UNIVERSITY OF LATVIA
FACULTY OF PHYSICS AND MATHEMATICS
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CENTRAL CORNEAL ENDOTHELIUM IN NORMAL TRENTINO'S POPULATION

BACHELOR THESIS

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ANOTĀCIJA

Bakalaura darbs uzrakstīts angļu valodā uz 30 lappusēm. Tas satur 18 attēlus, 8 tabulas un atsauces uz 23 literatūras avotiem.

Pētījuma mērķis bija novērtēt tādu faktoru kā smēķēšana un kontaktlēcu lietošana ietekme uz radzenes endotēlija fizioloģiju.

Metode: 45 pacientiem (vecumā no 29 līdz 58 gadiem) veikta aptauja par smēķēšanas ieradumiem un kontaktlēcu lietošanu. Ikvienam tika novērtēts endotēlijs, trīs reizes mērot ar Takagi 700GL spraugas lampu un EndoKer programmu.

Rezultāti: Darbā netika atrasta nozīmīga korelācija starp dzimumu un vecuma grupām attiecībā uz kādu no ietekmes faktoriem. Kā arī nebija nozīmīga atšķirība starp smēķētājiem, kontaktlēcu lietotājiem un normālo populāciju.

Secinājumi: Lielāka izlases grupa uzrādītu ietekmes faktoru saistību vai jāskatās arī uz citiem parametriem kā radzenes liekums un biezums.

Atslēgas vārdi: radzenes endotēlijs, Trentino populācija, EndoKer, smēķēšana, kontaktlēcu lietošanu.

ABSTRACT

The Bachelor thesis is written in English on 30 pages. It contains 18 images, 8 tables and 23 references.

The aim was to establish whether factors such as smoking and contact lens wearing or the combination of more can affect endothelial physiology of a normal healthy population.

Method. All the 45 patients (aged 29 to 58 years old) have filled up a questionnaire about their habit of smoking and contact lenses wearing. For each subject have been gathered three measurement, using a Takagi 700GL slit lamp and EndoKer software.

Results. No significant differences were found between genders or between age groups, for none of the considered parameters. Moreover, there were no significant difference between smokers, contact lens wearers or normal population.

Conclusion. A bigger sample could show different results, or we shall consider different parameters, such as corneal curve and thickness.

Keywords: corneal endothelium, Trentino's population, EndoKer, smoking, contact lens wearing.

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INTRODUCTION

The tiny piece of tissue, which is our cornea, is the only window on the external world for our brain. This delicate passage of precious information and sensations is kept in balance by a unique physiology, which places transparency as its main goal.

Corneal endothelial layer play an important role into the regulation of this physiology and in keeping our cornea transparent. This layer is made up of semi-permanent cells, which structure naturally changes throughout the course of our life. This natural decay can be enhanced by situations of physiological stress and hypoxia.

It is still an open debate about what factors of our lifestyle can play a significant role in altering corneal endothelial physiology and many studies have been made about the influence of continuous contact lenses wearing, with contrasting results, or pathological situations. My question was how much other diffused habits, such as smoking, can affect this delicate physiology. Differently from other studies, the measurements were taken using a Takagi 700GL slit lamp with endothelial biomicroscope and using EndoKer as software for the automatic count and morphometric evaluation of endothelial parameters.

The aim was to establish whether factors such as smoking and contact lens wearing or the combination of more can affect endothelial physiology of a normal healthy population. The results have been compared to studies conducted with specular microscopy.

Tasks

1. to evaluate the influence of age on the corneal endothelium parameters;
2. to evaluate the influence of gender on the corneal endothelium parameters;
3. to determine the possible correlation between smoking and endothelium parameters;
4. to determine the correlation between contact lens wearing period and corneal endothelium parameters.

1. REVIEW OF LITERATURE

1.1. Endothelial physiology

Endothelium is built up from a flat cell layer, with a normal area of 200-300 μm² per cell circa, mainly of hexagonal shape (around 60% at birth). This monolayer of cells is generally 6 μm thick and plays a crucial role in the maintenance of ocular physiology. As far as cornea needs to remain transparent, no blood vessels are allowed to pass through it, so the nutrients can reach the stroma only by diffusion, which is enhanced by the pressure that aqueous humour generates toward the endothelium. It acts as a semi-barrier: the junctions between each cell allow the passage of ions towards the aqueous humour, of nutrients to the stroma and avoid too much fluid exchange, which would lead to a corneal swelling (edema), and so keeping the stroma to its normal level of hydration with 70% of water content. The more the hexagonal structure is kept, the more effective this semi-barrier will be. As far as endothelial cells junctions cannot stop all the aqueous to flow to the stroma, water excess is carried out through a weak ion pump, like Na+/K+/ATPase pump, generated by endothelial cells themselves.

From the birth, when we can find a mean cell density up to 6000 cells/mm², the number of these cells start to decay (Nucci et al., 1990), as far as they are not able to replicate in vivo or without an external stimulation (Senoo & Joyce, 2000). When we reach the age of 5 years old their number is already 3500 cells/mm² and then decreases with a rate of 0,6% every year with an average of 3400 cells/mm² at the age of 15 years old up to 2200 cells/mm² at 85 years of age (Bourne, 2003) (see Table 1.1.).

Aging process is usually symmetrical between the two eyes; while little differences are tolerated, a disparity higher than 280 cells/mm² is considered abnormal (Thomas, 2009).

Table.1.1.

Table for an esteem of the normal mean cell density, according to Bourne (2003) and Thomas (2009).

Age Group	Bourne (2003)	Thomas (2009)	estimated SD
31-40	3006	2800	
41-50	2830	2700	± 400
51-60	2665	2500	

In response to a trauma (such as intraocular surgery) or to cell death due to a normal aging process, endothelial cells migrate towards the empty area and expand to cover up the space left from the dead cells. Then they create new junctions to re-establish the effectiveness of their barrier against aqueous humour. This mechanism leads endothelial cells to vary their shape from the original hexagonal one and creates a phenomenon that we know as polymegetism (see Fig.1.1).

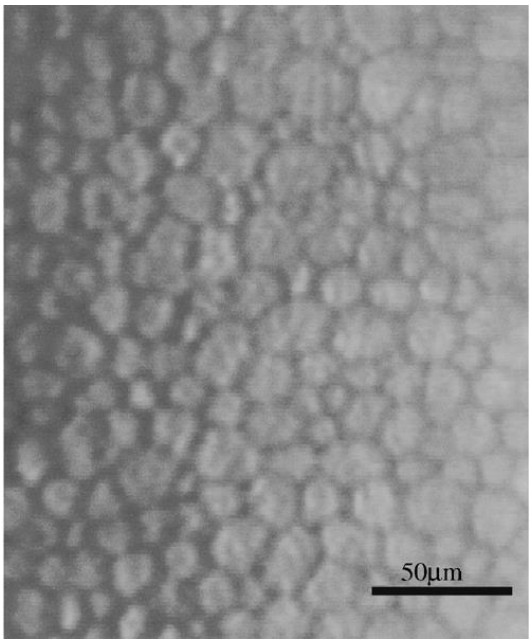

Fig.1.1. Polymegetism and pleomorphism in a long-term contact lens wearer (Bourne, 2003). CD=3081 cells/mm²; COV=44%; HEX=43%.

Clinically, polymegetism is represented by the Coefficient of Variation of cells (COV). As an effect of aging process, a COV from 20% to 30% is considered normal, while a rate over 40% is considered abnormal. Abnormal values can be found in presence of other ocular or systemic pathologies, such as glaucoma, uveitis or diabetes (Thomas, 2009).

Another important task of endothelium is that of producing collagen for the Descemet's membrane: since before our birth, endothelial cells start to produce banded collagen layers, which can reach a thickness of 2 µm circa. It will be the basis for a non-banded collagen membrane that will continue to grow throughout our life, reaching a thickness of 10 µm circa. Under physiological stress, some case have been reported of endothelial cells producing banded collagen layers after birth, and this can cause a further thickening of Descemet.

It is very difficult to assess normal parameters of corneal endothelium, as far as there still is much work to be done to analyse the differences between the world populations. The previously mentioned values come from American researchers, but was not underlined whether

only Caucasian subjects composed their sample or not. However, aa parameter that goes beyond the race is the cell density critical level: if mean cell density goes under 300 to 500 cells/ mm² circa, the integrity of their bounds can be compromised, as well as their semi-barrier function. Although, this function can be altered also from the irregularities in cells' shape, despite a normal cell density. This phenomenon is known as pleomorphism and is indicated by the percentage of hexagonal cells present in the central endothelium: a rate under 50% is usually considered at risk of developing other endothelial pathologies (Thomas, 2009), though some studies consider normal to find lower hexagonality values, up to 40% or even 30% if we consider the research of Snellingen et al. (2001).

As our sight mainly depends on the activity of these cells and as we still know so little about them, it is very important to continue the research on this topic and gather more data. Therefore, we will be able to assess better criteria on their role in the maintenance of a good ocular physiology and subsequently a transparent cornea.

1.1.1 Endotheliopathies

Endotheliopathies are generally divided between primary and secondary, determined by the fact that they can be independent pathologies or can be associated with other systemic factors or pathologies.

The most common irregularity considered as primary endotheliopathy, which we can find during endothelial screening, is corneal guttata. It is more frequent in women and often enhanced by age (present in 70% of population over 40 years old (Thomas, 2009). Zoega et al. (2006) also found a correlation between smoking more than 20 cigarette packs per year (which means more than one per day, if we consider normal packs contain 20 cigarettes) and the predisposition in developing cornea guttate (see Fig.1.2 and 1.3).

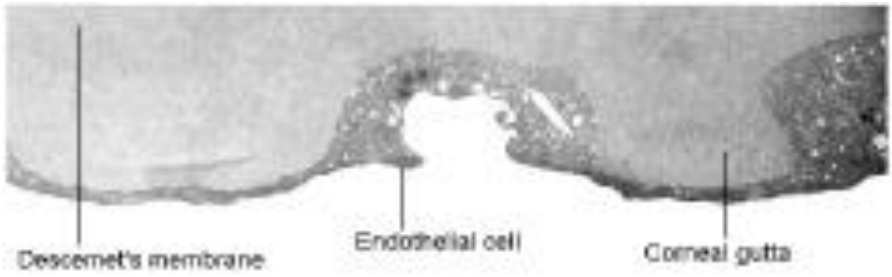

Fig.1.2. Transmission electron micrograph of corneal guttate from Thomas (2009).

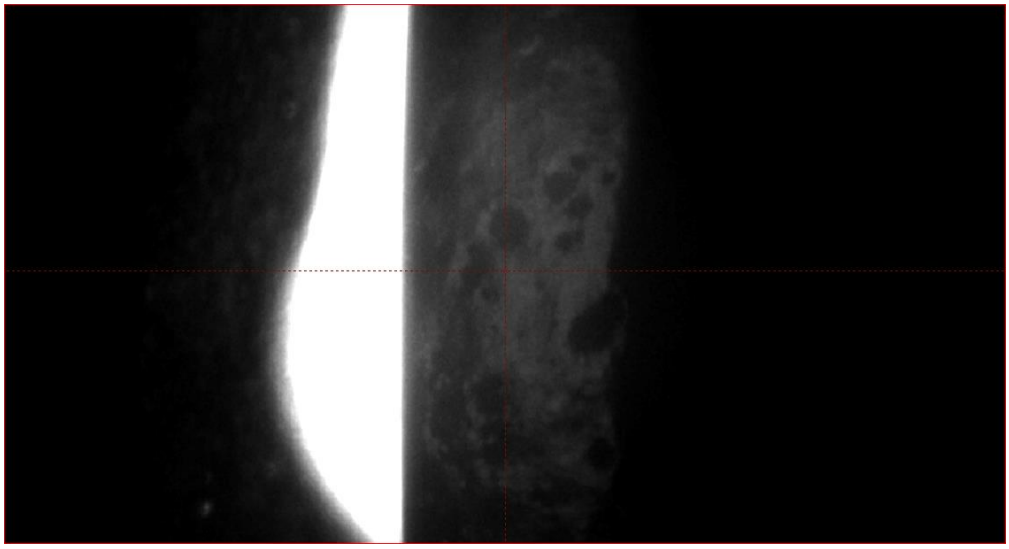

Fig.1.3. Corneal guttata of a patient of Centro Ottico Optometrico di Prezzi Sergio, found during the measurement for the present study. The patient is a female subject of 56 years of age. She had never complained anything related to her visual acuity or her eyes in general. She never smoked.

As said before, endothelial cells are supposed to produce a collagen layer that will form the Descemet's membrane. Under physiological stress, endothelial cells can produce abnormal amounts of collagen in a non-uniform distribution that creates a thickening of the Descemet membrane. These areas appear to the microscope as dark holes in the mosaic structure, though fortunately in most cases does not affect visual acuity. It generally starts from a single cell, then expanding to the neighbours, involving also a variation in shape of those cells adjacent to the guttata's borders in advanced phases. If associated with asymmetrical cell loss and significant levels of pleomorphism and polymegetism, it stays in the family of age-related endotheliopathies.

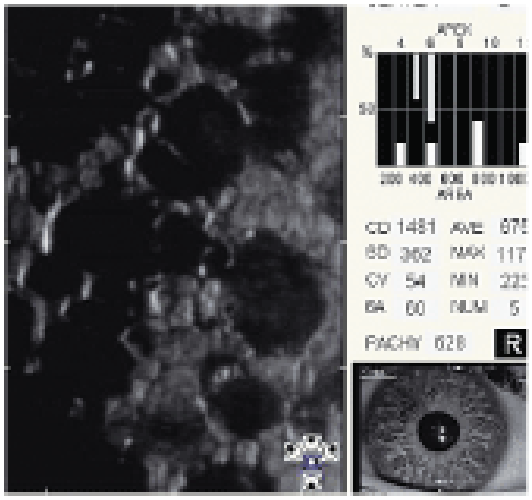

Fig.1.4. Fuch's dystrophy (Thomas, 2009).

More rarely, we can find bilateral corneal guttata: the endotheliopathy takes the name of Fuch's dystrophy (see Fig 1.4). This inherited pathology affects 4% of the population and can lead to a lack of transparency in central cornea due to the edema caused from the malfunctioning endothelium. It generally becomes manifest to the age of 40 or later. Like other inherited primary endotheliopathies, it seems to be correlated to a mutation in the gene for collagen (Bourne (2003)). Among more rare inherited endotheliopathies, we can find the Posterior Polymorphous Dystrophy (PPD), where some epithelial-like cells can be seen in the endothelium, and Congenital Hereditary Endothelial Dystrophy (CHED), where the entire endothelium presents epithelial-like cells at birth, so that the cornea cannot be transparent. The two are both bilateral.

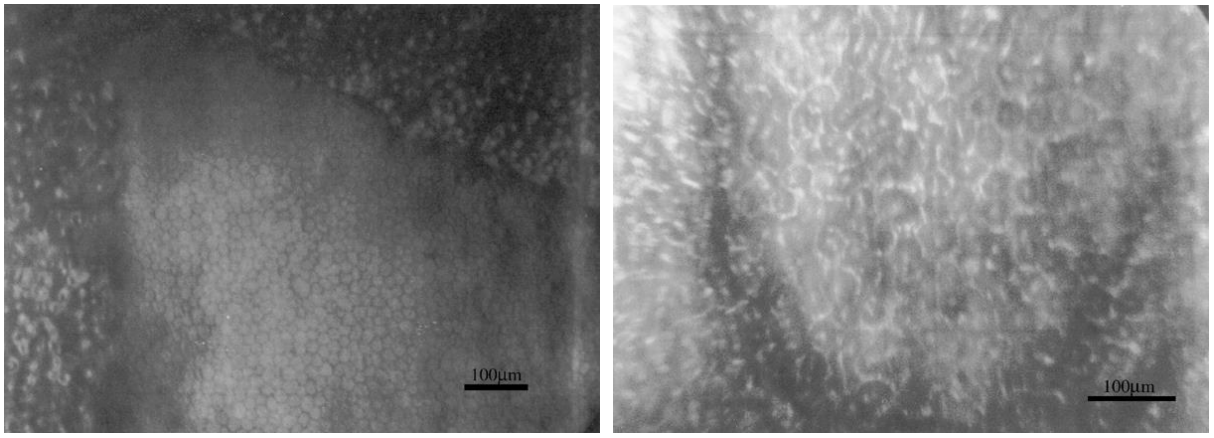

Fig.1.5. On the left: cornea with partial Chandler's syndrome on the left, with normal looking cells in the middle; On the right: closer view of cells affected by ICE (Bourne, 2003).

Another primary endotheliopathy is the iridocorneal endothelial syndrome (ICE, see Fig 1.5), also known as Chandler's syndrome. Here the peripheral cells are more likely to be involved: they appear to the microscope like black cells having white junctions. Either it remains stable or it can develop up to creating synechiae on the peripheral corneal's inner surface or to be attached to the iris, as far as this pathology is often characterized by an abnormal proliferation of endothelial cells. In last second case, Chandler's syndrome can also cause an increase in IOP and subsequently a glaucoma. ICE generally affects only one eye and it is not frequently widespread throughout the whole cornea: there often is a neat boundary between affected cells and smaller normal-looking ones, like a well-defined line (Bourne, 2003).

Among secondary endotheliopathies, we can find iatrogenic endotheliopathy (see Fig 1.6): because of surgeries like cataracts, endothelium can lose up to 10% of its elements. Moreover, the induced trauma will cause accelerated decrease of 2.5% per year of mean cell density up to the next 10 years, which can be enhanced from other systemic pathologies of the patient.

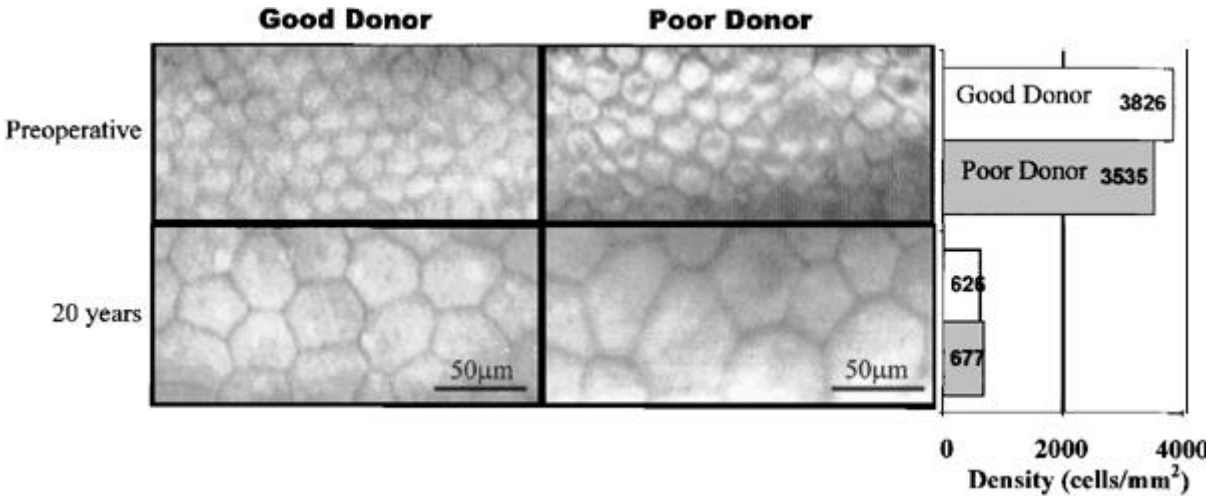

Fig.1.6. Preoperative and postoperative view of the endothelim in cornealtransplat, comparing cell loss in good and poor donors after 20 years. 10 years before the last picture, we could count 854 cells/mm² for the good donor and 1035 cells/mm² for the poor donor (Bourne, 2003).

As previously mentioned, also anterior uveitis can cause endothelial cell loss. This is because the leukocytes released in the aqueous humour by the immune response during the inflammation (see Fig 1.7), which can invade endothelium, while in high-pressure glaucoma an excessive intraocular pressure can lead to physiological stress and accelerate endothelial decay.

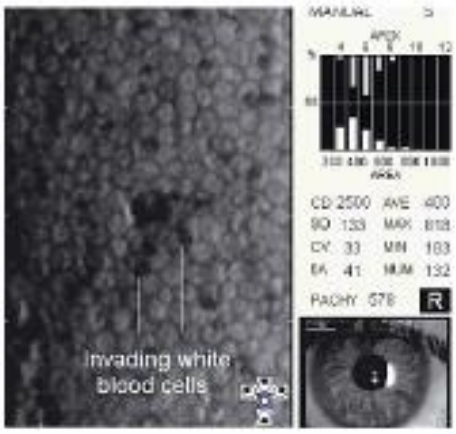

Fig.1.7. Blood cells among endothelial ones during the acute phase of an anterior uveitis (Thomas, 2009).

1.1.2 The effect of long term contact lens wearing on endothelial cells

According to the literature, almost every kind of contact lenses (Michael, 2016; Liesegang, 2002; Bourne, 2001) is responsible of a certain amount of pleomorphism and polymegetism in endothelial layer, though it is generally not symptomatic, with no effect on visual acuity and does not affect endothelial function. An exception is made for silicone hydrogel contact lenses: the results of studies such those of Norhani et al. (2014) and Carlson

et al. (1990) underline that there is no significant correlation with the long term wear of silicone hydrogel contact lenses and the presence of pleomorphism or polymegetism.

Mean cell density is more rarely affected both from silicone hydrogel contact lenses or any other type of soft or rigid gas permeable contact lenses, though in severe cases can be observed. As observed from Sibug et al (1991), discontinuing contact lens wearing after a long-term wear could help in re-establishing better endothelial coefficient of variation and hexagonality, though the recovery seems to be very slow.

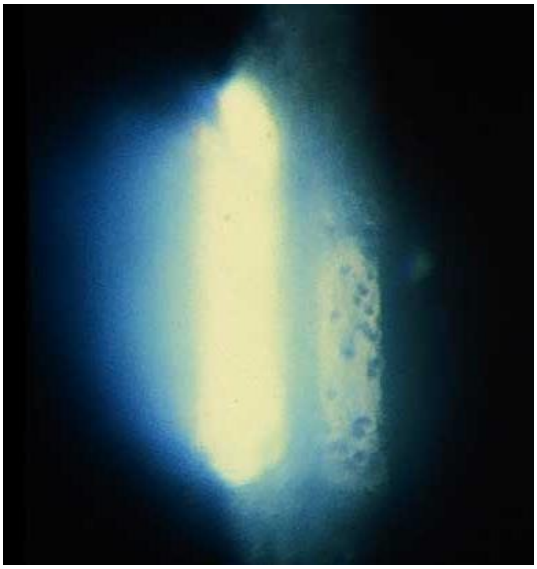

Fig.1.8. Endothelial blebs seen in slit lamp. Copyright © 2016 Alcon Laboratories, Inc.

A common finding that can be seen in patients wearing contact lenses for the first time or having discomfort with their contact lenses is endothelial blebs, which are illustrated in the above Fig 1.8. They were first observed by Zantos, Holden (1977) and look like small black spots in the microscope picture, not bigger than two or three cells (like a starting level of cornea guttata, but more widely present). In these cases, the maximum amount of blebs can be seen after 10 – 30 minutes after the lens insertion and then gradually disappear after some hours of continuous wear.

1.2. Used techniques in endothelial morphometric analysis

Alfred Vogt performed the first observation of corneal endothelium in vivo of which we have memory in 1919, through the specular reflection of a slit lamp. Unfortunately, this technique could detect only big changings in the endothelial structure, such as folds, big guttae, etc. It was only in 1970s that specular microscopy was introduced in medical research by Laing et al., so that allowed a clearer view of endothelial cells. At the beginning, this technique could provide relatively small pictures: 0.04 mm^2 was the size reached by Bourne, Kaufman (1976).

This rendered patient follow-up very difficult as rarely was possible to photograph exactly the same portion of endothelial cells.

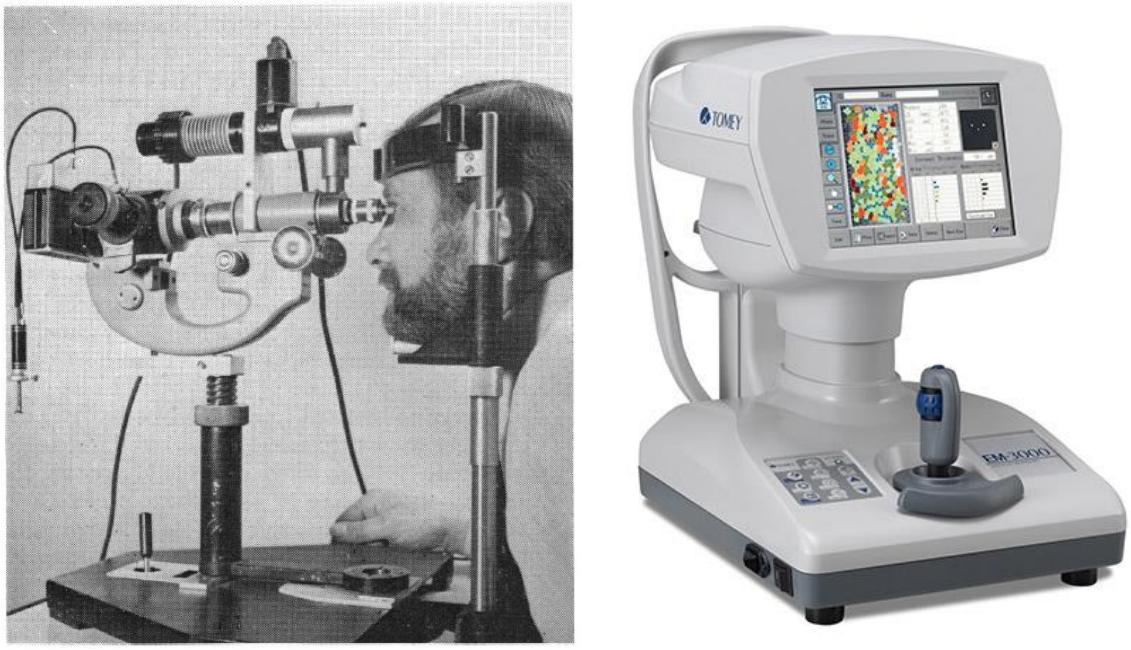

Fig.1.9. On the left: endothelial contact specular microscope (Price & Cheng, 1981); on the right: non-contact specular microscope Tomey EM3000.

As shows the study of Price & Cheng (1981), the non-contact specular microscope allowed the operator to take photographs even 0.20 mm² bigger than a contact microscope could, allowing a better analysis of cell density and morphometry of endothelia having a considerable rate of pleomorphism or polymegetism. Moreover, it provides a higher percentage of acceptable photos taken in the same time of a contact specular microscope.

Up to now many famous names of specular microscope producers, like Tomey or Topcon, added new digital technology to this technique to render endothelial cells screening always more precise and automatic. Although, all these innovations are space taking and rarely affordable for private ambulatories, so that endothelial screening was generally relegated to hospitals and clinics.

Recently, a new software for the automatic morphometric endothelial analysis has been developed, that can be applicable to a normal slit lamp with the external support of a computer. EndoKer system has been compared in the study of Ferraro et al. (2016) both with manual counting (mean accuracy found on the same picture for cell density = 0.2%, SD = 4.3%, while for HEX = 6.3%) and results expected by literature, finding great agreement in both comparisons. Comparing the EndoKer pictures with those taken with the Tomey EM3000 on the same patient, the results encountered more differences, as far as it was not possible to

measure precisely the same piece of the cornea. However, in addition to its practicality and affordability, this method proved to be also very reliable, and this is why it was chosen for the present study.

1.3. Endothelial morphometric analysis in normal world population

If we have a look to literature regarding endothelial cells normal parameters, it first comes to our attention the great differences among ethnical groups, in addition to smaller differences between genders and age groups. This makes us clearer why we still have such a poor understanding of the function of this small cell layer and even more compelling the need for further researches and the establishment of normal parameters.

Rao et al. (2000) conducted a study on endothelium of more than 500 Indian eyes from 20 to 87 years of age: cell density in the study population was 2525 ± 337 cells/mm², which decreased every year of 0.3% together with the HEX%, while mean cell area and COV increased with age. He also reported lower values of mean cell density and hexagonality compared to the equivalent age groups described in Japanese population analised by Matsuda et al. (1985). The same has been found comparing Indian eyes to American ones, though this difference was less evident than the one with Japanese population, and considered statistically significant only in age groups younger than 50 years old.

Padilla et al. (2004) found similar results in Filipino eyes, with 2798 ± 307.2 cells/mm² with a mean area of 360 μm² circa. In his study, the patients' sample was a little smaller (300 people circa) with almost the same age of Rao's one. Both Padilla and Rao considered the two eyes in their researches. Padilla notes that generally women show a meaningfully higher cell density compared to men. He also agrees with the rest of the literature about the decrease in cell density and, of course, increase in cell area and COV in relation to age, up to 60 years of age: after this age, the trend is the same, but enhanced. There was a slight decrease in hexagonality percentage with age increase, but it was not statistically significant.

Trying to asses normative datas for Iranian eyes, Hashemian et al (2006) selected the same age interval of previous reserchers, measuring more than 500 eyes. This study confirmed a rate of mean cell loss of 0.6%, but the mean cell density of this population was found to be extremely lower than the previously described: 1961 ± 457 cells/mm². Moreover, he did not find any particular difference between genders.

Wörner et al. (2011) tryed to find a common denominator among ethnic grups by studying cell pattern. This study starts with the hypothesis that endothelial cells follow the Mullins-von Neumann law for the two-dimensional growth of cells: this theory states that the perfect

equilibrium between cell growth and cell loss is given only by exagonal cells, as far as cells with less sides tend to become smaller or to die, while cells with seven sides or more tend to expand.

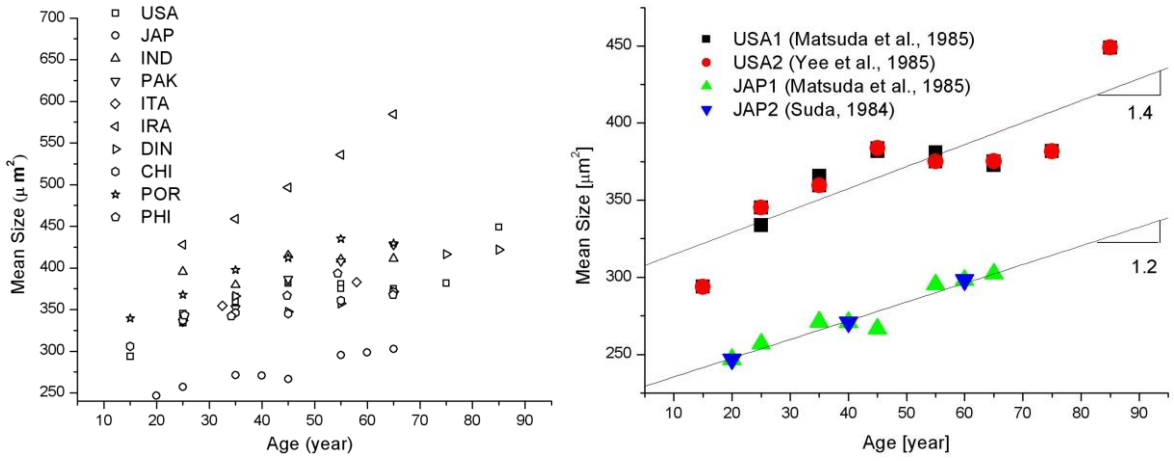

Fig.1.10. Diagrams showing the increase of cell size with the increasing of age among different ethnic groups. Wörner et al. (2011). The first compares the results of all previous studies considered by Wörner, the second show the different rate of increase in cell size for Japanese and Americans.

This could be an explanation of the different decay rate among different populations (see Fig 1.10): up to now, Iranian eyes have demonstrated to have the higher cell area (537.0 ± 137.4 μm²) and also the higher decay rate, while Japanese eyes with a mean cell area of circa 270 μm², show a much slower increase in cell size. Anyhow, comparing the slope of two very different populations like American and Japanese ones analysed by Matsuda et al. (1985), Wörner still finds very similar slopes in the increasing rate of cell area: 1.4 μm²/year and 1.2 μm²/year for Americans and Japanese respectively.

This would lead us to the hypothesis that a higher hexagonality percentage corresponds to a lower coefficient of variation, as it was found by Rao et al. (2000), Padilla et al (2004) and among the Thai population of Phramongkutklao hospital, examined by Narumon et al. (2008). Even in this study was found that increase in hexagonality leads to a decrease in the coefficient of variation, as confirmed by comparing the differences between male and female sample (mean cell density, COV and HEX: 2780 ± 362; 38%; 54% for male and 2578 ± 285; 41%; 50% for female respectively); however, even if the cell density has been found to be significantly lower in elderly patients, no significant changes of hexagonality and coefficient of variaton have been shown in this research. Narumon also evaluated the possible correlation between ednothelial parameters and other factors, such as intra ocular pressure, the presence of other diseases and dry eye, use of drugs through eye drops and smoking (in this case, not making differences

between people smoking more than 20 packs per year or less), not finding any significant relation.

Snellingen et al. (2001) is one of the wider study comparing different ethnic groups' endothelial cells. Even if the measurements were taken in different regions, the instrument used was the same as well as the analysis procedure, which makes this study even more important in assessing ethnical differences, because we do not have to consider the possible error caused by instrumental defferences. He had the possibility to compare Nepali, Bangladeshi and South Indian populations from 40 to 75 years old, finding statistically significant differences among these populations. For instance, Nepali eyes do not show any relevant difference in all three considered parameters (CD, COV and HEX) between male and female (p value > 0.05), while Bangladeshi and South Indians do (p value < 0.01). The results regarding South Indian population were surprisingly different to those gathered by Rao et al (2000): in fact, Snellingen's sample showed a higher cell density, of 2714 ± 360 cells/mm², despite the higher mean age. Moreover, the first two mentioned groups perfectly stick to the previously described hypothesis of Wörner et al. (2011), while Southern Indians show a higher COV, though they can boast the highest percentage of hexagonal cells among the three compared populations.

Other parameters, which have rarely been taken into consideration, are corneal thickness and steepness. The only study I found on the topic was the one of Müller (2004), who actually found a relation: the steepest and/or thinner the cornea is, the more endothelial cells decay is expected in elderly subjects (mean age of the involved sample 75.7 ± 10.9). Refractive error has never been considered relevat in the previously mentioned study, and this can be confirmed by the research of Sanchis-Gimeno et al. (2005), which compared mean cell density in relation to age for emmetropic, myopic and hyperopic subjects, not finding any substantial difference among the three groups.

All this said, the assessment of general criteria for a healthy and normal endothelium still looks very far from the end.

2. RESEARCH

2.1. Participants

This study evaluates only the right eye of 45 adult subjects, going from 29 to 58 years of age (mean 43 ± 8 SD), 22 male and 23 female (see table 2.1). Older people were not considered because of the lack of a pachimetry throughout the whole study: in fact, the natural decay of mean cell density in people around 70 years of age can be enhanced by corneal thickness or steepness, as suggested by the study of Müller (2004). Referring to the previously mentioned study and to the one of Sanchis-Gimeno et al. (2005), I did not take into consideration refractive error as a parameter as far as also in other studies it does not seem to affect endothelial cell density. Younger people were not considered, in order to avoid the influence of growth on the average cell density, as suggested by Bourne (2003).

Table.2.1.

An overview of selected patients.

	All	**M**	**F**
Tot. Patients	**45**	**22**	**23**
age	43 ± 8	43 ± 8	43 ± 8
smokers	16	11	3
contact lens wearers	24	10	14

All patients come from the area of Mori, Rovereto, Trento and Riva del Garda (Trentino, Italy) and most of them were recruited among the patients of Centro Ottico Optometrico di Prezzi Sergio, by which I work. Only people who grew up in this area have been considered, to minimize the influence of environmental differences. Most of them is also born here. None of them is affected by ocular diseases or suffered pathologies which could involve the right eye, neither in their past nor nowadays.

All patients could reach a visual acuity of 0.00 log Mar or better with their full correction. None of them presented neither scars nor corneal opacities: people who have had corneal transplant, cataract surgery or any kind of other ocular surgery were excluded from the present study. This information has been gathered before the measurement were taken, thanks to a topography, a visual acuity check, a slit lamp check of the anterior segment and a questionnaire.

All measurements have been taken in an 8-months period and totally, 55 people were enrolled for the measurement, however during the taking of the photographs two of them were excluded for the presence of endothelial structures resembling a corneal guttata, while other 8

people presented such a bad tear quality that it was impossible to gather reliable measurements. No blebs have been found during the measurements. Other subjects were excluded because of their bad tear quality, which made it impossible to take repeatable measurements.

The present study was undertaken with the written consent of each participant. The procedures followed the tenets of the Declaration of Helsinki. No tests that could possibly damage the people involved in the experiments has been carried out.

All the patients have been asked to fill up a questionnaire to evaluate the presence of factors that could alter endothelial physiology, increasing its normal deterioration process, and then, they were explained the aim of the photos we were taking.

The questions made were about:

- age, sex and birth and living place;
- the use of contact lenses;
- smoking period in their life (people who said they have smoked only in some sporadic occasions were not considered in this study, because of a lack of objective parameters. Only people who smoked every day more than one cigarette per day for at least two years are included in the "smokers" category, while the "non-smokers" have no history of nicotine dependence throughout their whole life. This parameter was taken into consideration because of the influence that smoking can have on the development of corneal guttata, as underlined in the study of Zoega (2006);
- the presence of any ocular pathology in the right eye, or other systemic pathologies that can have an influence on general physiology of endothelial cells (i.e.: diabetes, glaucoma, uveitis), nowadays or in the past (people who answered "yes" to this question were excluded from the present study).

2.2. Method

For every subject, I have been taken at least three measurements of central corneal endothelium for each eye, of an area of 0.111 ±0.020 mm². Only the three measurement more regular in size and with less artefacts have been taken into consideration.

The instrument used for this study is the same tested in the research of Ferraro et al. (2016): a Takagi 700GL LED (magnification set at 40x) with EndoKer software for the automatic morphometric analysis. The digital camera of the slit lamp is a TD-10 with a sensor of 1024 × 512 pixels and pixel size: 1.67 × 1.67 μm² (dynamic range: 8 bit; sensor exposure time: 10 ms; sensor frame rate: 60 fps). The disadvantages in using such an instrument, instead of a digital non-contact specular microscope are:

- the possibility to take only one picture at a time for the right and the left eye;
- the long waiting time of activation of the EndoKer software, which needs to be switched on and off every time you want to save a picture;
- for every picture, there are allowed to select, analyse and save only one area;
- no automatic eye tracking, so both the operator and the patient must stay very still. Unfortunately for this reason I had to exclude a certain number of patients, who were unable to keep fixating the mire for the brief instant required to take a picture;
- no automatic recognition of the pupil centre.

By the way, an advantage surely is its being space saving and the possibility to photograph large areas throughout the whole cornea and, as we do not need to touch the cornea to take pictures, allows full patient safety.

Fig.2.1. Taking pictures with Takagi 700GL slit lamp with EndoKer external elaboration system.

The lamp was placed 30° on the left (operator's perspective), while the microscope is placed 30° in the opposite direction. The slit is set at a width of 1 mm. Because of the need to have a reference point to make sure of measuring always the same central piece of the cornea, a long slit has been used, so that it could reach the upper eyelid and could be used as reference for the operator. The light intensity of the slit lamp was a quarter of the total intensity available. No diffused light was used. The room was dark, so that even presbyopic patients could better see the red luminous mire.

A mire at near has been placed as a fixation point at the same height of the lamp, but straight ahead of the patient's eye (it is a red movable light, currently available on the Takagi 700GL slit lamp). The first request made to the patients was that of finding a comfortable

position, so that he/she will stay as still as possible, and making him/her watch at the mire constantly.

The measurement starts setting the microscope at 10x, to find the correct position by focusing on the reflex of the slit on the central part of the cornea (during this step the entire eye can be seen, so that we can be sure of measuring precisely the centre, see Fig 2.2).

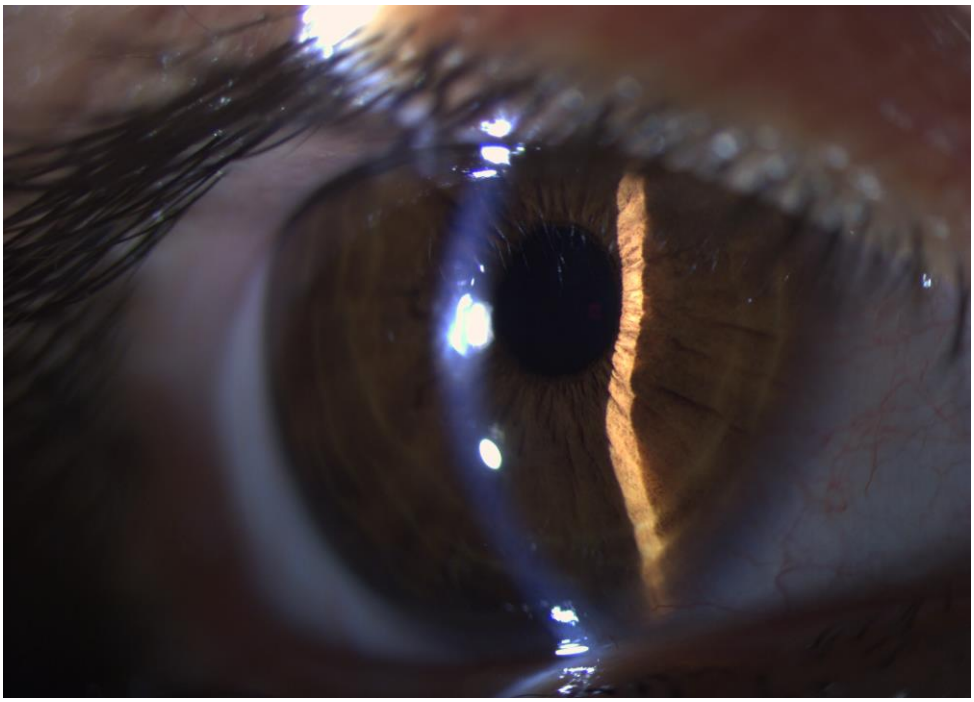

Fig.2.2. Picture taken with the Takagi 700GL slit lamp at 10x. The focus of the biomicroscope shall be on the white reflex instead of on the iris before switching to a higher magnification.

When the correct position is found, we will see the slit lamp's reflex on the screen with a greyish area on its right side, which is the place where the endothelial cells are visible. If it is so, we can enhance the magnification up to 16x and adjust the microscope's position again before setting the magnification to 40x (see Fig 2.3).

Note: it is the EndoKer software that enables the right magnification to photograph endothelial cells precisely, and all the images are managed through a screen out from the microscope itself.

Now we can try to take a picture, adjusting the position again and following the subtle movements of our patients until the image results clear on the screen: to avoid imperfections caused from a poor lacrimal film, the patients have been required to blink the eye several times before taking each photograph. When the picture is taken, we can select manually the area of

interest, which the software will elaborate, and the computer gives an immediate response of the dimension of sides in μm² and of the area in μm².

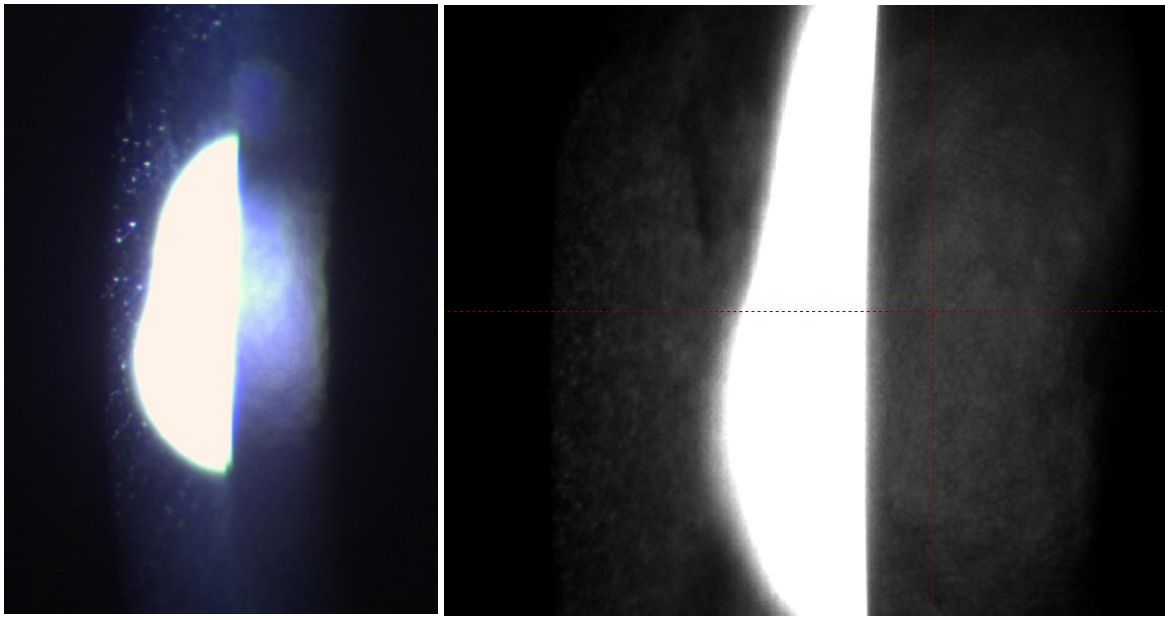

Fig.2.3. Picture of the endothelium at 40x seen through the Takagi 700GL slit lamp (on the left) and through the EndoKer magnification system on the computer screen.

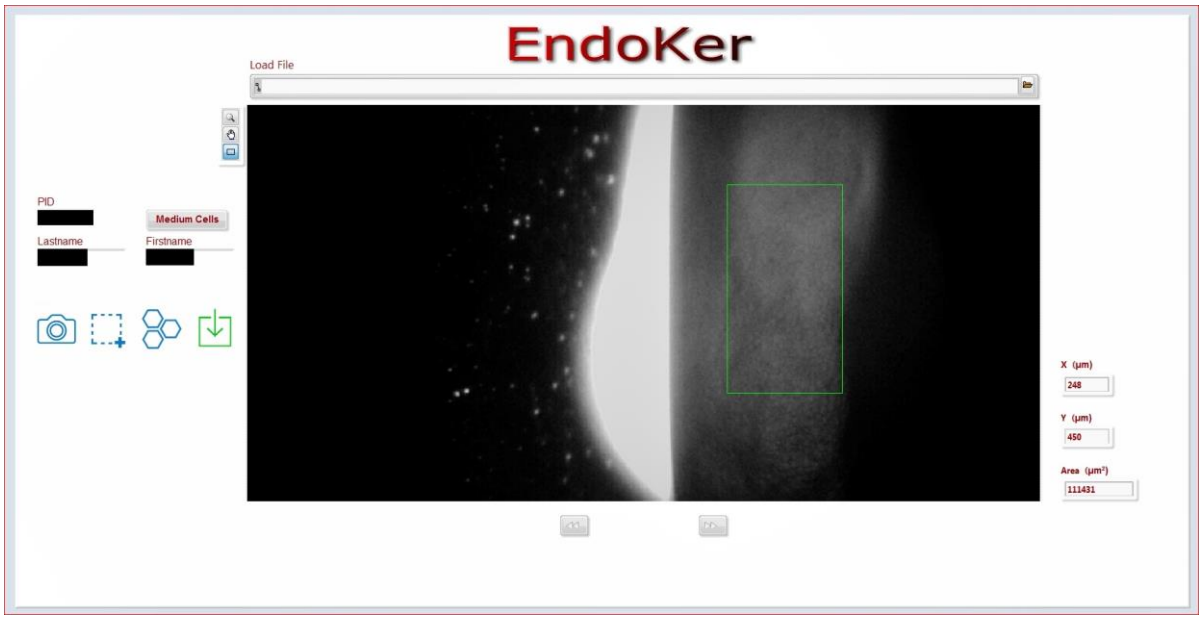

Fig.2.4. Picture of the endothelium seen through the EndoKer magnification system on the computer screen with the area selected for measurement in green and its dimensions on the right part of the screen.

As shows Fig 2.5, EndoKer elaborates the photo taken and the information gathered are:

- dimension of the selected area in mm²;
- total number of cells found in the selected area;
- mean cell density per mm² (CD);

- average cells' area ±SD;
- coefficient of Variation (COV), which is the ratio between the previously mentioned SD and mean cells' area;
- maximal and minimal cell area measured;
- percentage of hexagonality (HEX%);
- histogram on the frequency distribution of cells' areas;
- histogram on the frequency distribution of cells' sides.

The studied area is selected manually for each photograph, to avoid the computer taking into consideration unnatural images.

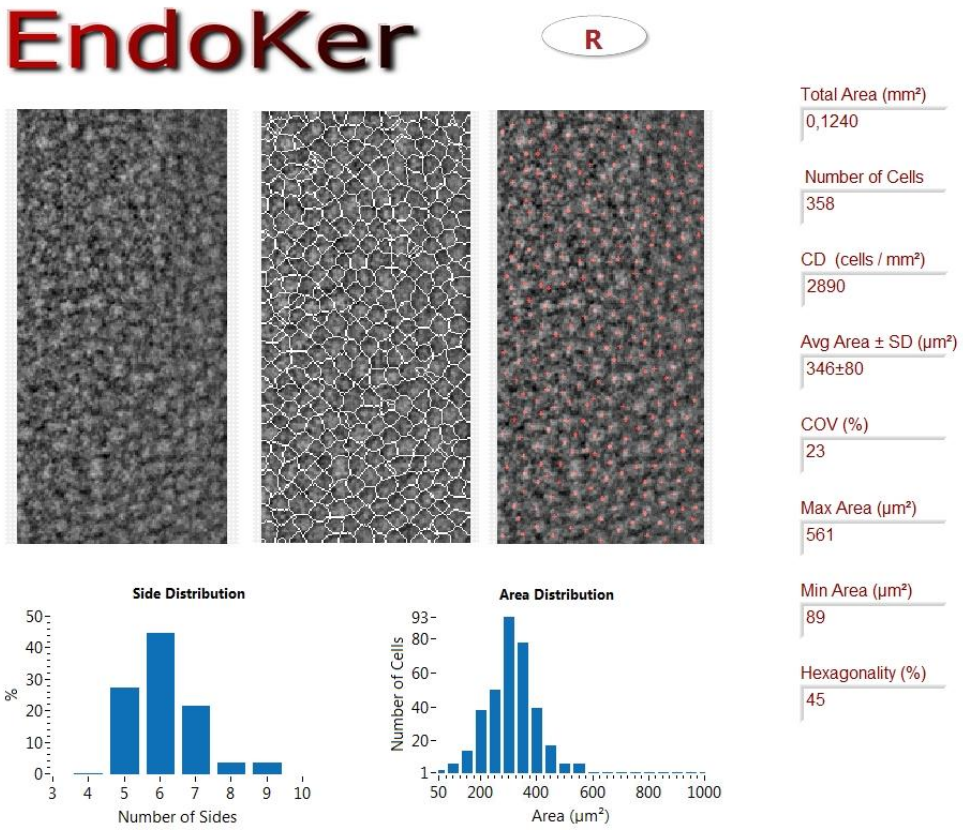

Fig.2.5. Picture of the endothelium analysed by EndoKer.

For each patient have been taken at least three pictures, mainly on the same day, with at least one minute of rest between the photos, due to the time needed to elaborate and save the data. Then, the data of the more clear and similar photos have been reported manually into an excel file, to elaborate them. The parameters taken into account are:

- age;
- sex;

- the use of contact lenses (years);
- smoking period (years);
- mean cell density per mm² (CD);
- coefficient of Variation (COV);
- percentage of hexagonality (HEX%)

2.3. Analysis of data and results

Data was analysed mainly with excel, using www.socscistatistics.com for the calculation of the Mann-Whitney U-test. Once the three measurements were inserted in the file, they were used to calculate an average value of:

- cell density (CD),
- percentage of hexagonal cells (HEX%),
- coefficient of variation of cell size (COV).

The result for every patient was the considered data for all further analysis. Unpaired t-test and Mann-Whitney U-test were used to compare couplets of data groups, like between genders, and p values less than 0.05 were considered significant. These tests were not used to analyse the correlation between endothelial parameters and age, smoking period and contact lens wearing, for which I used the Pearson correlation test. ANOVa test was used to compare more than two ranks of data, such as three age groups.

Table.2.2.

Overview of the gathered data for the entire population.

	Average	*SD*	*SE*	min	max	range
measured area (mm²)	0,111	0,020	0,002	0,079	0,198	0,119
cell density (cells/mm²)	3021	143,84	12,38	2500	3340	840
HEX (%)	43,3	4,42	0,38	31,0	58,0	27,0
COV (%)	36,2	7,33	0,63	24,5	57,6	33,1
mean cell area (µm²)	333	_	_	89	1396	1307

The measured area for every picture was quite large: 0.11 ±0.02 mm². The dimension of sides varies from time to time, to avoid selecting dark areas or artefacts. The whole population presents mean values corresponding with literature's expectation how far as COV and HEX are

concerned, for which I found respectively 36.2% and 43.3%. Always taking into consideration literature values, this population shows a higher mean CD of 3021 ± 143.8 cells/mm² and average cell area is of 333 μm² (see Table 2.2).

2.3.1 Age related differences

Initially, I analysed the data in function of the age, to verify its comparability with previous literature. First, I divided the population into three groups of age, as similarly done in other researches. The groups are:

- 29* to 40 years old, which counts 18 subjects;
- 41 to 50 years old, that includes 21 members;
- 51 to 60 years old, comprehending 6 patients;

As far as the groups are three and do not have the same number of elements, I analysed them using the ANOVa test and for each one of the three parameters (CD, COV and HEX) I made a separate analysis. The results for mean cell density are surprising: there is no significant relation between the mean numbers of cells per mm² and the age (p value = 0.473; significance with $p < 0.05$). Mean cell density per group of age: 2997 ± 112; 3042 ± 117; 3019 ± 91 for 31 to 40, 41 to 50 and 51 to 60, respectively. This indicates values 33% higher than Iranian population and 18% lower than Japanese population in the first group, and 55% higher than Iranian people in the last group, versus 10% lower than Japanese's eyes values (see Table 2.3 and 2.4 and Fig 2.6).

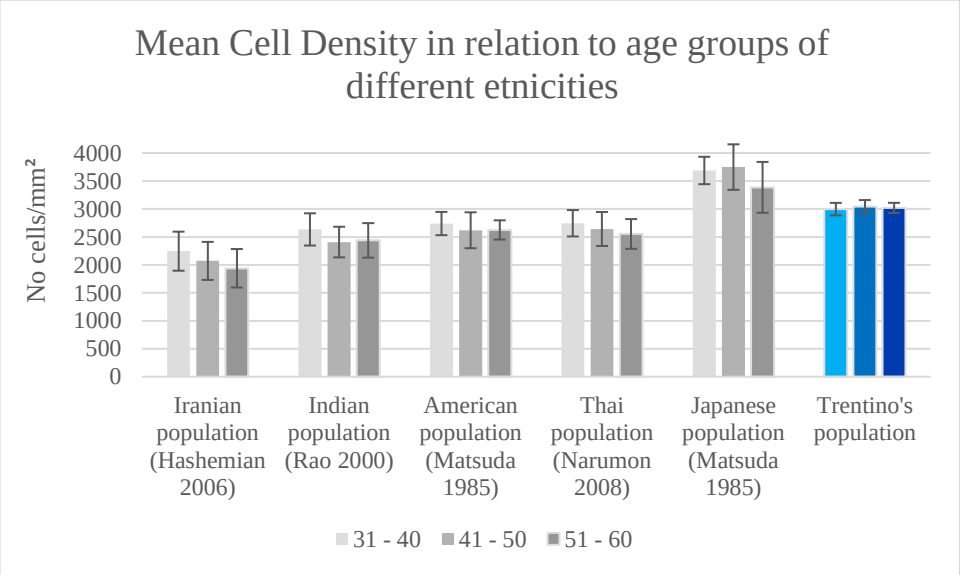

Fig.2.6. Histogram showing different mean cell density throughout various studies and ethnicity groups compared with the data gathered in the present study, with bars indicating standard deviation.

* Only one subject of this groups is 29 years of age and none of them is 30, therefore I consider it as comparable with previously analysed groups of 31 – 40 years old.

Comparing my results to those of Matsuda et al. (1985), we will find similar sample dimensions (see Table 2.3); although Trentino's population shows a much smaller standard deviation among the groups' members.

Table.2.3.

Overview of different mean cell density (cells/mm^2) throughout among ethnicity groups.

	Age groups								
	No eyes	**31 - 40**	***± SD***	No eyes	**41 - 50**	**± SD**	No eyes	**51 - 60**	**± SD**
Iranian population (Hashemian 2006)	*45*	2245	*349*	*66*	2071	*340*	*87*	1939	*344*
Indian population (Rao 2000)	*96*	2634	*288*	*97*	2408	*274*	*98*	2438	*309*
American population (Matsuda 1985)	*6*	2739	*208*	*11*	2619	*321*	*13*	2625	*172*
Thai population (Narumon 2008)	*50*	2744	*236*	*104*	2642	*304*	*78*	2553	*266*
Japanese population (Matsuda 1985)	*10*	3688	*245*	*10*	3749	*407*	*10*	3386	*455*
Trentino's population	*18*	2997	*112*	*21*	3042	*117*	*6*	3019	*91*

Table 2.4

Close-up of endothelial parameters' variation between age groups in Trentino's population

	29-40	**41-50**	**51-58**
CD (±SD) [cells/mm^2]	2997 ± 112	3041 ± 117	3018 ± 91
HEX (±SD) [%]	42,57 ± 2,61	43,39 ± 2,97	44,77 ± 1,66
COV (±SD) [%]	36,40 ± 5,70	35,88 ± 5,26	36,89 ± 5,11

For the Coefficient of Variation the possible differences with age groups are even smaller (p value = 0.906; significance with $p < 0.05$). Also Hexagonality did not show itself as a significant parameter ($p = 0.223$; significance with $p < 0.05$).

As a control test, I used the Pearson correlation test to verify the presence of possible relations as a function of age. The results confirmed the ones of the previously conduced AnOVa tests and underlined a countertendency of the percentage of hexagonal cells: in fact, Cell Density does not show a significant relation with aging ($r = 0.104$), as well as the Coefficient of Variation ($r = -0.012$). Surprisingly, Hexagonality seems to have slight improvements as a function of age ($r = 0.312$).

Table.2.5.

Overview of the results of ANOVa test among age groups (on the left). Significance with $p < 0.05$. On the right, overview of the Pearson correlation test's results. Positive results indicate a directly proportional correlation, while negative ones an inversely proportional correlation. Correlation is perfect when $r > 0.8$; strong when $0.8 > r > 0.5$; weak when $0.5 > r > 0.2$; is not relevant with $r < 0.2$

Results for ANOVA test between different age groups		Results for Pearson correlation test comparing parameters and age	
CD	p = 0,473	**CD**	r = 0,1043
COV%	p = 0,906	**COV%**	r = -0,0124
HEX%	p = 0,223	**HEX%**	r = 0,3127

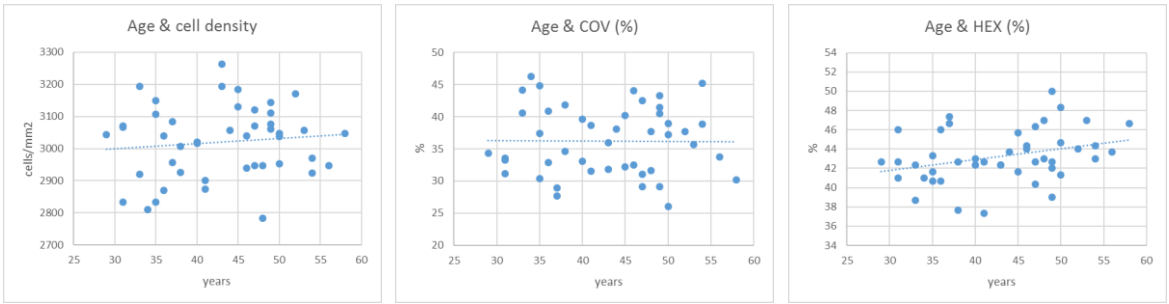

Fig 2.7 Graphs about the correlation between age and endothelial parameters' variation.

In Fig 2.7 we can find graphical representation of the correlation between age and endothelial parameters: this is very weak for what concernes cell density R is 0.082 and the P value of two-tailed T-test is 0.59. For the Coefficient Of Variation the value of R is -0.015 and the P value of two-tailed T-test is 0.92; while hexagonality shows a value of R = 0.35 and P value of two-tailed T-test = 0.02.

2.3.2 Relations between endothelial parameters and genders

Yet it is not clear, up to which point genders can affect normal parameters in endothelium. To discover possible relations in my sample, I divided the subjects between male and female and proceeded in evaluating each parameter separately with Mann-Whitney U-test. The results showed no significant differences between the two samples, for none of the considered parameters: Cell Density p = 0.631; Coefficient of Variation p = 0. 952; percentage of Hexagonal cells p = 0.904, as summarized in Table 2.6. A graphical representation can be seen in Fig 2.8.

Table.2.6.

Overview of the mean values characterizing these samples.

General parameters of male and female population		
	M	**F**
Nr.	22	23
CD (±SD) [cells/mm²]	3023 ± 127	3019 ± 98
COV% (±SD) [%]	36, 36 ± 5,82	36,10 ± 4,91
HEX% (±SD) [%]	43,21 ± 2,96	43,29 ± 2,56

Seeing these results, I found not relevant to analyse separately aging effect for male and female subjects, as well as for other factors relations.

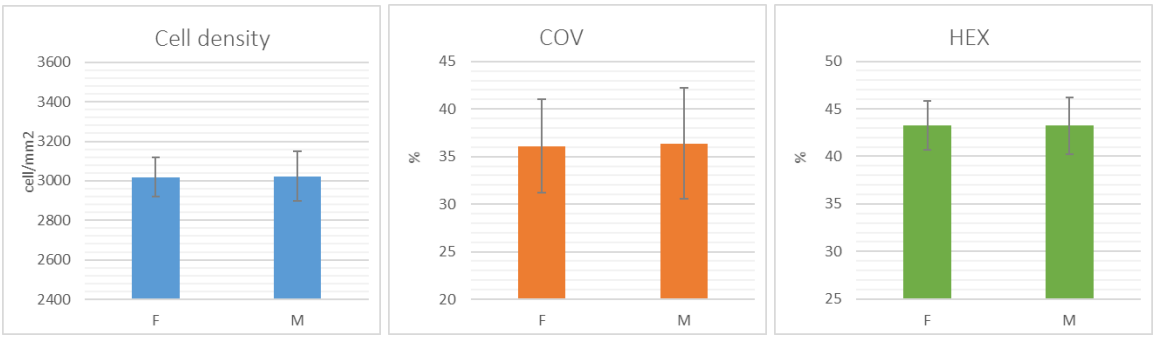

Fig 2.8 Comparison of endothelial parameters between genders.

2.3.3 Relations between endothelial parameters and other factors

As before largely explained, it is in the interest of this study to verify whether lifestyle factors such as smoking and contact lens wearing can affect endothelial physiology. I proceeded carrying out the Pearson correlation test, to find out whether there is any correlation between the years of contact lens wearing (mean period of port 9.57 years ± 12) and the possible decay

of endothelial parameters. The test was conducted between years of contact lens port and every parameter, separately. The results excluded the possibility of any correlation with Cell Density ($r = 0.113$) and percentage of Hexagonality ($r = 0.047$), while bring to our attention the possibility of an inversely proportional interdependence with the Coefficient of Variation in cells' size. In fact the value $r = -0.249$, underlines a weak, though significant correspondence, meaning that the more years the subjects have worn contact lenses, the smallest this coefficient is.

The same tests have been done for smoking (mean smoking period 6 years ± 9.52), finding no significant association at all. I want to underline that only people smoking more than 20 cigarettes pack per year were classified as smokers. Being endothelial semi-permanent cells, it was not considered as relevant if the subject still smokes or has quitted. As before, all three endothelial parameters have been compared to the years someone smoked, separately for every parameter using the Pearson correlation test. The results were respectively: $r = 0.067$ for Cell Density; $r = -0.017$ for the Coefficient of Variation; $r = 0.015$ for percentage of Hexagonal cells.

As a counter test, I divided the whole population into four groups:

- people who never smoked nor worn contact lenses (11 members);
- people who smoked but never used contact lenses (10 members);
- people who used contact lenses but never smoked (18 members);
- people who used contact lenses and smoked (6 members).

Table.2.7.

Overview of the mean values and results of ANOVa test among the groups of: smokers; contact lenses wearers; smokers and contact lenses wearers; non-smokers and non-contact lenses wearers. Significance is given by $p < 0.05$.

	Smokers	Contact Lenses Wearers	Non smoking and Contact Lenses Wearing	Smoking and Contact Lenses Wearing	Results for ANOVA test
CD (±SD)	3000 ± 122	3032 ± 87	3013 ± 111	3037 ± 176	p = 0,877
HEX% (±SD)	43,60 ± 3,47	43,20 ± 2,87	43,54 ± 1,74	42,27 ± 2,88	p = 0,900
COV% (±SD)	35,76 ± 6,12	35,94 ± 5,04	37,31 ± 5,52	35,85 ± 5,49	p = 0,799

I analysed each parameter in function of this last classification with the ANOVa test, in search for something that can influence the outcome. The results were $p > 0.05$ for all parameters (Cell Density, Coefficient of Variation and > 0.05 Hexagonality), underlining once

more that no group was significantly different from the others and so that no factor can really influence endothelial cells parameters (see Table 2.7).

In conclusion, Trentino's normal population endothelium seems to be regardless of any effect of age, except a slight improvement of hexagonal percentage with the increase of age, and shows no differences between male and female sample. Moreover, external factors such as contact lens wearing or smoking for several year did not demonstrate to have any particular influence on the results (see Table 2.8).

Table.2.8.

Summary of the relevant values found in the present study.

Legend: ŧ = Mann-Whitney U-test; r = Pearson correlation test.

Factors	**CD**	**COV%**	**HEX%**
Gender ŧ	0,63	0,95	0,90
Age *r*	0,10	-0,01	**0,31**
Smoke *r*	0,07	-0,02	0,02
Contact lens wearers *r*	0,11	**-0,25**	0,05

CONCLUSIONS

1. The study results present no statistically significant differences for Trentino's small population between all three age groups, both for corneal endothelial cell density parameters and coefficient of variation. Trentino's population (aged 29 to 58) average values are for cell density ± SD = 3021±112 cells/mm^2 and for coefficient of variation ± SD = 36±5 % .

2. Analysing hexagonality data, we notice that the age has weak but significant impact on changes of corneal endothelial cells structure (r=0.31; p=0.01), however, unlikely to what our expectation would be, the percentage of hexagonal cells slightly increases among older age groups. This should be further investigated to understand whether it is a widespread phenomenon or it is just a consequence of the small size of the population's sample considered.

3. Gender is no relevant factor for this sample, in none of the considered parameters:
    a. male average data:
        i. cell density ± SD = 3023±127 cells/m^2;
        ii. hexagonality ± SD = 43±3 %;
        iii. coefficient of variation ± SD = 36±6 %;
    b. female average data:
        i. cell density ± SD = 3019±98 cells/m^2;
        ii. hexagonality ± SD = 43±3 %;
        iii. coefficient of variation ± SD = 36±5 %.

4. Despite previous hypothesis given by the literature of smoking as a factor that can influence endothelial cells' health, in the present study was no validation of those values. Smoking people corneal endothelial cell density (3000±122 cells/mm^2) is not different from non-smoking people data (3013±111 cells/mm^2), as well as other parameters.

5. Endothelial parameters seem to be not affected by the usage of contact lenses, in contraposition to every finding in literature on this topic. Corneal endothelial cell density in people wearing cntact lenses (3032 ± 87 cells/mm^2) is not different from non-users data (3013±111 cells/mm^2), as well as other parameters.

FINAL WORDS

The data gathered in this study show a very deep difference with what can be found in literature on this topic. As largely described in literature review, the instrument used is highly reliable and its measurements can be compared with more diffused non-contact specular microscopes, so the reason for these differences shall not be searched among instrumental errors.

Bigger samples would give us a clearer image of the trend of this population, although many other studies, one for all Matsuda et al. (1985), have shown reliable results among small samples.

A personal hypothesis, is that we shall look at the particular environmental conditions of the examined population, who can boast an especially low pollution level, or at some other genetic or structural factors (i.e.: corneal thickness and steepness), which could not evaluated in this study.

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Author: /Barbara Prezzi /

Recommend/unrecommend thesis for defensing
Supervisor: prof., Dr.phys. Gunta Krūmiņa

Reviewer: MOptom. Sanita Līduma

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Dean’s authorized person: _____

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