hypothesize that these areas create pockets of mesenchymal cells against the IOL. If these mesenchymal cells are maintained between the Soemmerring's ring and the IOL, they could eventually migrate behind the IOL. This could explain cases in which PCO suddenly appears on the posterior capsule after years of unobstructed vision.

The expression of Pax6 in our cultured capsules and less developed IOL capsules showed that these tissues have many cells with the potential to form lens fiber cells (Figure 4.13). It is interesting to note that this potential is not lost even after the effects of cataract surgery on LECs. Pax6 distribution in more developed IOL capsules and in intact lenses was very similar. It showed high expression at the equator that decreased drastically as LECs differentiate at the lens bow (Figure 4.14). These results are similar to a previous study[279] that demonstrated that LECs in the germinative zone of regenerated infant lenses showed intense proliferation in the first week after cataract surgery, but these LECs lost Pax6 expression at the initiation of differentiation and migration from the lens equator. We found that, Pax6 was positive in LECs on the anterior pole of intact lenses, showing that LEC's from any part of the lens can have the potential to form lens fiber cells, not only LECs at the lens equator (Figure 4.13 and Figure 4.14).[212, 280]

More developed IOL capsules in general present morphologies less similar to cultured capsules but more similar to intact lenses. Presenting better cellular organization, such as a monolayer of LECs on the anterior capsule and a newly formed bow region at the

equator, characteristic for cells undergoing differentiation towards fiber cells (Figure 4.11 and Figure 4.14). A very similar reorganization of lens morphology has previously been seen in *in vivo* regenerated rabbit lenses 8 weeks after lens extraction through medium and large anterior capsulorhexis.[281] Recent research indicates that reciprocal inductive interactions between LECs and fiber cells may be integral for proper fiber cell differentiation, organization and lens regeneration.[282, 283] This might explain why there is always an intact LEC monolayer present when fibers can be distinguished in the Soemmerring's ring.

Also, in our more developed IOL capsules, even when easily distinguishable fiber cells are not formed, the expression of Vimentin without α-SMA in the large globular cells, again seemed to indicate that these cells are developing lens fiber cells. Similar globular cells were also seen in the previously mentioned regenerated rabbit lenses near more normal regenerated lens morphology.[281] In addition, just like in intact lenses, Vimentin expression is highest at the equator, and is gradually lost towards the center of the lens, indicating the fiber cell transition zone (Figure 4.10). It has been shown that Vimentin is expressed during fiber cell elongation and that expression stops some 2-3 mm into the lens.[284] Furthermore, the apparent loss of nuclei in these fiber cells in some developed IOL capsules, correlated with normal lens development.[279]

After analyzing all of our samples, we noticed that there were 5 common types of cellular morphologies (Figure 4.15). Interestingly, some of these were shared between cultured capsules (Figure 4.15A-C) and less developed IOLs (Figure 4.15A-D), and others were shared between less developed IOLs and more developed IOLs (Figure 4.15D, E), this seems to support the idea that these are all parts of the same continuous process.
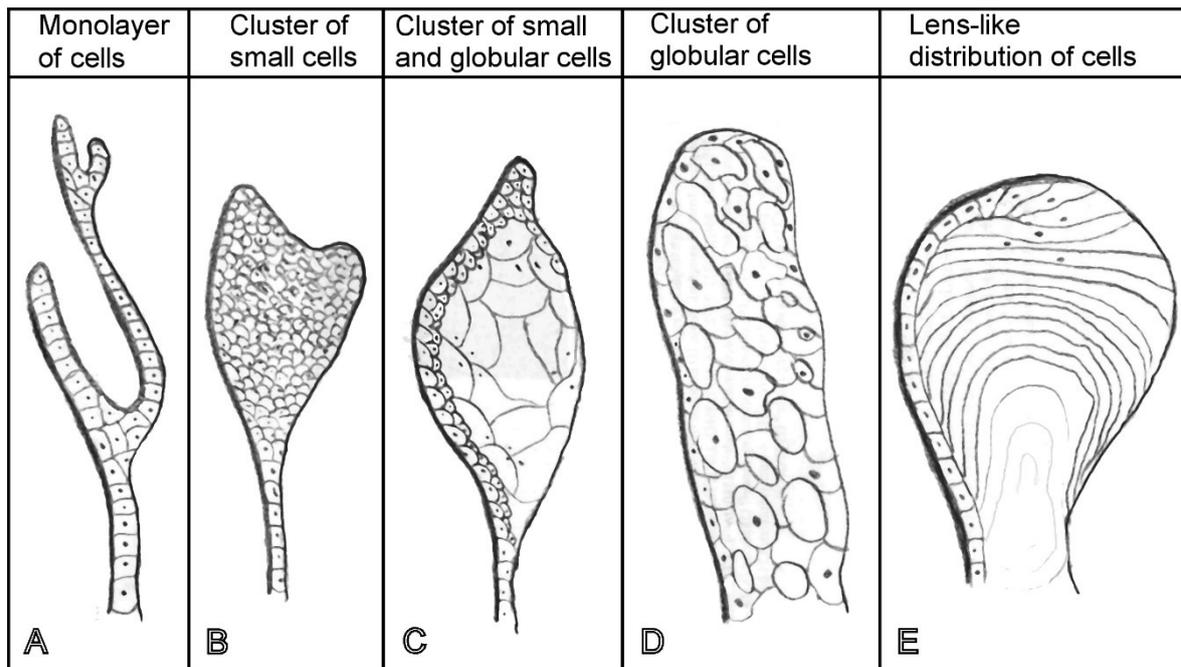
Figure 4.15 Schematic illustrations of common cellular morphologies found at the equatorial region. Drawings are based on our samples stained with Hematoxylin and eosin and Vimentin. (A) Monolayer of cells: adhered capsules with small cells grown mainly in a monolayer. (B) Cluster of small cells: area of high small cell proliferation. (C) Cluster of small and globular cells: uneven globular cells with a couple of layers of small cells on the anterior capsule. (D) Cluster of globular cells: large uneven globular cells with a monolayer of small cells on the anterior capsule. (E) Lens like distribution of cells: distinguishable LEC monolayer, bow region, fiber cells and a central cell mass. Illustrations not to scale.

The most probable order of events during PCO development may be that LECs undergo epithelial-mesenchymal transition and migrate to seal and fill the capsule, then cells revert to LECs reforming the LEC monolayer, after which, fiber cell differentiation starts, beginning as globular lens fiber precursors which eventually become normal fibers. Thus, proper organization and morphology is slowly regained, although not transparency. However, we cannot confirm this, because it was not possible to estimate the time between cataract surgery and tissue donation in our samples.

The morphological similarities we have observed between more developed IOL capsules and intact lenses (Figure 4.16), seems to indicate that the Soemmerring's ring formation is an attempt at regenerating the lens. However, these Soemmerring's rings appeared opaque which might be due to a lack of alignment of the fibers[264, 285]; disruption of the packing of cortical fiber cells, either due to cellular swelling or dilation of the normally tight spaces between the cells, increasing light scattering[267, 286], a foreign body or scaring reactions to the IOL (collagen and α-SMA), or due to the circular scar induced by the anterior capsulorhexis.[281] Recently, the investigation of lens regeneration as a treatment

for cataracts has been studied, in rabbits[281] where it has been shown that a relatively complete and transparent lens can be regenerated, depending on the rhexis size during surgery. Interestingly, the samples with the largest rhexis sizes (6 mm), similar to our cultured capsule samples, showed very similar cellular morphologies, such as layers of mesenchymal cells where the capsules were closer together and reformed bow regions with differentiating fibers at the equator. Furthermore, an *in vivo* study in human infants, regenerated a partially transparent doughnut shaped lens 2 years after phakic cataract surgery, by having extracted the lens through a small peripheral rhexis.[212]
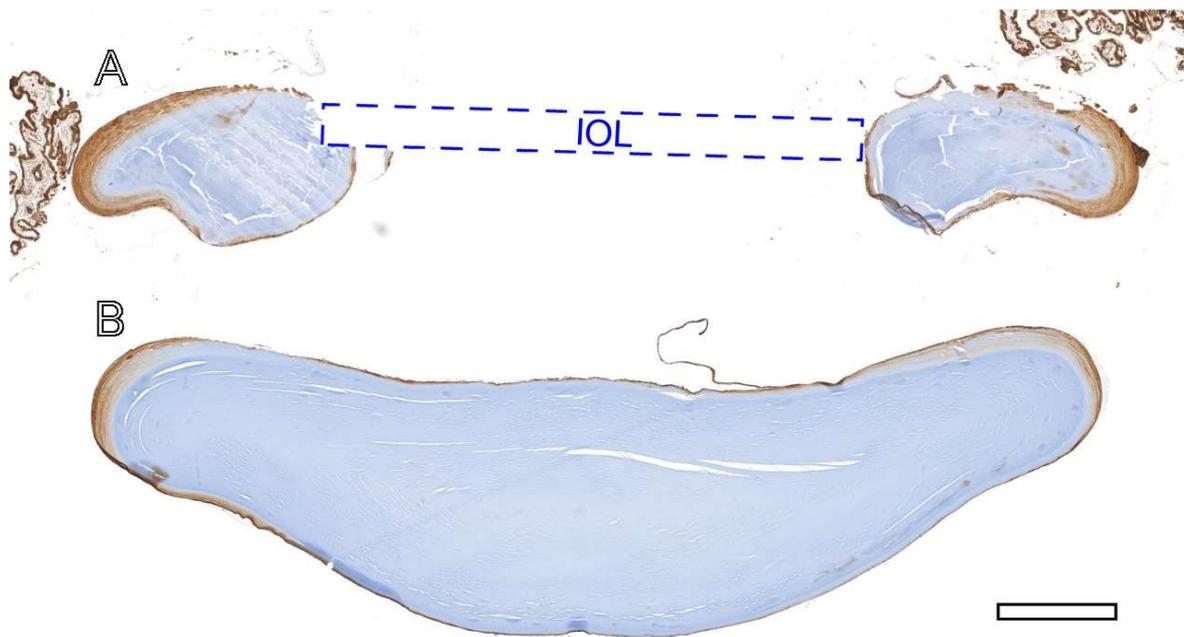
Figure 4.16 Complete histological sections of (A) more developed IOL capsule (618a) and (B) young intact lens (M84b). Both samples stained with Vimentin. Depth of stain below the lens capsule at the equator in both samples was about 0.23 mm. Blue dashed rectangle represents the IOL cross-section. Scale bars = 1 mm.

There still remains the question of why do we find IOL capsules with such varying Soemmerring`s ring sizes and morphologies (Figure 4.9). Based on our results alone, we cannot answer this question. It might depend on the length of time since the cataract surgery or on how many LECs and fiber cells remain after polishing the lens capsule. Cataract type and capsulorhexis size might also have an effect, although we did not see an effect of the later in our samples since, the rhexis sizes were all similar. IOL type, material and shape probably affects central PCO the most and to a lesser extent peripheral PCO, such as when square edged optics create a mechanical barrier for the migration of LECs.

In summary, the similarities between cultured capsules and less developed IOL capsules indicate that our *in vitro* developed PCO is comparable to early *in vivo* developed PCO. The presence of Pax6 throughout all sample types showed that there are many cells with the potential to develop into lens fibers even after cataract surgery. The expression of Vimentin and lack of α-SMA in globular cells suggests that these are developing lens fiber cells. And finally, the similar morphological distribution of LECs and lens fiber cells in more developed IOL capsules and intact lenses, could be considered attempts at lens regenerations. Thus, even though recent studies have been focusing on lens regeneration in infants, we believe that our results show that there is a potential for lens regeneration even in adults.

Appendix. Control tissue

When visualized with bright field illumination, Picro Sirius Red (PSR) should stain collagen type I, III and IV red and cytoplasm yellow. Under polarized light, due to the birefringent nature of PSR, collagen type I is seen in yellow-orange, collagen III in green and collagen IV does not show birefringence. Cytoplasm is seen in yellow-white with polarized light. The absence of a red stain in bright field even with minor yellow-white appearance in polarized light means there is an absence of collagen. The same holds for a pink background stain in bright field together with absent birefringence.

We have confirmed these staining patterns with human tissue from the ciliary body and the cornea which were used as positive controls (Appendix Figure 4.1). As expected, the ciliary body showed Collagen types I and III (yellow-orange and green color in polarized light) in the connective tissue among the muscle bundles.[287] Our positive control also confirmed that the basal membrane of corneal epithelium consists only of collagen IV (red in brightfield and no birefringence with polarized light).[288] The corneal stroma on the other hand showed predominantly collagen I and some III (yellow-orange and some green color in polarized light) as was to be expected [289].
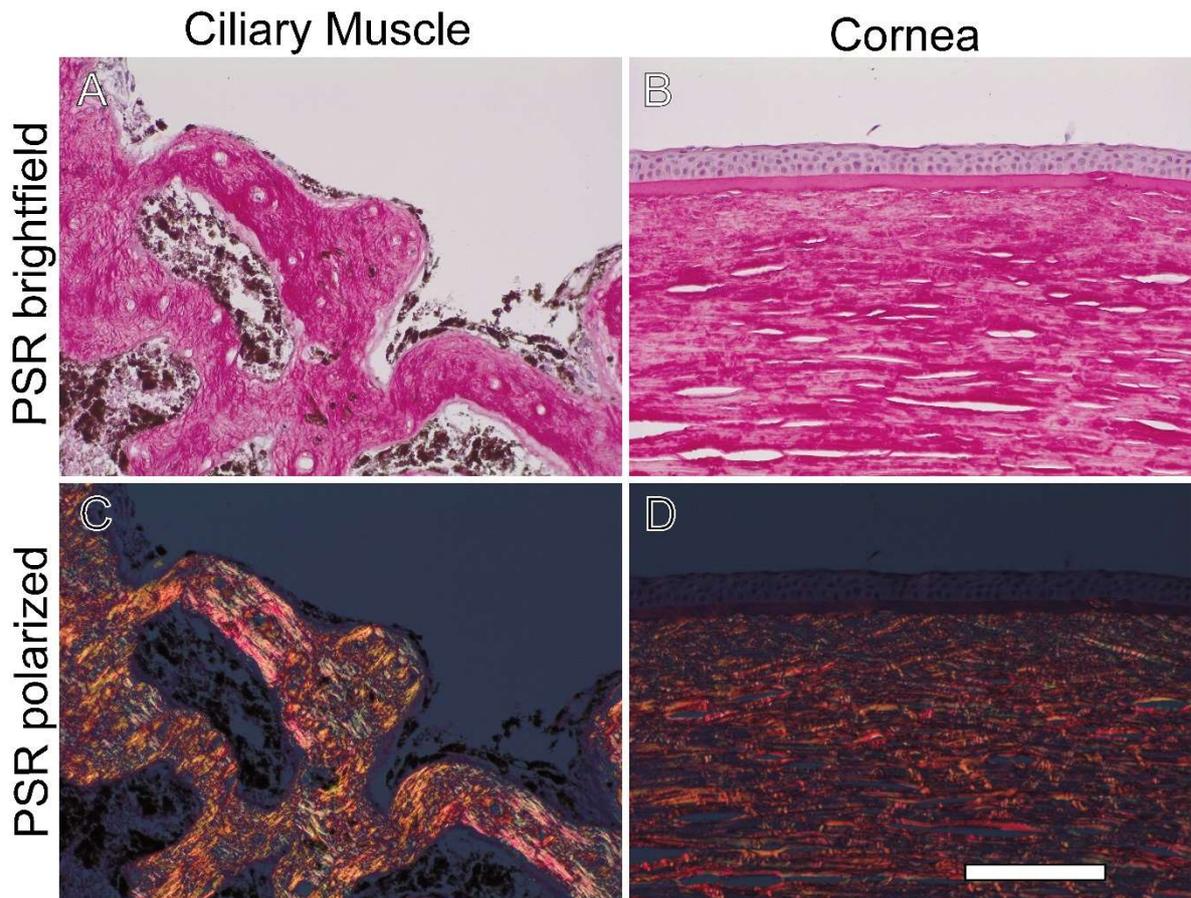
Appendix Figure 4.1 Microscopic images of the histological sections of two of our control samples, (A & C) ciliary muscle (C040) and (B & D) cornea (624a), stained with Picro Sirius Red (PSR), shown with brightfield (A & B) and polarized light (C & D). Scale bar = 150 μm.

Our 3 cataract lenses showed the same staining patterns for Vimentin and alpha smooth muscle actin (α-SMA) as the 3 young intact lenses (Figure 4.10E & J and Appendix Figure 4.2B & C). PSR stain showed undulated fiber bundles near the lens cortex and no collagen I, III or IV was observed (Appendix Figure 4.2D). The yellow stain in the bright field was characteristic for cytoplasm. When observing the stains for Vimentin, α-SMA and PSR side by side, an obvious distinction between the lens cortex and the nucleus can be seen, this is probably the area of transition of developing and fully elongated lens fibers. The loss of stains is probably related to the loss of organelles and membrane integrity, which would also explain the intense cytoplasmic staining. The undulated fiber bundles in the lens cortex (Appendix Figure 4.2D) probably correspond to the cortical cataract [39].
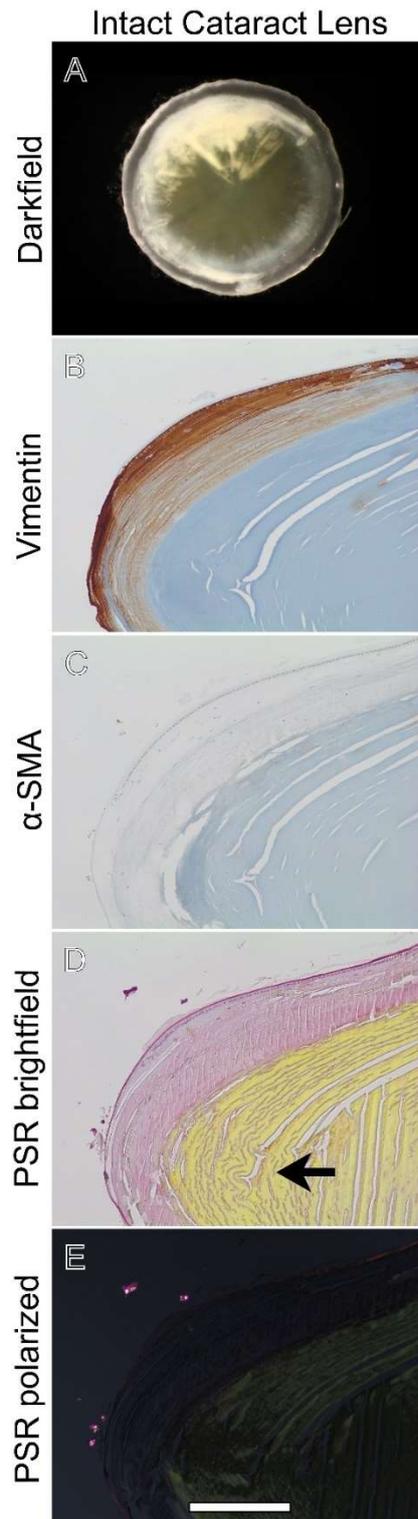
Appendix Figure 4.2 Images of an intact lens with cortical cataract (sample 615a). (A) Frontal dark field. (B to E) Microscopic histological sections, showing (B) Vimentin, (C) alpha smooth muscle action (α-SMA) and (D) Picro Sirius Red (PSR) with brightfield (E) and with polarized light. Undulated fiber bundles seen in (D) are marked with a bold arrow. Scale bar = 300 μm.

Acknowledgements

Author Contributions

Competing Interests: The authors declare no competing interests.

4.3. Lens cell regeneration capability studies (unpublished)

4.3.1. Soemmerring's ring transparency analysis

Abstract

Soemmering's rings are a late stage development of posterior capsule opacification, that develop outside of the visual axis. They consist of a ring of lens epithelial derived cells that grow around the IOL. These rings when visualized frontally, appear opaque, even though in some cases, the cells that compose these rings are organized just like those in transparent lenses. Based on this, we hypothesize that maybe only some layers of the Soemmerring's ring are opaque, but when visualized frontally it seems like the ring is opaque throughout. If this is the case, it would support the possibility of lens regeneration.

In order to study this, and which morphological factors affect transparency in Soemmerring's rings, we extracted 17 Soemmerring's ring and 2 lens samples from donor eye globes. After extraction and frontal imaging, we thickly sectioned these samples sagittally in order to analyze the degrees of transparency of the different layers with dark field illumination.

We also intend to histologically section and stain the adjacent halves of all samples. We will prepare light microscopy stains for alfa smooth muscle actin (α-SMA) and Vimentin and immunofluorescent stains for wheat germ agglutinin (WGA) to stain cell membranes and DAPI to stain nuclei.

The thick cross-sections of the Soemmerring's rings showed that almost all samples had some transparent layers, ranging from thin transparent layers between the very opaque nucleus and cortex to samples that were mostly transparent aside from the outer most cortex. We suspect that these sections will also show well organized lens fibers and LECs when analyzed histologically.

In conclusion, adult *in vivo* developed Soemmerring's rings, maintain the ability to produce organized and transparent layers of cells.

Introduction

Soemmerring's rings are late stage developments of posterior capsule opacification.[70] However, since they develop outside of the visual axis, there is no need to remove them unless they cross the mechanical barrier of the IOL. Even in these cases, only the growth on or behind the IOL is cleared, usually using posterior laser capsulotomy. This process will still leave the areas around the IOL intact, allowing the Soemmerring's ring to continue developing.

When these Soemmerring's rings are extracted from ex-vivo donor globes, they mostly appear opaque and milky. Despite this, when sagittally sectioned for histological analysis in many cases very organized lens fiber layers can be observed.[290] (Figure 4.11 B; Figure 4.14 A-D; Figure 4.16 A)

Our previous article, Koch, C.R. & D'Antin, J.C. et al. 2019,[290] highlighted the surprising degree of organization that some more developed Soemmerring's rings could obtain. As we had mentioned, the morphology of some of our samples was comparable to normal transparent lenses (Figure 4.16). Interestingly, when we observed the remaining halves of the Soemmering's rings that were still in paraffin, different degrees of opacification were evident, as seen in Figure 4.17A and these seemingly more transparent areas did align with the more organized fibers observed in the H&E stained histological section of the same samples (Figure 4.17B). However, all Soemmerring's rings when viewed frontally in a dark field, appear opaque. Since one would expect well organized lens fiber cells to be transparent, we hypothesized that maybe some layers of the Soemmerring's ring are transparent while other less organized layers are opaque.
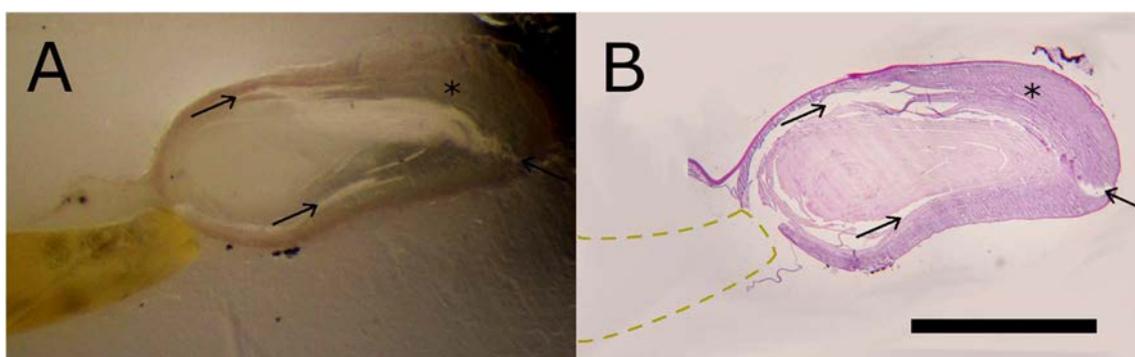
Figure 4.17 Images of a Soemmering's rings sample (548) A) fixed in a paraffin block and B) sectioned histologically and stained with H&E. Arrows mark the more opaque areas and the asterisk marks the supposedly more transparent area. Scale bar = 1mm.

The relationship between lens fiber cell organization and lens transparency has been well documented and studied. In 2008 Michael R. et al.[39] showed the relation between cortical cataract opacifications and lens fiber disorder or damage (Figure 4.18). This highlights how one of the major factors in lens transparency is the proper organization of lens fibers.
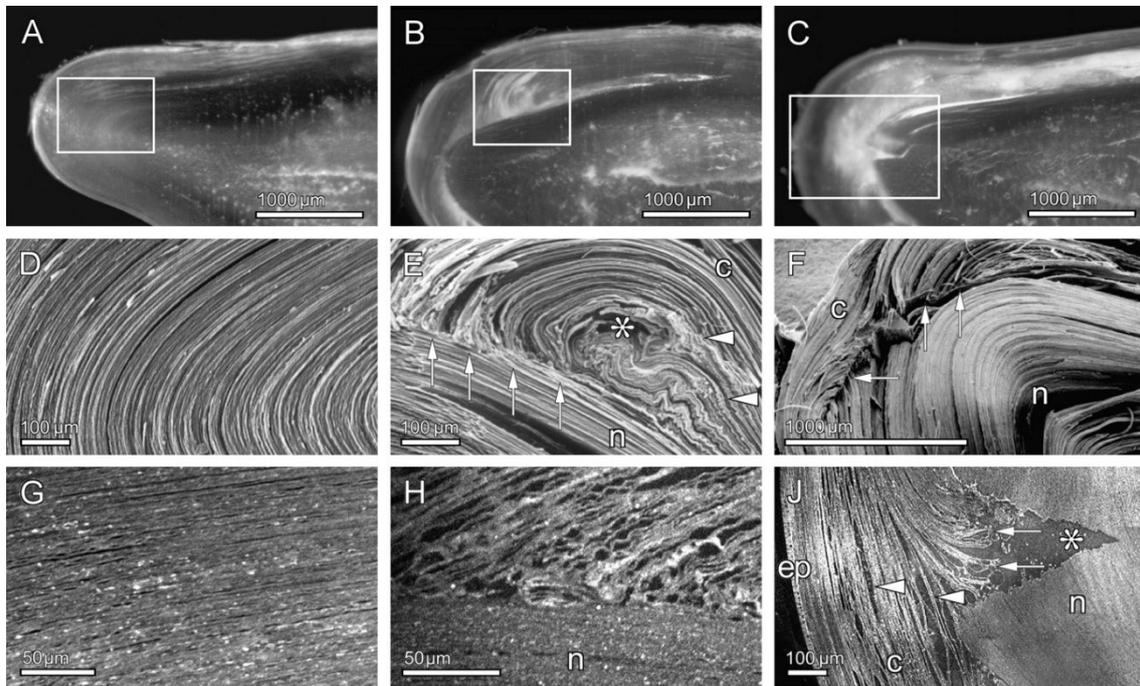
Figure 4.18 Fiber organization in a lens without cataract (A) and in two cases of cortical opacities (boxed areas in B and C), as visualized by SEM (D–F) and fluorescence histology (G, H, and J). It is shown in (E) that fibers at the border zone between the nuclear and cortical lens regions is broken (arrows) and that the broken ends are directed against the nuclear fibers, which maintain a regular, uninterrupted organization. Further, note the curled (asterisk) and folded (arrowheads) fibers in the region adjoining the broken fibers. Fluorescence histology of this type of opacification (H) shows the oblique orientation of the broken fibers on the regularly organized nuclear fibers. The dark regions in the fluorescence micrograph indicate the presence of spaces likely filled with fluid. The SEM micrograph of (F) shows broken fibers at several places (arrows) in the border zone between the cortex and nucleus. The nuclear fibers are regularly organized, as are the more superficial cortical fibers bridging the break zone. The fluorescence micrograph (J) shows the nuclear fibers and the most superficial fibers, which are regularly organized. The fibers between these layers are partly separated by water lakes (arrowheads). Some are broken (arrows), and there is a large triangular region (asterisk) (cf. F) free of fibers, most likely filled with fluid. Note: Ep, epithelium, n, nuclear side; C, cortical side. **Michael R. et al. 2008 [39]**

Thus, based on our previous results and publications like this one, we hypothesize that maybe only some layers of the Soemmerring's ring are opaque, but when visualized frontally it seems like the whole ring is opaque. If these transparent layers exist, it would further support the idea that Soemmerring's develop due to the incomplete process of lens regeneration, as we have previously stated.[290] Furthermore, if residual cells in the lens capsule can be transparent, this would reduce the negative effects of PCO.

In order to test this hypothesis, we collected various ex vivo Soemmerring's ring samples from human donors with the intention of thickly sectioning them in order to analyze if there are visibly transparent areas. We also intend to histologically section the adjacent tissue samples in order to stain and visualize the cellular morphology and organization to see if there is a relation to transparency.

Objective

Our objective is to test whether any part of the Soemmerring's ring is transparent, despite appearing opaque when viewed frontally, and how this could relate to morphological factors.

Materials and methods

17 Soemmerring's rings and two transparent lenses were extracted from 19 human donor eye globes from the "Banc d'Ulls per a Tractaments de Ceguesa". Only human eye globes classified as non-suitable for transplantation were used. Written informed consent for the removal and use of the eye globes for diagnostic and research purposes was obtained from patients and/or relatives. This experimental study follows the tenets of the Declaration of Helsinki.

Lenses and Soemmerring's rings when extracted from the eye globe were photographed frontally, in a dark field with an operation microscope, in order to analyze the degree of transparency (Figure 4.19).
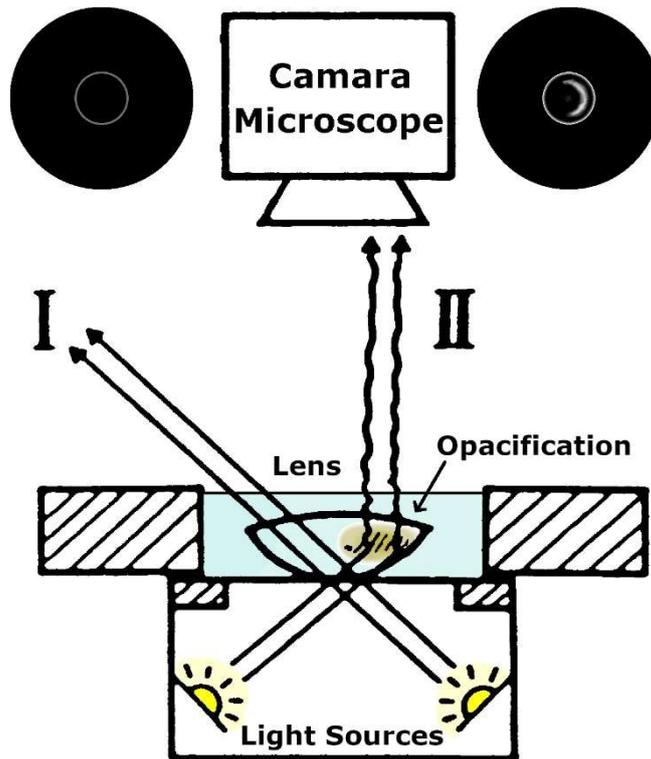
Figure 4.19 Schematic representation of how transparency is observed with darkfield illumination. I) Shows how when light travels through a transparent medium or sample, it is not affected nor deflected toward the microscope or camera. Thus, as seen in the top left corner, transparent lenses only show a vague outline in a darkfield. II) Shows how when light travels through an opaque medium or sample, it is partially deflected toward the microscope or camera. Thus, as seen in the top right corner, lenses with cortical cataracts illuminate opacities in a darkfield.

The next step was to obtain thick sections of all the samples in order to observe the variations in transparency. In order to obtain these thick sections, we designed a custom blade holder. Which consists of two metal plates that can be screwed together in order to hold 3 blades and 2 separators in place (Figure 4.20). The metal plates have gaps through which only the blades protruded in order to section the samples (Figure 4.20C). Lenses were sectioned with 400µm separators between the blades and Soemmerring's rings were sectioned with 200µm separators. When attempting to section the lenses with thinner separators, the tissue lost its cohesion.
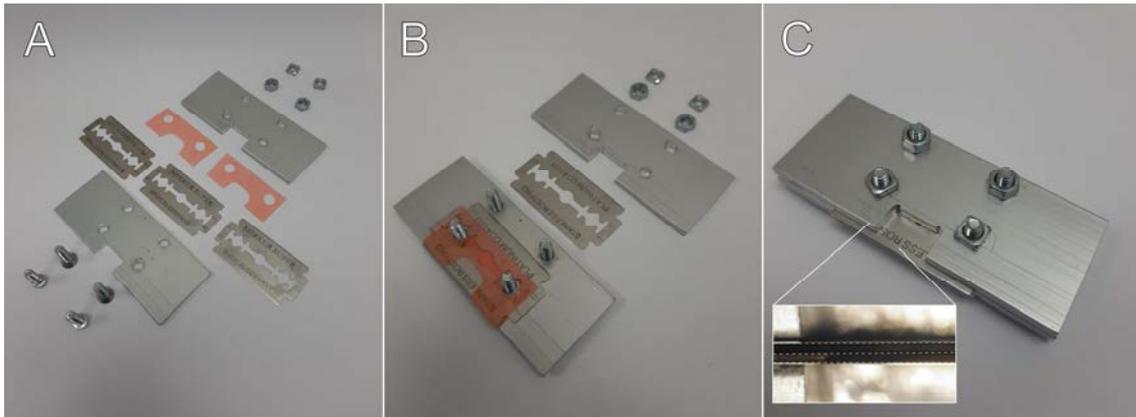
Figure 4.20 Images of our metal blade holder designed for making thick sagittal sections. A) Blade holder separated into all of its pieces. From bottom left to top right: 4 screws, back plate, 3 blades, 2 separators, front plate and 4 nuts. B) Blade holder partially assembled. C) Blade holder fully assembled with zoomed in view of the three blades evenly separated (highlighted with dotted lines).

Lens samples, once frontally imaged, were placed in 4% paraformaldehyde for 6 days in order to fix them and make thick sectioning possible (Figure 4.21A & C). Transparent lenses were thickly sectioned through the center of the lens.

An important factor we noted was that, if samples were sectioned without sufficient fixation time, lens consistency was not uniform and the nucleus of the lens was to soft and lost its consistency when sectioned (Figure 4.21B & D). The remaining adjacent ends of the lenses, after thick sectioning, were embedded in paraffin for histological analysis.
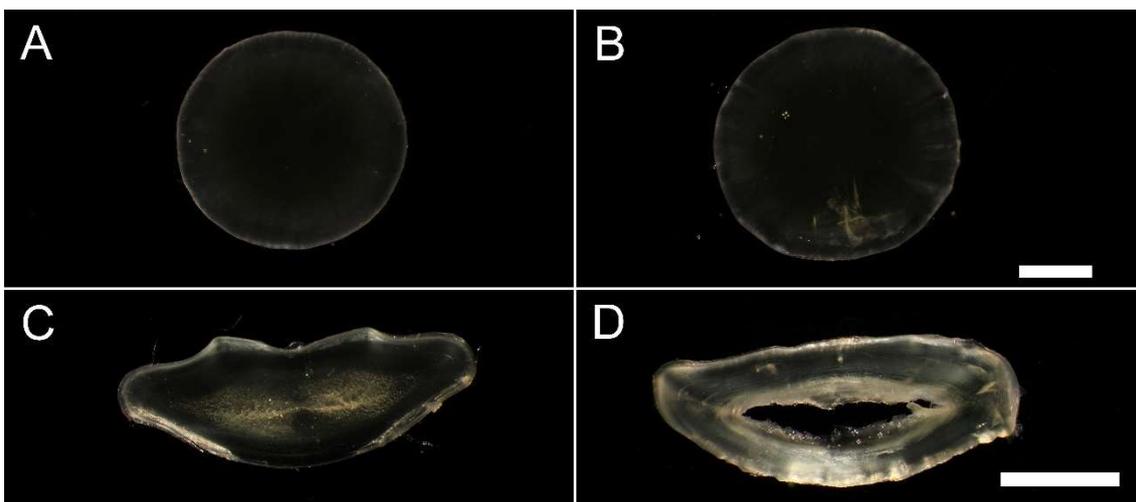
Figure 4.21 Darkfield images of whole fresh (A,B) human lenses (A=706A; B=705B) and the same lenses sectioned after 6 (C) and 3 (D) days of fixation. These images highlight how despite being transparent, if not sufficiently fixed, sectioning softer lenses leads to inconsistent results. Scale bars = 3mm.

Soemmerring's rings, once frontally imaged, were fixed in 4% paraformaldehyde for around a week and then embedded in paraffin for storage and for future histological analysis. Samples that were ready for analysis, were cut in half through the thickest part of the Soemmerring's ring. One half was kept in the paraffin block for future histological analysis and the other half was removed from paraffin and rehydrated in order to thickly section it and analyze transparency. In order to rehydrate samples, excess paraffin was first melted away and then samples were placed in Xelene for 12h, then fresh Xelene for 12h, then 100% EtOH for 1h, then fresh 100% EtOH for 1h, then 30min in 95% EtOH and finally 70% EtOH for 30min. After this point, this half of the Sample was thickly sectioned along the cut edge.

The processing of all samples is summarized in Figure 4.22.

Once the thick sections of the samples had been obtained, they were placed in an optic petri dish and submerged in BSS for 30min to allow samples to hydrate. After which they were photographed in a dark field in order to visualize the degree of transparency.

The adjacent halves of all samples, still in paraffin, will be histologically sectioned at 5μm using a microtome. Once sectioned, they will be DAB stained with alfa smooth muscle actin (α-SMA), Vimentin and immunofluorescently stained with wheat germ agglutinin (WGA) and DAPI.
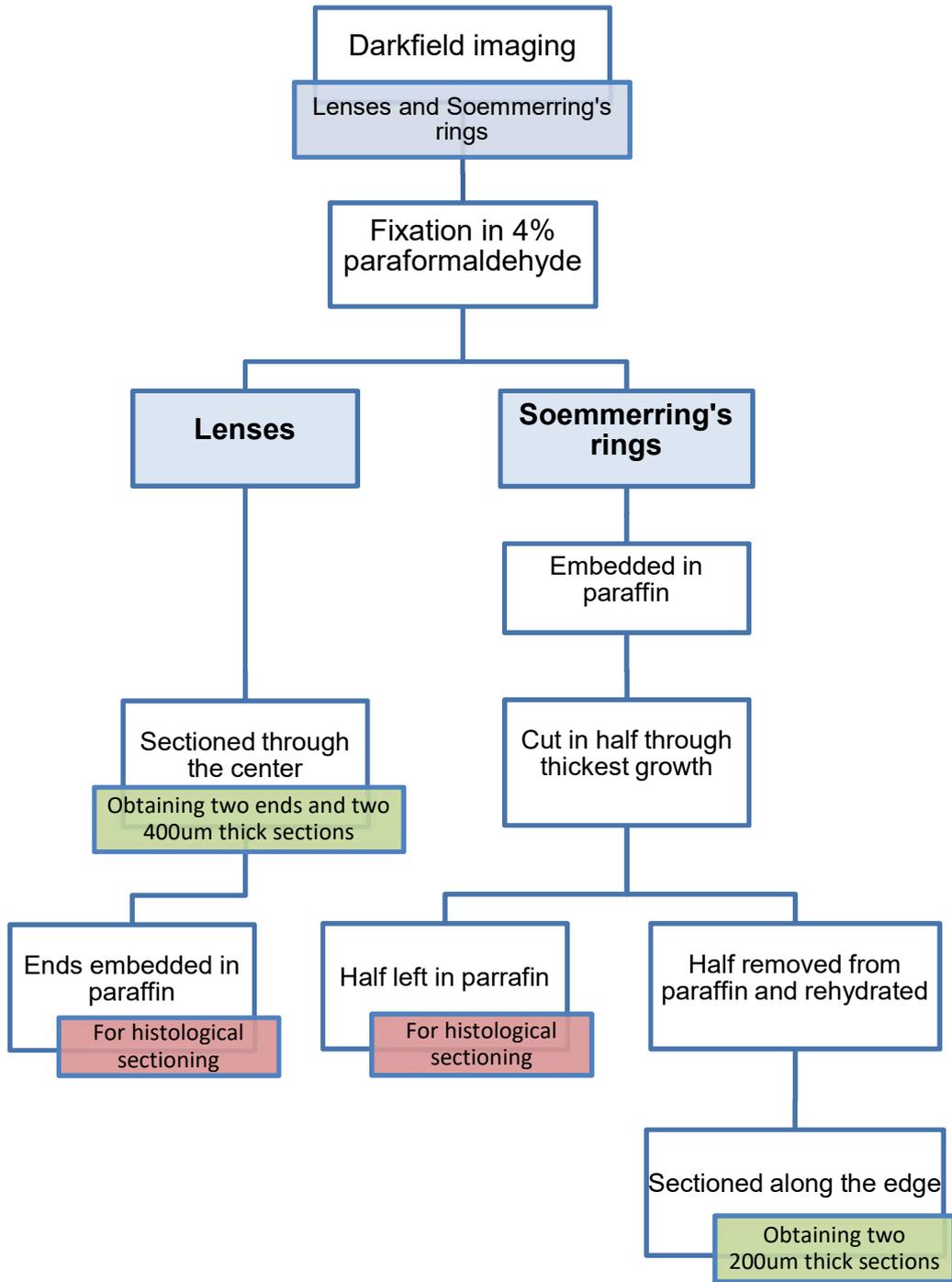
Figure 4.22 Diagram explaining how after frontal imaging, samples were processed in order to obtain both thick (green) and histological (Red) sections.

Vimentin will help visualize LECs and early lens fiber cells. α-SMA marks cells that have undergone epithelial to mesenchymal transition. Vimentin (clone V9, Ventana) and α-SMA (clone 14A, Cell Marque) will be performed with the BenchMark® ULTRA device (Ventana Medical Systems, Inc.) following the manufacturers protocol and counterstained with Hematoxylin.

WGA (L4895, Sigma-Aldrich) and DAPI (MBD0015, Sigma-Aldrich) will be used to better visualize fiber cell order and general cell morphology in order to relate this to the degree of transparency. Both immunofluorescent stains will be performed together by hand.

First, WGA at a concentration of 10ug/ml will be applied onto the sample, covered with a coverslip and then incubated at room temperature for 2h in a humidified dark chamber. After which, they will be washed three times with PBS.

After this, samples will be stained with DAPI at a concentration of 1µg/ml, covered with a coverslip and then incubated at room temperature for 30min in a humidified dark chamber. After which, they will be washed three times with PBS.

Finally, samples will be covered with Fluoromount (F4680, Sigma-Aldrich) and a coverslip, and sealed with transparent nail polish.

Fluorescent images will be obtained with the Leica TCS-SP5 Confocal Microscope located in the microscopy service of the UAB.

Preliminary Results

Disclaimer: Due to the unforeseen global pandemic of COVID19, we were unable to perform all of the desired analysis for this study. However, we still fully intend to continue and complete this study after the completion of this thesis.

The 400μm thick sections of the transparent lens when observed in a darkfield, showed clearly transparent cortical regions, while the nucleus showed slight aberrations (Figure 4.21C). These distortions were due to cutting artifacts and protein aggregations in the nucleus due to the fixation process. These were not due to nuclear cataracts, since when the lens was viewed frontally in the darkfield, before fixation, it was completely transparent (Figure 4.21A).

We had extracted 17 Soemmerring's ring samples from their eye globes, fixed them in paraffin and thickly sectioned them at 200 μm. When viewed frontally in the darkfield, all Soemmering's rings, showed a relatively high degree of opacification, with some variation, but nothing near transparent (Figure 4.23).

However, after sectioning, almost all samples showed some clearly transparent areas (Figure 4.23 B, C, D). The degrees of transparency observed in the thick sections of the different samples are summarized in Table 4.1.

Three common distributions of transparency could be distinguished. The most common, 8 of the 17 samples, presented an opaque outer cortex with a transparent or translucid nucleus and inner cortex such as in Figure 4.23 C & D. Another seen in 5 of the 13 samples, presented opaque or cloudy layers with some transparent layers in between, such as in Figure 4.23 B. The final type, seen in 4 of the 13 samples, consisted of samples that were opaque or cloudy throughout, such as in Figure 4.23 A.

Also, samples seemed to develop in to 3 general shapes, either long and thin (Figure 4.23 C), tear drop shaped (Figure 4.23 A) or round (Figure 4.23 B). No obvious relation between shape and degree of transparency was evident.
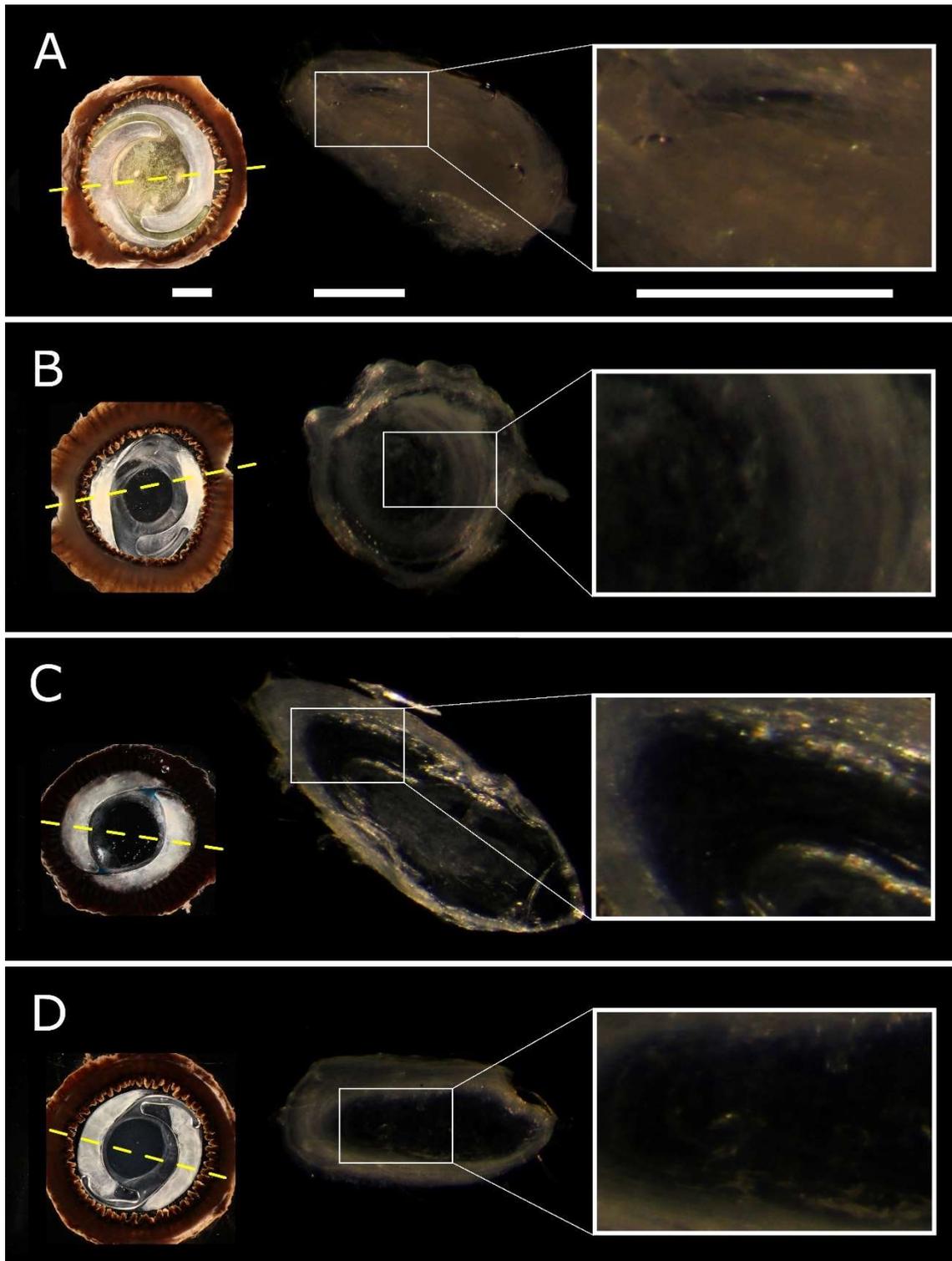
Figure 4.23 The left images (**A** 622A; **B** 613B; **C** 590A & **D** 577B) show frontal darkfield images of fresh human Soemmerring's rings. The yellow dotted lines show where the samples were sectioned. The central dark field images show the same samples sectioned 200 μm thick. The right images are magnifications of areas of interest. Scale bars from left to right = 3mm, 500μm & 500μm.

Tissue	Degree of transparency		
	Outer cortex	Inner Cortex	Nucleus
561a	XXX	XX	X
563a	XX	XXX	XX
577b	XXX	O	O
590a	XXX	O	X
600a	XXX	XX	X
605a	XXX	XXX	XX
607b	XXX	X	O
608b	XXX	O	O
612b	XXX	XX	X
613b	XXX	X	XX
616a	XXX	O	O
617a	XX	O	X
618a	XXX	O	X
621a	XXX	XXX	XX
621b	XXX	X	XX
622a	XXX	XXX	XXX
625a	XXX	X	XXX

Table 4.1 Summary of the different degrees of transparency observed in the three main layers of the thick sections of each sample. **XXX** = Opaque, **XX** = slightly translucid, **X** = translucid & **O** = Transparent.

Discussion

For now, we have only obtained the preliminary results of this study. However, these results are promising, since we obtained thick cross-sections of Soemmerring's rings that showed significant portions that were clearly transparent (Figure 4.23). We suspect that these sections will also show well organized lens fibers and LECs when analyzed histologically.

Of our 17 samples, 7 showed clearly transparent areas, this was mostly apparent in samples that presented an opaque outer cortex with a transparent nucleus. This is probably due to the fact that transparency is linked to lens fiber compaction.[291, 292] Thus, it would make sense that the inner fiber would be transparent as they are pushed together as new fibers grow around them. Plus, the fact that the cortex is opaque also explains why Soemmering's rings are always seen as opaque when viewed frontally.

What is important, is that the residual cells left in the lens capsule after cataract surgery can regain their transparency, if this could be promoted it would reduce the negative effects of PCO.

We have proven the main point of our hypothesis that there are transparent layers within the Soemmerring's ring. However, we still intend to study the reasons of the differences in transparency of different portions of the Soemmerring rings. If we can discern the reasons behind these discrepancies, maybe we could help guide the development of Soemmerring's rings *in vivo*, which could lead to the possibility of lens regeneration. However, this is still a far-off point and many more in depth studies of the mechanisms involved need to be performed.

Conclusions

Adult *in vivo* developed Soemmerring's rings, maintain the ability to produce organized and transparent layers of lens cells.

4.3.2. In vitro lens regeneration experiment

Abstract

In order to test whether a regenerative Soemmerring's ring like growth could be obtained *in vitro*, we designed a new tissue culture method that we tested, to see whether it would favor LEC development towards lens fibers while reducing EMT.

We based our new tissue culture protocol on other published works that had achieved partial regeneration *in vivo*. We focused on the changes they made to the surgical aspects of the lens extraction process and tissue culture.

We changed the method of performing the anterior rhexis in order to reduce the size of the opening, increase the amount of residual anterior capsule, reduce the trauma to the LECs and increase the initial number of residual LECs. We also developed new silicone support pieces to favor contact between the anterior and posterior capsule.

Once we had established the new protocol, we prepared 7 human and 10 porcine lens capsule samples and cultured them for up to 67 days. During culture, we used darkfield microscopy to monitor cell growth, migration and transparency. To monitor changes in thickness, we photographed the samples with a slit lamp, and after culture all samples were histologically sectioned and stained for α-SMA and vimentin.

All samples except one did not develop wrinkles within their capsule, and slit lamp images showed that the anterior and posterior capsule of all samples had adhered together within the first week. However, in most samples, LECs escaped from the capsules and migrated to the petri dish. Furthermore, in all pig samples, residual pigment cells from the iris-ciliary body, migrated and proliferated, making darkfield analysis very difficult. Vimentin and α-SMA expression were mostly positive in all samples, indicating that most LECs had undergone epithelial-mesenchymal transition. Finally, despite some samples developing volumetric peripheral growths, many showed signs indicative of apoptotic events.

In conclusion, we did not achieve lens fiber regeneration, but we showed the benefits of the contact between the anterior and posterior capsules to avoid wrinkles and that residual lens fiber cells do not survive as well as LECs.

Introduction

Based on the results of our previous article, Koch, C.R. & D'Antin, J.C. et al. 2019[290], we wanted to test whether a Soemmerring's ring like growth could be obtained *in vitro*. For this we needed to develop a new culture method that would favor LEC development towards fibers and reduce scarring.

We started by extending the bibliographical research from our previous publication in order to further understand which factors have been proven to promote fibrosis, specifically during cataract surgery. We found two recurring factors that we could modify in our tissue culture model in order to promote regeneration. First, some authors have highlighted the negative effects of stressing the residual LECs during cataract surgery by polishing the anterior capsule.[293-296] Second, other authors have shown that there seems to be a clear relation between the size of the rhexis and the level of organization of the Soemmerring's ring. They show that with smaller rhexis sizes, more transparent Soemmerring's rings are obtained [281, 297-300].

Objective

Our objective was to test whether our new mock cataract surgery protocol could promote the regeneration of lens like structures from the residual LECs in adults lens capsules.

Materials and methods

Establishment of new mock cataract surgery protocols

Our first new protocol was based on the work published by Lin, H. et al. in 2016 in Nature [212], where the authors partially regenerated the lenses of young dogs, rabbits and human infants. The main difference between that technique and normal cataract surgery, was that they extracted the lens through a peripheral 1.0–1.5 mm micro-rhexis instead of through the normal 6mm rhexis and that they did not implant an IOL.

We tested this with the aid of two anterior segment expert surgeons from our clinic (Dr. Rafael Barraquer & Dr. Milan Pešić). They removed the lenses of three human donor globes through 2 microrhexes (Figure 4.24A) using phacoemulsifiers. In order to test a simplified version of this surgery, that we could perform unaided in the lab, we also designed a method where the lens could still be hydroexpressed. To this end, we prepared four more samples where the lens was removed through a "smile" rhexis (a

curved incision connecting two opposing microrhexes) (Figure 4.24B). Afterwards these seven samples were cultured in the same conditions as all our previous samples.

Although cellular progression was initially very similar, we eventually noticed that the cells had migrated out of the capsules and on to the petri dish, further highlighting the resilience of these cells. Furthermore, in the case of the "smile" samples, the anterior capsule would roll up (Figure 4.24C).

Figure 4.24 A) Frontal darkfield image of a lens capsule emptied through two microrhexes. B) Frontal darkfield image of a lens capsule emptied through a "smile" rhexis. C) A slit lamp image of a rolled up "smile" rhexis anterior capsule.

After the results of our first tests, we noticed that an important factor for better *in vitro* development would be assuring that the LECs could not migrate outside of the lens capsule. With this in mind, we started thinking of ways to seal the capsule after extracting the lens and decided that the simplest way would be to sandwich the capsule between two pieces of silicone.

We tested three different designs, first, we simply tried to close our smile rhexis with two semicircles of silicone, one below the capsule and another above, connected by two 30G needles (Figure 4.25A). However, this approach had the disadvantage of needing to unroll the anterior capsule flap resulting from the "smile" rhexis, which would damage many of the LECs on the anterior capsule.

In order to avoid this problem, in our second design, we decided to make a straight cut across the anterior capsule to impede anterior capsule roll up. This obviously wouldn't be an option in an *in vivo* surgery, as it crosses the visual axes, however we considered this to be acceptable in an experimental setting, as it was for a proof of concept. This cut was sealed with two rectangular pieces of silicone (Figure 4.25B). This design damaged the LECs less, however, we noticed that there was poor contact between the anterior and posterior capsules.

With this we reached our third and current design, which is similar to the previous design with a minor change to the bottom silicone piece. In this design, the bottom silicone piece has two thin arms creating a cross shape (Figure 4.25C). We did this in order to lift the posterior capsule and improve the contact between the anterior and posterior capsule. We did this since it has been shown that a lack of contact between the capsules prevents LEC development [301-304].

Figure 4.25 Frontal darkfield images of three of our silicone sandwich designes. A) a capsule with a "smile" rhexis sealed with two semicircular silicone pieces connected with two 30G needles. B) a capsule with a straight cut connecting two microrhexisis, sealed with two rectangular silicone pieces connected with two 30G needles. C) a capsule with a straight cut connecting two microrhexisis, sealed with a cross shaped silicone piece under a rectangular silicone piece ontop, connected with two 30G needles.

Since the objective of this new culture protocol was to produce Soemmerring's ring like growths, changes in thickness would need to be monitored with the aid of a slit lamp.

Implementation of new mock cataract surgery protocols

Sample obtainment

Seven human donor eye globes from the "Banc d'Ulls per a Tractaments de Ceguesa", Barcelona, and eight porcine eye globes from "Patel, S.A.U." Barcelona, were obtained. The human eye globes had been classified as non-suitable for transplantation. Written informed consent for the removal and use of the eye globes for diagnostic and research purposes had been obtained from patients and/or relatives. This experimental study was approved by the Ethical Committee for Clinical Research of the Centro de Oftalmología Barraquer and followed the tenets of the Declaration of Helsinki.

As in our previous studies, we started by removing the corneoscleral disk from the eye globes with a circular trephine, allowing us to access and dissect the iris–ciliary body–lens complex in a single piece.

These pieces were transferred to sterile petri dishes and any residual adherent vitreous was removed from the posterior surface of the lenses. We noted that the vitreous in the porcine lenses was attached more firmly than in the human samples, this was probably due to the age difference of the samples.

Once clean, each sample was placed on a silicone ring (Figure 4.26A), anterior surface facing upward, and attached by passing 8 30G needles through the ciliary body. Once fixed, the iris is removed with forceps and the sample is quickly moistened with balanced salt solution (BSS) to avoid drying of the capsule, which would make it stiff and opaque.
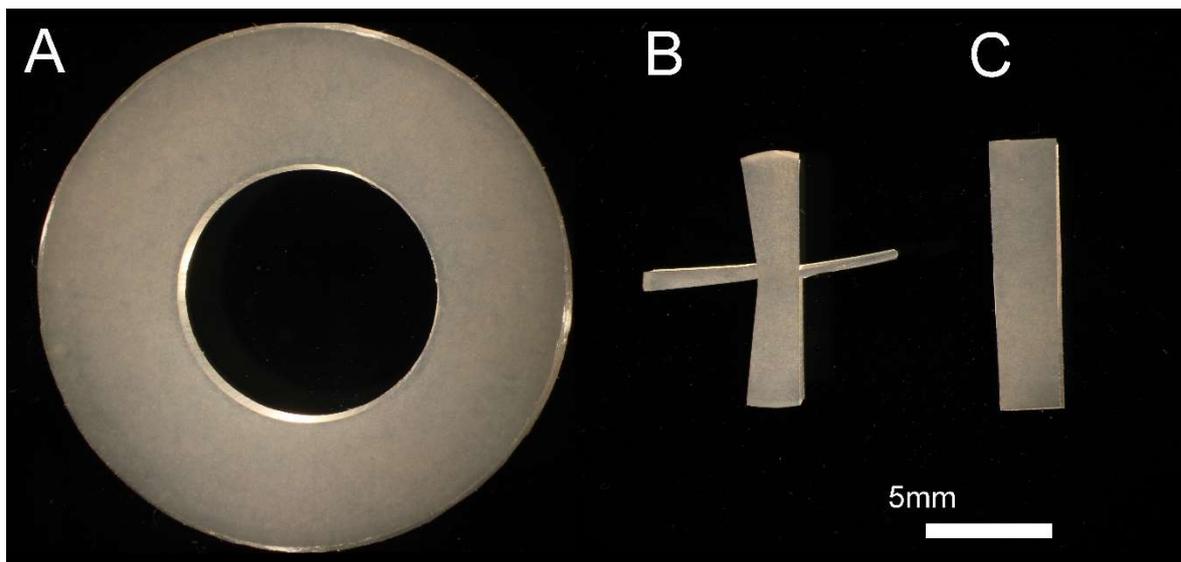
Figure 4.26 Frontal images of the silicone pieces used to support and seal the lens capsule. A) Silicone ring with an inner diameter of 10mm. B) Silicone cross piece that goes under the posterior capsule. C) Silicone rectangle that seals the straight cut rhexis.

Next, two microrhexes where performed opposite each other and as close to the lens equator as possible, careful not to be too close to the zonules as this severely complicates the microrhexis procedure. The easiest method we found to perform a microrhexis was to use a 21G needle to pierce the anterior capsule. Due to the shape and size of the needle, a small "c" shaped flap is formed on the anterior capsule. With the help of capsulorhexis forceps we pulled on this flap in a circular fashion, to finish the

circle that the initial puncture created, thus obtaining a continuous curvilinear rhexis of around 1-2mm. Once both microrhexes were performed, they were connected with a straight cut, using iris micro scissors (Figure 4.27A).
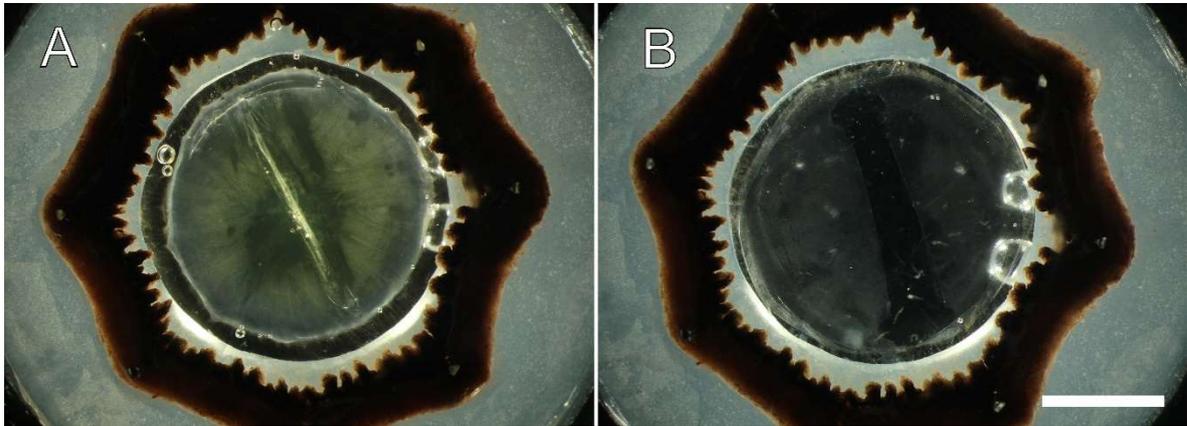
Figure 4.27 Frontal darkfield images of human lens samples during different stages of lens extraction. A) The 2 microrhexes connected by the straight cut. B) The lens capsule emptied of lens fibers but with the anterior LECs intact. Scale bar = 5 mm.

In order to damage the LECs as little as possible, careful hydroexpression was employed to detach the lens from the capsule and out through the opening. Residual lens fibers were removed through aspiration with a Simcoe cannula, although less thoroughly than in previous studies, in order to damage the LECs as little as possible (Figure 4.27B). At this point, the 4 uncultured controls (2 human and 2 porcine) were fixed in 4% paraformaldehyde, for histological analysis. We did this in order to observe the starting point of our cultures.

Finally, we placed a silicone cross piece (Figure 4.26B) under each sample and a rectangular silicone piece (Figure 4.26C) over the sample, sealing the rhexis cut. These two pieces were pinned together using two 30G needles (Figure 4.28).
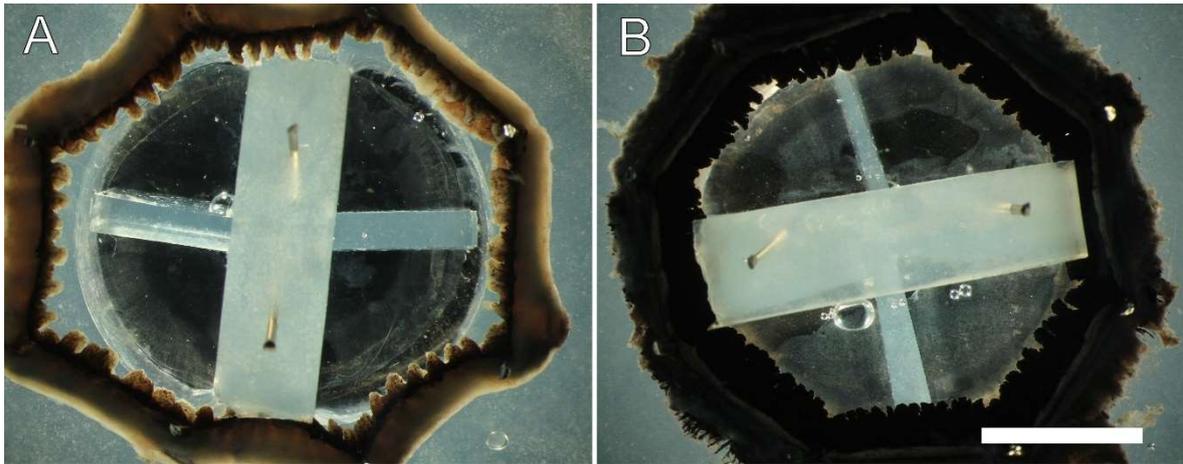
Figure 4.28 Frontal darkfield images of finished A) Human and B) Pig samples, prepared with our new protocol. Scale bar = 5 mm.

Tissue culture

All samples, once prepared, were transferred to a biosafety cabinet (Bio-II-A, Telstar®) and decontaminated with a BSS and antibiotic-antimicotic solution, then submerged in RPMI-1640 culture medium (R8758 Sigma-Aldrich) supplemented with 5% fetal bovine serum (F7524 Sigma-Aldrich) and 1% antibiotic-antymycotic (A5955 Sigma-Aldrich) and finally placed in an incubator. Culture medium was exchanged every 3 to 4 days. Samples were cultured for up to 67 days.

Culture monitoring

Every time the culture media was changed, photographs were taken to monitor cell growth, migration and transparency. As with our previous experiments, we used an inverted microscope, phase contrast (Ph2) illumination and a normal 2.5x Plan-objective, for dark field images. With the same microscope we also took photos with the phase contrast illumination ring slightly misaligned in order to obtain better contrast images of the individual cells. These images were taken in each of the four quadrants made by the silicone cross piece.

Furthermore, in order to visualize changes in growth thickness, we used a slit lamp together with a camera to obtain Scheimpflug cross sectional images. Samples in their petri dishes were placed on a holder that allowed us to move the sample in order to focus the light on the area of interest. (Figure 4.29)
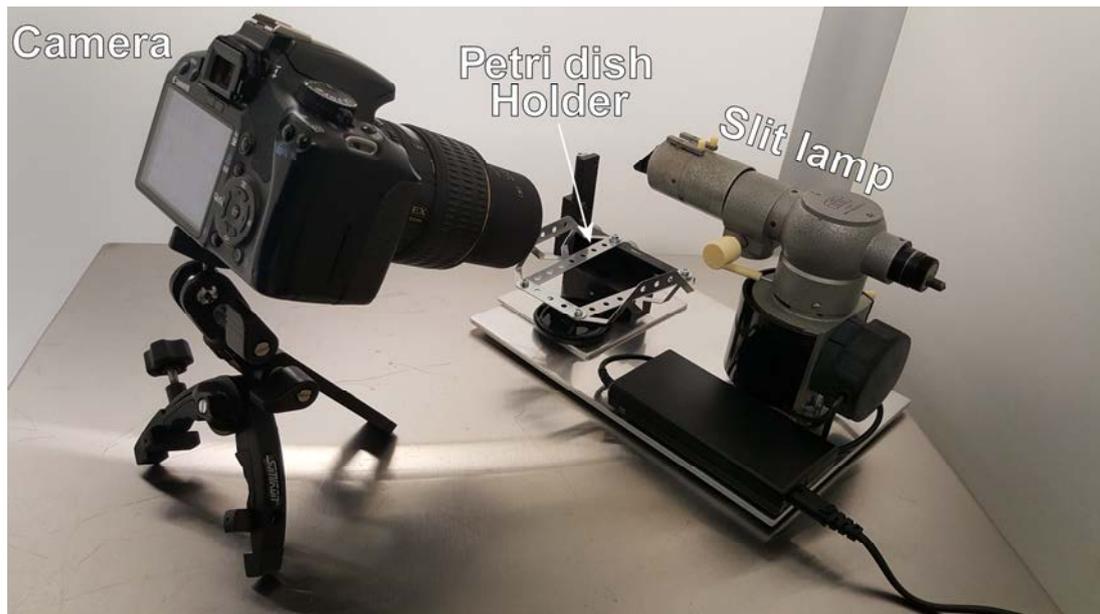
Figure 4.29 Image showing the distribution of our slit lamp set up.

Histological staining

Immediately after samples were removed from culture, they were placed in 4% paraformaldehyde for fixation for at least one week and then embedded in paraffin. Once all samples were in paraffin, they were sectioned along the sagittal plane and stained. Tissue were stained with α-smooth muscle actin (α-SMA) (clone 14A, Cell Marque) for myofibroblast formation and intercapsular adhesion and vimentin (clone V9, Ventana) a marker of both undifferentiated lens epithelium as well as differentiating fiber cells. Stains were made on adjacent sections in order to compare results. All immunostainings were done with the BenchMark® ULTRA device (Ventana Medical Systems, Inc.).

Results

Macroscopically, none of the cultured capsules presented clear wrinkles within their capsules, except for one human lens capsule which had a partial rupture of its posterior capsule, produced during lens extraction. However, slit lamp images showed that the anterior and posterior capsule of all samples had adhered together within the first week.

In samples with residual lens fiber cells, there were visible accumulations of cells around the fibers and some of these, over time, seemed to develop in to Elschnig pearls (Figure 4.30).
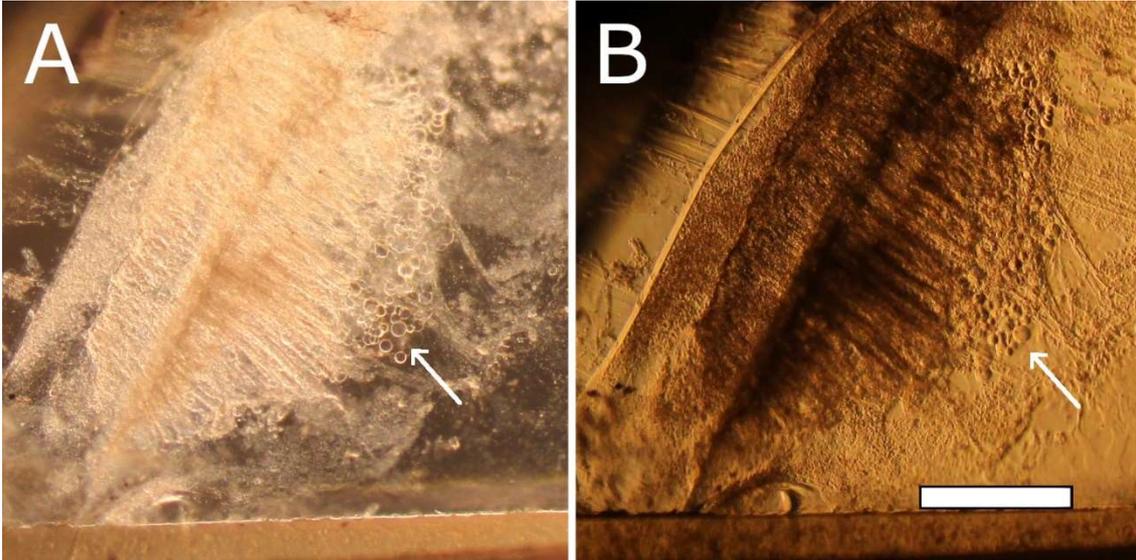
Figure 4.30 A) Darkfield and B) Pseudo-darkfield images of one quadrant of a human sample during culture. Arrows indicate what seem to be Elschnig pearls growing on and around residual lens fibers. Scale bar = 500μm.

Despite the silicone seal, LECs escaped from the capsules and migrated to the petri dish in all samples except in one human sample. This sample was also the one with the most significant peripheral growth (Figure 4.31).
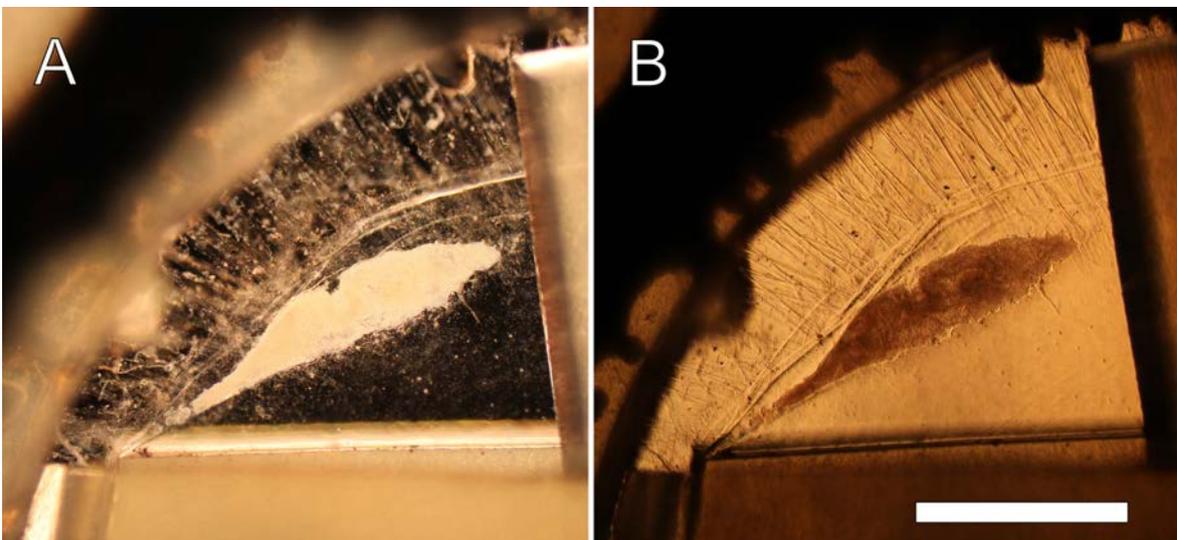
Figure 4.31 A) Darkfield and B) Pseudo-darkfield images of one quadrant of a human sample during culture with a relatively large peripheral growth. Scale bar = 2 mm.

In all pig samples, residual pigment cells from the iris-ciliary body, migrated and proliferated significantly on and behind the capsule, making proper analysis very difficult (Figure 4.32).

Furthermore, in pig samples, large areas of LECs would detach in sheets from the anterior capsule during lens extraction. Even once the capsule was sealed, some parts of the anterior LEC layer could detach and float around inside the capsular bag.
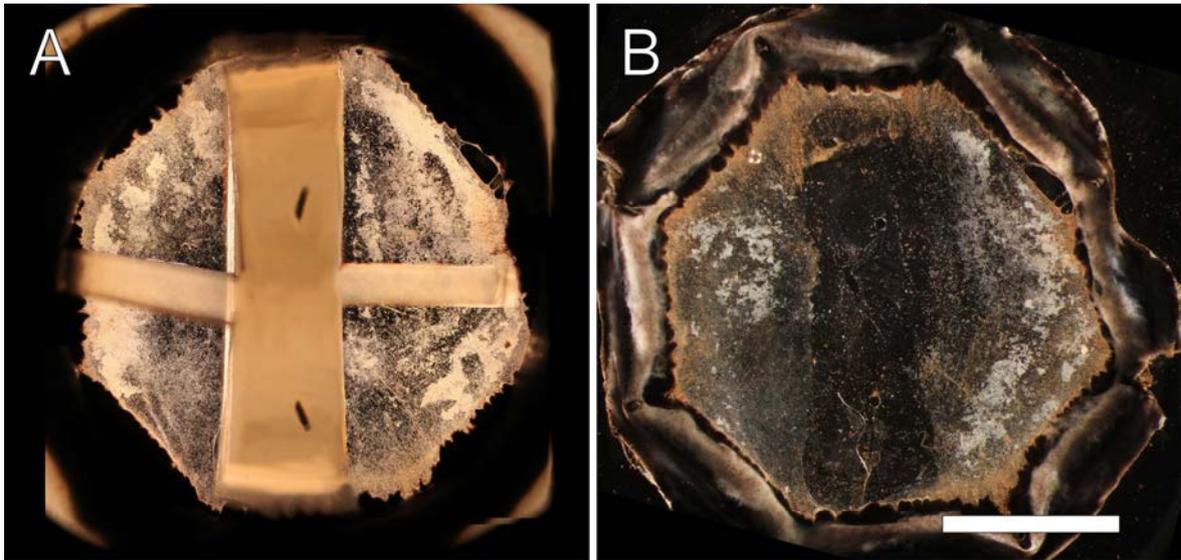
Figure 4.32 Frontal A) pseudo-darkfield and B) darkfield of a pig sample after two months of culture. The different imaging techniques highlight the difficulty of differentiating between lens cell and pigment cell progression. A) seems to show an extensive growth of lens cells, but B) shows that most of it is pigment cells. Scale bar = 5 mm.

Histological sections of controls showed a LEC monolayer on the anterior capsule and layers of lens fiber cells at the equatorial regions. Control samples were negative for α-SMA staining and positive for Vimentin throughout (Figure 4.33).
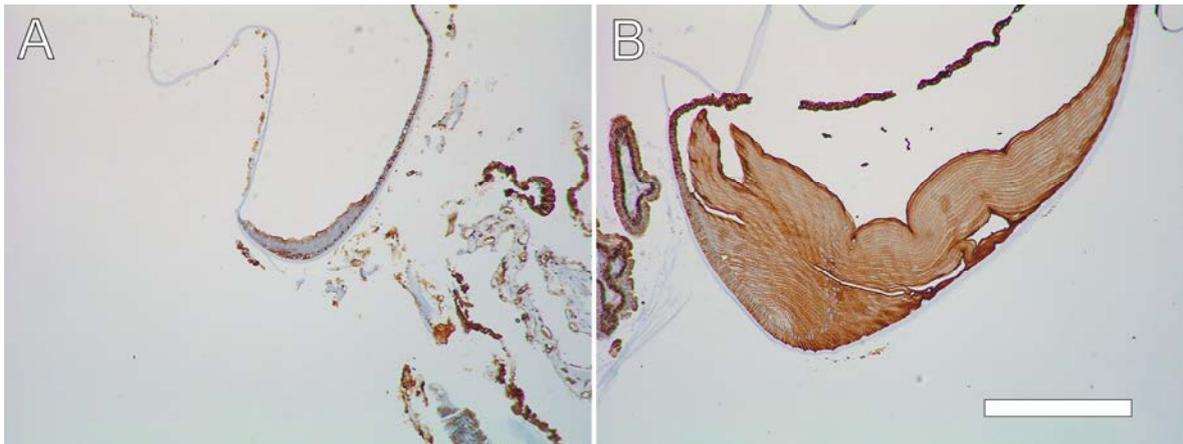
Figure 4.33 Microscopic images of the histological sections of the equatorial region of two control samples, showing staining for Vimentin. A) shows a human sample and B) shows a pig sample. Scale bar = 300 μm.

Cultured capsules consisted of a monolayer of cells along the central part of the capsule, with many samples showing some volumetric growth at the equators. Interestingly, when pig samples were sectioned, capsules separated, this was probably related to the weak adhesion of LECs that we previously noted.

Vimentin and α-SMA expression were mostly positive in all cultured capsule samples, indicating that the LECs had undergone epithelial-mesenchymal transition (Figure 4.34A, C, D, E, G & H). However, two human samples showed a lack of α-SMA within the globular cells of their peripheral growths while these were still positive for Vimentin (Example Figure 4.34B & F). These could be areas where lens fiber precursors were forming.

However, many peripheral growths showed what seemed to be broken up cells agglomerated together, which is indicative of an apoptotic event. Based on the lack of either stain and their morphology, we deduced, with the help of a pathologist, that these areas are of necrotic tissue that had undergone accidental cell death[305] (Figure 4.34B & F).
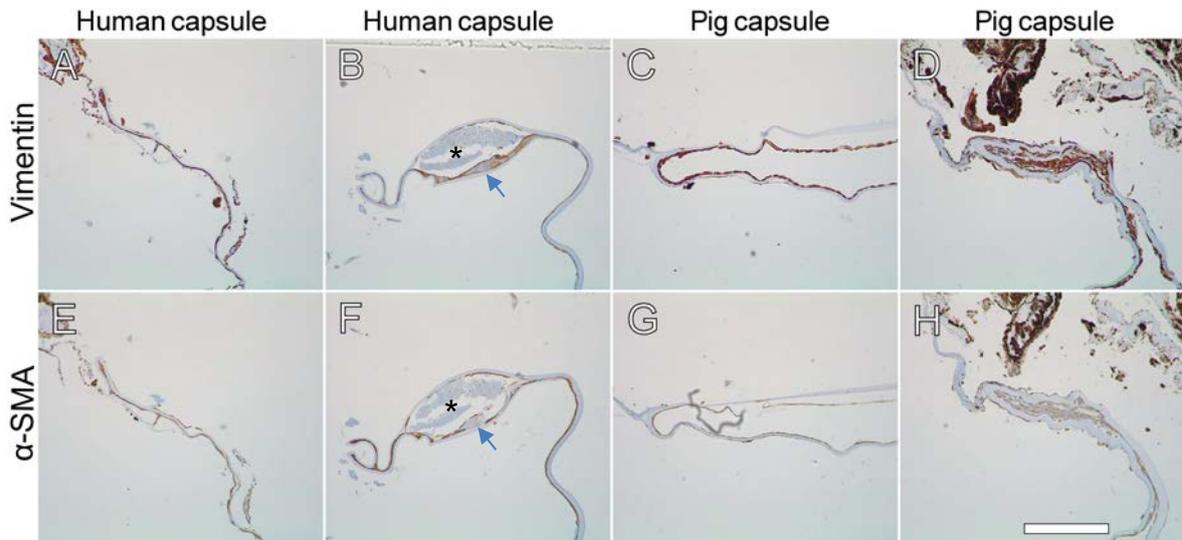
Figure 4.34 Microscopic images of the histological sections of the equatorial region of some of our samples, showing staining for Vimentin (A-D) and alpha smooth muscle actin (α-SMA) (E-H). Images (A & E) show a cultured human capsule, with only a monolayer of growth and an absence of wrinkling. Images (B & F) shows a cultured human capsule sample with necrotic tissue (asterisk) and globular cells (arrow). Images (C & G) shows a cultured pig capsule sample with poor adhesion. Images (D & H) shows a cultured pig capsule sample with some layers of growth. Scale bar = 300 μm.

Discussion

Although no clear sign of regeneration was obtained, we did observe some interesting results.

The more restrictive lens extraction and polishing had a clear effect on the starting conditions, as some layers of fibers were left at the equator. These layers in our previous culture tests were meticulously removed. Our initial thought was that these fiber cells could act as a scaffold or guide along which new fiber cells could develop. However, after seeing our results, it seems like this was not the case. Furthermore, the necrotic tissue we observed was most likely the result of the death of the residual lens fiber cells. We had not left these residual fiber layers in previous studies, nor had we seen these bundles of necrotic cells. This probably negatively affected the LECs and impeded proper cell development and growth.

Another interesting result was the reduction or even absence of wrinkles on the posterior capsule (Figure 4.34A & E). This seemed to be due in part to the contact and rapid adhesion of the anterior and posterior capsules. Probably because, although LECs had undergone epithelial to mesenchymal transition, as evidenced by the positive α-SMA

stain, they did not need to migrate from one capsule to the other, as these two were already in contact.

The "Elschnig pearls" we observed around fibers (Figure 4.30) would make sense since Elschnig pearls have been described as fiber cell precursor cells, much like our previously mentioned globular cells.

The fact that we still had cells migrating on to the posterior capsule shows that our sealing method was not efficient enough and that in future experiments we will have to develop a new method.

Ex-vivo porcine lenses have proven to be inadequate study models as they do not retain their monolayer of LECs well, which also affects intracapsular adhesion. They also do not seem to grow faster than human samples despite the age difference. Furthermore, the excessive proliferation of pigment cells makes proper analysis of LEC development very difficult.

Conclusions

In conclusion, we did not achieve lens fiber regeneration, but we showed the benefits of the contact between the anterior and posterior capsules to avoid wrinkles and that residual lens fiber cells do not survive as well as LECs. Thus, many aspects of this mock cataract surgery protocol need to be improved in order to prove the natural limits of adult lens regeneration in optimal *in vitro* conditions.

5. General discussion

The general aim of this thesis has always been to better understand the development of PCO. We originally intended to use this knowledge in order to prevent PCO from developing. However, after observing the extreme resilience of LECs, we decided to take a different approach to PCO prevention. Instead of eliminating all LECs, maybe we could control LEC development after cataract surgery in order to achieve transparent organized cells or even, regenerate the lens.

5.1. Posterior Capsule Opacification prevention

We initially had the idea of preventing PCO development through the use of substances to interfere with the biological processes underlying PCO. Variations on this approach had already been extensively studied for over 20 years in various models.[59] Three main approaches have been followed: induction of LEC death, prevention of cell proliferation/migration and interfering with the immune response (Table 1.1).

5.1.1. Selection of Study model

Our first step in studying PCO prevention, was to select a useful model. Our bibliographic research highlighted that the reason PCO development is still not fully understood, seems to be due in part, to the variations in the developmental factors involved in PCO between humans and most of the cell and animal models used to study it.[93, 168, 169, 171-173, 175, 193-195] Not only are there variations between species, it has also been proven that there are variations based on the age of the subjects.[212, 214] Many published works use young animal models, which increases the difficulty of establishing a relation PCO developed in adult humans. Therefore, since we wanted to study the development of PCO in adult humans, we used tissue samples obtained from adult human donors.

Furthermore, the conservation of LECs on their natural substrate, the lens capsule presents advantages, it simulates the natural environment more faithfully than cell line cultures and avoids the differences due to the capabilities of the LECs of smaller mammals which are not comparable to those of adult humans. Also, the lens capsule has a protective effect on LECs against apoptosis inducing situations, because the lens capsule is a source of essential survival factors for LECs[237].

5.1.2. Testing study model

Once we had selected our study model, we needed to test our capability to develop PCO in a fashion comparable to previously validated and published *in vitro* lens capsule studies. To this end, we prepared our capsular bag samples using the protocol proposed by Cleary G, et al. in 2010[194].

Once prepared, samples were cultured with culture medium consisting of: RPMI-1640 media (R8758 Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS) (F7524 Sigma-Aldrich) and 1% antibiotic/antimycotic solution and placed in a humidified CO2-incubator at 37 °C with 5% CO2.

When we compare the development of PCO in our control samples over time to other published works, the results are comparable, as can be seen in Figure 5.1.
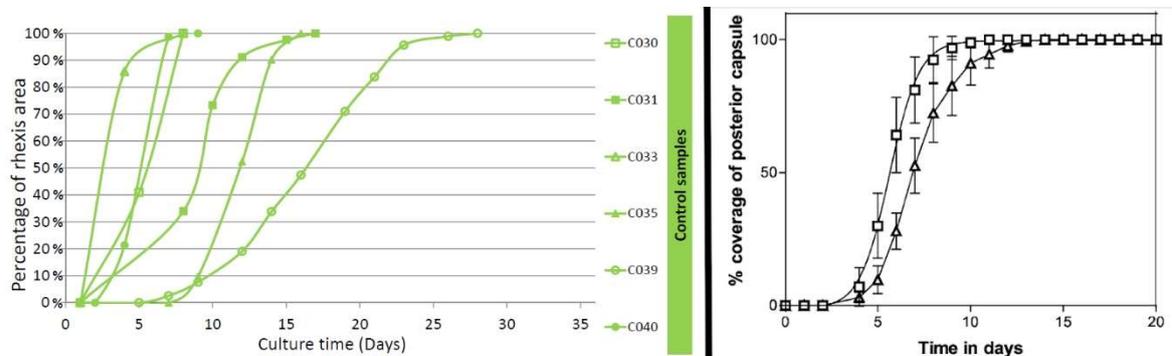
Figure 5.1 Two diagram of cellular confluence progression during cell culture. Showing percentage of confluence on posterior capsule against culture time. 0% means no signs of cell growth on posterior capsule within visual limits of the rhexis and 100% means total confluence. **Left graph** is a modified version of our Supplementary Figure 5.1, only showing control samples. **Right graph** is from El-Osta 2003. Growth curves derived from young donor lenses (<50 years, n = 10), represented by squares, and old donor lenses (>50 years, n = 37), represented by triangles, are shown (error bars = SEM) [203].

This indicates that both our study model and culture protocol are correct and can give valid and reproducible results.

5.1.3. Selection of preventative substance

We wanted to use a substance with a clear effect on LECs but that could still be tolerated by the surrounding tissue.

Based on our bibliographic research[227-229], we chose hydrogen peroxide (H_2O_2), because it is naturally found in normal human lenses and aqueous humor at concentrations of

approximately 20 to 30 μM. However, many cataract patients have elevated H_2O_2 levels ranging from 2- to 7-fold higher than the normal range in the lens, and 30-fold greater than normal in aqueous humor[306].

Oxidative stress and the generation of reactive oxygen species (ROS) are the most common damaging factor that can accelerate cataract development through damage to lens epithelial cells.[23] Excessive generation of reactive oxygen species (ROS) from either the environment or from mitochondria of the LECs can damage cellular macromolecules such as proteins, DNA, and lipids, leading to cell death through apoptosis or necrosis, compromising lens transparency[23].

As a positive control of total LEC death, we used a very aggressive concentration of 30mM of H_2O_2.

Our intention was to subsequently reduce the concentration to a level more easily tolerated by the surrounding tissue. Surprisingly, despite this aggressive treatment, we still observed PCO develop in half of our cases. This result highlighted the difficulty in attempting to prevent PCO by eliminating or severely damaging residual LECs.

Nevertheless, more aggressive treatments would still present problems. In an *in vitro* experimental study[158], where all cells within the lens capsule had been eliminated using 1 μM thapsigargin, negative effects on IOL stability were observed. The authors observed that in untreated capsules, the residual LECs growing around the IOL, help to stabilize and fix it in place. In the treated samples, without cells, the IOL pivots and wobbles, producing intolerable visual symptoms. Furthermore, another potential concern is that a lack of cells will lead to degeneration of the capsule, possibly accelerated due to enhanced mechanical abrasion from IOL movement; this in turn could lead to increased fragility and IOL subluxation or dislocation. The sum of these observations show that completely preventing residual LEC growth, in order to prevent PCO after cataract surgery, is not a viable option.

5.2. Developmental potential of residual lens epithelial cells

Despite the difficulties in finding a useful substance for the prevention of PCO, the general incidence of cases has been drastically reduced over the past years, in large part, thanks to the mechanical barrier effect of modern IOL designs[95].

However, even in these cases where residual LECs are mechanically blocked from growing over the visual axis, PCO still occurs, since the residual cells still develop around the IOL, forming the Soemmerring's ring.[167, 307, 308] Because there will always be residual LECs after cataract surgery, there will always be some degree of Soemmerring's ring development, and thus infiltration of cells on to the posterior capsule, is only a matter of time[55].

Based on these articles and our first results, we decided to move away from preventing PCO, in order to study what would happen if LECs were allowed to develop unimpeded. How would long term culture develop? What could we use as a control?

The easiest answer to long term developed LEC, was naturally occurring *in vivo* PCO. Thanks to our ready access to donor globes, we could extract lens capsules containing IOLs (Figure 5.2A), from donors who had previously *in vivo* undergone cataract surgery.
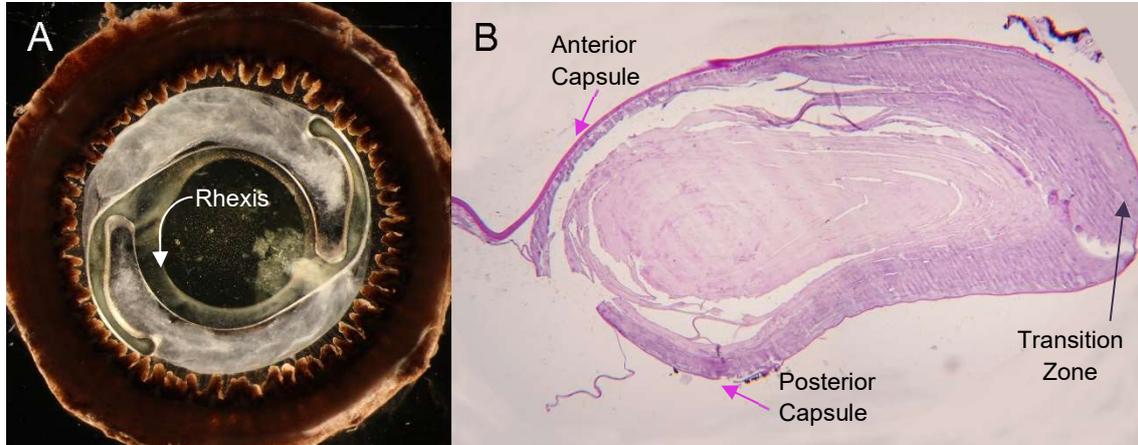
Figure 5.2 Post cataract surgery capsule sample with advanced Soemmerring's ring formation around an IOL. A) Frontal dark field photo of a sample highlighting the extent of the Soemmerring's ring. B) Histological cross section of a Soemmerring's ring sample stained with H&E showing the monolayer of LECs, the transition zone and layers of lens fibers.

In order to get an idea of what we could see in these samples, we fixed and embedded them in paraffin for histological sectioning. The first samples were stained with H&E (Figure 5.2B) and already gave us some very interesting results.

Based on this we wanted to perform a more in-depth analysis of the mechanisms involved in the development of PCO in *ex vivo* samples compared to our cultured control samples, leading to our second article.

5.2.1. Soemmerring's ring development

The results of our second article highlighted the true potential of adult LECs to regenerate lens structures and solidified the concept that PCO and especially the formation of Soemmerring's rings arise from the failed innate process of lens regeneration.

In that article, we analyzed the progression and morphology of PCO in three types of samples that could represent 3 different points along the continuous developmental process of PCO (Figure 4.9A-C).

The first point, represented by control lens capsules, showed normal early PCO development, consisting mainly of monolayers of cells that have undergone proliferation and epithelial to mesenchymal transition (EMT). The second point was represented by the less developed (early) Soemmerring's rings, samples where some areas still consisted of monolayers of mesenchymal cells, but others start to show volumetric growth at the equator around the IOL. The morphology of these two types of samples presented many similarities (Figure 4.13). One of the control samples even started showing volumetric growth, with a morphology surprisingly similar to our second type of sample (Figure 4.10B,D,G and I). These results agree with the idea that these two points are part of the same continuous process.

Finally, the third point was represented by more developed (late) Soemmerring's rings, samples where the cells within the capsules have been developing around the IOL for a long period of time. In these cases, volumetric growth at the equator was significantly increased, being either less or more organized (Figure 4.11A and B). However, in both cases, the monolayer of LECs was recovered and cells express less mesenchymal cell markers. This indicates that in either case, at this stage, a degree of reorganization is taking place. Furthermore, the more organized samples were so morphologically similar to normal lenses, that they can be considered as partially successful attempts of lens regeneration (Figure 4.14 and Figure 4.16).

An interesting observation of these results was the interaction between the cells in these more developed Soemmerring's rings and their IOL. While the cells closer to the equator recovered their normal cell type and function, the cells that are directly in contact with the IOL, maintain their mesenchymal phenotype and express collagen (Figure 4.12). This

indicates two negative effects of IOLs, which impede lens regeneration and favor future clinically relevant PCO. First, the cells seem to develop a foreign body or scaring reactions to the IOL by producing collagen at the areas of contact, impeding the progression of regenerated cells. Second, since pockets of mesenchymal cells are maintained between the IOL and the collagen layers, these can eventually breach the mechanical barrier of the IOL and migrate on to the posterior capsule. This can produce clinically relevant PCO that negatively affects the vision of the patient. This can explain many cases of late onset PCO that need Nd:YAG laser posterior capsulotomy.

5.2.2. Soemmerring's ring transparency

Another interesting observation was that, despite the high degree of cellular organization in some Soemmerring's ring samples, they weren't transparent. Our histological results showed that despite the correct distribution of LECs in a monolayer and the organized layers of lens fiber cells, these almost always grow around an amorphous central mass of cells. We suspected that these cluster of cells were most likely the origin of the opacities seen in the more organized Soemmerring's rings.

However, when viewing the results of our transparency experiments, in most cases, it was actually the central part of the Soemmerring's ring cross sections that were transparent (Figure 4.23). This leads us to conclude that it is most likely the compaction of fiber cells that allows its transparency, and the newer less compact layers of fiber cells are the cause of the opacities.

It has been shown that the space between fiber cells plays a major role in lens transparency and that lens fibers form interdigitations in order to compact together as much as possible, reducing the intracellular space in order to increase transparency and optical properties[291, 292].

According to the literature, one of the most important lens fiber proteins for transparency is Aquaporin 0 (AQP0). It is necessary to compactly pack the fiber cells and reduce extracellular space to minimize light scatter as well as to establish lens refractive index gradient and biomechanics for proper image formation[291, 292].

AQP0 has roles on water permeability, cell-to-cell adhesion, regulation of gap junctions, and establishment and maintenance of refractive index gradient and lens biomechanics. AQP0 is also involved in fiber cell elongation, migration and maintenance of cell shape during differentiation[309, 310].

This protein will be the focus of our continued studies in to both Soemmering's ring transparency and proper lens regeneration.

Another factor that might play a role in transparency is the elastic nature of the lens capsule, which probably aids in maintaining cells tightly compacted together as the lens grows and develops.[311] In the case of Soemmerring's rings, the capsule is loose and can only start constraining the Soemmerring's ring after it has gained some volume and is trapped behind the IOL.

5.3. Lens regeneration

The idea of lens regeneration is not a new one, and its relation to PCO has been alluded to for a long time.[159] The ability of some mammals to regenerate the lens was already published back in 1827 by Cocteau & Leroy-d'Etoille [163, 312] when they showed that rabbits could regenerate transparent lenses after lens extraction. Interestingly, some physicians of that time did not accept that the lens could regenerate as that contradicted the theory that only the liquid humors of the body could regenerate.[313]

After Cocteau's publication, there was an upsurge in publications studying the same regenerative process in rabbits and other animals[314-318], until the 1900s. After which, the topic was drop until the 1960s when lens regeneration was again studied in primates and other animals.[319-321] Interestingly, around this time was also the beginning of the use of PMMA IOLs.[322, 323] Over time, with the increase in the use of IOLs and the continued study of lens development and regeneration in animals, a relationship between PCO after cataract surgery and lens regeneration could be seen. Although it was mainly studied in animals, it was also observed and studied in human infants.[212]

After analyzing human cases of adult PCO both in our published work[290] and in our transparency study, we believe that even adults have the capability to regenerate, at least partially, the lens. In order to test this in an *in vitro* environment, we concluded that we would have to modify our current tissue culture model in order to promote lens fiber cell differentiation and organization.

We started by modifying two aspects of our traditional tissue culture model, residual LECs polishing and rhexis size. We chose these two factors, since they are related and easy to implement, and based on our bibliographic research[281, 293-300], they directly affect residual LECs development. On one hand, we reduced the anterior rhexis size, in order to leave as much of the capsule intact as possible since the capsule is a source of essential survival factors for LECs[237], and it should give structural order to the

regenerating lens[281, 297-300]. On the other hand, we reduced the stress to the residual LECs by avoiding polishing of the anterior capsule and limiting polishing[293-296] of the residual fibers at the equator. This should avoid a more inflammatory and scarring reaction from the residual cells and promote a more regenerative development. The changes introduced in both aspects, increased the initial amount of viable LECs, when compared to our previous tissue culture model or even, routine cataract surgery.

However, despite our changes to the mock cataract surgery protocol, we could not obtain newly developed lens fiber cells in our culture model. This could be expected, at least to a certain degree, due to the relative simplicity of our changes. But the factors we had changed still needed to be analyzed and tested.

The lack of fiber cell differentiation, could be due to a variety of factors, such as inadequate culture media, lack of specific growth factors that would be present *in vivo*, culture time and/or conditions, etc. Furthermore, it has already been mentioned that lens fiber differentiation in tissue culture models is very difficult or directly not considered possible[168] (Table 1.2).

We still strongly believe that lens regeneration in adult humans is possible, however, it is clearly not an easy nor straight forward process and the current *in vitro* approach for studying it is inadequate. Major changes to the current *in vitro* capsular bag model are necessary, such as, varying specific growth factors over time in order to better imitate *in vivo* conditions[205] and changing the surgical procedure. In any case, the best and most reliable results of lens regeneration potential would be obtained *in vivo*, however, due to the preliminary nature of our results, this is something that will only be possible in the future, after further study and testing.

5.4. Future perspectives

Based on our results, analysis and conclusions, lens regeneration should be the focus of future studies for the treatment of cataracts and possibly even presbyopia. Furthermore, if transparent lenses are obtained, PCO would no longer be an issue.

Thus, just as we have started to propose changes to the *in vitro* tissue culture model, in the future, changes and innovations to the current cataract surgical procedure will have to be proposed and studied, in order to regenerate the lens and prevent PCO.

Even though our original objectives did not extend beyond the *in vitro* prevention and analysis of PCO, we would like to speculate on the future of cataract surgery. To this

end, we would like to analyze which aspects of current cataract surgery might hinder lens regeneration as a possible future treatment option.

5.4.1. Current and future treatment of cataracts

The treatment of cataracts has evolved and improved vastly over the years, but due to the relative effectiveness and simplicity of the current surgical procedure, progress in this direction seems to be reaching its limit. The current best surgical option of extracapsular cataract extraction (ECCE) through phacoemulsification with IOL implantation, does not favor the continued progress of cataract treatment towards its possible next step of lens regeneration.

Currently, the main approaches for improving cataract surgery outcomes and patient's quality of life, are centered around improving the IOL design.

The discovery of the barrier effect might be the aspect that has most reduced the prevalence of PCO. Thus, many components of the IOL itself have been improved to further increase this effect. IOL haptics have been elongated and angulated upwards to increase contact and pressure between the back of the IOL optic and the posterior capsule. Changes to IOL material have been implemented to improve its adhesiveness to the capsule.[122] Furthermore, as we have previously mentioned, most modern IOLs have sharp square-edge optic designs in order to form stronger mechanical barrier against the LECs.[123, 124]

Other studies seek to improve the design by testing new materials that can help prevent PCO or reduce surface haze and roughness.[324, 325] Some groups have designed IOLs with improved functionality, such as an extended functional range of vision[326-328], or even IOLs that can accommodate[113, 329]. Accommodating IOLs, are designed to allow both good distance and near vision. They attempt to accomplish this either by changing their curvature or slightly moving the optical portion of the IOL, in response to the contraction of the ciliary muscle.

In any case, the real objective of these innovations, to the IOL design, are to simulate the functionality and characteristics of the young natural lens. Unarguably, this would be best achieved by regenerating the lens. However, in part, it is the implantation of these IOLs themselves that make lens regeneration impossible, since they occupy the space the regenerating lens would need.

The lack of incentive to change the current standard of cataract surgery with IOLs could be related in part to its simplicity and profitability. An estimated 26 million cataract

surgeries were performed worldwide in 2017.[330] The average prices of cataract surgery range from 164€ in India to 736€ in Europe to 2324€ in the United states.[331, 332] This gives a global cost range for cataract surgeries of around ~4.23 billion to 60.4 billion euros. Thus, investment in a new surgical procedure, that does not use these very profitable IOLs, will be difficult.

However, based on our analyses and study of Soemmerring's rings, total unaided lens regeneration in adults would take a prohibitively long time. Thus, it would most likely be necessary to add promotors or growth factors that would accelerate fiber formation, for lens regeneration to be a viable treatment for cataracts in humans. These promotors could be the focus of future studies and could be marketable pharmaceuticals. This might be the incentive needed to invest in this possibly revolutionary future surgical option.

6. Conclusions

The general conclusion of this thesis is:

That PCO is a complex and multifactorial process that is both very difficult to prevent and predict. However, our results together with recent publications indicate that PCO is the result of a failed innate process of lens regeneration. Furthermore, given the right circumstances, residual LECs can regenerate lens like structures, and this should be the focus of future PCO prevention and cataract surgery.

The specific conclusions are:

1. Normal clinical PCO can be correctly simulated in a lens capsular bag tissue culture model.

2. Hydrogen peroxide is a possible treatment for the delay of posterior capsule opacification *in vitro*, but the resilient nature of LECs makes this a difficult task.

3. Tissue culture models can develop the same PCO morphologies as those seen in less developed *in vivo* samples, including the same cell types and markers. While more developed *in vivo* samples, are morphologically more similar to normal adult lenses.

4. The morphological distribution of LECs and lens fiber cells in Soemmerring's rings can be similar to that in normal human lenses and seem to arise from an incomplete process of lens regenerations.

5. Adult *in vivo* developed Soemmerring's rings, can be made of organized and transparent layers of lens fiber cells, further supporting the idea of lens regeneration.

6. Changes only to the surgical process of the tissue culture model are not enough to promote lens fiber cell formation and regeneration. The addition of growth factors should be the focus of future studies.

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