In vivo corneal confocal microscopy in diabetes

PhD Thesis Summary

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I. INTRODUCTION

The selection of topic defines a research project, and the selection of title defines the expectation of readers. Reading the title of my thesis, thus getting the first glimpse at my research presented, one could ask the following questions: Why the cornea? Why diabetes? Why diabetic keratopathy? And: why in vivo confocal microscopy?

Why the cornea?

The cornea is one of the most interesting and most remarkable tissues in the human body. The healthy cornea is transparent, a window to the world enabling vision. The transparency of the cornea stems from its layered structure, with every layer having a strictly organized, uniform cell structure. Transparency depends on the delicate balance of the components of this highly complex structure. If regularity breaks down due to any disease condition or other pathology the transparency decreases, thus deteriorating vision.

Another very remarkable property of the cornea is that it is one of the most densely innervated tissues in the body, having the highest number of sensory nerve fibers in a given volume. It is estimated that there are approximately 7 000–16 000 nerve fiber endings per mm$^2$ within the superficial epithelial layer. For comparison, the corneal innervation is 20–40 times denser that of tooth pulp and 300–600 times that of skin.

Why diabetes?

Diabetes, especially its most prevalent type, type-2 diabetes mellitus (accounting for 90-95% of all diabetes) is endemic in developed nations but also getting more prevalent in the developing countries, affecting 5-6% of the global adult population. Its importance is growing, as it occurs not only in aged population, but an increasing number of middle-aged and young adults are affected by the disease, in both developed and developing nations. With its increasing incidence and prevalence and the improving healthcare services the life expectancy of diabetic patients increases, thus they have to live longer with the condition and also with its complications.

Why diabetic keratopathy?

Diabetes mellitus affects all tissues of the eye, but its most well-known and most investigated complication is diabetic retinopathy. The previously underrated diabetic keratopathy and keratoepithelopathy have also become to the focus of attention, lately. Changes in diabetic corneas are believed to be connected to
peripheral diabetic neuropathy; moreover they also lead to impaired (worse and prolonged) wound healing.

There are numerous ophthalmological and optical interventions affecting the cornea, e.g. contact lenses, corneal transplantation, etc. Also, the novel methodology of cataract surgery leads to the widespread application of corneal incisions. Moreover, cataract surgeries are performed at younger age than done previously. By the same token, the widespread use of modern excimer laser refractive surgeries also became an important procedure affecting the cornea.

Diabetes used to be relative contraindication for most ophthalmological interventions. However, the longer life expectancy of diabetics, and the steady and rapid improvement of medical technologies lead to an increased number of ophthalmologic interventions in diabetic patients, lately.

It is important to stress, however, that diabetic keratopathy can worsen wound healing after otherwise successful cataract and refractive surgeries. Accordingly, diabetic keratopathy can in one hand diminish visibility during long vitrectomies, and on the other hand it can cause prolonged reepithelization.

And: why in vivo confocal microscopy?

In vivo confocal microscopy is an ideal approach for the investigation of the cornea in vivo. It can visualize the structure, layers, and cells of the cornea with high resolution, in real time, and non-invasively. In principle, it is a histological examination without invasiveness or staining, and most importantly in a living person.

In vivo confocal microscopy can help investigating the background and mechanisms underlying diabetic keratopathy, follow-up of its progression, and assess the efficacy of therapeutic approaches. The future clinical potential of the technique lies in its possible prognostic value for the evaluation of diabetic population before interventions affecting the cornea.
II. AIMS

1. Corneal cell density measurement in vivo by scanning-slit confocal microscopy: method and validation

Our aim was to develop and validate a non-invasive, fast, reliable, and reproducible method for in vivo quantitative (cell counting) measurements of corneal cell layers using a commercially available scanning-slit confocal microscope (SSCM).

2. In vivo confocal microscopy in diabetic keratopathy: quantitative and qualitative examinations

2.A. Corneal cell densities in six corneal layers in diabetes

We aimed to describe qualitative and quantitative changes in the human cornea induced by diabetes, in vivo, in early stage of the disease.

We also aimed to quantify the corneal cell densities in six corneal layers and compare the differences between diabetic patients and healthy controls using our previously developed and validated cell counting method utilizing in vivo confocal microscopy.

It is known, that the average corneal autofluorescence (CAF) in diabetic patients is higher than that of healthy controls. Moreover, it increases with the severity of diabetic retinopathy. We aimed to evaluate whether corneal cell density differences could be the cause of the increased corneal autofluorescence in diabetics.

2.B. The subbasal nerve plexus in diabetes

We aimed to assess the corneal innervation, particularly the subbasal nerve plexus in diabetes, to observe early pathologic changes caused by the disease using in vivo confocal microscopy.

Very highly reflective cells (HRC) were observed immediately beneath the basal epithelial cells’ layer, typically located in close vicinity of subbasal nerves. We aimed to describe the features of these cells, both qualitatively and quantitatively.
2.C. The corneal stroma in diabetes, stromal nerves

Vast majority of cells visible on confocal microscopy images of the corneal stroma are keratocyte nuclei. However, besides the keratocyte nuclei, other structures, e.g. stromal nerves, can also often be visualized on stromal images.

We aimed to observe and describe the properties of stromal nerves in diabetic patients and healthy controls.

We have also found structures consisting of a highly reflective head and a vague tail (straight or undulated) in the stroma. We aimed to describe the features of these structures in diabetic patients and healthy controls, qualitatively and quantitatively.
III. METHODS

Setting. The studies were conducted in the Center of Ophthalmology, Institute of Biomedical Research on Light and Image (IBILI), University of Coimbra, Coimbra, Portugal.

Participant selection. Patients and controls were recruited in a consecutive way, between July 2001 and July 2002. They underwent a slit-lamp examination and epithelial abnormalities were checked by fluorescein staining. All participating corneas were transparent and free from any visible ophthalmic pathology. Patients wearing contact lenses, having an acute eye disease or previous ophthalmic history, except due to diabetes, were excluded.

1. Corneal cell density measurement in vivo by scanning-slit confocal microscopy: method and validation. Twenty healthy controls with age between 28 and 74 years (average: 53.7) without previous ophthalmic or systemic disease were recruited. All healthy controls underwent a prior slitlamp biomicroscopy examination. In vivo confocal microscopy was performed according to the protocol; cell densities were determined in six corneal layers.

2.A. Corneal cell densities in six corneal layers in diabetes. Fifteen type-2, non-insulin dependent diabetic patients (9 men, 6 women) with level 20 of retinopathy according to the Early Treatment of Diabetic Retinopathy Study (ETDRS), aged between 39 and 69 years (mean age 60.9 ± 8.5 yr) were selected, at their first visit to the Ophthalmology Clinic at the Coimbra University Hospital after referral by the physician. Diabetes duration ranged between 2 and 20 years (mean duration of diabetes: 11.3 ± 6.2 yr). Fifteen healthy controls (4 men, 11 women) aged between 35 and 74 years (mean age 59.3 ± 11.0 yr) were selected for comparison.

2.B. The subbasal nerve plexus in diabetes. Twenty type-2, non-insulin dependent diabetic patients, aged between 50 and 75 years (11 men, 9 women), 10 with mild (level 20 ETDRS, DR20) and 10 with moderate (level 35 ETDRS, DR35) retinopathy were selected. Ages in the patient groups (DR20: 58.9 ± 9.37 yr, DR35: 60.1 ± 5.65 yr) and in the ten age-matched healthy controls (mean age 60.1 ± 7.98 yr) were similar.

2.C. The corneal stroma in diabetes, stromal nerves. Eight type-2, non-insulin dependent diabetic patients, aged between 50 and 75 years (4 men, 4 women), 4 with mild (level 20 ETDRS, DR20) and 4 with moderate (level 35 ETDRS, DR35) retinopathy were selected. Ages in the patient groups (DR20: 60.2 ± 8.37 yr, DR35:
62.1 ± 5.43 yr) and in the 8 age-matched healthy controls (mean age 62.33 ± 6.98 yr) were similar.

**Instrumentation.** The confocal microscope used for *in vivo* examination of human corneas was a SSCM (Confoscan P4, Tomey, Fortune Technologies, Vigonza, Italy) equipped with the standard Achromplan non-applanating water immersion objective (Zeiss, Oberkochen, Germany, 40x/0.75 numeric aperture). This instrument, based on the confocal principle, visualizes *in vivo*, on a computer screen, yellow light reflected by corneal structures in the measurement volume (optical section).

**Examination protocol.** After administration of a drop of topical anesthetic in the eye (0.4% oxybuprocaine chlorhydrate, Anestocil®, Oftalder, Oeiras, Portugal) and one drop of clear gel (Sodium hyaluronate 10 mg/ml, Healon®, Pharmacia & Upjohn, Uppsala, Sweden) as immersion fluid for optical coupling between objective lens and cornea the patient was seated in front of the microscope. Head and eye movements were minimized with the aid of a chin rest and a flashing fixation lamp for the contralateral eye.

During image acquisition optical sections were displayed in real-time (25 frames per second) on a computer screen and were also recorded on S-VHS videotape. Image acquisition was performed by scanning the cornea along the optical axis, in perpendicular slices. Several (6-10) entire depth scans were recorded from epithelium to endothelium. *In vivo* examination of one eye took about two-three minutes, resulting in the recording of approximately 3000 – 4500 video frames per eye.

Image analysis, i.e. the qualitative and quantitative analysis of the recorded images was performed already without the patient present. The best-focused images (with no motion blur) recorded on videotape were selected for later analysis. The selection of the images (based on cell layer classification) and analysis of the recorded data took about one hour per eye.

**Classifications of the different corneal cell layers.** Classification of corneal layers was based on the morphological features described in the thesis.

Six corneal layers were considered for cell density measurements: superficial epithelial, basal epithelial layer, anterior-, mid-, posterior stroma and endothelium.

For subbasal nerve evaluation, particular attention was paid to the layer of the subepithelial nerve plexus and the neighboring layers.

**Counting method.** Counts were performed semi-manually using the best focused images (with no motion blur) recorded on videotape. Each defined cell or
nucleus in a predefined rectangular frame was marked on the computer screen. An unbiased counting frame of Gundersen was used: all cells even partially inside the frame were marked provided they did not intersect the two exclusion edges of the frame.

The precise area of the counting frame and the surface cell density (number of cells per mm$^2$) were calculated by the Confoscan P4 microscope’s integrated software (Confo-Commander v2.7.1, Tomey, Erlangen, Germany).

For the superficial epithelial, basal epithelial and endothelial cells, surface density (number of cells per mm$^2$) was used since these cells are assumed to form monolayers. For the (anterior, mid and posterior) stromal layers, volumetric density (number of nuclei per mm$^3$) was determined. The surface density of stromal keratocyte nuclei was first determined with the Confoscan software using the counting frame and the marked cells. The volumetric density required for stromal keratocytes was calculated using stereologic principles. Total stroma cell density was calculated using a weighted mean of anterior, mid-, and posterior stroma cell densities, with the weights being the relative thickness of each layer.

For the measurement of cell densities in human corneas, five different, independent images were evaluated from each corneal cell layer.

Validation

Point spread function (PSF). The optical sectioning property of the microscope depends on its axial resolution. The axial resolution can be evaluated by the full-width at half maximum (FWHM) of the PSF. We measured the PSF using the technique of moving a perfect reflector axially through focus. The experimental set-up comprised a Melles-Griot (Irvine, CA, USA) model 01 MFG 011 plane mirror mounted on a precision positioner (Melles-Griot MicroBlock™ 17 AMB 003). The microscope was fixed, focused on a given plane, and the mirror was scanned through that plane at 1 µm steps. For each step an image was recorded. The intensity values obtained from each image were used to calculate the PSF.

Image size and distortion. On screen image distortion was evaluated using a USAF 1951 Chromium test target (Melles-Griot model 04TRP003) positioned at various places in front of the microscope. The dimensions of a calibrated square on the target (55.2 µm × 55.2 µm) were measured using the Confoscan P4 software. The counting area reported by the Confoscan P4 software was also validated with the test plate.
Validation of the image classification: The criteria used to classify images, based on the layer definitions given above, were validated by measuring the position of the measurement volume along the optical axis. The relative position of such an optical section was visualized on a digital voltmeter connected to a precision potentiometer coupled to the calibrated microscope position wheel. This method requires an immobile eye between the measurement of a reference layer and that of the optical section. We used a whole donor eye (two hours post mortem) and one eye of a healthy volunteer. Confocal corneal microscopy was performed as usual and the positions of the selected images were compared to those of the layer classification. The total thickness of the cornea was confirmed in both cases by ultrasonic pachymetry.

Repeatability of the measurements. The intra-session repeatability was assessed by repeating measurements on a given cornea, using different images selected from the same recording session. The inter-session repeatability was assessed by repeating measurements on the same cornea after 14 days. The measurements were done by the two observers on three healthy volunteers. The intra- and inter-session repeatability coefficients were calculated according to the definitions issued by the British Standards Institution.

Validation of cell densities. Validation of the use of confocal microscopy for density measurements was performed, in each corneal layer, by comparing the results obtained on three human donor corneas by confocal microscopy in optical sections perpendicular to the optical axis of the cornea with those obtained by a conventional light microscope (Nikon Eclipse TE 300, Tokyo, Japan) on the same cornea in sections cut perpendicularly to the optical axis. The donors were free of ophthalmic diseases; biomicroscopic observations of the donor corneas did not show any morphological changes.

After collection of the eye (two hours post mortem), all corneal layers were examined with confocal microscopy. Immediately after the examination the cornea was excised, cut with an 8 mm diameter calibrated corneal trephine and fixated with 10% buffered formalin. After fixation, the cornea button diameters were measured with calipers. Shrinkage was measured by comparing the button diameters before and after fixation. The button was prepared for histology by embedding in paraffin and cutting 8 µm thick frontal sections. The sections were stained with hematoxylin-eosin. Volumetric cell densities were calculated from the corresponding surface densities using the same stereologic technique applied in confocal microscopy, with correction for tissue shrinkage, which was assumed equal for all corneal layers.
IV. RESULTS

1. Corneal cell density measurement in vivo by scanning-slit confocal microscopy: method and validation - results

Point spread function, image size and distortion. The measured PSF had a FWHM value of 8 µm. The image of a 55.2 µm × 55.2 µm (= 0.00305 mm²) calibration target measured at five locations on the screen yielded 57.4 ± 0.9 µm as average width, 53.0 ± 1.4 µm as average height, and 0.00304 ± 0.000094 mm² as average area. The maximal deviation was less than 3%.

Validation of the image classification: All layers, as defined by the classification, were indeed imaged at their expected depth, except the in vivo measured basal layer which was 3% below the range limit. Our depth measurement results showed that a morphology based layer and cell classification method is a viable solution for repeatable and consistent layer selection in corneal confocal microscopy.

Repeatability of the measurements. The average (all corneal layers, both observers) intra-session repeatability coefficient was 5.8 % (range: 0.4 – 10.2%) and the average inter-session repeatability coefficient was 8.3% (1.9 – 16.9%). In both cases the best repeatability was observed on the endothelium.

Validation of cell densities. The cell densities measured with the confocal microscope were validated by histology. The results were obtained on three donor corneas. The difference between both methods was on an average -1.3% and ranged between -24.1% (posterior stroma) and +16.4% (basal layer). The mean difference between stromal cell densities by the two methods was -2377 cells/mm³. The 95% confidence limits of agreement were -10742 cells/mm³ and 5989 cells/mm³. Mean differences for the superficial, basal and endothelial cell densities were -22.1, 899.1 and 231.1 cells/mm², respectively. The measured mean tissue shrinkage was 6.0%.

Cell density measurements in healthy controls. Superficial epithelial cell density was 759 ± 162 cells/mm² (mean ± SD). In the basal epithelial layer, the cell density was 5817 ± 632 cells/mm². The volumetric cell density in the mid-stroma (19578 ± 4421 cells/mm³) was significantly lower than that in the anterior (28616 ± 3081 cells/mm³) or posterior stroma (26073 ± 5962 cells/mm³). Endothelial cell density was 2743 ± 285 cells/mm².
2.A. Corneal cell densities in six corneal layers in diabetes - results

No visible difference between diabetic patients and healthy controls was found in the morphology of cells in any of the corneal layers.

The calculated cell densities in each layer along the optical axis of the cornea were normally distributed both in healthy controls and diabetic patients according to the D’Agostino’s test for departure from normality. Since the inter-observer repeatability was on average 7.2%, the results of both observers were averaged.

The number of cells per unit area of superficial, basal epithelial, and endothelial layers in diabetic patients were 815 ± 260, 5060 ± 301 and 2660 ± 364 cells/mm², and in healthy controls were 725 ± 171, 5950 ± 653 and 2690 ± 302 cells/mm² respectively. The number of keratocytes per unit volume of the anterior-, mid- and posterior stroma of diabetic patients were 27560 ± 3880, 21930 ± 2110 and 25790 ± 3090 cells/mm³, in healthy controls were 26300 ± 4090, 19390 ± 3120 and 25700 ± 3260 cells/mm³, respectively.

A highly significant lower density was found in the basal layer (-15.0%, p = 0.0004) in diabetic patients compared with healthy controls. No significant differences in cell densities between healthy controls and diabetic patients were found in the other layers (p > 0.07).

The mid-stromal cell density was significantly lower than both the anterior and posterior stroma cell densities (p < 0.0001).

2.B. The subbasal nerve plexus in diabetes - results

The number of subbasal nerve fiber bundles per confocal image in mild and moderate diabetic retinopathy patients were similar to each other (DR20: 1.25 ± 0.52 and DR35: 1.22 ± 0.60, p = 0.996), and was significantly lower in both diabetic groups than in healthy controls (3.90 ± 1.13, p<0.001). In diabetic corneas we have also observed increased subbasal nerve tortuosity.

Although there was no significant difference in the density of basal epithelial cells between level 20 and level 35 diabetics (DR20: 5145 ± 237 cells/mm² DR35: 5042 ± 236 cells/mm², p = 0.6) a significant reduction in the number of basal cells was observed in both groups compared to the healthy controls (5648 ± 236 cells/mm², p < 0.001).

Furthermore, very highly reflective cells (HRC) were observed immediately beneath the basal epithelial cells’ layer, typically in close vicinity of subbasal nerves. Since the morphology of HRC differs from that of keratocytes, we consider
them as another type of cells, most probably Langerhans- or dendritic cells. The number of HRCs was significantly higher both in level 20 and level 35 diabetics (DR20: 2.36 ± 1.12 and DR35: 3.10 ± 0.97) than in healthy controls (1.13 ± 0.72, p < 0.02). No differences were found between level 20 and level 35 diabetics (p = 0.207).

2.C. The corneal stroma in diabetes, stromal nerves - results

We observed elongated, thick, bandlike and reflective structures besides keratocyte nuclei on several confocal images recorded from the anterior and mid stroma. These structures are stromal nerves. Healthy stromal nerves are straight entities, with occasional branching. Undulated structures with dimensions similar to large stromal nerves were also observed in diabetic patients.

Structures consisting of a highly reflective head and a vague tail (straight or undulated) were also found mainly in the anterior and mid-stroma of some diabetic patients and healthy controls. They were often observed near the stromal nerves and were rare in posterior stroma.

The average number of these structures found during entire-depth scans (from epithelium to endothelium) was 52.5 ± 9.3 and 40.0 ± 14.0 in level 20 and 35 diabetic patients, respectively. There were significantly less of these structures present in healthy controls (6.8 ± 2.1, p < 0.001).
V. CONCLUSIONS AND SUMMARY OF NEW RESULTS

1. Corneal cell density measurement in vivo by scanning-slit confocal microscopy: method and validation

We developed and validated a non-invasive, fast, reliable and reproducible method for in vivo quantitative (cell counting) measurements of corneal cell layers using a commercially available scanning-slit confocal microscope.

We described the classification of corneal layers based on in vivo confocal microscopic morphologies, detailed the methods of image acquisition and the semi-manual counting method, explained the determination of surface and volumetric cell densities, and proved the repeatability and reliability of the measurements also by validation of all densities by histology.

We showed that valid, repeatable quantitative measurements of corneal cell density can be performed with the SSCM Tomey Confoscan P4 in conscious humans based on the cell layer classification presented, and using semi-manual cell counting technique. The cell densities measured with the confocal microscope were also validated by histology.

Taken together, our method provides reliable cell density measures in six layers (depths) along the optical axis of the cornea.

The significance of our validation study is shown by the fact that it gained steady interest in the literature; we are often contacted by colleagues interested in cell density measurements of the cornea. Until the submission of this thesis (April 2009) we have tracked 17 independent citations, including a textbook chapter.

2. In vivo confocal microscopy in diabetic keratopathy: quantitative and qualitative examinations

From all the ophthalmological complications of diabetes, we contributed to the body of clinical and scientific knowledge regarding diabetic keratopathy by showing that changes occurring already in the early stages of disease can be visualized and evaluated non-invasively using in vivo confocal microscopy.

The importance of the topic and our work is shown by the numerous studies conducted in parallel with our investigation and the several papers published since then.
2.A. Corneal cell densities in six corneal layers in diabetes

We performed in vivo cell density estimation and comparison in six layers along the optical axis of the cornea of type-2 diabetic patients and healthy controls using the non-invasive cell counting method we developed and validated earlier.

We found significant difference between cell densities of diabetic patients and healthy controls in the basal layer (the cell densities of diabetics being lower) but not in the other layers.

A significant lower cell density was found in the mid-stroma of both diabetic patients and healthy controls, in comparison with anterior and posterior stromal layers, however this decrease was less prominent in diabetic patients (15.0% versus 24.6% of healthy controls, p = 0.053), the difference being on the borderline of significance.

We also investigated whether corneal cell density differences could be the cause of the increased corneal autofluorescence in diabetics. We showed that changes of cellular density in the stroma are probably not responsible for the increased autofluorescence. Role of changes in the basal epithelial cellular density can be excluded, as well, because of the homogeneous autofluorescence distribution over the corneal axis.

2.B. The subbasal nerve plexus in diabetes

Using in vivo confocal microscopy, we demonstrated that corneal innervation is impaired in diabetes already at an early stage of the disease. The number of subbasal nerve fiber bundles is significantly decreased in type-2 diabetic patients, when compared to healthy controls, likewise the basal epithelial cell density. The decrease in the number of subbasal nerve fiber bundles and of basal epithelial cells in the corneas of diabetic patients is characteristic of the disease and might be related to each other.

Furthermore, we observed very highly reflective cells immediately beneath the basal epithelial cells’ layer, typically in close vicinity of subbasal nerves. The number of these cells was significantly higher in diabetic patients than in healthy controls. We consider them most probably Langerhans- or dendritic cells.
1. 2.C. The corneal stroma in diabetes, stromal nerves and other highly reflective structures

According to our knowledge we were the first to investigate and describe corneal stromal nerves in type-2 diabetic patients using in vivo corneal confocal microscopy.

In diabetic patients, undulated structures with dimensions similar to large stromal nerves were also observed. We consider them as degenerated or pathologically regenerating stromal nerves.

In the corneal stroma, structures consisting of a highly reflective head and a vague tail (straight or undulated) were also found. The origin of these structures is yet unknown, nevertheless we observed that they are significantly less prevalent in healthy controls than in diabetics.
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INDEPENDENT CITATIONS RELATED TO THE TOPIC OF THE THESIS


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