Department of Ophthalmology

University of Helsinki

Helsinki, Finland

Corneal recovery after uncomplicated and complicated PRK and LASIK

Jukka Moilanen

Academic Dissertation

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Supervisors

Timo Tervo

Professor

Department of Ophthalmology

Helsinki University Central Hospital, Finland

Minna Vesaluoma

Adjunct Professor

Department of Ophthalmology

Helsinki University Central Hospital, Finland

Reviewers

Ismo Virtanen

Professor of Anatomy

Institute of Biomedicine/Anatomy

University of Helsinki, Finland

Steven E. Wilson

Professor of Ophthalmology

The Cole Eye Institute

The Cleveland Clinic, Cleveland, Ohio, USA

Opponent

Hannu Uusitalo

Professor of Ophthalmology

Department of Ophthalmology

Tampere University Central Hospital, Finland

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To Hannaleena, Verner, Kiira and Eevert

Contents

1 List of Original Publications

This thesis is based on the following original publications, which will be referred to in the text by Roman numerals:

I. Moilanen JAO, Vesaluoma M, Vesti E, Vaajoensuu T, Partinen M and Tervo T. Photorefractive keratectomy in ophthalmic residents. *J Refract Surg 2000;16:731-738*

II. Moilanen JAO, Vesaluoma M, Müller L, Tervo T. Long-term corneal morphology following PRK by *in vivo* confocal microscopy. *Invest Ophthalmol Vis Sci*, 2003;44:1064-1069.

III. Moilanen JAO, Holopainen JM, Vesaluoma MH, Tervo TMT. Corneal recovery after LASIK for high myopia. A 2-year prospective confocal microscopic study. Br J Ophthalmol, submitted 2007

IV. Moilanen, J., Holopainen, J., Helintö, M., Vesaluoma, M., Tervo, T. Keratocyte activation and inflammation in DLK after formation of an epithelial defect. *J Cataract Refract Surg*, 2004;30:341-349

2 Abbreviations

BSCVA	Best (spectacle) corrected visual acuity
BM	Basement membrane
CM	Confocal microscopy
CMTF	Confocal microscopy through focusing
D	Diopter
DLK	Diffuse lamellar keratitis
ECM	Extracellular matrix
HOA	Higher order aberrations
IL-1α	Interleukin-1α
ivCM	in vivo confocal microscopy
LASIK	Laser assisted keratomileusis in situ
LOA	Lower order aberrations
PRK	Photorefractive keratectomy
RK	Radial keratotomy
SNFB	Subbasal nerve fiber bundle
TGF-β2	Transforming growth factor β2
UCVA	Uncorrected visual acuity
VA	Visual acuity
WF	Wave-front

3 Abstract

Background

Refractive errors, especially myopia, seem to increase worldwide. In some areas it is a major health problem, as the prevalence of myopia turns to be almost 75 %. Many myopic patients would need correction of their refractive error, yet cannot afford spectacles or are out of the reach of public health care.

During the last three decades, the correction of refractive errors with excimer laser surgery has changed our view from surviving with spectacles to living without them. Thus, the number of refractive corrections performed has increased rapidly, with several million procedures performed worldwide annually. However, as the procedure is targeted on an otherwise healthy, clear cornea, it is crucial that the final outcome is extremely predictable and safe. Excimer laser surgery was introduced after a limited number of studies done with animals and to date there still are only few long-term follow-up studies of the results.

Objective

The present thesis aims to evaluate the safety and functional outcome of, as well as to quantify the cellular changes and remodelling in the human cornea after, photorefractive keratectomy (PRK) and laser assisted in situ keratomileusis (LASIK). These procedures are the two most common laser surgical refractive methods. Special attention is given to long-term tissue recovery and complications.

Means

In all, the present study comprised 40 patients, 19 of whom underwent PRK and 21 LASIK procedures.

In Study I, myopic ophthalmic residents at Helsinki University Eye Hospital were offered a refractive correction by PRK. Five patients were followed up for 6 months to assess their subjective experience in hospital work and their performance in car driving simulator and in other visuomotor functions. Corneal changes were assessed by in vivo confocal microscopy (ivCM).

Study II comprised 14 patients who had undergone a PRK operation during the first years it was introduced in Finland i.e., 1993-1994. Visual acuity was examined and ivCM examinations performed 5 years postoperatively.

Study III was designed as a 2-year prospective follow-up comprising 15 patients, who underwent LASIK refractive correction for moderate to high myopia. Their corneal morphology, with special reference to the recovery of corneal sensory innervation, was examined by ivCM.

Diffuse lamellar keratitis (DLK) is a common but variable complication of LASIK. Yet, its aetiology remains unknown. In Study IV we examined by ivCM six patients who had developed DLK as a consequence of formation of an intraoperative or post-LASIK epithelial defect, to assess the corneal and conjunctival inflammatory reaction.

Results

In the whole series (Studies I-IV), where the mean refractive correction was -6.46 diopters (D), visual acuity (BSCVA) improved slightly: Ten patients gained one line of BSCVA, one patient gained two lines, whereas three patients lost one line and one patient lost two lines. The latter appeared to be due to pigment glaucoma. In fifteen patients there was no change in the best corrected visual acuity. The mean achieved refraction was 0.35 D undercorrected compared to the attempted refraction. Biomicroscopy showed signs of mild haze in half of the PRK patients, whereas after LASIK all corneas were clear throughout the follow-up, except for scar formation observed at the flap margin.

IvCM was used to assess morphological changes in post-surgical corneas. After PRK, the stromal scar formation was highest at 2 to 3 months postoperatively and subsequently decreased. Subbasal nerve fiber regeneration could be observed already at 2 months in 2 patients after PRK. At the final visit (7-12 months postoperatively), subbasal nerves were observed in 7 out of 9 corneas (Study I).

At 5 years (Study II) confocal microscopy still revealed increased reflectivity in the subepithelial extracellular matrix, keratocyte nuclei and processes in all patients. Interestingly, no Bowman's layer was detected in any patient after PRK. All corneas presented with a subbasal nerve fiber layer, the density of which, however, was still lower than in control corneas.

LASIK induced a hypocellular area on both sides of the flap interface (Study III). Adjacent to this, thin areas of keratocyte activation were observed in the first postoperative weeks. Throughout the follow-up, small bright particles in the interface were detected by ivCM. In the corneas that developed DLK (Study IV), inflammatory cell-type objects were present in the interface in three patients. The other three patients presented only with keratocyte activation and highly reflective extracellular matrix. These changes resolved completely with medication and time.

Subbasal nerve regeneration was first detected at one month post-LASIK, but still after two years the density of subbasal nerve plexus, however, was only 64 % of the preoperative values. A decrease of the most anterior keratocyte density was also observed. Night driving simulator tests did not show any changes pre- vs. post-PRK performance (Study I). The performance of ophthalmological examinations and microsurgery without spectacles was easier postoperatively, which was appreciated by the residents.

Conclusion

Both PRK and LASIK showed moderate to good accuracy and high safety. IvCM still revealed morphologic alterations in the corneal structure even 5 years after PRK. A decrease of anterior keratocyte density was also found 2 years after LASIK. This might be related to the slow recovery of the corneal nerves. The most common complications, such as DLK, were mild and had no effect on the final postoperative visual acuity. In terms of visual perception and subjective evaluation, few patients stated any complaints in the whole series of studies. Instead, the majority of patients experienced a marked improvement in everyday life and work performance.

PRK and LASIK have shown similar results, with good long term morphological healing. It seems evident that, even without the benefit of over-20-year follow-up results, these procedures are sufficiently safe and accurate for refractive corrections and corneal reshaping.

4 Introduction

Ametropia, a deviant refractive ability of the eye, results in reduced visual acuity and is associated with an increased risk of certain eye diseases, such as glaucoma and rhegmatogenous retinal detachment. Refractive errors have major health and economic burdens, for example as regards visual requirements at work. It has been calculated that 2.5-4.3 billion dollars are spent each year in the USA merely for the inspection and correction of myopia.

The prevalence of myopia varies with age, ethnic group and the level of education. In Western populations, about one out of four subjects is estimated to present with myopia (Sorsby et al., 1960; Sperduto et al., 1983; Kempen et al., 2004), but in some Asian populations myopia prevalence can be as high as 70% to 90% (Chow et al., 1990; Wong et al., 2000). Accordingly, at a conservative estimate there are about two billion myopic individuals in the world. The exact cause of myopia is not clear but both genetic and environmental factors may play a role (Mutti et al., 1996; Fredrick 2002).

Spectacles are the most commonly used and the safest choice for correction of refractive errors, followed by contact lenses and refractive surgery. Risks such as accidental breaking of spectacles (Sinclair et al., 2006) and contact lens-associated infections (Driebe 2003; Holden et al., 2003) can be attributed to the use of eyewear. Refractive surgery allways possess a risk for complications, such as keratectasia, but the complication rates are considered low. Although spectacles do not correct the so called higher order aberrations of refraction, the impact of the aberrations to visual performance remains to be elucidated.

Corneal refractive surgery aims at a controlled alteration of the shape of the anterior surface of the cornea. Such surgery on a completely healthy eye sets high demands on the control of wound healing. It has been estimated that several million laser surgery operations are performed annually, and an increasing number of myopes are seeking refractive laser correction. In Finland, an unofficial survey concluded that ca. 7,000 operations were performed in 2006.

Recent developments in refractive surgery include wave front sensing technologies (Walsh and Charman 1985; Liang et al., 1994), which enable measurement of the aberrations of

the light traversing the eye's optical system. This is then converted to the corneal plane by creating a neutralizing inverse wave front aberration profile to the corneal surface. Theoretically, the procedure allows for better accuracy than the traditional methods of correction of ordinary sphere and cylinder (1-2 order) errors. There are numerous reasons why the correction of the human eye does not yield "super-vision", as one might expect. Changes of aberrations related to variation in the accommodative status, pupil size, wave length (color) of light, and aging have been known for a long time (Guirao et al., 1999; Oshika et al., 1999; McLellan et al., 2001), and neuronal processing modulates perception based on the generation of optical images (Holopainen et al., 2004). Furthermore, aberrations may fluctuate. Examples of such aberrations are tear fluid-based abnormalities and alterations in the constantly renewing corneal epithelium. Besides aberrations, also the diffraction of light and the optical clarity of the transmitting media affect the quality of vision. The latter is important during the healing of refractive surgical wounds induced in the cornea.

Basically, two different types of laser refractive surgery procedures are performed: surface or stromal/lamellar ablation. In PRK (Trokel et al., 1983), ablation is performed to the most anterior part of the corneal stroma after removal of the corneal epithelium. PRK has mostly been overtaken by LASIK (Pallikaris et al., 1990), in which laser ablation is performed under a stromal flap. LASIK yields faster visual recovery, less postoperative pain and haze formation. However, for at least up to -6.00 diopters (D), the final results in terms of accuracy and uncorrected visual acuity (UCVA) after myopic correction are identical for PRK and LASIK (AAO 1999; Azar and Farah 1998; Hersh et al., 1998; Shah et al., 1998; El-Maghraby et al., 1999; Hersh et al., 2000; Pop and Payette 2000; Shortt et al., 2006a and 2006b). LASIK also requires more surgical skill and may cause more severe complications (Azar and Farah 1998; Dubbs et al., 2006; Shortt and Allan 2006; Kymionis et al., 2007), e.g. keratectasia (Perez-Santonja et al., 1997; Chayet et al., 1998; Melki et al., 2001; Netto et al., 2005; Schallhorn et al., 2006), which have again changed the attitudes in favour of PRK and, most recently, epi-LASIK (Pallikaris et al., 2003; Katsanevaki et al., 2007). In epi-LASIK, a microkeratome creates an epithelial flap that is placed back onto the wound after laser ablation, instead of scraping the epithelium off, as is done in PRK. However, despite some inaccuracy of correction, high patient satisfaction is achieved by both methods.

Despite the tremendous popularity of refractive corrections with excimer laser, there are only some long-term results published (Kim et al., 1997; Matta et al., 1998; Stephenson et al., 1998; Pietilä et al., 2004; Rajan et al., 2004; Erie et al., 2005; Dawson et al., 2006; Erie et al., 2006; O'Connor et al., 2006; O'Doherty et al., 2006; Rajan et al., 2006; Sekundo et al., 2006; Condon et al., 2007; Kymionis et al., 2007; Patel et al., 2007). Furthermore, the observed cellular alterations induced by excimer laser were initially based on animal studies, which represent only to some extent the wound healing mechanisms in the human eye. Fortunately, severe late complications are rare. Yet, short term complications are much more common: Myopic regression, haze, overcorrection, undercorrection, corneal stromal opacification, dry eyes, and DLK. However, most of these problems can be corrected and treated. Excimer laser surgery has provided an extremely precise method of removing corneal tissue.

In vivo confocal microscopy (ivCM) has proved to be a valuable tool for the visualization of corneal pathology and wound healing after refractive surgery (Linna et al., 1997, 1998 and 2000; Alio et al., 2000; Vesaluoma et al., 2000a and 2000b; Tervo et al., 2003). It enables the evaluation of tissue and cell responses, as well as epithelial and stromal thickness analysis of human corneas in vivo (Masters and Thaer 1994; Nagel et al., 1995; Linna and Tervo 1997; Moller-Pedersen et al., 1997; Slowik et al., 1997; Boehnke et al., 1998; Frueh et al., 1998; Linna et al., 2000; Vesaluoma et al., 2000). In addition, the degree of regeneration of the subbasal nerve fiber bundles (SNFB); the occurrence, density, and thickness of post-PRK haze; and the morphologic alterations in keratocytes can be assessed. IvCM can also be used to visualize and quantify leukocyte rolling and extravasation in conjunctival blood vessels following inflammation (Kirveskari et al., 2001).

The aim of the present study is to evaluate the morphological alterations induced by PRK and LASIK to the human cornea in relation to clinical outcome. The main interest is on subjective observations, complications, and side effects. The aim is also to elucidate the mechanisms behind the inflammatory reaction following epithelial detachment -associated DLK after LASIK.

5 Review of the literature

5.1 Anatomy

5.1.1 Tear film

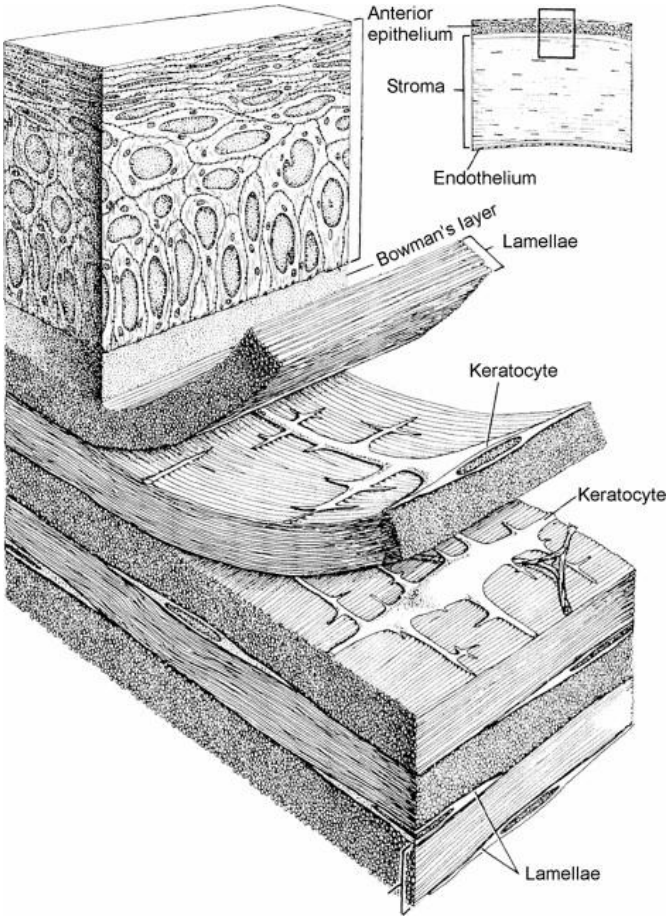
The optical properties in an otherwise healthy eye determine the quality of vision. Frontmost, the precorneal tear film forms a prerequisite for optimal vision, protecting and maintaining the health of the epithelium. The thickness of this aqueous film is estimated to be from 3 to 40 µm (Ehlers 1965; Ehlers and Hjortdal 2006; Maurice 1973; Benedetto et al., 1975; Prydal et al., 1992; Prydal and Campbell 1992; Danjo et al., 1994; King-Smith et al., 2000). Tear film is composed of three continuous, concentration gradient-dependent layers (Prydal et al., 1992): 1) a hydrophilic mucous layer anchored to surface epithelial cells and produced by conjunctival goblet cells and conjunctival and corneal epithelial cells (Chao et al., 1980); 2) an intermediate protein-rich aqueous layer produced by the main and accessory lacrimal glands, and; 3) a hydrophobic lipid enriched layer produced by the meibomian glands. The latter of these prevents the evaporation of the tear film.

The protein composition of tear film has been recently studied using mass spectrometric techniques. Li et al. (2005) identified 54 proteins. Somewhat surprisingly, 491 proteins were identified by de Souza et al. (2006). This seems to be an overestimate as the tear fluid proteosome contains several intracellular proteins. Moreover, the tear fluid lipid composition remains largely unknown.

Tears also contain white blood cells, which increase in number during inflammation, and several growth factors (e.g. EGF, TGF-α, HGF) (Li et al., 1996; van Setten et al., 1990 and 1996), which may influence the post surgery wound healing mechanisms in the cornea. The role of leukocytes in the event of complications, e.g. diffuse lamellar keratitis (DLK), remains to be studied. Upon the decrease of tear secretion after refractive surgery, changes in tear film composition and volume may have a long lasting effect on postoperative quality of vision.

5.1.2 Corneal structure

Figure I.



Block diagram of cornea illustrating stromal lamellae and flat keratocytes. [Reprinted from Ehlers N and Hjortdal J. The cornea: Epithelium and stroma. Advances in Organ Biology. 10, 83-111 (2006), with the permission from Elsevier].

An avascular, perfectly shaped and clear cornea is the most powerful refractive lens of the eye, comprising in average 45 D of the approx. 60-70 D total refractive power of the eye. The smallest errors in its shape and transparency impair visual performance. Tear fluid changes, tissue remodulation and scar formation in the cornea or other refractive media (lens, vitreous) following e.g. corneal laser surgery, or in dry eye syndromes, keratitis, or hereditary degenerative diseases, can reduce the optical quality of the cornea.

The cornea consists of three distinctive layers (Figure I): 1) The ca. 50 µm thick epithelium; 2) the ca. 450-500 µm thick stroma; and 3) the endothelium. the epithelium and the stroma are divided by the epithelial basement membrane (BM) and the 8-10 µm

thick Bowman's layer posterior to the BM (Ehlers and Hjortdal 2006). Furthermore, between the stroma and the endothelium is the Descemet's membrane. On average, the cornea is thinner centrally (500-550 µm) than peripherally (600-700 µm).

The cornea is one of the most densely innervated organs of the human body (Müller et al,. 2003). Nerve injury delays or even arrests corneal wound healing, which may lead to formation of optical aberrations related to corneal irregularities, corneal ulcers and even perforations (Bonini et al., 2003). Diseases and surgical operations can lead to permanent and/or long standing neuronal injuries. The correlation between nerve loss and cellular alterations remains unknown.

5.1.2.1 Epithelium

The non-keratinized squamous stratified epithelium consists of three morphologically different cell types (Beuermann and Pedroza 1996; Ehlers and Hjortdal 2006): 1) an average of 2-3 layers of flat polygonal surface cells, located most superficially, containing apical microvilli in contact with the tear film. These cells are joined by tight junctions, adherens junctions and desmosomes, restricting the entry of tears into the intercellular spaces and providing mechanical strength between adjacent cells (Petroll et al., 1999; Ban et al., 2003); 2) 2-3 layers of intermediate wing cells; and 3) a single layer of columnar basal epithelial cells. These basal cells are approx. 20 µm tall and show a limited division capacity (Ehlers and Hjortdal 2006). Basal cells serve as the source for differentiation into wing and superficial cells. Hemidesmosomes attach basal epithelial cells to the underlying basement membrane (BM) (Gipson et al., 1987). The 0.05 µm thick basement membrane is composed mainly of type IV collagens and laminins produced by the basal epithelial cells (Tuori et al., 1996).

Stem cells are located in Vogt's gridles in the corneal limbal area (Tseng 1989; Lavker et al., 2004; Sun and Lavker 2004). These cells are continuously proliferating, providing a resupply for shedding epithelial cells, thus maintaining corneal integrity. A complete turnover of corneal epithelial cells occurs in ca. 7 to 10 days (Hanna et al., 1961, Cenedella and Fleschner 1990; Ehlers and Hjortdal 2006).

5.1.2.2 Bowman's layer

The uppermost part of the corneal stroma is the Bowman's layer, which is an acellular, unorganized array of fibrils of collagen types I, III, V, and VI, and is ca. 8-12 µm thick (Marshall et al., 1993). It develops from processes of the superficial mesenchymal cells of the corneal stroma (Sevel and Isaacs 1988) and connects to the adjacent basement membrane through anchoring fibrils and plaques (Gipson et al., 1987).

5.1.2.3 Stroma

The corneal stroma makes up ca. 90% of the corneal thickness and is composed of a heterodimeric complex of type I, III, and V collagen bundles arranged parallel to the corneal surface in specific lamellae (Ihanamäki et al., 2004). Additionally, the corneal stroma contains non-lamellar collagen of types IV, VI, and VII (Assil and Quantock 1993). Keratocytes are the major cell population located between the collagen lamellae. Additionally, there are bone-marrow derived dendritic antigen presenting cells (APC cells), and Langerhans cells (Latina et al., 1988, Hamrah et al., 2003), in the corneal stroma that are participants in immune and inflammatory responses. Also macrophages have been found at least in mouse corneas and act as potent APC cells as well (Hamrah et al., 2003; Novak et al., 2003; Yamagami and Amano 2003). The special quality of these cells in the cornea protects against pathogens but also prevents inflammatory and immunological damage to the eye.

Keratocytes are quiescent, mesenchyme-derived fibroblast-like cells of the mature cornea (Hay 1979; Ehlers and Hjortdal 2006; West-Mays and Dwivedi 2006). However, keratocytes contain many stacks of rough endoplasmic reticulum and large Golgi fields suggesting high activity in protein synthesis and storage (Müller et al., 1995). Furthermore, keratocytes contain twice as many mitochondria in the anterior stroma as in the mid- or posterior stroma. Keratocyte density is also significantly higher in the anterior stroma [(50,000 – 60,000 cells/mm^3 (Moller-Pedersen et al., 1997; Erie et al., 2006), 800 cells/mm^2 (Prydal et al., 1998)] than in the posterior stroma [23,000 cells/mm^3 (Erie et al., 2006), 65 cells/mm^2 (Prydal et al., 1998)]. Keratocytes are organized in a clockwise, spiral manner so as to be evenly distributed and located in the cornea (Muller et al., 1995). This specific organization may be beneficial in that light traverses through a similar system in every part of the cornea.

The integrity of the corneal basement membrane is critical in minimazing the fibrotic response of keratocytes and subsequent scarring and loss of corneal clarity (West-Mays and Dwivedi 2006). When injured, keratocytes either undergo rapid apoptosis or transform into repair phenotypes of migrating keratocytes/myofibroblasts (Jester et al., 1999; Fini and Stramer 2005; West-Mays and Dwivedi 2006). These cells eventually form a fibrotic scar. This, together with a decreased expression of crystallins (Jester et al., 1999) in altered/migratory keratocytes contributes to decreased transparency in wounded, healing corneas. A physiological 0.3 % annual decline of keratocyte density has been observed during aging (Moller-Pedersen 1997). There is also evidence that, after LASIK, stromal keratocyte density decreases (Vesaluoma et al., 2000; Mohan et al., 2003; Erie et al., 2006). This type of keratocyte loss might affect the integrity of the cornea, but the reason for keratocyte loss remains elusive.

5.1.2.4 Descemet's membrane and the endothelium

The main function of the corneal endothelium is to dehydrate the corneal stroma to maintain its clarity by an active pump mechanism, compensating the leak to the stroma from the aqueous humor (Maurice 1972; Waring et al., 1982). At birth, the endothelium consists of ca. 400,000 hexagonal cells, arranged uniformly in a continuous 5-µm-thick monolayer. The density of the endothelial cells decreases with age but considerable variation occurs: Under age 5, the endothelial cell density is ca. 3,000 cells/mm^2, by age 50 the range is usually from 1,000 to 3,500 cells/mm^2, and by age 80 the variation can be from 900 to 4,000 cells/mm^2 (Hiles et al., 1979; Hoffer and Kraff 1980; Waring et al., 1980).

Interposed to the stroma, endothelial cells produce a basement membrane called the Descemet's membrane. It is composed of regularly arranged, stratified layers of predominantly type IV and VIII collagen, laminins and glycoproteins (Marshall et al., 1993; Beuerman and Pedroza 1996). The Descemet's membrane thickens with age, from some 2 µm in childhood to 10 µm in adults (Johnson et al., 1982).

5.1.3 Corneal innervation

The nerve fibers in the human cornea are mostly sensory by type, but also autonomous nerves exist. The sensory innervation derives from the ophthalmic and maxillary branch of the trigeminal nerve (Vonderahe et al., 1928; Ruskell et al., 1974; Müller at al., 2003). Anatomically, the corneal sensory nerves can be divided into the stromal, the subbasal and the intraepithelial (Müller et al., 2003). Functionally, there are three different modalities of sensory nerves: 1) polymodal nociceptors that respond to mechanical energy, heat, and exogenous irritants and endogenous chemical mediators, 2) mechano-nociceptors that react to mechanical forces of a magnitude close to that required to damage corneal epithelial cells, and 3) cold-sensitive receptors that respond to decreases in the normal corneal temperature (Belmonte et al., 2004).

5.1.3.1 Stromal nerves

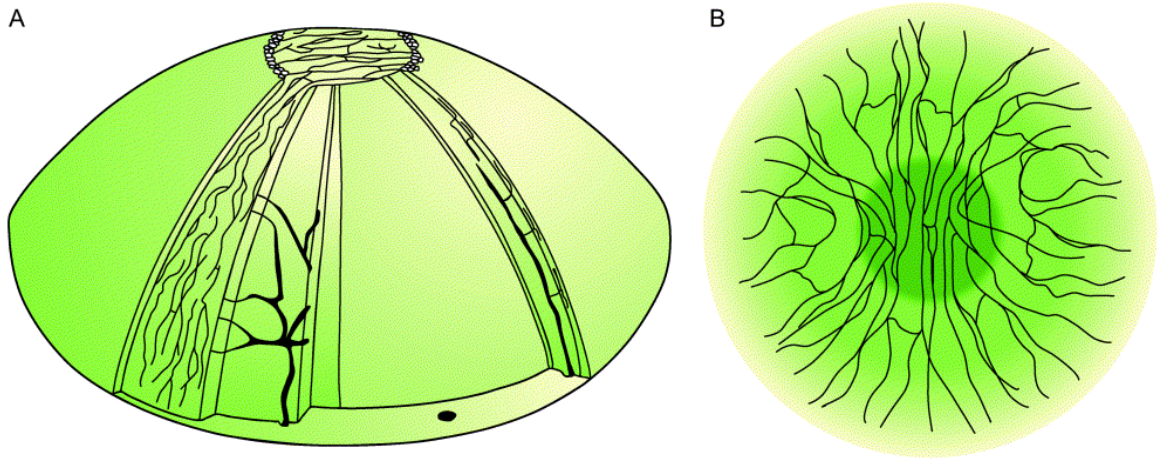
Through animal experiments (Morgan et al., 1978; Tervo and Palkama 1978a and 1978b; Tervo et al., 1979; Marfurt et al., 1989; LaVail et al., 1993), it has been estimated that some 200 to 450 nerve bundles from the ophthalmic and maxillary branches of the trigeminal nerve enter the cornea at the corneal periphery. These nerve bundles lose their perineurium and myelin sheaths ca. 1 mm from the limbus, but retain their Schwann cell sheaths. The radially orientated thick stromal nerve trunks run towards the corneal apex in the middle third of the corneal stroma (Muller et al., 1996 and 2003; Radner and Mallinger 2002). During their course, they send out thinner fibers which innervate individual keratocytes directly. Stromal nerves divide dichotomously or trichotomously, bend anteriorly 90 degrees and proceed towards the corneal surface, lose their Schwann cell sheath, and penetrate the Bowman's layer at points throughout the peripheral and central cornea (Muller et al., 1996 and 2003). Large nerve bundles divide into several smaller nerve fiber bundles after penetrating the Bowman's layer and bend again 90 degrees to form the subbasal nerve plexus between the Bowman's layer and the basal epithelial cell layer (Muller et al., 2003).

5.1.3.2 Subbasal nerve plexus

The subbasal nerve plexus contains ca. 5,400-7,200 nerve bundles (Muller et al., 2003). Each subbasal nerve fiber bundle, consisting of 3 to 7 individual straight or beaded nerve fibers (Muller et al., 1997), may bifurcate or branch. This means that the total number of axons in the subbasal plexus is between 20,000 and 45,000. Beaded fibers of these bundles separate and course obliquely in between the epithelial layers eventually to terminate at the superficial epithelial cell layers. As an individual nerve fibre gives rise to at least 10-20 nerve terminals, it has been estimated that there are nearly half a million nerve terminals in the cornea.

The orientation of subbasal nerve leashes has been examined only in the corneal apex. Studies have revealed that nerve bundles preferentially cross the apex in the 6-12 direction. Leashes from the 5-11, 7-1, 9-3, 2-8 and 4-10 directions extend towards the apex without reaching it or crossing to the other side (Figure II). It has, however, been shown that there is a vortex pattern of the subbasal nerves in the apical cornea (Patel and McGhee 2005).

Figure II.



(A) Schematic distribution of nerves in the stroma (Adapted from D. Maurice after data from R.W. Beuerman, 1984) and (B) organization of the subbasal plexus (by Müller et al., 1997) in human corneas. [Reprinted from Müller LJ, Marfurt CF, Kruse F, Tervo TMT. Corneal nerves: structure, contents and function. Exp Eye Res. 76, 521-542 (2003), with the permission from Elsevier]

5.1.3.3 Autonomous innervation: sympathetic and parasympathetic nerves

Only a scarce sympathetic innervation has been demonstrated in humans. These fibers originate from the superior cervical ganglion, penetrate through the corneal stroma and end in the epithelial layer (Sugiura and Yamaga 1968; Toivanen et al., 1987). A trophic effect of sympathetic innervation on the corneal surface, its integrity and wound healing has been demonstrated in rats and cats, but not in humans. Furthermore, a modest parasympathetic innervation has been demonstrated also in other mammals, but the evidence still remains unclear for humans.

5.2 Refractive errors and Wave front

There are three sources of image blur that affect an optical system, such as a human eye: 1) optical aberrations, chromatic or monochromatic, 2) light scattering from the media and its changes in cornea and lens, and 3) diffraction e.g. from pupillary edges. Chromatic optical aberrations affect the quality of vision. Among these, monochromatic aberrations can be treated; there are several alternative methods, as discussed below. Scattering of light occurs in every optical system. When caused by corneal scars or lens irregularities, it can induce deterioration of optical quality. Diffraction becomes problematic at small pupil sizes of 2 mm or less.

An additional limitation to visual acuity comes from the retinal photoreceptor diameter and density, which in an ideal system would give acuity of 20/8 to 20/10 (Applegate et al., 2001). Moreover, neural processing and its plasticity may vary between individuals, with age, and due to surgery (Artal et al., 2003; Pepose and Applegate 2005)

Any ray of light that is misdirected from its desired image point in an imaging system is called an optical aberration. These aberrations are classified further as a) lower order aberrations (LOA) and b) higher order aberrations (HOA). Lower order aberrations, e.g. defocus (myopia and hyperopia, described as sphere) and regular astigmatism (cylinder), can be corrected with spectacles. Higher order aberrations, however, cannot. As diffraction hampers visual perception in smaller pupil sizes, the significance of higher order aberrations increases in line with pupil diameter (Wang et al., 2003).

Adaptive optics was first introduced in the 1950s to improve the telescopic visualisation of stars (Babcock 1953). This technology developed into human optics to correct monochromatic aberrations (Liang et al., 1994). Conventionally, light has been conceived of as rays that traverse the eye's optical system. In wave front imaging, however, light could be considered as a wave, a plane of light that enters the various optical units of the eye. Depending on the properties of these units, the velocity of the wave can either decrease or increase. Such shifts in spatial phase from various locations of the optical system may further induce deterioration of visual perception.

Wavefront aberrometers measure the refractive index and linear path variations of the eye and, consequently, define the difference between an unaberrated reference wavefront and the actual wavefront formed by the eye's optics. Knowing the aberrations of an optical system allows one to reconstruct an image point by point by using mathematical processes. Thus, it is possible to produce a spectacle — a lens that corrects the patient's optical aberrations preoperatively and simulates the postoperative result.

Initially, laser refractive surgery was used to correct defocus and regular astigmatism by reshaping the cornea. In 1999, the first wavefront corrected LASIK procedure was performed (Seiler et al., 2000) to correct higher order aberrations as well. Since then, it has been estimated that more than half of North American refractive surgeons routinely perform wavefront-guided ablations (Duffey and Leaming 2005). However, while the treatments are customized and directed at patient-specific aberrations, not infrequently the same treatments lead to unpredictable visual outcomes attributable to variability in wound healing and biomechanical factors related to the cornea. The reported results of wavefront customized treatments have been mostly encouraging, but due to methodological flaws, conflicts of interest, or other limitations, peer-reviewed studies directly comparing conventional and wavefront-guided operations are still scarce: One report showed a smaller increase in HOAs after customized PRK compared to conventional PRK, but no other significant changes in visual acuity parameters were observed (Mastropasqua et al., 2004). Another study reported significantly greater total aberrations over a 7 mm pupil after customized LASIK compared to customized PRK (Oshika et al., 1999). Furthermore, bilateral comparisons in a 24- patient group showed a reduction in induced HOAs in wavefront-guided LASIK compared to conventional (Kim et al., 2004), but no subjective differences were observed.

While, overall, customized treatment in correcting refractive errors has shown good results (Phusitphoykai et al., 2003; Kim et al., 2004; Kohnen et al., 2004; Mastropasqua et al., 2004), one should take into consideration that in most cases higher order aberrations comprise only a small fraction of the total refractive error and that the clinical effect may be insignificant for the patient. Furthermore, the diameter of these small errors may be far smaller than the diameter of a single corneal epithelial cell, which in conjunction with other wound healing mechanisms tends to smooth features applied to the corneal stroma (Netto and Wilson 2004).

5.3 Refractive surgery

Surgical procedures to the cornea are not a new idea. Barraquer showed in his thesis in 1949 that corneal curvature could be reshaped and the refractive power of the eye could be modulated. Although many fundamental discoveries were made in the 1950s and 1960s, including radial keratotomy (RK), only the development of laser gave a larger impact to reshaping the cornea. Conventional laser ablations use data obtained from manifest and cycloplegic refractions. Wavefront-guided treatments allow corrections of higher order aberrations in addition to spherical and cylindrical defocus. This is accomplished with wider and, accordingly, deeper ablations.

5.4 Excimer laser

In order to create an effective laser beam, an excimer laser typically uses two gases: one inert gas, such as Argon, Krypton, or Xenon, and another reactive gas, such as Fluoride. Under electrical stimulation, these gas molecules can form an excited dimer. Upon returning to the non-activated state it emits laser light in the UV-range. This type of light beam is exceptionally well focused and, more importantly, is avidly absorbed by biological matter. Laser light provides sufficient energy to disrupt the molecular bonds of the tissue, thus causing the molecules to evaporate. As the laser does not heat the tissue nor cause structural changes in the remaining adjacent matter, it can be used for tissue reconstruction.

5.4.1 Photorefractive keratectomy, PRK

Reshaping the cornea by a surface photoablation, such as in PRK, was the first form of corneal laser surgery (Trokel et al., 1983). Following epithelial removal by laser or by mechanical or alcohol-assisted scraping, excimer laser photoablation of the stroma is performed, removing the epithelium, subbasal nerves, the Bowman's layer and a variable depth of stromal tissue. The ablation might either correct sphere and cylinder error or be wavefront-guided. In contrast to LASIK, the most anterior portion of the corneal stroma is ablated by excimer laser, leaving a thicker residual bed so as to retain the cornea's biomechanical strength (Müller et al., 2001). The main problems associated with PRK are transient subepithelial haze, short term regression of myopia, and dry eyes. Early studies often reported a relatively long-lasting regression of the induced correction and the development of a post-operative anterior stromal scarring or "haze", especially after the correction of moderate to high myopia (Frueh et al., 1998; Moller-Pedersen et al., 1998a-c; American Academy of Ophthalmology 1999; Jester et al., 1999; Moller-Pedersen et al., 2000; Mohan et al., 2003). Improved post-operative management and medication, along with newer lasers with smoother or otherwise modified ablation profiles may decrease haze.

Patients who undergo PRK typically have moderate to severe pain for one to four days after the procedure, and the recovery of visual performance usually takes one to two weeks (Jackson et al., 1998; Williams 2000; Nagy et al., 2002).

Several other techniques have also been introduced, such as Epi-LASIK and LASEK, but these are merely modifications of PRK. In Epi-LASIK, a special microkeratome, epikeratome, dissects the epithelium from the Bowman's layer instead of mechanical scraping, while in LASEK the central epithelium is exposed to alcohol, after which an epithelial button can be removed.

Long-term (>10 years) studies have been published only very recently. These show that PRK is a safe and predictable procedure in correcting low and moderate refractive errors (Rajan et al., 2004; O'Connor et al., 2006).

5.4.2 Laser in situ keratomileusis, LASIK

LASIK is close to Barraquer's invention of the early 1950s, where a corneal button was frozen and modified after removal with a microkeratome. In LASIK, a mechanical microkeratome, or more recently a femtosecond laser, creates a corneal flap of 100-200 µm in the anterior part of the stroma and epithelium. The microkeratome has an oscillating blade to cut the flap after the immobilisation of the cornea with a suction ring. A narrow portion of the stroma, either at a superior or nasal position, is left undetached and serves as a hinge for the flap, which is then folded aside. A laser ablation is then performed on the midstroma. After the laser ablation the stromal bed is irrigated and the corneal flap repositioned. Recently, flaps have been created with femtosecond lasers, but the acclaimed higher accuracy of this method is still controversial compared to newer mechanical microkeratomes (Javaloy et al., 2007; Netto et al., 2007).

LASIK results in faster improvement of uncorrected visual acuity, and has significantly less postoperative discomfort than in surface ablation. As there is no epithelial-stromal interaction, except at the flap margin, haze is minimal after LASIK. This enables deeper ablations and thus corrections for higher myopic errors compared to PRK. However, deeper ablation results in a thinner corneal bed, which puts the maintenance of corneal curvature at risk (Condon et al., 2007). There is a consensus regarding a sufficient stromal bed thickness of 250 µm, but many ophthalmic surgeons prefer a more conservative value of 300 µm so as to avoid keratectasia. Futhermore, the suction ring induces an increase of intraocular pressure to over 100 mmHg, usually for 15 to 45 seconds. Such an elevated IOP poses a potential risk for occlusion in blood flow and thus hypoxemia in the macular region.

Long-term follow-up studies after LASIK have shown that this procedure is safe and predictable (Sekundo et al., 2003; O'Doherty et al., 2006). It seems that over the long-term the results of PRK and LASIK are very similar.

5.4.3 Unwanted corneal responses to refractive surgery

5.4.3.1 Scarring

Anterior stromal fibrosis (haze) is a result of increased cellular reflectivity (Moller-Pedersen et al., 2000) and the synthesis of extracellular matrix (ECM) by (activated) keratocytes (Moller-Pedersen et al., 1998; West-Mays and Dwiveni 2006). Injury to the basement membrane leads to synthesis and release of interleukin-1α (IL-1α) and transforming growth factor β2 (TGF-β2) from corneal epithelial cells. IL-1α induces the anterior keratocytes to either undergo cell death by apoptosis or transformation into fibroblast-like, activated keratocytes that proliferate and secrete matrix metalloproteinases (MMP). These activated keratocytes continue to secrete IL-1α, which remodels the extracellular matrix (ECM). TGF-β2 induces a subpopulation of keratocytes to transform into myofibroblast-like cells that secrete ECM (Fini et al., 1999; Stramer et al., 2003; Fini and Stramer 2005; West-Mays and Dwiveni 2006). These cells, which represent the active wound healing response, have been shown to cause most of the postsurgical light scattering (Moller-Pedersen et al., 1998 and 2000). During the differentiation, keratocytes lose the ability to highly express corneal crystallin proteins (Pei et al., 2004 and 2006). The decrease of water soluble proteins has been associated to increased light scattering from keratocytes and thereby to loss of corneal transparency.

Immunohistochemical studies have shown that the haze comprises hypercellular scar tissue and a dense network of collagen type III (Dawson et al., 2005). Corrections of high myopia (i.e., deeper ablations) enhance the formation of haze (Carson and Taylor 1995). Haze reaches its maximum between the second and third months (Corbett et al., 1996), but then resolves spontaneously in months to years. PRK-induced haze may continue to recede even after the first postoperative year. That being said, cases of late-onset haze have also been described (Lipshitz et al., 1997).

A LASIK-induced stromal keratocyte reaction is less pronounced than in PRK. IvCM shows a maximum reflectivity in the stroma adjacent to the interface 3 days to 2 weeks after the procedure, but biomicroscopically the reflectivity is hardly detectable. Two years after the procedure the interface is merely hypocellular and shows no signs of increased reflectivity (Vesaluoma et al., 2000; Erie et al., 2006). This is in accordance with cadaver

studies, which show hypocellularity in the stroma adjacent to the interface (Dawson et al., 2005).

5.4.3.2 Regression

Regression of myopia after PRK is thought to result from the resynthesis of ECM by activated fibroblasts and altered keratocytes (Moller-Pedersen et al., 2000). This new tissue appears to contribute to the reformation of the photoablated anterior stroma, thus leading to corneal resteepening. Furthermore, Erie (2003) found that there was a 21% increase in epithelial thickness by 12 months after PRK that remained unchanged thereafter. In some cases, the regression of myopia may continue for as long as 5 years after PRK (Kim et al., 1997). However, the central corneal thickness has been shown to become stable at one year after PRK (Patel et al., 2007).

Regression after LASIK is minor but possible for up to five years postoperatively (Kato et al., 2007) and occurs in 20% of patients (Chen et al., 2007). It is dependent on the preoperative ocular values, i.e., refraction, keratometric values, size of optic zone, and age (Chen et al., 2007). A rapid increase has been shown in central epithelial thickness during the first postoperative month, probably due to epithelial hyperplasia. Thereafter, the thickness persists for at least 7 years (Patel et al., 2007). This suggests that the epithelium has a role in myopic regression during the early postoperative period.

5.4.3.3 Diffuse lamellar keratitis (DLK)

DLK, or “Sands of Sahara”, was first discovered by Smith and Maloney (1998). It is characterized by the appearance of diffuse white opacities at the LASIK flap interface in an inflamed cornea in the first week after refractive correction. The first visible manifestation of DLK on slitlamp examination is the migration of dot-like objects resembling white blood cells into the interface during the first postoperative day. Linebarger (2000) graded DLK in 4 stages according to the density and location of the corneal infiltration. While the condition seems to be sterile (Johnson et al., 2001; Ambrosio and Wilson 2001) a number of etiologies have been proposed for DLK: Microkeratome oil (Kaufman et al., 1998), bacterial endotoxins released from sterilizer reservoir biofilms (Holland et al., 2000), metallic debris from the blade (Kaufman et al., 1998), cleaning solutions (Nakano et al., 2002; Yuhan et al., 2002), carboxymethylcellulose drops (Samuel et al., 2002), Meibomian

secretions (Johnson et al., 2001), and the activation of endogenous chemotactic systems (Shah et al., 2000; Wilson et al., 2002a). Johnson (2001) reported a significant association between peroperative epithelial defects and DLK. Epithelial defects may also aggravate the risk of epithelial ingrowth, as shown in an ivCM study by Sachdev et al. (2002). Johnson et al. (2001) reported an incidence of 1.3% for DLK, but the incidence was considerably higher in cases where an epithelial defect occurred during or immediately after LASIK, compared to cases where there was no epithelial defect. While the aetiology of DLK has remained unclear, some authors have proposed a theory of "multiple factors" (Johnson et al., 2001). A number of studies have revealed accumulations of ovoid cells in the flap interface at the acute stage followed by some degree of keratocyte activation near the interface (Vesaluoma et al., 2000b; Buhren et al., 2001; Chung et al., 2002). The ovoid cells have been interpreted to be inflammatory cells. Alternatively, they may represent macrophages or some form of keratocyte, which later turn into activated keratocytes. IvCM studies of DLK (Bühren et al., 2001 and 2002; Kymionis et al., 2007) have concluded that mononuclear leukocytes and/or granulocytes occupy the interface. That being said, immunohistochemical studies in humans have not been performed to confirm the type(s) of cells residing in the interface. Holzer (2003) and de Rojas Silva (2007) showed in experimental rabbit DLK models that granulocytes and lymphocytes exist in the corneal stroma around the interface. Rabbit corneas also show enhanced production of prostaglandins (Phillips et al., 1993; Tomas-Barberan et al., 1997) and growth factors (Vesaluoma and Tervo 1998, Wilson et al., 1999a) after excimer laser photoablation.

Prostaglandins can act as chemoattractants for inflammatory cells (such as leukocytes or lymphocytes) (Srinivasan et al., 1980); this chemotaxis is reduced by corticosteroids (Phillips et al., 1996). While polymorphonuclear leukocytes secrete proteolytic enzymes that may interfere with stromal healing and eventually lead to an impaired visual outcome, it seems reasonable to avoid the accumulation of inflammatory cells under the flap. Consequently, topical and peroral corticosteroids seem to be beneficial and are commonly used in post-LASIK regimens, despite the limited clinical data confirming their efficacy (Price et al., 2001). Rabbit models confirm the advantages of corticosteroid therapy in the treatment of experimentally induced DLK (Holzer et al., 2002). Nevertheless, only a fraction of DLK patients present clinically demonstrable flare and/or inflammatory reactions in the anterior chamber. This suggests that the inflammation remains localized in the flap interface.

5.4.3.4 Corneal infections and inflammation

A bacterial ulcer is an uncommon complication of LASIK (Ambrosio and Wilson 2001). It is usually treated with topical antibiotics and, if necessary, by lifting the flap and cleaning and rinsing the wound surfaces, or occasionally, in case of serious melt, by removal of the flap. The final outcome of bacterial keratitis is variable.

Staphylococcus aureus has been reported to cause infectious keratitis after LASIK (Quiros et al., 1999; Rubinfeld et al., 2001). Furthermore, other sporadic bacterial and fungal keratitis incidents have been reported (Pache et al., 2003; Hamam et al., 2006; Moshirfar et al., 2007). Only few cases of bacterial ulcers on the LASIK flap have been reported (Quiros et al., 1999; Chung et al., 2000; Karp et al., 2000; Rubinfeld et al., 2001; Lindbohm et al., 2005; Park et al., 2007).

5.4.3.5 Interface debris

Vesaluoma et al. (2000a) showed that, on average, there are ca. 600 particles per square milllimeter in the interface 3 days after LASIK. The particles may include cells, cell fragments (apoptotic bodies), debris, metal particles, plastic particles (Ivarsen et al., 2004), and salt precipitates from tear film (Perez-Gomez and Efron 2003). One month after surgery, the particle count was lower. Improved cleaning procedures adopted after the study seem to have decreased the particle counts. The significance of interface particles to LASIK complications, such as DLK, seems to be minimal. Even if the flap is created with a new femtosecond laser, there still are interface particles after the surgery (Ramirez et al., 2007).

5.4.3.6 Flap striae and folds

Flap striae, or macrofolds, are indications for lifting the flap as soon as possible after LASIK (Linna et al., 2000b; Ambrosio and Wilson 2001; Sugar et al., 2002). Macrofolds can easily be detected under a slit lamp, especially when using retroillumination. Microfolds seem to be almost unavoidable (Vesaluoma et al., 2000a), but their impact on visual performance appears to be minimal. They may, however, induce higher-order aberrations after flap formation (Pallikaris et al., 2002).

5.4.3.7 Buttonhole

Buttonhole results from a central resurfacing of the microkeratome blade (Johnson et al., 2000; Wilson et al., 2001b). It is more common in steep corneas (*K*>47 D), where a doughnut-formed flap may be created. The formation of an epithelial defect allows epithelial–stromal interaction and facilitates scar formation (Li and Tseng 1995; Wilson et al., 2001b). As a result, these corneas show variable degrees of subepithelial haze. IvCM can be used to detect cases with buttonhole flaps and to differentiate them from very thin central flaps, which also tend to be associated with corneal irregularities and subepithelial haze (Vesaluoma et al., 2000a; Gokmen et al., 2002; Erie et al., 2002).

5.4.3.8 Epithelial ingrowth

Large epithelial cysts (Perez-Santonja et al., 1998; Alio et al., 2000; Linna et al., 2000b; Vesaluoma et al., 2000b) can easily be imaged with ivCM and are found frequently following LASIK. Similar phenomena have been found both in RK wounds (Jester et al., 1999b) and in relaxing incisions. Epithelial cells may persist for months or years in wound clefts (Vesaluoma et al., 2000b). The condition may also lead to flap melt (Alio et al., 2000; Holland et al., 2000; Vesaluoma et al., 2000b), which is a rare complication of LASIK (Wang and Maloney 2001). It is clinically known that any epithelial tissue under the flap must be removed if the melt proceeds (Holland et al., 2000; Johnson et al., 2001; Sugar et al., 2002; Ambrosio and Wilson 2001). Such areas show keratocyte activation, which is probably associated with the expression of proteolytic enzyme cascades (Ambrosio and Wilson 2001).

5.4.3.9 Interface fluid

Interestingly, the use of topical steroids, as recommended for DLK therapy, may cause — if the individual is a steroid responder — elevation of intraocular pressure (IOP) and accumulation of “interface fluid” (Lyle and Jin 1999; Belin et al., 2002; Galal et al., 2006) in the stroma. The condition is characterized by regression of the induced refractive result, by haze at the flap interface, and by stromal swelling.

5.4.4 Preoperative assessment of refractive surgery

A complete eye examination, as well as proper anamnesis, is necessary to exclude any ocular or systemic pathologies that could affect the postoperative result.

5.4.4.1 Refraction

The assessment of the refractive error to be corrected with laser is preferably a summary of several methods, which should be used properly before any procedure. The methods used are automated refraction, objective refraction with a retinoscope, subjective manifest refraction, and cycloplegic refraction. Furthermore, wave front devices measure the total refractive errors of the eye. The more consistent the different measurements, the easier it is to make a decision on the final intended correction.

5.4.4.2 Corneal topography

Pathology in corneal curvature is a contraindication to corneal refractive surgery, unless the procedure has a therapeutical aim. One example is LASIK for keratoconus, to postpone the need for keratoplasty. Keratometry is also needed for assessing the suitability of the cornea to microkeratome, as K-values of less than 39 D (shallow cornea) and more than 47 D (steep cornea) pose a risk for LASIK flap complications.

5.4.4.3 Pupillometry

In the early days of laser assisted refractive surgery, the standard treatment diameter was 4 mm. Yet, it was found that in dim light there was a potential risk of postoperative glare and halos, if the scotopic pupil size was large. Accordingly, the laser ablation zone was increased to 6 mm. With wave-front technology, the diameter can be adjusted up to 9 mm. Hence, the pupil diameter should be measured preoperatively under both bright and dim illumination. Widening of the treatment zone deepens the ablation, thus inducing a greater risk of postoperative haze (after PRK) or LASIK-induced ectasia.

5.4.4.4 Pachymetry

Ultrasonic pachymetry, or an equivalent method, is essential for measuring the central corneal thickness preoperatively, so as to ascertain sufficient residual stromal thickness postoperatively. It is recommended to measure the corneal thickness also during the operation: First, the total thickness in the beginning of the procedure, secondly, the stromal bed thickness after the flap creation, and thirdly the residual bed thickness after the ablation.

5.4.4.5 Schirmer test

Tear secretion usually decreases after refractive surgery and almost half of the patients suffer from dry eyes after PRK and LASIK (Siganos et al., 1997; Ozdamar et al., 1999; Lee et al., 2000). The Schirmer test, with or without anesthetic, and the biomicroscopically observed quality of the tear meniscus are essential assessments for informing the patient of the possible dry eye symptoms, and even to decide on the method of surgery. In practice, LASIK induces more dry eye problems than PRK (Lee et al., 2000).

5.5 Corneal imaging and in vivo confocal microscopy

Means of imaging corneal or other tissue in the eye are being constantly developed. Optical Coherence Tomography (OCT; Humphrey-Zeiss Medical Systems) was developed for imaging of the retina and optic nerve, but it can also be used for imaging of the anterior segment and cornea and estimation of central corneal thickness (Bechmann et al., 2001; Muscat et al., 2002; Neubauer et al., 2002; Wirbelauer et al., 2002; Avila et al., 2006). More recently, a corneal module for HRT II (Heidelberg Retinal Tomography) has been developed. It is comparable to the image quality of ivCM (Bochert et al., 2005; Kobayashi et al., 2006; Stachs et al., 2007; Szaflik et al., 2007; Zhivov et al., 2007). Even epithelial wing cells and a lamellar interface between the anterior stroma and the Bowman's layer can be observed. The Nidek EAS-100 anterior segment analysis system also enables imaging of the whole anterior segment including the cornea, as well as the objective measurement of tissue opacities, such as cataracts (Ito et al., 1999; Vinciguerra et al., 1998).

5.5.1 In vivo confocal microscopy

The basic idea of ivCM is to minimise the scattering of light from structures outside the focal plane, enabling the optical sectioning of thin layers of the cornea (or other tissue). This technique provides images on *x*-, *y*-, *z*-axes and/or time. The images can be manipulated to produce two- or three-dimensional images or numerical data. While thin (9–40 μm) optical sections are used, opacities in the corneal tissue are less destructive for image formation than with the standard techniques.

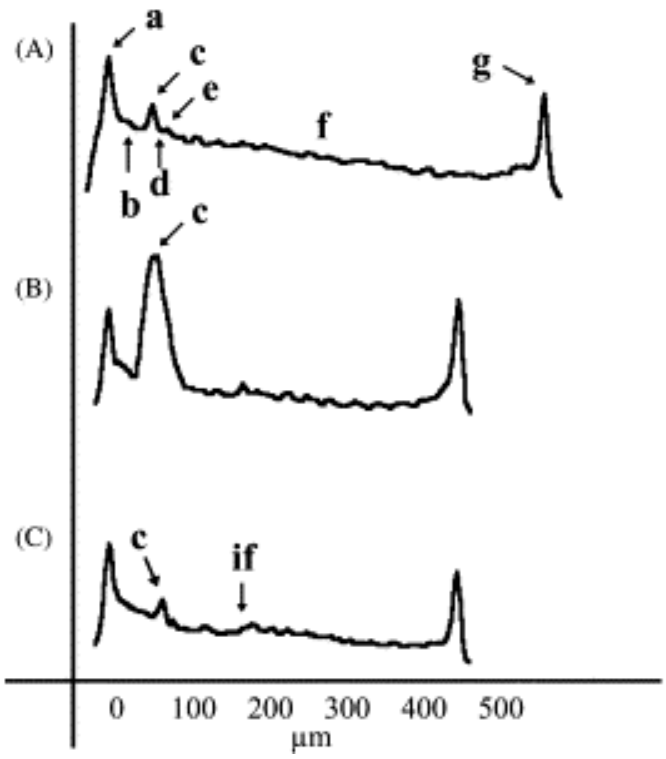
In 1957 Minsky patented the first confocal microscope (Minsky 1957). In 1968, Petran et al. developed the tandem scanning confocal microscope (TSCM), which employed the idea of a dual light path (for illumination and detection) through a rotating disc. This Nipkow disc contains optically conjugate pinholes arranged densely along Archimedian spirals. The rapid rotation of the disc provides scanning rates suitable for video microscopy. The microscope was adapted for the imaging of in vivo cornea by Lemp (1985) and was later developed by Cavanagh and co-workers (Cavanagh et al., 1990, 1993, 1995, and 2000; Jester et al., 1992; Petroll et al., 1993 and 1998). The microscope is equipped with

ultraviolet and heat filters (to prevent injury to the human eye from the mercury lamp serving as the light source), and an applanating objective with a motorised drive to enable *z*-axis scanning. The objective also has "suspension" and an alarm to minimize the risk of corneal erosions if the patient moves the eye. The limitations of the technology are its relatively poor light transmission (0.25–2%), and the necessity for an applanating objective, which may cause distorted images.

5.5.1.1 Z-axis measurements and pachymetry

The estimation of the thickness of the various layers of the cornea (the epithelium, the Bowman's layer, the stroma, the endothelium) or of the thickness of a LASIK flap, wound bed or depth of injury or post-PRK haze is possible with special features and software built in confocal microscopes. A confocal microscopy through-focusing (CMTF) system was developed by Li et al. (1997) to translate the four-dimensional (*x*, *y*, *z*, time) information obtainable from living cells and tissues. The system is based on moving the focal plane rapidly through the entire corneal thickness while producing a stack of two-dimensional images from which a *z*-axis intensity profile can be calculated by averaging the pixel intensity in the centre of each image and plotting it against *z*-axis depth. The CMTF scan is depicted in Figure III. The system also quantifies the amount of backscattering of light from corneal structures. The unit of measurement (U) is defined as μm×pixel intensity. Finally, the system can be used to create 3-D images from 2-D images (Petroll et al., 1993 and 1998; Cavanagh et al 2000; Li et al., 2000).

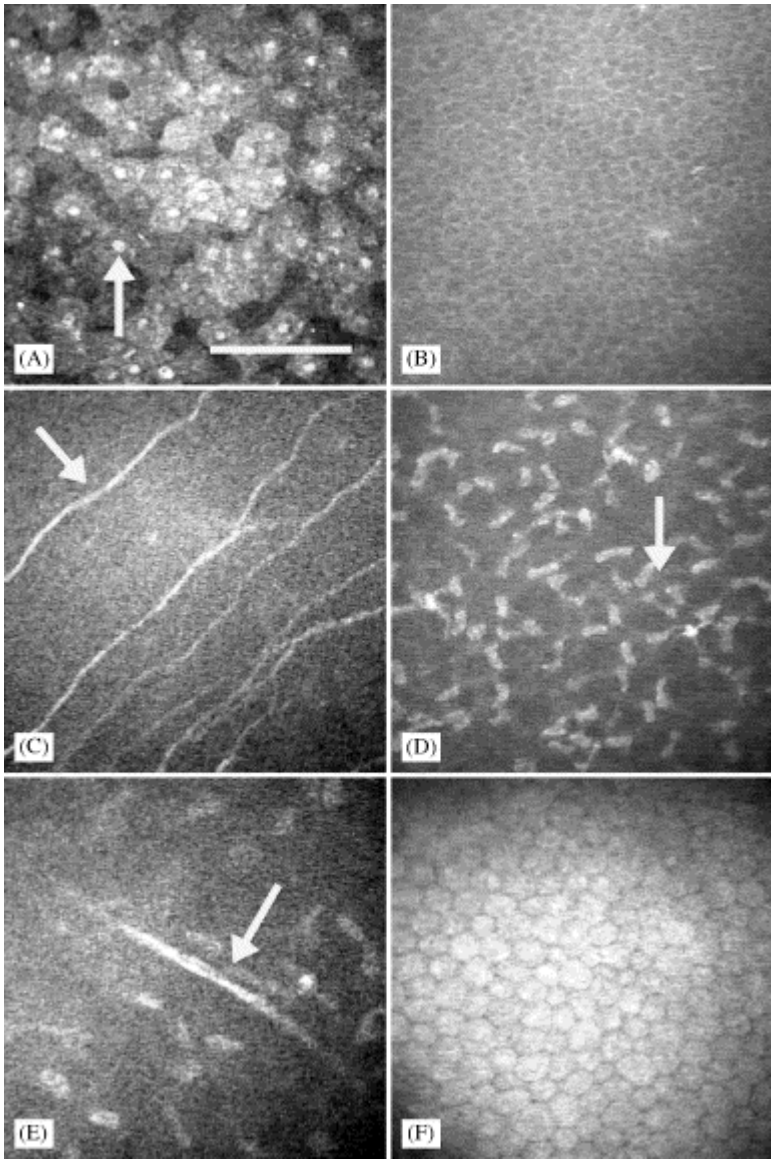
Figure III.



(A) Normal CMTF intensity profile of the author's cornea (JM). Peaks in the scan correspond to: (a) surface epithelium; (b) basal epithelial cells; (c) subbasal nerve fibre bundles; (d) notch of the non-reflecting Bowman's layer; (e) the most anterior keratocytes; (f) midstromal keratocyte area; (g) endothelium. The height of a peak reflects the intensity (U) of the backscattered light from different sublayers of the cornea. (B) CMTF-scan 3 months after myopic PRK (−3.75 D). The *c*-peak includes the partially regenerated subbasal nerves but most of the intensity of the backscattered light (2321 U) originates from the activated keratocytes and altered extracellular matrix, which constitute the post-PRK the haze. (C) CMTF-scan 3 months after myopic LASIK (−10.0 D, intended flap thickness 180 µm) with only minor keratocyte activity at the interface area (if; intensity 126 U), measured flap thickness 187 µm. [Reprinted from Tervo T and Moilanen J. In vivo confocal microscopy for evaluation of wound healing following corneal refractive surgery. Prog Retin Eye Res. 22, 339-358 (2003), with the permission from Elsevier].

5.5.2 IvCM of the cornea

Figure IV.



Normal corneal sublayers imaged by in vivo confocal microscopy: (A) surface epithelium with prominent, bright nuclei (arrow); (B) Basal epithelial cells showing only cell borders; (C) subbasal nerve fibre bundles (arrow); (D) the most anterior keratocytes with bright nuclei, typically forming clusters (arrow); (E) midstromal keratocytes and a stromal nerve (arrow); and (F) endothelium showing mild polymegetism, bar 100 μm. [Reprinted from Tervo T and Moilanen J. In vivo confocal microscopy for evaluation of wound healing following corneal refractive surgery. Prog Retin Eye Res. 22, 339-358 (2003), with the permission from Elsevier]

5.5.2.1 Tear film

Prydal and Campbell (1992) have used ivCM to measure the thickness of tear film in animals. They found that the precorneal tear film thickness is between 9 and 15 μm. However, the applanating objective and use of Goniosol® gel may disturb the estimation of tear characteristics.

5.5.2.2 Epithelium

Irrespective of the type of the microscope, both superficial (Fig. IV A) and basal epithelial cells are easily visualised (Fig. IV B). The Bowman's layer appears as a cell free area, but its thickness (around 16 μm) can be measured using ivCM (Cavanagh et al., 2000). Wing cells can be imaged with a scanning slit ivCM only (Masters and Thaer 1995). The subbasal nerve plexus appears as long leashes of branching nerve fibre bundles. The non-reflecting Bowman's layer is imaged in the same optical section (Fig. IV C).

5.5.2.3 Stroma

Normal or "quiescent" keratocyte nuclei can readily be imaged (Figs. IV D and E). Consequently, a confocal microscope is not as accurate as histological techniques (Jester et al., 1992a, 1994, 1996, 1997, and 1999b; Poole et al., 1993) or laser confocal microscopes (Cavanagh et al., 1995) in assessing cellular changes. Alterations in the keratocyte phenotype as well as their response to wound healing can also be perceived (Jester et al., 1999a). Collagen laminae are beyond the resolution of ivCM but in wounded or healing corneas the newly formed extracellular matrix (ECM) can easily be detected as an unorganized scar (Jester et al., 1999b). The increased back scattering of light can be translated into numerical values by CMTF, providing a means for grading corneal scarring (Moller-Pedersen 1997 and 2000; Jester et al., 1999a; Linna et al., 2000b; Vesaluoma et al., 2000a; Tuominen et al., 2001). Nerves in the mid-stroma can readily be visualised (Fig. IV E).

IvCM is an excellent tool for measuring stromal cell densities in different corneal layers. However, limitations to the cell count arise from the small image area (0.16 mm^2), and the relatively low effective illumination.

5.5.2.4 Endothelium

Endothelial cells (Fig. IV F) and alterations in their size and shape can usually be seen in like manner as with specular microscopes even in relatively opaque corneas (Koester et al., 1993; Masters and Thaer 1994; Masters and Thaer 1995; Cavanagh et al., 2000).

5.6 Corneal recovery after refractive surgery

The wound healing mechanisms and corneal response to surgery differ markedly between PRK and LASIK. In PRK, wound healing is primarily affected by epithelial-stromal interactions, owing to loss of the epithelium and basement membrane, and the Bowman's layer (Li and Tseng 1995).

LASIK, however, induces epithelial-stromal interaction only at the flap edge. On the other hand, epithelial thickening occurs after LASIK, the etiology of which phenomenon is poorly understood.

Both PRK and LASIK severely affect the nerves in the anterior cornea, which leads to decreased sensitivity (Murphy et al., 1999; Gallar et al., 2004), impaired tear fluid secretion, and dry eye symptoms (Ozdamar et al., 1999; Lee et al., 2000; Siganos et al., 2000) and may induce the development of relative neurotrophic epitheliopathy (Wilson 2001a and 2001b). The absence of the Bowman's layer in corneas after PRK (Seiler and McDonnell 1995) may impair neural orientation and regrowth. Furthermore, the absence of neural control on keratocytes (Wilson et al., 2001b) may also interfere with wound healing.

Stromal nerves are cut deeper in the stroma in LASIK. However, a fraction of stromal and subbasal nerves are preserved in the hinge area. PRK ablates the subbasal nerves in the whole treated area, but more superficially, resulting in less stromal nerve destruction. Thus, the neural recovery after PRK is usually faster. PRK performed on a cornea with previous neural damage, such as after penetrating keratoplasty (Tuunanen et al., 1997), or after LASIK (Carones et al., 2001), appears to lead to an excessive formation of haze. It has recently been observed, however, that several years after LASIK a PRK on the LASIK flap is possible without excessive scar formation (Neira Zalentein et al., 2008, in press). This suggests the importance of sufficient neural regeneration to the postoperative result.

The mechanical stabilizing properties of the Bowman's layer appear to be insignificant (Seiler et al., 1992). Muller et al. have suggested, however, that ablation of the most anterior interwoven collagen lamellae may reduce corneal stability (Muller et al., 2001).

There have been no signs of changes to the posterior stromal keratocytes or to the endothelium due to either surgical method.

5.6.1 Wound healing after PRK

Both a simple epithelial scrape and PRK have been shown to induce death of anterior stromal keratocytes by apoptosis within minutes. Cell death continues for at least a week, but necrosis contributes significantly to it within 24 hours of injury (Helena et al., 1998; Mohan et al., 2003; Wilson et al., 2007). Transepithelial PRK photoablation has been reported to induce less stromal cell apoptosis than PRK performed after the surgical scraping of the epithelium (Kim et al., 1998). Even a simple epithelial scrape induces keratocyte apoptosis, followed by the activation of the keratocytes lining the apoptotic zone (Helena et al., 1998; Mohan et al., 2003; Wilson et al., 2007), but only wounds traversing the Bowman's layer into the stroma induce keratocyte activation followed by long-lasting myofibroblast-like transformation (Helena et al., 1998; Jester et al., 1999b; Mohan et al., 2003; Wilson et al., 2007). The magnitude of these changes is also related to the amount or depth of photoablation (Moller-Pedersen et al., 1998a and 2000). One explanation could be the progressively more severe neural damage with deeper photoablations. Corneas with neural damage from any source, such as penetrating keratoplasty (Tuunanen et al., 1997) or disease (American Academy of Ophthalmology 1999), tend to develop an intense haze and regression following PRK.

5.6.1.1 Epithelium

The morphology of the human cornea during the first days after PRK is poorly understood owing to the persistent epithelial defects and the use of bandage contact lenses. Epithelial healing occurs usually in 2 to 4 days. The epithelial thickness does not seem to be related

to post-PRK regression, since postoperative values do not differ from the preoperative thicknesses (Moller-Pedersen et al., 1997; Patel et al., 2007).

5.6.1.2 Stroma

Immediately after PRK, the anterior stromal keratocytes undergo programmed cell death, apoptosis (Dubbs and Wilson 2006). This is followed by repopulation of the anterior stroma through keratocyte proliferation and infiltration, presumably with inflammatory cells (Van Setten et al., 1992; Helena et al., 1998; Jester et al., 1999b; Mohan et al., 2003). Migratory, spindle-shaped keratocytes can be observed even with IVCM during the early stages of healing (Jester et al., 1999b). After the repopulation is completed, these cells increase in size, express alpha smooth muscle actin and thus attain the phenotype of myofibroblasts (Jester et al., 1999b; Mohan et al., 2003). Keratocyte activation and the deposition of new ECM can be observed as early as 7 days post-PRK (Linna and Tervo 1997) and may persist for years (Dawson et al., 2005). However, normal-looking keratocytes start to reappear 3–6 months after PRK. Immunohistochemical and EM studies have revealed that in the ablated area there is a hypercellular fibrotic stromal scar formation, which is composed of dense network of collagen type III (Dawson et al., 2005).

In the post-PRK stroma there occurs true stromal regrowth, which can be elegantly shown in three-dimensional images (Moller-Pedersen et al., 1998; Jester et al., 1999b). During the first 2 weeks, four hyperreflective areas can be observed: The epithelium, photoablated stromal surface, a layer containing migratory, spindle-shaped keratocytes and located under the area of initial stromal cell death, and the endothelium. The second and fourth zones merge after about 3 weeks. Subsequently, the regrowth continues and may lead to a complete regression of the induced laser surgical correction (Moller-Pedersen et al., 2000). As rethickening and the amount of the haze in the CMTF scans do not correlate, the authors believed that haze formation and stromal regrowth represent separate phenomena.

PRK is associated with increased or altered synthesis and the release of a number of cytokines in the cornea, tears or lacrimal gland (Li and Tseng 1995; Tervo et al., 1995; Vesaluoma and Tervo 1998; Wilson et al., 2001b). Cytokines are thought to mediate epithelial–stromal interactions (Li and Tseng 1995), which induce e.g. the change in keratocyte phenotype into activated/altered, scar-forming keratocytes. The major

candidate cytokines leading to the development of fibrosis are TGF-β2 and PDGF-BB (Moller-Pedersen et al., 1998b; Jester et al., 1999b; Wilson et al., 2001b). However, Wilson et al. (2001b) has reported that tears may not be needed for the induction of keratocyte apoptosis.

Epithelial scrape injury, such as PRK, in animal models have shown the accumulation of leukocytes in the anterior stroma (Wilson et al., 2001b and 2004; Mohan et al., 2003; Carlson et al., 2006). Yet, in humans the evidence is scarce, mainly owing to methodological difficulties. Our own data on inflammatory reactions in conjuctival venules on day one post-PRK have failed to reveal leukocyte rolling, thus suggesting that the possible leukocyte accumulation comes from tears or from limbal vasculature. Based on studies with a scanning slit CM, Böhnke and Masters (1999) have reported that there was a stromal edema immediately after PRK. An increase of cells in the anterior stroma was demonstrable already on day one. Anterior stromal keratocyte density was elevated for up to 4 months. Confirming these findings, Erie et al. (1999) has reported that on day one after PRK the keratocyte density increased by 9%, and remained elevated for 3 months. Knowing that there should be initial keratocyte death after PRK (Helena et al., 1998; Mohan et al., 2003), these findings might be related to the initial appearance of leukocytes and macrophages, followed by keratocyte proliferation and repopulation.

An increasing evidence supports that the integrity of epithelial basement membrane serves as the most important barrier against entry of epithelium derived cytokines into the stroma. Defects in the basement membrane allows TGF-β2 and other possible cytokines from the epithelium to enter the anterior stroma thus allowing the initiation of anterior stromal cell necrosis and apoptosis, which is followed by keratocyte transformation into myofibroblasts. Early signs of apoptosis may appear immediately after the injury, but apoptosis markers may be demonstrable even years after surgery (Wilson et al., 2007). Smoothening of the surface irregularieties by PTK with methylcellulose may also decrease haze formation after PRK (Netto et al., 2006).

5.6.2 Wound healing after LASIK

The fast clinical recovery after LASIK is based on the epithelium being only slightly disturbed. The epithelial-stromal interaction at the flap edge and the hypocellular stroma adjacent to the flap interface centrally express only minor changes but also a relatively weak tensile strength of the LASIK flap wound.

5.6.2.1 Epithelium

Despite the fact that LASIK induces a less pronounced wound healing response (Helena et al., 1998; Perez-Santonja et al., 1998; Alio et al., 2000; Mohan et al., 2003) and less postoperative haze than PRK (El-Maghraby et al., 1999), both the flap and the epithelium may undergo rethickening (Lohmann and Guell 1998; Spadea et al., 2000). Recent ivCM studies have shown that epithelial thickness increases gradually after LASIK (Erie et al., 2002; Patel et al., 2007).

5.6.2.2 Stroma

Experimental LASIK has been shown to increase keratocyte apoptosis that can be induced on both sides of the flap within minutes (Helena et al., 1998; Wilson et al., 2001b; Mohan et al., 2003). Acellular zones have been found on both sides of the flap interface (Vesaluoma et al., 2000b; Erie et al., 2004). These zones were thickest in corneas investigated 3 days after LASIK, whereas beyond 3 weeks keratocytes appeared in the proximity of both interfases. However, even when compared with shallow PRK, LASIK induces far less stromal cell apoptosis and subsequent keratocyte proliferation and transdifferentation to myofibroblasts (Mohan et al., 2003). In human corneas investigated 3 days after LASIK, both highly reflecting oval cells and larger cells with interconnecting prominent processes and some highly reflective extracellular microdots could be observed (Vesaluoma et al., 2000b). Microdots have also been observed in corneas subjected to PRK (Böhnke et al., 1998). These oval cells may represent large inflammatory cells, such as macrophages, or a transitional form of keratocyte, characteristic for activated keratocytes. Another explanation was suggested by Ivarsen et al. (2004), who found that the microdots originated at least partly from plastic material of the microkeratome.

After LASIK, the CMTF haze score was found to be highest on day three (i.e., the earliest time point analysed), and achieved normal levels by 1 month. It correlated with the occurrence of activated keratocytes and the formation of new scar-type ECM at the flap interface, rather than with the number of unidentified debris and particles in the interface (Vesaluoma et al., 2000b). This cross-sectional study also suggested that the postoperative keratocyte density in the flaps is lower than it was preoperatively. The observation was later confirmed by an ivCM study by Mitooka et al. (2002). The studies showed that the keratocyte density decreased from 20% to 40% in the flap and from 16% to 30% in the anterior retroablation layer under the flap and remained so at least for 12 months. Keratocyte counts in the deeper stromal layers remained unchanged. This is in agreement both with our data, which showed the keratocyte-free layers on both sides of the flap 3 days after LASIK (Vesaluoma et al., 2000b) and with the experimental data by [Helena et al., (1998) and Mohan et al. (2003). The keratocyte-free layers supposedly represent zones where cells underwent apoptosis/necrosis.

5.6.2.3 Estimation of the flap thickness

The biomechanical strength of the cornea changes following stromal photoablation. The thickness of the post-LASIK stromal bed is critical when LASIK surgery is planned (Sugar et al., 2002; Dupps and Wilson 2006). The prevention of complications, such as post-LASIK keratectasia, is thought to require at least 250 μm of stromal bed under the flap (Ambrosio and Wilson 2001; Sugar et al., 2002; Condon et al., 2007). Standard pachymetry is routinely used to measure corneal thickness pre-operatively. Different microkeratomes, however, cut flaps with variable precision (Jacobs et al., 1999; Behrens et al., 2000; Yildirim et al., 2000; Gokmen et al., 2002; Shemesh et al., 2002; Solomon et al., 2004; Pietilä et al., 2006). IvCM provides a precision (resolution 2.6 μm) that is superior to optical coherence tomography for the estimation of flap thickness postoperatively (Vesaluoma et al., 2000b; Pisella et al., 2001; Gokmen et al., 2002). We showed that flaps cut with an ACS corneal shaper were thinner than intended (Vesaluoma et al., 2000b). Using the CMTF scan, Gokmen et al. (2002) compared two microkeratomes and showed significant difference in their precision: Some microkeratomes may also cut thicker flaps than intended. Cases with a thin stromal bed due to an overly thick flap cannot be detected by standard pachymetry, unless the pachymetry is performed during the operation, after the

creation of the flap. Postoperatively, such cases can be detected by IVCM equipped with CMTF or some similar feature.

5.6.2.4 Wound edge

The wound edge shows normal scar reaction that makes it easily visible under a slit lamp. Many of the features in the healing of the flap edges resemble those of linear incision wounds (Jester et al., 1992b and 1999b). While epithelial–stromal interaction (Li and Tseng 1995; Taliana et al., 2001) is possible at the edge area, there is a lot more deposition of fibronectin and tenascin at the flap edges (Perez-Santonja et al., 1998), and changes in the composition of corneal basement membrane may occur as well (Maguen et al., 2002). Consequently, Dawson et al. (2005) showed that the cellular reactions at the flap edges resemble those in the central corneal haze after PRK. Furthermore, an IvCM study by Vesaluoma et al. (2000b) contained similar findings.

5.7 Nerve regeneration after refractive surgery

Both PRK (Campos et al., 1992; Ishikawa et al., 1994; Trabucchi et al., 1994; Kohlhaas et al., 1995; Perez-Santonja et al., 1999; Benitez-del-Castillo et al., 2001; Tervo and Moilanen 2003; Erie et al., 2005) and LASIK (Kanellopoulos et al., 1997; Chuck et al., 2000; Perez-Santonja et al., 1999; Matsui et al., 2001; Linna et al., 2000a; Ang et al., 2001; Tervo and Moilanen 2003; Gallar et al., 2004; Erie et al., 2005) sever corneal nerves and lead to a loss of corneal sensitivity.

PRK ablates the Bowman's layer and the subepithelial nerve plexus. There is a considerable amount of data on nerve regeneration following PRK: Histological sections have revealed regenerating nerve fibres already on day one post-PRK (Tervo et al., 1994; Trabucchi et al., 1994). The first regenerating subbasal nerves can be observed at seven days post-PRK using ivCM (Linna and Tervo 1997). Thereafter, there is a slow increase of fibres and some restoration of subbasal plexus. However, histological studies (Tervo et al., 1994) have revealed remarkable long-term morphological alterations and the confocal morphology of the human subbasal plexus is not completely regenerated at 12 months

(Linna et al., 2000a). By two years most corneas have reached the preoperative subbasal nerve density, which remained unchanged to five years (Erie et al., 2005).
Accordingly, corneal mechanical sensitivity is markedly decreased after the procedure, first due to the removal of the corneal epithelial nerve supply and a substantial portion of the underlying stromal nerves during the operation, and then, after 2 weeks, probably due to the newly regenerated epithelium acting as a barrier (Murphy et al., 1999). Later, a gradual recovery of sensitivity follows, but one year postoperatively sensitivity has still not reached the preoperative values.

In LASIK, the automated microkeratome cuts the subbasal nerve fiber bundles and the superficial stromal nerves in the flap margin, with the exception of those at the flap hinge. Subsequently, the excimer laser photoablation obliterates the nerves of the stromal bed. The Bowman's layer remains intact centrally after LASIK. Consequently, the subbasal nerves retain their topographical environment. Most studies report an early sensory decrease, and recovery during 3–7 post-operative months, but sensitivity remains subnormal at 6 months after LASIK (Kim and Kim 1999; Patel et al., 2001b; Chung et al., 2002; Matsui et al., 2001; Linna et al., 2000a; Donnenfeld et al., 2003; Gallar et al., 2004; Bragheeth and Dua 2005). However, a correlation between the regeneration of subbasal nerve morphology and mechanical sensitivity was observed in an ivCM study by Linna et al. (2000a): First, regenerating fibres appeared as short subbasal leaches. Then, by 3 months, they were elongated, but interconnections were not observed during the first 6 months. Interestingly, longitudinal studies done using confocal microscopy (Lee et al., 2002; Erie et al., 2005) suggest that morphological recovery of corneal innervation may also take up to five years after LASIK. Corneal immunohistochemical studies in humans showed that 3 months after LASIK the corneal nerves had not fully recovered (Anderson et al., 2002) and even after 20 months only a few epithelial nerves were found (Latvala et al., 1996).

The neuronal system may have an important role during the healing phase after PRK and LASIK. The disturbance of the sensory reflex arch between the cornea and the lacrimal system may induce ocular surface diseases related to tear fluid abnormalities and/or neurotropic phenomena (Wilson et al., 2001a and 2001b), which represent the most common adverse effect of PRK and LASIK (Sugar et al., 2002; Ambrosio and Wilson 2001; Wilson et al., 2001b; Breil et al., 2002). Furthermore, the generally known

difference in the wound healing response between superficial and deeper stromal wound models (Jester et al., 1999b; Wilson et al., 2001b; Mohan et al., 2003) might result from the interference of neural systems in the healing process. Hyperopic PRK may induce even more profound sensory denervation, since it damages the thick peripheral nerve bundles, thus leading to more haze and keratocyte activation in the corneal periphery, as well as in the central area. These sensory disturbances may persist for up to 2 years. Interestingly, the regeneration of subbasal nerves has been reported to correlate with epithelial thickness (Tuominen et al., 2001). Post-LASIK corneas, which have undergone a significant sensory denervation, tend to present with thicker epithelia (Lohmann and Guell 1998). Thus, epithelial hyperplasia may be one cause of post-LASIK regression.

6 Aims of the study

The general aim of the present study was to investigate the eyes' functional recovery, as well as corneal cellular recovery, after the most common refractive surgical procedures, PRK and LASIK, in regard to the postoperative intended and pathological conditions. To achieve the aim, four approaches were adopted:

1. The aim of Study I was to find out how an ophthalmologist working on demanding visual tasks, such as ophthalmic microsurgery, experiences a refractive correction with PRK. The effect of potential side effects such as haze formation, anisometropia, and dryness was also evaluated.

2. The aim of Study II was to reveal the long-term cellular changes after PRK. Special attention was paid to subbasal nerve regeneration and anterior stromal haze.

3. Complementary to Study II, the aim of Study III was to reveal the long-term cellular and functional changes in the eye after LASIK.

4. The aim of Study IV was to investigate the effect of a common complication, namely diffuse lamellar keratitis (DLK), after LASIK to corneal wound healing and visual performance.

7 Patients and methods

7.1 Patients

In the present study, all examined corneas were of human controls or patients who had undergone PRK or LASIK. The study was carried out according to the tenets of The Helsinki Declaration. It was approved by the Ethical Review Committee of the Helsinki University Eye Hospital. All patients and controls gave an informed consent and participants were informed about the examinations.

In all, out of 40 patients, 23 eyes were treated with PRK and 21 with LASIK. The best spectacle corrected visual acuity (BCVA) preoperatively was 1.07 $\pm$ 0.17 (SD, range 0.63-1.25). Six patients had preoperative BCVA of less than 20/20; five of these 20/25 and one 20/32 (Preoperative refraction from -3.0 to -13.88 D). The mean preoperative refractive error in spherical equivalent (SE) was -6.64 D $\pm$ 3.36 (range -1.63 - -13.88 D). The intended correction (-6.46 $\pm$ 3.27, range -1.63 – -13.63) was to achieve emmetropy in 34 eyes (77%). Four of the patients desired undercorrection due to presbyopia and 6 patients were deliberately undercorrected so as to maintain sufficient stromal bed depth or to avoid overcorrection. A more detailed description of patient demographics is given in Studies I – IV.

7.2 Methods

7.2.1 Study protocols

7.2.1.1 Study I

The patients (n=5) were examined preoperatively, and at 1 and 6 months after PRK. Uncorrected visual acuity (UCVA), best spectacle-corrected visual acuity (BSCVA), manifest refraction, corneal biomicroscopy, videokeratography, perimetry, contrast sensitivity, pattern visual evoked potential - test, car driving simulator -test, and confocal microscopy were performed. Patients completed a questionnaire designed to evaluate the benefits and problems associated with the surgery.

7.2.1.2 Study II

The preoperative and postoperative 1-month examinations included refraction, uncorrected (UCVA), and best corrected (BSCVA) visual acuity, and slit lamp biomicroscopy. At 5 years after PRK, the patients (n=14) were examined on slit lamp to estimate corneal haze (Fantes 1990), and UCVA, BSCVA, and refraction were measured. Furthermore, confocal microscopy was performed. Subjective comments of the patients were also collected.

7.2.1.3 Study III

Each patient (n=15) was examined preoperatively and at time points 1 day, 5 days, 2 weeks, and 1, 3, and 6 months, and at 2 years (mean 27.3, range 24-37 months) postoperatively. Examination at every time point included visual acuity measurement and refraction, biomicroscopy, and confocal microscopy.

7.2.1.4 Study IV

All six identified DLK patients were examined carefully with biomicroscopy to exclude any pathology that could affect the wound healing process. Manifest and cycloplegic refraction were measured, corneal pachymetry and topography studied, and fundus inspected. The morphological changes were examined by confocal microscope.

DLK classification

The classification of DLK followed the 4-stage system of Linebarger et al. (2000): Stage 1 changes were defined as peripheral, and stage 2 as central granular, white objects under the flap. Stage 3 included more dense and clumped granular objects involving the visual axis and thus decreasing visual acuity. Five eyes, of 5 patients, were examined by corneal ivCM. One eye (Study IV, Table 1, patient 4) was examined twice, 7 and 18 months postoperatively. Two of the six DLK patients were examined by confocal microscopy of the conjunctival venules.

7.2.2 PRK Procedure, Study I and II

In Study I the patients were instructed to stop wearing their contact lenses 2 weeks before surgery. Photorefractive keratectomy was performed after a surgical abrasion of the epithelium using a Beaver eye blade (Becton Dickinson, Franklin Lakes, NJ). The mean refractive correction was -3.70 D (range -1.63 - -6.5 D). The laser ablation diameter was 6 mm, performed using a VISX 20/20B excimer laser (VISX Co, Sunnyvale, CA) equipped with software version 4.02 (Study I) or version 2.7 (Study II). In Study II the mean ablation depth was 54.64 ± 14.98 µm (range 30–80 µm), and the purpose of each operation was correction to attain emmetropy. The eyes were patched for 2 to 3 days following PRK. The patients changed the patch and added chloramphenicol ointment (Oftan Chlora; Santen, Tampere, Finland) twice a day for 3 days after PRK. Treatment with chloramphenicol ointment was continued after the removal of the patch three times daily for 3 days, and subsequently for 3 nights. Postoperative medication also included fluorometholone drops (Liquifilm-FML; Allergan, Irvine, CA), starting on the fourth postoperative day, three times a day for 1 to 4 months, or alternatively dexamethasone (Maxitrol; Alcon Laboratories, Inc., Fort Worth, TX), or prednisolone acetate (Pred Forte; Allergan). Oral diclofenac sodium, 25 mg, (Voltaren; Ciba-Geigy, Basel, Switzerland) 30 minutes before the operation and two to three times a day for the first 2 days after PRK (Study I), and oral diazepam, 5 to 10 mg (Diapam; Orion, Helsinki, Finland) for the first postoperative night were also used. The patients were on sick leave for 4 days postoperatively. If the other eye was to be operated, the interval between the procedures was 1 to 3 months.

7.2.3 LASIK Procedure, Study III and IV

In the LASIK procedure, the eyes were anesthesized with topical 0.4 % oxibuprocaine hydrochloride (Oftan Obucain; Santen, Tampere, Finland). A Hansatome microkeratome, model HT 230 (Bausch & Lomb Surgical, Inc., San Dimas, CA) was used to create a corneal flap with a superior hinge (thickness 160 or 180 µm, diameter 8.5 or 9.5 mm). An excimer laser (VISX Star 2, software version 2.5, VISX; Santa Ana, CA, USA) was used to ablate the stroma. A balanced salt solution (BSS, Alcon Laboratories Inc., Fort Worth, TX) was used to irrigate the cornea before and after the flap formation and after photoablation.

The treatment after the operation included a bandage soft contact lens on the first night. All patients self-administered topical 0.1% fluorometholone (Liquifilm-FML®, 1 mg/mL, Allergan Inc., Irvine, CA) twice a day, topical ofloxacin (Exocin®, 3 mg/mL, Allergan Inc., Irvine, CA) three times per day for one week, and artificial tears (Oculac®, povidone).

The additional medication for DLK patients with epithelial defects included topical chloramphenicol ointment (Chloromycetin® 10 mg/g), fluorometholone or hydrocortisone, and peroral prednisolone (Prednison®) or a soft therapeutic contact lens (Dk 43, Polarlens 75%, Polarlens). A detailed description of patient medication appears in Study IV.

7.2.4 Examinations

7.2.4.1 In vivo Confocal Microscopy, Study I, II, III and IV

After clinical examination, the corneas were examined by a tandem scanning confocal microcope (TSCM; Model 165A; Tandem Scanning Corp., Reston, VA) for morphologic evaluation and for measurement of the thickness of the different sublayers of the central cornea. The setup and operation of the confocal microscope has been described previously (Petroll et al., 1996; Li et al. et al., 1997; Moller-Pedersen 1997). Briefly, the corneas were anesthesized with one drop of topical 0.4% oxibuprocaine hydrochloride (Oftan Obucain; Santen, Tampere, Finland). A 24× (numerical aperture 0.6) objective lens was used, with a variable working distance from 0 to 1.5 mm, including a floating tip retraction mechanism. Hydroxymethylcellulose 2.5% gel (Goniosol, Iolab Pharmaceuticals, Claremont, CA) served as an optical coupling medium between the tip of the lens and the cornea. A 100 W mercury lamp supplied the illumination, and a low-light video camera (VE-1000 Sit System, Dage-MTI, Michigan City, IN) captured the real-time images to an S-VHS tape. With this camera and objective, the field of view was 450 x 360 μm and the optical slice thickness (z-axis resolution) was 9 μm. A motorized focusing device (Oriel 18011 Encoder Mike TM Controller, Stratford, CN) adjusted the internal lenses of the objective interfaced to a personal computer to vary the focal plane relative to the objective tip.

In each visit, a manual mode of the confocal microscope was used to scan slowly the central corneal epithelium, the subbasal nerve plexus, the most anterior keratocytes, the midstromal keratocytes and interface area, and the posterior stroma with endothelium, in

order to obtain the best possible view of that particular area for cell and nerve fiber calculations. Additionally, confocal microscopy through focusing (CMTF) mode was used to scan the cornea four to six times from the epithelium to the endothelium and back for thickness measurements, and for keratocyte density calculations at various corneal depths (see below). The intensity of the backscattered light representing haze formation and altered keratocytes in the interface area were measured from CMTF scans with manual camera gain mode. Some of the CMTF scans were rejected because of occasional eye movements, and 2 to 4 scans were analyzed for the calculations.

Subbasal nerve fiber bundles

The density of the subbasal nerve fibers, i.e. the nerve plexus anterior to the Bowman's layer extending along the basal aspect of the basal epithelial cell layer, were measured in each visit from the best image acquired. From these images (coronal section 450 x 360 μm, area 0.162 mm^2), one examiner marked all visible nerve fibers and measured the total length of the nerves (Adobe Photoshop 7.0). The nerve density was then expressed as $\mu m/mm^2$. The number of long (>200 μm) subbasal nerve fibers were manually counted from the same images and expressed as number/mm^2.

Keratocyte density

Due to the fact that keratocyte density measured from a confocal image is always an estimate, the focus was on the differences in two-dimensional cell density compared to preoperative values. One to two best quality images with no motion artefact from each corneal layer were selected: Most anterior keratocytes, midstromal keratocytes 120 μm (i.e. in the mid flap) and 240 μm (ca. 80 μm posterior to the interface) from the epithelium, 10 μm anterior and 10 μm posterior to the interface, and posterior stroma just anterior to the endothelium. Keratocyte nuclei were manually counted from the selected images. Keratocyte density was then expressed as cells/mm^2.

Thickness measurements

CMTF intensity profile curves exhibit the intensity of the backscattered light from the corneal subunits, where the epithelium, subbasal nerves, most anterior keratocytes, and the endothelium comprise the areas of the highest intensity. Additionally, the LASIK interface shows as a drop of intensity after the operation and also exhibits bright small interface

particles. With these landmarks, the thickness of the epithelium, the stroma, the LASIK flap, and the whole cornea were calculated.

The thickness of the area that expressed altered/migratory keratocytes and increased backscattering of light both anterior and posterior to the flap interface was measured, as well. The CMTF intensity curve showed so little variation at these areas that the depth was set manually in each scan.

7.2.4.2 In vivo confocal microscopy of the conjunctival vessels (Study IV)

An analysis of the confocal microscopy videos of the conjunctival vessels was also performed (Kirveskari et al., 2001). Briefly, the number of vessels, vessel diameter, and analysis time were adjusted to be equal before and after LASIK, and thus the possible differences in leukocyte velocity could be interpreted to result from the inflammatory reaction caused by LASIK. To quantify leukocyte rolling, the camera was adjusted to give a face view of the bulbar conjunctival vessels. The video stream from the camera was captured directly by a video card (Matrox Marvel G-400, Matrox Electronic System) in a second computer with Matrox PC-VCR software and saved in Windows AVI format. The final video analysis was performed with Adobe Premiere 5.1 (Adobe Systems), and image analysis and processing were performed with Adobe Photoshop 5.0.

The venous in vivo Confocal Microscopy was performed twice in each control subject. The conjunctival vessels of the temporal conjunctiva were imaged 3.0 to 6.0 mm lateral to the limbus. Vessel diameters, mean centerline flow velocity, the number of rolling cells, and the velocity of rolling cells were counted in all vessels. The mean centerline flow velocity was counted with 3 to 5 freely moving bright cells by measuring the average movement in 4 consecutive frames. Vessels with flow above 500 μm/s were included in the analysis. The number of rolling cells was counted in each vessel with continuous flow, and a sharp image from the cells passing an imaginary horizontal line in the vessel, which was fixed on one of the local landmarks in the vessel area to eliminate the effect of small movements. Extravasated leukocytes in the focal plane of the conjunctival stroma were also quantified from an area adjacent to the vessels where rolling had been analyzed.

7.2.4.3 Car driving simulator test (Study I)

The aim of the simulator test was to reveal possible problems associated with driving after PRK. The driver wore best spectacle correction on the unoperated eye while driving. The simulator, based on the STIsim-program (Systems Technology Inc., CA), and designed at the Haaga Neurological Research Center, Helsinki, is set up in a normal car and with three computers linked together. The driver has a 135° view on a white curved screen in front of the car. The controls (steering wheel, brakes, etc.) are connected to the computer, which processes and files all the maneuvers of the driver. The driving simulator test was carried out four times with each patient (preoperatively and at 1, 3, and 6 months postoperatively). One patient missed a test at 1 month after PRK. One day before the first test each patient visited the simulator laboratory and drove two practice tests to lessen the stress (excitement) caused by the simulator and to minimize the learning effect.
The driving test is described in detail in Study I.

7.2.4.4 Perimetry,contrast sensitivity, videokeratography, and pattern visual evoked potential (Study I)

Perimetry was examined using the program 24-2 and the the SITA algorithm of the Humphrey Field Analyzer Model 750 (Humphrey Instruments, San Leandro, CA) preoperatively and at 1 and 6 months after PRK. Contrast sensitivity was evaluated with Vistech VCTS 6500 (Dayton, OH) distance charts preoperatively and at 6 months. The contrast sensitivity test was administered under photopic illumination at a 3-meter test distance. Videokeratography was performed with TMS-1 (Computed Anatomy, Inc., New York, NY). The spatial frequencies of the gratings were 1.5, 3.0, 6.0, 12.0, and 18.0 cycles per degree (c/deg). Maximum contrast sensitivity for each spatial frequency was determined. The test was conducted monocularly with patients wearing trial lenses fitted for the testing distance. Pattern visual evoked potential was recorded using a Cadwell Quantum 84 device preoperatively and at 6 months. The field size was 14 x 12 degrees, the check size 37 min of arch, the contrast 96%, and the temporal frequency 2.1 Hz. The band pass was set to 1 to 100 Hz and 100 responses were averaged. The latency of the positive P100 was measured.

7.2.4.5 Biomicroscopy

The biomicroscopically observable haze, i.e., opacity of the cornea, was graded according to Fantes haze scale (Fantes et al., 1990): Grade 0, totally clear, no opacity could be seen by slit-lamp biomicroscopic examination; grade 0.5, trace of haze, a faint corneal haze seen only by indirect broad tangential illumination; grade 1, haze of minimal density seen with difficulty with direct and diffuse illumination; grade 2, a mild haze easily visible with direct focal slit illumination; grade 3, a moderately dense opacity that partially obscure the iris details; grade 4, a severely dense opacity that obscures completely the details of intraocular structures.

7.3 Statistics

Statistical analyses were performed (SPSS for Windows, ver. 7.0. SPSS, Chicago, IL). Continuous variables were tested by Mann-Whitney or Student's *t*-test. The Pearson or the Spearman correlation coefficient (r) was used to evaluate the correlations between variables. Data are expressed as the mean ± SD. Mean values of the CMTF measurements were used for all statistical calculations (Study II).

All measured parameters were compared individually across time using repeated measures of variance (ANOVA) with post hoc Bonferroni-Dunn correction. A p value of less than 0.02 was considered significant (Study III).

8 Results

8.1 Clinical findings

8.1.1 Visual acuity and refraction

In Studies I-IV (Table 1.), there were 23 PRK treated eyes and 21 LASIK operated eyes. At the study endpoint BCVA was 1.11 $\pm$ 0.17 (range 0.63 – 1.25). Only four eyes achieved BCVA of less than 20/20: three eyes had maintained 20/25 visual acuity, one PRK treated eye had lost two lines and had 20/32 acuity. The mean postoperative refraction was -0.55 D $\pm$ 1.16 (range -5.75 - +0.75).

DLK had no effect on the visual acuity at end point, but during DLK the mean decrease in visual acuity was 2.1 Snellen lines (range 1 – 4 lines).

8.1.2 Safety

At the end of the follow-up, ten patients had gained 1 line of BCVA [(six PRK patients, refractive corrections mean -4.48 D (range from -3.0 to -7.0); four LASIK patients, refractive corrections mean 11.03 D (range from -9.75 to -13.88)], and one patient had gained two lines (Study III, patient 2 with refractive error -13.13 D preoperatively). Three patients lost one line of best corrected visual acuity from 20/16 to 20/20 (Study I, one PRK patient, preoperative refraction -3.0; Study III, two LASIK patients, preoperative refractions -5.13 and -7.38) and one PRK patient with -6.25 correction from Study II had lost two lines from 20/20 to 20/32. At five years, this patient presented also with ptosis, pigmentary glaucoma and gradus 0.5 haze in the cornea; his visual acuity had been 20/25 one month postoperatively.

8.1.3 Predictability

In all, the deviation of the postoperative refraction from the attempted correction was -0.35 D $\pm$ 1.07 (range -5.25 - +1.0). Within 0.5 D range were 28 eyes (14/23 (61 %) PRK, 14/21 (67 %) LASIK eyes), within 1.0 D range were 39 eyes (22/23 (96 %) PRK, 17/21 (81 %) LASIK eyes).

8.1.4 Stability

In Study II, 13 PRK operated eyes examined at 1 month and 5 years postoperatively. During that time the mean myopic regression was -0.47 D (range +1.0 - -1.87 D). After LASIK (Study III) the mean myopic regression was -0.4 D. 10 eyes showed mild myopic regression (range +0.25 - -1.12) and one patient regressed -2.25 D from 6 months to two years. This patient showed an overall regression of -5.25 D from the intended correction.

8.1.5 Biomicroscopy

At six months after PRK (Studies I and II) six corneas out of 23 were clear in biomicroscopy. Ten eyes presented with grade 0.5 haze (Fantes haze scale). Four eyes showed grade 1 haze, and two eyes had denser grade 2 haze. Five years postoperatively (Study II) only 4 eyes presented with trace of haze (gradus 0.5), while 10 corneas were clear. At this point, central superficial linear brown ferritin deposits were observed in four corneas.

In two year follow-up after LASIK (Study III) all corneas healed without clinical evidence of epithelial or interface abnormalities or inflammation, except one patient (Table 1, Study III, Patient 2), who developed a steroid induced rise in IOP and interface fluid three weeks postoperatively, accompanied with marked regression. Biomicroscopical examination showed a clear central cornea at all time-points for each patient. Mild epitheliopathy, i.e., small punctate lesions, was seen in 6 out of 15 patients 6 months postoperatively, and in two patients at 2 years. Biomicroscopically observable microfolds were present in the LASIK flap in 11 out of 15 patient at both 6 months and 2 years. A ferritin-line was observed in all 15 patients 2 years postoperatively.

DLK (Study IV) presented with small, white, dot-like changes in the interface under the epithelial defect. The defects were 2.0 to 5.0 mm in diameter and were located in the flap periphery in 5 cases and in the middle in 3 cases. There was no stromal melting or permanent corneal scarring in any of the DLK patients.

8.2 In vivo Confocal Microscopy

8.2.1 The appearance of corneal layers after PRK

8.2.1.1 Thickness of corneal layers after PRK

The mean epithelial, stromal, and total corneal thicknesses are shown in Table 2, Study II . As expected, the stroma was significantly thinner in the corneas that had undergone PRK compared to controls. The epithelial thickness did not differ from that in control corneas. Three patients exhibited regression during follow-up. Two showed normal epithelial thicknesses (47 and 53 µm), but no CMTF reading could be obtained in the third.

8.2.1.2 Corneal morphology

Cell morphology was evaluated in the central cornea of control subjects and PRK patients. Preoperative ivCM examinations were performed on three patients (Study I) and nine controls (Study II). These corneas presented with normal sublayers, where anterior keratocytes showed a typical clustering. Their evenly reflected nuclei were oval-shaped and were well separated from the surrounding cells, as cell processes were not visualized and the extracellular space reflected only minimally.

In the postoperative examinations, the surface epithelial and basal epithelial cell layers appeared normal (Study I and II).

Anterior stromal haze

Study I: Alteration of the most anterior keratocytes, characterized by brightly reflecting nuclei and visible keratocyte processes, was observed in the first examination 3 to 4 weeks after PRK. The presumed keratocyte activation contributed to the haze observed throughout the follow-up. The haze was densest at 2 to 3 months. At 9 to12 months the corneas still showed some keratocyte alteration subepithelially, although less than at earlier timepoints.

Study II: Five years post-PRK, the most anterior keratocytes appeared to be unevenly distributed, and even clinically clear corneas still showed signs of opacity: Increased fibrosis-like ECM reflectivity, bright keratocyte nuclei, and visible keratocyte processes.

Subtle morphologic alterations (i.e., thin, faint keratocyte processes) in the post-PRK keratocytes were also visualized posterior to the area with enhanced ECM reflectivity. However, these changes were so subtle that they could not be considered to differ significantly from the controls.

The clinical haze score correlated positively with the thickness of the haze area ($r= 0.71$, $p< 0.01$) and with the depth of the laser ablation ($r=0.63$, $p= 0.02$). However, there was no correlation between the clinical haze score and the CMTF haze estimate ($r=0.31$, $p= 0.30$).

Posterior stroma and the endothelium

The posterior stromal keratocytes, ECM, and the endothelium appeared morphologically normal and identical to the control corneas (Study I and II), except in six post-PRK corneas (Study II) that showed microdots in the anterior or posterior stroma or both. The significance of these particles remains unclear.

8.2.2 LASIK induced changes in corneal layers

8.2.2.1 Thickness of corneal layers after LASIK

The corneal thickness in LASIK patients was 533±23 μm (range 500-573 μm) preoperatively. As expected, this was reduced to 481±26 μm 6 months postoperatively. From 6 months to 2 years postoperatively the corneal thickness remained unchanged (470±34 μm). Preoperatively the thickness of the corneal epithelium was 49±6 μm and at day 1 this increased to 55±8 μm. Subsequently the epithelial thickness increased gradually to 62±6 μm at 2 years. This finding is highlighted also in the analysis of the thickness of the LASIK flap, showing a gradual increase in its thickness. No changes in the thickness of the stromal bed were found during follow-up.

8.2.2.2 *Corneal morphology after LASIK*

Keratocyte densities in the whole stroma as well as in its different sublayers are presented in Study III, Table 2. The postoperative anterior keratocyte density decreased to 92.5 % by 3 months, and continued to decrease after 6 months (86.9 %), and 2 years (81.9 %). This decrease was statistically significant at 2 years postoperatively ($p=0.002$). The

keratocyte densities, at 120 µm and 240 µm post epithelial surface, and just anterior to the Descement's membrane, remained unaltered during the follow-up. Yet, keratocytes 10 µm posterior to the interface were found to have decreased.

Altered/migratory keratocytes were observed both anterior and posterior to the LASIK interface. The thickness of the stromal area where increased back scattering of light, keratocyte processes, and bright keratocyte nuclei were present was found to be highest at postoperative day 1 and showed then a gradual decline throughout the follow-up. In accordance with the decrease in the thickness of the stromal layer where these altered cells were observed, the intensity of the back-scattered light decreased from 201±93 (day 1) to 10±33 intensity units (2 years postoperatively). Brightly reflecting particles were observed at the interface. No decline in the number of these particles was observed throughout the follow-up (data not shown).

8.2.3 Comparison of neural regeneration after refractive surgery

Subbasal nerves were observed in every control and preoperative cornea. The subbasal nerve plexus consisted of branching and interconnecting nerve fiber bundles. In control corneas, the number of the long subbasal nerve fiber bundles (SNFB's) in the field of view ranged from 3 to 6 (mean, 4.9 ± 1.1), being slightly higher than in the surgically treated corneas (range, 1–7; mean, 4.2 ± 2.1). At 9 to 12 months after PRK the subbasal innervation was well regenerated in seven of nine corneas (long nerve fiber bundles with or without interconnecting fibers; Study I). At 5 years, on the other hand, SNFB's were observed in all corneas. Yet, five (36%) corneas had only one or two long nerve fiber bundles, and in 10 out of 14 corneas branching was estimated to be normal. The number of the SNFB's showed no correlation with the ablation depth (r = -0.13, p = 0.66), CMTF haze estimate (r = 0.15, p= 0.62), or the thickness of the haze area (r = 0.08, p= 0.79) (Study II).

Following LASIK, the SNFB density was lower at all postoperative follow-ups with the greatest reduction (83%, from preoperative 15.1 mm/mm^2 to 2.6 mm/mm^2) evident at day 5 following LASIK. A gradual increase in the SNFB length was observed up to the last follow-up. However, even in the last follow-up the length 9.7 mm/mm^2 was significantly lower compared to the preoperative figure. The number of long (at least 200 µm) SNFB's was 36.2±14.5 nfb/mm^2 preoperatively. Postoperatively, significantly fewer long nfb's

were found at all follow-up visits: 19.2, 8.3, 5.2, 6.7, 10.4, 11.7, and 21.25 SNFB/mm^2 at postoperative visits 1 day, 5 days, 2 weeks, 1 month, 3 months, six months and 2 years, respectively. A gradual increase in long nfb's was seen after 3 months.

Taken together, after refractive surgery, the regeneration of SNFB's seems not to be complete even 5 years after PRK and 2 years after LASIK.

8.2.4 IvCM detected changes in DLK patients' corneas after LASIK

IvCM revealed two patients with interface changes resembling inflammatory-cell-like infiltration. The DLK 1-patient (Study IV, Table 1, DLK stage 2) had 2 interfaces: The first LASIK procedure 19 months earlier was discontinued because of the formation of a free cap (flap thickness 80 μm; first interface); intraoperative epithelial detachment occurred during the reoperation (flap thickness 180 μm; second interface) and led to the development of DLK, which was detected on day 1. IvCM revealed a few small, round, cell-like objects in the anterior (first) interface. The second interface presented with a marked activation of keratocytes and an increased formation of extracellular matrix.

The interface in the DLK 2-patient (Study IV, Table 1, DLK stage 2 to 3) showed numerous round objects resembling inflammatory or epithelial cells. The other 2 patients examined by ivCM during the first 3 days after the onset of DLK as well as the DLK 5-patient, 4 days after DLK onset, showed signs of keratocyte activation on both sides of the interface. The DLK 3-patient also presented with bright oval objects approximately 13 μm in diameter of unknown origin. No changes corresponding to inflammatory-cell-like structures were observed.

The DLK 1-patient was re-examined a month after the development of DLK. The anterior interface presented with numerous bright particles (<5 μm). The reaction in the posterior interface was less evident, and some increased backscattering of light resembling haze formation was observed.

8.2.5 Inflammatory response in conjunctival vessels detected by ivCM

In the control subjects, there were no rolling leukocytes in the conjunctival venules before LASIK (0.03 ± 0 rolling leukocytes/min). One day postoperatively, rolling cells were observed more frequently (4.4 ± 3.9 rolling leukocytes/min), but the difference was not significant (p= 0.10, Wilcoxon signed rank *t* test). The rolling velocity showed a tendency to increase 1 day after LASIK (110 ± 0 μm/s versus 204 ± 56 μm/s, p=0.18). No cell-like structures were present in the conjunctival stroma before or 1 day after LASIK.

Patients 2 and 6, who were examined by VivCM 1 day after the development of an intraoperative epithelial defect, showed no signs of inflammation in the conjunctival venules. One day after LASIK, there were 0.7 ± 1.0 rolling leukocytes/min and the rolling velocity was 198 ± 24 μm/s. No leukocyte-like cells were present within extravascular conjunctival tissues 1 day after LASIK.

8.3 Complications following PRK and LASIK

Study I

Patient 2 ended up with +1.0 and +0.62 overcorrection on both eyes, but fortunately this patient was scheduled an intended undercorrection, and the postoperative refraction was -0.5 and +0.62, respectively.

Study II

One patient, who also presented with ptosis and pigmentary glaucoma, lost two lines of BCVA at 5 year control after uneventful PRK. Another patient (PRK 9) still complained about glare and shadows in the temporal field more than five years post surgery. Two other patients suffered of myopic regression of -1.0 and -1.25 D.

Study III

One epithelial erosion occurred during the LASIK operation, which prevented the Day 1 confocal microscopy examination (Study IV, LASIK 7). This erosion healed without complications. During the two year follow-up, four patients developed a marked myopic regression: -1.5, -1.75, -2.0, and -5.25 D. The latter patient developed a steroid induced rise in IOP and interface fluid three weeks postoperatively. This resulted in significant

myopic regression of -3.5 D at 6 months, which continued to -5.25 D at two years, post LASIK.

Study IV

DLK after LASIK following corneal epithelial detachment presumably induced leukocyte accumulation in the flap interface and keratocyte activation. These alterations disappeared during follow-up and they had no effect on the final outcome.

The overall complication frequency was thus 10 out of 38 (26 %, DLK patients excluded), when regression or overcorrection of $\geq$ 1.0 D was considered significant. However, only one patient complained about glare and halos after PRK (Study II), another lost two lines of best corrected visual acuity, which was not related to PRK (Study II), and one patient suffered from marked myopic regression after LASIK, which gives a rate for significant complications of 3 out of 38 (8 %)

9 Discussion

Although corneal refractive surgery has recently gained popularity, many ophthalmologists still prefer wearing spectacles or contact lenses. Patients often ask why ophthalmologists have not had refractive surgery themselves. Approximately 12 million laser refractive procedures (PRK, LASIK, LASEK, or epi-LASIK) have been performed so far. Yet, long-term follow-up studies and results are still scarce: Only eight studies of more than ten years have been published, four on PRK (Rajan et al., 2004; O'Connor et al., 2006; Alio et al., 2008a and 2008b), and four on LASIK (Condon et al., 2007; Kymionis et al., 2007; Alio et al., 2008c and 2008d). Even relatively long-term prospective studies are just a few (Kim et al., 1997; Matta et al., 1998; Stephenson et al., 1998; Pietilä et al., 2004; Erie et al., 2005; Dawson et al., 2006; Erie et al., 2006; O'Doherty et al., 2006; Rajan et al., 2006; Sekundo et al., 2006; Condon et al., 2007; Patel et al., 2007). These studies do not confirm the long-term effects of laser surgery to visual quality or the possible alterations in corneal stability. However, laser surgery is expensive and demands high technical skills and maintenance. Furthermore, refractive procedures are sometimes perceived as cosmetic surgery, and, thus, entail an element of vanity. Numerous short-term studies have been published, however, and these studies have shown that the current methods are safer and more predictable than other surgical procedures to correct ametropia.

Corneal alterations after PRK and LASIK

In vivo confocal microscopy is a relatively new tool for analyzing corneal morphology and innervation, in both diseased and postsurgical corneas (Linna and Tervo 1997; Moller-Pedersen et al., 1997; Boehnke et al., 1998; Frueh et al., 1998; Linna et al., 2000; Rosenberg et al., 2000; Tervo and Moilanen 2003; Erie et al., 2005). The method has been used to examine the long-term effects of myopic PRK and LASIK on corneal thickness, keratocyte density, and nerve recovery. The present confocal microscopic study clearly reveals that even biomicroscopically clear corneas show morphologic changes in the subbasal nerve plexus, subepithelial keratocytes, and extracellular matrix still 5 years after PRK and 2 years after LASIK. The IVCM results also showed changes in cellular morphology and ECM during active wound healing processes both in normal and in pathologic DLK conditions.

Study I

In this study, free PRK surgery was offered to seven myopic ophthalmic residents. Three of them preferred spectacles. Four residents as well as a medical engineer were operated on. All refractive corrections were moderate and one resident was operated in one eye only. All patients were very satisfied with the outcome of their refractive surgery. The 6-month results revealed good visual outcome, acceptable accuracy, and minimal haze formation. Negative experiences included pain a few days after surgery, slow recovery of visual acuity after the first operation, dryness of the eyes for 1 to 3 months postoperatively, and a period of anisometria between the operations. Yet, highly demanding visual tasks without spectacles, such as an eye examination using a slit lamp or indirect ophthalmoscope, and ophthalmic microsurgery, became significantly easier. No significant changes in perimetry, contrast sensitivity, or car driving simulator were observed. Also, pattern visual evoked potential was not changed, in line with the results of Spadea et al. (1996). In this series of patients, all ablations were well centered, which might partly explain why no decrease was observed in low spatial frequency contrast sensitivities.

Study II

Despite the biomicroscopically clear corneas of most patients, ivCM demonstrated a marked stromal hyperreflectivity in every cornea even 5 years after PRK for myopia. Furthermore, their subbasal nerve density was lower than in control subjects at 5 years, suggesting a very slow neural regeneration or a permanent shortage in the number of SNFB's. While the keratocyte and ECM alterations seem stable, the decrease in neural density may have long-lasting effects on possible future corneal wound healing properties.

Study III

A prospective 2-year follow-up study of 15 highly myopic patients receiving LASIK revealed a slow but constant subbasal nerve recovery, which presumably was not completed by the final visit. A gradual decrease in the anterior stromal keratocyte density was noticed, as well, which indicates a continuous wound healing/keratocyte alteration process in the stroma. This finding is somewhat surprising as the recovering neural system would be expected to assist the stromal remodelling towards a stabile and endurable state.

Study IV

DLK, a common and usually easily treated, but potentially sight-threatening complication of LASIK, yields often a remarkable overtreatment with antimicrobial drops due to its unknown aetiology. It was shown by confocal microscopic study that DLK induced by corneal epithelial erosion during or after LASIK may be an inflammatory process or a keratocyte activation process only, which is treated with topical or systemic corticosteroids. Treated properly, the prognosis as to visual function is good.

Epithelial and stromal thickness after PRK and LASIK

Because CMTF scans were not available in 1993-4 when the PRK patients were operated on, we could not evaluate changes in corneal layers. Yet, compared to normal controls, the epithelium thickness was the same in both groups. As expected, the stroma was thinner after PRK.

Although statistically not significant, the total thickness of the cornea seemed to decrease slightly from 6 months to 2 years after LASIK. A relatively minute epithelial hyperplasia accompanied this finding. The importance of this hyperplasia for myopic regression after LASIK remains to be explored. Yet, at the same time a myopic regression of -0.4±0.6 D was observed. In contrast to previous studies (Chayet et al., 1998; Lohmann et al., 1998; Hjortdal et al., 2005; Ivarsen and Moller-Pedersen 2005), it seems feasible to suggest that myopic regression is not caused by changes in the corneal thickness. Accordingly, changes in the axial dimensions of the globe and/or subtle keratectasia may cause the observed regression. Alternatively, corneal viscous and elastic properties may have changed, causing altered biomechanics after LASIK (Pepose et al., 2007). Furthermore, there was no measurement of the possible corneal forward shift, which could affect the postoperative refraction (Miyata et al., 2004). Hence, it may be suggested that altered corneal biomechanical properties account for the observed regression, rather than alterations in axial lengths of the globe.

Morphological changes after PRK and LASIK

The absence of the Bowman's layer in every cornea after PRK (Seiler and McDonnell 1995) may be envisaged to hamper neural orientation and regrowth. Most probably, however, the Bowman's layer is not essential, and merely a newly formed basement membrane is sufficient to serve as a substrate for the migration of regenerating nerves. Unfortunately, a confocal microscope cannot be used to visualize a nonreflecting basement

membrane. The mechanical stabilizing properties of the Bowman's layer have appeared to be insignificant (Seiler et al., 1992). Muller et al. (2001) has suggested, however, that ablation of the most anterior interwoven collagen lamellae may reduce corneal stability. After LASIK, the Bowman's layer remains intact.

The transformation of keratocytes into highly reflective, altered, myofibroblast-like cells (Moller-Pedersen et al., 2000), as well as newly formed and irregularly deposited ECM (Lohmann et al., 1991), cause more backscattering of light, which contributes to the formation of subepithelial haze. The haze appears centrally after PRK, whereas after LASIK the central cornea is clear and haze is evident only at the flap margin. The question remains why after PRK the epithelium regains its original morphology and the anterior stroma becomes so hazy. Imbalance in the interaction between epithelial and stromal cells (Wilson et al., 1999b) and neural dysfunction (Muller et al., 2003; Wilson 2001a) are presumed to be involved in these changes.

With biomicroscopy, 4 out of 14 of PRK patients and none of the LASIK patients showed any haze, but with confocal microscopy, areas of hyperreflective keratocytes and ECM were identified in all of them. Because CMTF reflectivity in the control subjects was comparable with that in PRK-treated corneas, it can be concluded that the regeneration of corneal tissue must have been relatively complete. There were similar findings in LASIK patients. The present study indicates that corneal morphology is not reconstructed completely after PRK.

Keratocyte density

The most anterior keratocyte density after PRK could not be assessed because of significant stromal haze. After LASIK, the anterior keratocyte density decreased from 3 months postoperatively, as noted also by Vesaluoma et al. (2000a). In that study, significant changes in keratocyte cell density manifested from 6 months postoperatively. Yet, Erie et al. (2004 and 2006), in a prospective LASIK series, found that the decrease in anterior keratocyte density was more rapid and evident already 1 month after LASIK. The keratocyte density in the pre-IF stroma had not changed during the follow-up up to 2 years. A significant decrease in keratocyte cell density was, however, observed at the post-IF stroma at ≥3 months. No changes in the mid-stromal, post-stromal or posterior keratocytes were observed, in accordance with the aforementioned studies.

The reason why the anterior keratocyte density in the flap decreases after LASIK is unknown. It has been proposed, by us and others (Vesaluoma et al., 2000a; Mitooka et al., 2002), that this finding may be related to the denervation of the corneal flap, leading to a loss of neurotrophic influence on keratocytes. If this would be the reason, then we could expect that the decrease in cell density would manifest relatively rapidly, in weeks, rather than after several months when the nerves are already regenerating. Alternatively, the keratocytes might undergo intentional and uneventful cell death by the activation of the apoptotic pathways (Adrain et al., 2006). Yet, at least two independent observations are contrary to this suggestion. First, after an epithelial scrape or PRK/LASIK wound, almost immediate keratocyte apoptosis is observed and this apoptotic massacre of keratocytes is rapidly down-regulated (Wilson 2002b). Secondly, the keratocytes would need a specific signal for apoptosis to be manifested over a long term, because this process is an "all or nothing" type of reaction. It is arguable that such a signal would be elicited months after the injury when most of the cornea has already healed (Dawson et al., 2005). Erie et al. (2005) has speculated that the origin for the death signalling could be derived from viable corneal epithelial cells captured in the interface. The suggestion in the present study is that keratocyte loss is not due to the specific removal of cells by apoptosis. Yet, anterior keratocytes have been subjected to excess stress arising from the excimer procedure and they may become prematurely senescent. Accordingly, in this "one hit too many" scenario the ability of keratocytes to protect themselves against and to correct harmful events (such as UV radiation) diminishes after excimer laser procedure and thus these cells are more prone to permanent damage and therefore die too easily. The clinical significance of the gradual loss of keratocytes is unknown and it may be that a critical density of keratocytes is needed to maintain corneal clarity and strength. At least in the present study and in a previous study (Erie et al., 2006), this limit was not exceeded as the corneas in both studies were clear. Alternatively, high densities of keratocytes may be related to the protection of the cornea, as suggested by Wilson et al. (2001b).

Dawson et al. (2005) has shown that several months (≥6 months) after LASIK the altered/migratory keratocytes become quiescent and a hypocellular scar is formed. This finding is also highlighted in my study, showing that the activated/migratory keratocytes are gradually lost and the backscattered light is lost even more rapidly after LASIK. It seems that the early phase altered/migratory keratocytes are metabolically very active and participate in a normal wound-healing response. They then slowly return into their normal

quiescent phenotype, although they contain a higher number of intracellular vacuoles (Dawson et al., 2005). It should be underlined that these cells are not myofibroblasts, since such cells would most likely cause persistent stromal haze and scarring (Jester et al., 1999; Dawson et al., 2005).

Regeneration of subbasal nerves after PRK and LASIK

Reinnervation studies on corneal grafts suggest that even if epithelial innervation is restored, only minimal neural regeneration takes place in the stroma (Tervo et al., 1985). In previous CM studies faint subbasal nerves were observed in the central cornea already at 1 to 4 months after PRK (Latvala et al., 1996; Frueh et al., 1998; Erie et al., 2005). Linna et al. (2000) has shown that, after LASIK, corneal sensitivity improves as the subbasal nerves regenerate so that both function and nerve morphology seem to be close to normal in 6 months. Later, Erie et al. (2005) found that subbasal nerve density does not recover to near preoperative densities until 5 years after LASIK.

PRK has been shown to induce morphologic alterations in nerves up to 3 years after surgery (Linna and Tervo 1997; Erie et al., 2005). After 5 years, 71% of post-PRK corneas showed a branching pattern of regenerated subbasal nerves closely resembling that observed in normal control corneas. This suggests that after such a long period many patients have regained centrally the original pattern of innervation. As some surgical corneas still showed a reduced nerve fiber bundle density, it is likely that in those cases complete neural recovery will not be reached.

As shown in several studies, corneal sensation to mechanical stimulus is restored within 6 months after LASIK (Donnenfeld et al., 2003; Gallar et al., 2004; Bragheeth and Dua 2005; Tuisku et al., 2007). Yet, permanent or at least very long-term stromal nerve damage may follow as evidenced in Study III and this might affect the future healing of corneal wounds and e.g. corneal infections. In rabbits, corneal epithelial healing is almost completely arrested by sensory denervation (Tervo et al., 1985). Kim and Kim (1999) have shown that the depth of corneal ablation affected the extent of corneal sensitivity loss and recovery after LASIK. In man, nerve regeneration after LASIK continues beyond two years and our unpublished results 6 years after LASIK show that SNFB density is statistically not different from preoperative nerve density (Moilanen, unpublished). PRK enhancements after LASIK operations have recently been analyzed (Neira Zalentein

unpublished). The results suggest that these enhancements become safe only if the secondary operation is performed more than two years after the first operation.

In light of the results, it seems feasible to suggest that a critical nerve density and nerve recovery has to be maintained before the wound healing of the cornea will be accomplished. Compromised nerves do not allow for proper wound healing to take place. This is also highlighted in our earlier study which showed that surface photoablative procedures are not safe for corneal grafts due to the development of postoperative haze (Tuunanen et al., 1997).

Complications after LASIK

Despite the presence of some conjunctival hyperemia and a presumed leukocyte accumulation in the LASIK flap interface, corneas with DLK did not show inflammation in the conjunctival vessels. This finding is in contrast to cataract patients, which show significant inflammatory response in the conjunctival venules after the operation (Kirveskari et al., 2001). There were no observations of anterior chamber flare under the biomicroscope. These findings suggest that DLK represents a localized reaction within the cornea.

Development of an epithelial detachment during otherwise uneventful LASIK was probably due to basement membrane dystrophy that remained clinically undetectable, as has been reported by others (Jester et al., 1999; Rojas and Manche 2002). This is supported by the observation that both eyes of the same individual (Study IV, Table 1, patients 4 and 6) tend to behave the same way; the second eye will develop an intraoperative erosion if the first one did. For these patients, PRK might be a better procedure than LASIK (Ambrosio and Wilson 2001). Later spontaneous epithelial defects were probably associated with dry eye/neurotrophic keratitis (Dastgheib et al., 2000) and loose epithelial adhesion. Consequently, it is postulated here that the defect facilitates the epithelial stromal interaction known to eventually lead to changes in cytokine expression by stromal cells (Wilson 2001a) and eventually to keratocyte activation (Li and Tseng 1995) and inflammatory cell chemotacsis (Wilson et al., 2004; Carlson et al., 2006), possibly through limbal vasculature and/or tears.

In contrast to findings reported in previous studies (Linebarger et al., 2000; Ambrosio and Wilson 2001; Johnson et al., 2001; Melki et al., 2001; Bühren et al., 2002), in the present

study we found that the inflammatory interface reaction typical for DLK is not always associated with the presence of inflammatory cells in the flap interface. We hypothesize that DLK, at least in mild cases following an epithelial injury, may represent only an activation of keratocytes and formation of altered ECM through epithelial–stromal or inflammatory cell–keratocyte interactions on both sides of the interface. However, in clinically more clear cases repopulation of the flap interphase with leukocytes is clearly visible even by confocal microscope. These confocal microscopic observations are based on the size and morphology (Bühren et al., 2001 and 2002), of these cells. This interpretation is also supported by the findings in the present study (patients 1 and 3). Furthermore, corticosteroids are known to reduce chemotaxis (Phillips et al., 1996) and inflammatory reaction (Kirveskari et al., 2002), and have also been reported to improve symptoms of DLK in clinical (Shah et al., 2000; Hoffman et al., 2003) and experimental studies (Holzer et al., 2002; Holzer et al., 2003). This supports the suggestion that inflammatory cells may act as a trigger in the pathogenesis of DLK. Interestingly, the presence and density of dendritic cells in the central cornea and at the limbus is not affected by PRK (Mastropasqua et al., 2006).

It was expected in the present study that the presence of inflammatory cell-like objects, as imaged by Bühren et al. (2001 and 2002) and in this study, would induce a notable inflammation in conjunctival venules or in the anterior chamber. This study, however, showed that conjunctival vessels do not appear to mediate the extravasation of leukocytes into the flap interface. The area examined by confocal microscopy was 3.0 mm to 6.0 mm from the limbus and thus represents superficial vessels derived from the anterior ciliary artery branches (Warwick 1976). The unimaged pericorneal vasculature derived from the deeper peripheral arcade might also serve as a source for leukocytes. Furthermore, it does not seem likely that the inflammatory cells would enter the flap from the clear and noninflamed anterior chamber. Although tear fluid secretion and dynamics are impaired immediately after LASIK due to neural damage, inflammatory cells in the LASIK flap interface may derive from tear glands or deep pericorneal vessels and enter the cornea through the epithelial defect.

10 Conclusions

The confocal microscope is a unique instrument to examine corneal structure in vivo in humans. For over a decade, it has been an invaluable tool for the evaluation of corneal wound healing and long-term stability after refractive surgical procedures. Furthermore, ivCM has markedly increased our ability to assess complications after PRK and LASIK. Tandem scanning confocal microscopy measures corneal morphology and organization from the areas needed accurately and flexibly. It can detect micro-organisms, such as acanthamoeba, microsporidium and fungi, and inflammatory cells in a noninvasive manner in the human cornea. These all highlight the unique capabilities of tandem scanning confocal microscopy, to which newer instruments have only added some sharpness of the images. One must, however, remember the confocal microscope's small area of observation, which makes it obligatory to interpret the findings in accordance with the clinical and biomicroscopical status of the patient.

PRK and LASIK are accurate and considerably safe methods to correct refractive errors. Human ivCM studies have shown, however, that neural recovery is still incomplete after several years postoperatively, and furthermore, that the decrease of anterior stromal and LASIK flap keratocyte density exceeds that of normal aging. These findings warrant special attention on the long-term corneal integrity, and especially, on occasions of other ocular operations or reoperations. Furthermore, PRK and LASIK are still far too expensive and the laser equipment too sensitive to environmental factors to be a real solution for refractive correction in the areas where their need is highest. Laser surgical methods (wavefront technology, femtosecond laser) are continuously undergoing refinement, which may improve the accuracy and safety of the procedures, but makes them also more expensive

An optimal surgical method to conquer refractive disorders has not yet been found. Further studies are needed to solve the principal mechanisms of wound healing behind the morphological drawbacks.

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13 Original publications