Charles University in Prague

First Faculty of Medicine

The Biology and Pathology of the Cell

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Význam a funkce stromálních enzymů v patogenezi keratokonu

The role and function of stromal enzymes in keratoconus pathogenesis

Ph.D. Thesis

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DECLARATION

I, Ľubica Ďuďáková, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. I also confirm that the work presented hasn't been used to achieve another or the same university degree.

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ABSTRAKT

Keratokonus (KC) je nezánětlivé onemocnění rohovky, při kterém se rohovka ztenčuje a vyklenuje pravděpodobně v důsledku poruch ve vazbě kolagenních vláken. Je jednou z nejčastějších indikací k transplantaci rohovky. KC je multifaktoriální onemocnění, na jehož vzniku se podílí i genetické faktory, nicméně přesná příčina onemocnění ani její mechanismus nebyly zatím objasněny.

Cílem práce bylo porovnat výskyt a aktivitu enzymů lysyl oxidáz (LOX a LOX-like enzymy), které katalyzují vznik vazeb mezi elastinovými a kolagenními vlákny v kontrolních lidských rohovkách a v explantátech získaných při transplantaci od pacientů s KC. Zaměřili jsme se i na onemocnění asociované s výskytem KC a pokusili se najít jejich společné znaky. Provedli jsme studii ověřující asociaci jednonukleotidovych záměn (SNPs) v genech pro *LOX* a hepatocytární růstový faktor (*HGF*) s výskytem KC. V našem výzkumu jsme použily metody buněčné a molekulární biologie (tkáňové kultury, imunohisto- a imunocytochemie, mikroskopie, měření aktivity enzymů pomocí fluorometrie, genotypování a přímé sekvenování) a statistickou analýzu.

Prokázali jsme přítomnost celé rodiny lysyl oxidáz v kontrolní i KC rohovce, ve které jsme pozorovali pokles intenzity a nepravidelné rozmístění LOX, propeptidu LOX, LOXL2 a LOXL3. Zjistili jsme, že u KC dochází ke 2,5 násobnému poklesu celkové aktivity LOX enzymů. Zjistili jsme, že k podobným strukturním změnám jako u KC dochází i u prolapsu mitrální chlopně (PMC), což naznačuje podobný mechanizmus vzniku obou onemocnění. Upozornili jsme rozdíly mezi KC a makulární dystrofií rohovky (MDR), dosud vedené jako onemocnění asociované s KC. Prokázali jsme asociaci rs2956540-C v oblasti genu *LOX* s protektivním účinkem a rs3735520-A v *HGF* jako rizikový faktor pro rozvoj KC. Publikovali jsme hypotézu o vlivu disbalance mědi na rozvoj KC, v které jsme spojili všechny dosud popsané dílčí změny pozorované u tohoto onemocnění do jedné společné dráhy. Přesný způsob jakými se asociované SNPs a lokální deficience mědi podílejí na vzniku KC, zůstávají neobjasněny.

Potvrdili jsme, že u KC dochází k poruchám enzymů tvořících vazby mezi kolageny a nastínili, že podobný mechanismus se zřejmě uplatňuje i při vzniku PMC. U našich pacientů jsme vyloučili asociaci MDR a KC.

Klíčová slova: *lysyl oxidáza, keratokonus, imunohistochemie, aktivita enzymů, rohovka, asociační studie, SNPs*

ABSTRACT

Keratoconus (KC) is a non-inflammatory disease of the cornea, in which ectasia and thinning occur probably due to defects in the collagen fibres binding. It is one of the most common indications for corneal transplantation. KC is a complex disorder with the involvement of both genetic and environmental factors; however the exact pathogenic mechanisms leading to the disease development have not been elucidated.

The main aim of our work was to compare the presence and enzyme activity of cross-linking enzymes lysyl oxidases (LOX and LOX-like enzymes), in control human cornea samples and explanted cornea gained from patients with KC. We also focused on diseases previously described to be associated with KC with the aim to identify common signs among them. Furthermore, we replicated association of single nucleotide polymorphisms (SNPs) in *LOX* and hepatocyte growth factor (*HGF*) with KC risk. We attempted to link all pathophysiological disturbances observed in KC into one common pathway. We have used a wide spectrum of methods (cell culturing, immunohisto- and immunocytochemistry, microscopy, fluorimetric enzyme activity measurement, genotyping and direct sequencing, statistical analysis).

We demonstrated the presence of entire family of LOX enzymes in control and in KC corneas, with decrease and the irregular pattern for LOX, LOX propeptide, LOXL2 and LOXL3. In average, 2.5-fold decrease in total LOX enzymes activity was detected. We found that mitral valve prolapse (MVP) and KC share structural alterations, indicating similar pathogenic mechanism(s) were involved in the development of both diseases. In our cohort of patients, we have excluded association of KC and macular corneal dystrophy (MCD). We demonstrated the association of rs2956540-C in *LOX* genomic area with protective effect and rs3735520-A in *HGF* genomic area as a risk factor for the development of KC. A theory about involvement of copper imbalance in KC development has been published. The contribution of SNPs and copper imbalance on KC development remains unclear.

In summary, we confirmed our hypothesis that impairment of cross-linking enzymes occurs in KC and that the same mechanism could be involved in MVP pathogenesis. We excluded association of MCD and KC in our group of patients.

Key words: *lysyl oxidase, keratoconus, cornea, immunohistochemistry, enzyme activity, genotyping, SNPs*

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ABBREVIATIONS

α-SMA	α-smooth muscle actin
AGEs	advanced glycation endproducts
BMP-1	bone morphogenic factor 1
CHST6	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6
CI	confidence interval
CK	cytokeratin
CRL	cytokine receptor-like domain
CXL	corneal cross-linking
DALK	deep anterior lamellar keratoplasty
DHLNL	deH-dihydroxylysinonorleucine
ECM	extracellular matrix
GWAS	genome-wide association study
HGF	hepatocyte growth factor
HLNL	deH-hydroxylysinonorleucine
Hyl	hydroxylysine
KASP	Kompetitive Allele Specific PCR
KC	keratoconus
LNL	deH-lysinonorleucine
LOPP	lysyl oxidase propeptide
LOX	lysyl oxidase
LOXL	lysyl oxidase-like
LTQ	lysyl-tyrosyl quinone residue
lys	lysine
MAF	minor allele frequency
MCD	macular corneal dystrophy
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
PCR	polymerase chain reaction
PK	penetrating keratoplasty
SNP	single nucleotide polymorphism
SRCR	scavenger receptor cysteine-rich

1 Introduction

1.1 Cornea

The cornea is a unique connective tissue that combines transparency, refractive power for correct vision, tensile strength, and protection against infections (Chakravarti, 2001). It is comprised of 6 different layers: epithelium with its basal membrane, Bowman layer, corneal stroma, Descemet membrane and endothelium (Figure 1).

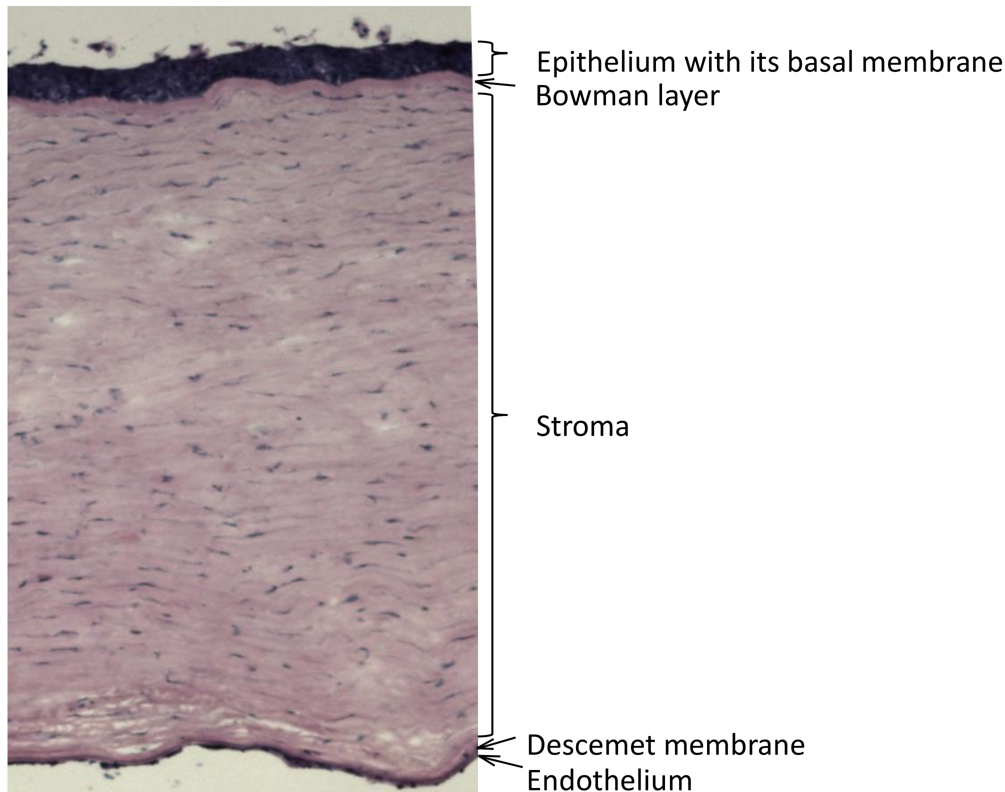

Figure 1: Human cornea stained with hematoxylin and eosin, magnification 40x

The corneal epithelium is approximately 50 µm thick and consists of five to six layers. The differentiation process requires about 7 to 14 days, when the superficial cells are desquamated into the tear film (Hanna *et al.*, 1961).

The corneal endothelium consists of monolayer of cells that lines the posterior corneal surface (Bahn *et al.*, 1984). The endothelium serves two functions to maintain the health and clarity of the stroma: by controlling the hydration of stroma and the permeability of nutrients and other molecules from the aqueous humour because these are not supplied by blood vessels as they are in other tissues (Bourne, 2003).

Stroma is located between anterior and posterior limiting membranes and constitutes 90% of the corneal thickness (Birk *et al.*, 1986). At the microscopic level, stroma appears as an organized, dense, avascular and relatively acellular connective tissue comprising collagen and proteoglycans (mainly decorin, lumican and keratan sulphate). The central thickness of the human cornea is approximately 0.5 mm, increasing towards the periphery where it measures around 0.69 mm.

How the corneal curvature is controlled and maintained is not well-understood. However, there is evidence that corneal shape evolves over time in a correlative manner with other ocular geometric changes to facilitate/maintain emmetropization (Carroll, 1982; Grosvenor, 1987) - the process by which the refraction of the anterior ocular segment and the axial length of the eye tend to balance each other to produce perfect vision. The mechanisms responsible for these changes in the cornea are not known.

Normal human corneal stroma is rich in type I collagen, but it also contains relatively large amounts of type V (Lee and Davison, 1984) and type VI collagens (Zimmermann *et al.*, 1986). Collagen type III is represented much less but during wound healing, inflammation and several pathological conditions it increases. Corneal collagen fibrils are composed of type I collagen molecules incorporated together with those of type V collagen into heterotypic fibrils (Birk *et al.*, 1988).

Stroma consists of approximately 200 parallel organized lamellae. Each lamella is 1.5-2µm thick and contains regularly arranged collagen fibrils 20-25 nm in diameter that are embedded in a ground substance composed of proteoglycans. The stroma also contains thin and flat keratocytes (corneal fibroblasts) between the lamellae that synthesize and regulate the extracellular corneal matrix constituents. Keratocytes form an interlinking network throughout the whole cornea and occupy between 3 % and 5 % of the stromal volume (Maurice, 1957; Nyquist, 1968).

There are two preferred orientations of collagen fibrils, which are orthogonal and alternate between successive lamellae. The cornea provides an example of well-ordered fibres in precise layers at a defined angle to each other (Muller *et al.*, 2004). The regular arrangement of collagen fibrils in each lamella allows for the corneal transparency and focussing light onto the retina with minimum scatter and optical degeneration. Abnormalities in corneal composition or structure due to damage or disease may lead to severe vision impairment.

1.2 Cross-linking of collagens

The understanding of collagen and elastin cross-linking is important in many disciplines, as stabilized extracellular matrix (ECM) is essential for all animal forms higher than protozoans. Given this key role, it is not surprising that abnormalities of collagen and elastin cross-linking may affect every tissue and organ in the body (Reiser *et al.*, 1992). Reported abnormalities range from hypertrophic scar formation and fibrosis to heritable diseases, such as Menke's disease, cutis laxa, or certain forms of Ehlers-Danlos syndrome (Prockop and Kivirikko, 1984).

Changes in cross-linking lead to differences in tissue mechanical properties (Bailey *et al.*, 1998). One of the factors determining the mechanical properties of the tissue is the diameter of the collagen fibres. As it increases, the flexibility of the tissue decreases resulting in lower ability to resist crack propagation. The variation in diameter of collagen fibres between tissues is illustrated by tendon (200 nm), skin (approx. 100 nm), cartilage (approx. 50 nm) and cornea (20 nm) (Parry *et al.*, 1978). There are two mechanisms of cross-linking (Figure 2).

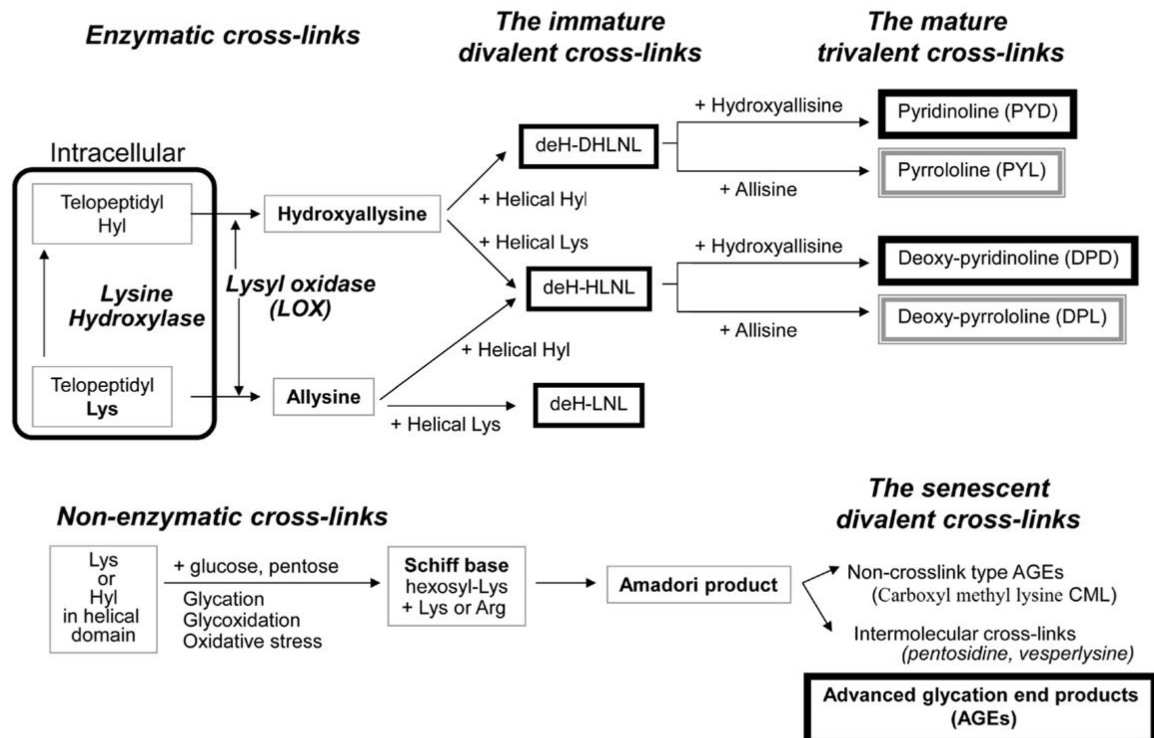

Figure 2: Schematic representation of cross-link formation (Saito and Marumo, 2013)

Enzymatic cross-linking

Enzymatic cross-link formation of collagens is regulated by the action of two types of enzymes: the **lysine hydroxylases** and **lysyl oxidase (LOX)**. Lysine hydroxylases regulate tissue specific enzymatic cross-link patterns intracellularly (Mercer *et al.*, 2003). After intracellular hydroxylation of lysine (**Lys**), collagen molecules are secreted into the extracellular space where LOX binds to

specific Lys or hydroxylysine (**Hyl**) residues and promotes conversion of ε-amino groups to become **allysine** and **hydroxyallysine**, respectively following extracellular aggregation of collagen molecules into fibers (Eyre *et al.*, 1984). Then these precursors of enzymatic cross-linking react and condensate with Lys or Hyl residues in the triple helical region of an adjacent collagen molecule to form **divalent immature cross-links** called deH-dihydroxylysinonorleucine (**DHLNL**), deH-hydroxylysinonorleucine (**HLNL**), and deH-lysinonorleucine (**LNL**). The divalent cross-links undergo a spontaneous reaction with another telopeptide Lys or Hyl aldehyde to form **trivalent mature pyridinium** or **pyrrole cross-links** (Brady and Robins, 2001).

Non-enzymatic cross-linking

Non-enzymatic cross-linking increases with age and induces a cascade of biochemical processes, collectively known as the **Maillard reaction**, which involves a spontaneous interaction between an **extracellular sugar-derived aldehyde** groups and for example, the ε-amino **group of** collagen-bound **hydroxylysine or lysine**. The resulting glucosyl-lysine rearranges to form an **Amadori product or Schiff base** adduct, both of which undergo further reactions with other amino groups (Saito and Marumo, 2013). These processes produce a family of molecules known as advanced glycation endproducts (**AGEs**) that form as cross-links within and across collagen fibres.

1.3 LOX and LOX-like enzymes

Lysyl oxidases are extracellular enzymes that catalyse the formation of lysine- and hydroxylysine-derived cross-links in collagens and lysine-derived cross-links in elastin (Kagan and Li, 2003) (Figure 2). These cross-links are essential for the tensile strength of collagens and the rubber-like properties of elastin. Both of them are abundant ECM proteins necessary for the structural integrity and function of connective tissues (Kagan and Li, 2003; Myllyharju and Kivirikko, 2004).

In contrast to other copper-containing amine oxidases (like vascular adhesion protein-1 or diamine oxidase (McGrath *et al.*, 2009)), much less is known about the structure and molecular function of the LOX and LOX-like (LOXL) enzymes (Figure 3). Approximately 40 years after the discovery and isolation of LOX from bovine aorta, crystal structure has not been solved for any member of the human LOX family, and very few biochemical studies have been conducted, except from those on LOX. Consequently, while numerous associations between LOX family members and various diseases have been identified (Barker *et al.*, 2012) (novel pathological roles are discovered yearly), the molecular functions of the LOX and LOXLs and the degree to which their functions overlap remain unsatisfactorily understood.

In total, five genes belonging to the LOX family of proteins have been identified in human genome: *LOX* (on chromosome 5), *LOXL1* (chromosome 15), *LOXL2* (chromosome 8), *LOXL3* (chromosome 2), and *LOXL4* (chromosome 10).

The LOX family of proteins can be divided into two subgroups based on the nature of their N-terminal domains (Figure 3):

- LOX and LOXL1 contain a highly basic peptide at their N-termini (termed the propeptide),
- LOXL2, LOXL3 and LOXL4 each contain four scavenger receptor cysteine-rich (SRCR) domains.

There is a conserved bone morphogenetic protein-1 (BMP-1) cleavage site between the propeptide and the LOX catalytic domain of LOX and LOXL1 (Uzel *et al.*, 2001), but this site is not conserved in LOXL2, LOXL3 and LOXL4 (Moon *et al.*, 2014).

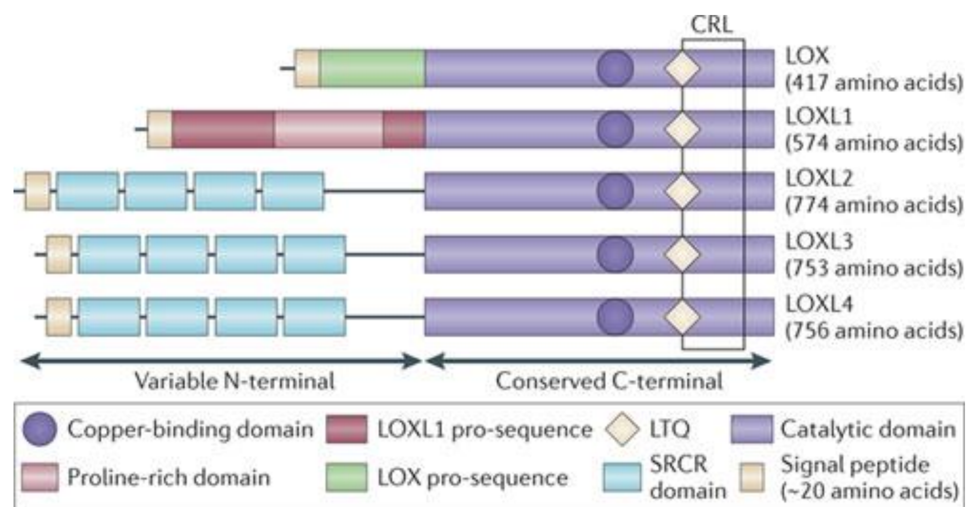

Figure 3: Structure of LOX and LOXL enzymes (Barker *et al.*, 2012)

SRCR - scavenger receptor cysteine-rich domain; **CRL** - cytokine receptor-like domain; **LTQ** - lysyl-tyrosyl quinone residue

LOX and LOXL1 share at the C-terminal which contains the catalytic domain 77% identity and 88% homology, while the C-terminal of LOXL2, LOXL3 and LOXL4 exhibits 71–72% identity and 84–88% homology (Moon *et al.*, 2014).

The precursor residues of the lysine tyrosylquinone cofactor and the predicted Cu^{2+}-binding site (His-*X*-His-*X*-His) are conserved at the C-terminal in all five family members (Moon *et al.*, 2014) (Figure 3). Additionally, all LOX family members possess an N-terminal secretion signal, but lack predicted transmembrane domains; therefore, they are generally considered to be secreted proteins (Kagan and Li, 2003).

The existence of a putative signal sequence for secretion and four SRCR domains suggests that the LOXL2-4 isoenzymes are extracellular proteins. SRCR protein super

family is involved in quite different functions, such as pathogen recognition, modulation of the immune response, epithelial homeostasis, stem cell biology and tumour development (Resnick *et al.*, 1994). Therefore LOXL2-4 may be involved in the binding and cross-linking of cell surface and extracellular matrix proteins.

1.3.1 LOX

LOX (OMIM 153455) is expressed highly in the heart, placenta, skeletal muscle, kidney, lungs and pancreas (Kim *et al.*, 1995). This amine oxidase is synthesized as an inactive 50 kDa proenzyme that is N-glycosylated in the endoplasmic reticulum and the Golgi complex. Then LOX proenzyme is secreted into the extracellular environment, where it is processed by pro-collagen C-proteinases (mammalian Tolloids), particularly by BMP-1, into an active enzyme (28-32 kDa) and an 18 kDa propeptide (LOPP) that can be N-glycosylated intracellularly into a ~35 kDa form (Trackman *et al.*, 1992; Uzel *et al.*, 2001; Guo *et al.*, 2007). Published data have shown that active LOX can subsequently be translocated from the extracellular matrix into the cytoplasm and nucleus of cells (Nellaiappan *et al.*, 2000) (Figure 4).

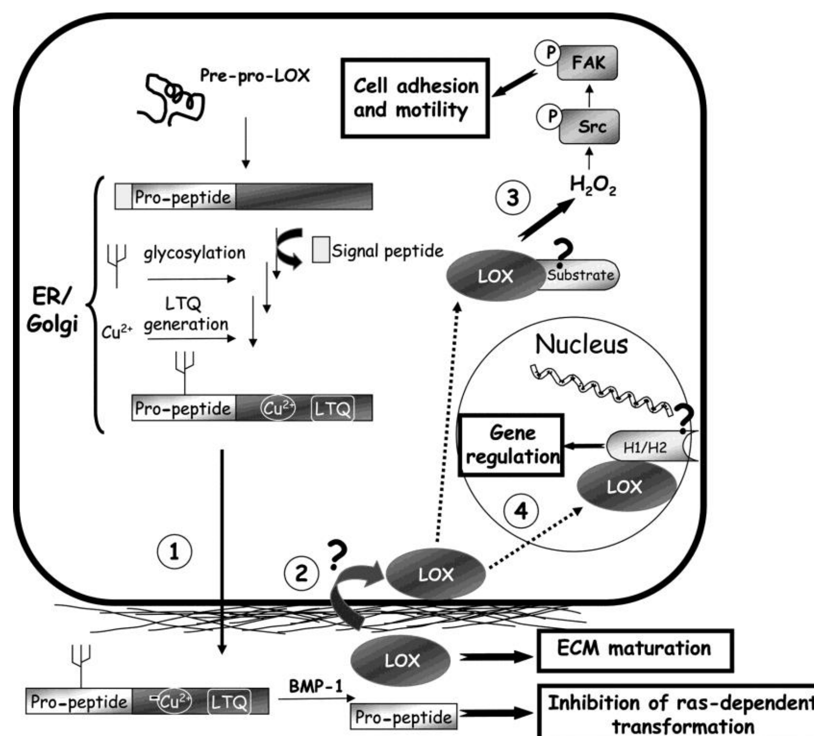

Figure 4: LOX synthesis and intra- and extracellular functions (Rodriguez *et al.*, 2008)

LOX is synthesized as a pre-protein, and after signal peptide hydrolysis, enzyme glycosylation, Cu^{2+} incorporation, and LTQ generation, the enzyme is released into the extracellular space (**1**). Then, BMP-1 cleaves the pre-protein into active LOX stabilizing the ECM via cross-linking of collagen and elastin chains and its pro-peptide function in inhibition of ras-dependent

transformation. Extracellular LOX can translocate to intracellular compartments (**2**). In cytosol, LOX controls cell adhesion and motility (**3**). Nuclear forms of LOX control gene expression (**4**). Histones H1 and H2 could be nuclear substrates of this enzyme.

1.3.2 LOXL enzymes

LOXL1 (OMIM *153456) has been shown to be expressed in a number of ocular tissues, including the ciliary body, lens, optic nerve, retina, and especially in the iris (Schlotzer-Schrehardt *et al.*, 2008). LOXL1 has gained some attention due to the fact that single nucleotide polymorphisms (SNPs) within this gene are associated with exfoliation syndrome in Scandinavian males over 60 years old showing 99% susceptibility (Thorleifsson *et al.*, 2007). A role for LOXL1 in elastic fibre renewal in adult tissues was proposed, based on the fact that mice lacking LOXL1 do not deposit normal elastic fibres in uterine tract, develop pelvic organ prolapse, loose skin and have vascular abnormalities with concomitant tropoelastin accumulation (Liu *et al.*, 2004).

LOXL2 (OMIM *606663) can be found in many tissues, with the highest expression observed in reproductive tissues, e.g. the placenta, uterus and prostate (Jourdan-Le Saux *et al.*, 1999). LOXL2 is generally expected to function similarly to LOX in regard to ECM cross-linking and stiffening. LOXL2 expression is linked to upregulation of tissue inhibitors metalloproteinase-1 and matrix metalloproteinase-9 (as also proposed for LOX), thereby promoting ECM degradation and dissemination of metastatic breast cancer cells (Barker *et al.*, 2011). However, there has been no *in vitro* biochemical study to directly compare the respective activities of LOX and LOXL2 in ECM stiffening.

LOXL3 (OMIM *607163) expression has been detected in many tissues and is most highly expressed in the placenta, heart, ovary, testis, small intestine and spleen (Jourdan-Le Saux *et al.*, 2001; Lee and Kim, 2006).

LOXL4 (OMIM *607318) is expressed mostly in the skeletal muscle, testis, pancreas, and cartilage (Asuncion *et al.*, 2001; Maki *et al.*, 2001). LOXL4 has a variety of recognized roles in human diseases. Similarly to LOXL1, LOXL4 is epigenetically silenced in bladder cancer cells, and overexpression of either protein in bladder cancer cells has been shown to inhibit Ras/ERK signaling pathways (Wu *et al.*, 2007).

1.4 Keratoconus

Keratoconus (KC) is a non-inflammatory disease characterized by progressive corneal thinning and ectasia manifesting as myopia and irregular astigmatism (Krachmer *et al.*, 1984) (Figure 5). It is typically diagnosed in the patient's adolescent years, usually affecting both eyes (Rabinowitz, 1998). The prevalence of KC in population of European descendent varies from 5.5 to 8.6 per 10,000 inhabitants (Kennedy *et al.*, 1986; Nielsen *et al.*, 2007) which makes it a common disorder. Males are affected more often than females with variable ratio 1.7/1.0 to 3.0/1.0 depending on population studies (Ihalainen, 1986; Weed and McGhee, 1998). Current understanding of KC supports a complex aetiology involving both genetic and environmental factors (Abu-Amero *et al.*, 2014).

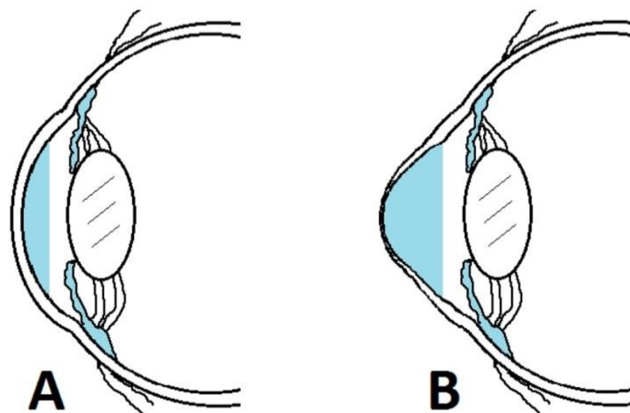

Figure 5: Normal (A) and keratoconic cornea (B)

1.4.1 Management of keratoconus

The clinical diagnosis of KC is based on the presence of typical corneal signs such as thinning, protrusion, Vogt striae, Fleischer ring (deposition of iron) and scarring. Diagnosis is confirmed ideally by the assessment of both the anterior and posterior corneal curvature maps along with pachymetry using devices such as Pentacam (Oculus, Germany) working on the principle of Scheimpflug imaging.

The management of KC depends on the state of disease progression. In the very early stages, spectacles lenses are an option; however, as they do not correct irregular astigmatism, vision may not be satisfactory. Rigid gas permeable contact lenses provide usually much better correction in these cases and in the past management with contact lenses was preferred by up to 90% of patients with KC (Rabinowitz *et al.*, 1991).

Currently, corneal stabilization, which does not however cure the disease, achieved by corneal collagen cross-linking (CXL) has become the golden standard of treatment in the early disease stages (Wollensak, 2006). The method is based on the principle that riboflavin as a photosensitizer generates reactive oxygen species that create covalent bonds between collagen molecules by photopolymerization on exposure to ultraviolet-A light (Wollensak, 2006) thus enhancing corneal rigidity and preventing further ectasia. However, not all patients can be treated using this method as some cases are still detected in an advanced stage when CXL is contraindicated due to insufficient stromal thickness and possible damage to the corneal endothelium.

The CXL approach leading to formation of covalent bonds between collagen fibrils, enhancing corneal rigidity (Wollensak, 2006), imitates physiological cross-linking catalysed by LOX (Kagan *et al.*, 1986).

In advanced stages, commonly used surgical options for KC include penetrating keratoplasty (PK) and deep anterior lamellar keratoplasty (DALK) (Gomes *et al.*, 2015).

PK has been the mainstay of KC treatment for many decades. The visual rehabilitation is often slow, influenced by high degrees of postoperative astigmatism. Main risks of PK include graft rejection or failure, infection and recurrence of the disease. Despite these negative influences, in advanced cases, especially with healed scarring or corneal hydrops (stromal edema due to leakage of aqueous through a tear in Descemet membrane) PK may be the only option for restoration of useful visual acuity (Gomes *et al.*, 2015).

Over the last few years there has been move from PK towards DALK in KC cases without significant corneal scarring or corneal hydrops. DALK technique aims to remove all or near total corneal stroma up to the Descemet membrane (Figure 6). The benefits of DALK are that it is an extra-ocular procedure preserving the host Descemet membrane and endothelium so that there is no risk of endothelial rejection. The main complication associated with DALK is intraoperative perforation of Descemet membrane with subsequent forced switch to PK (Fogla, 2013).

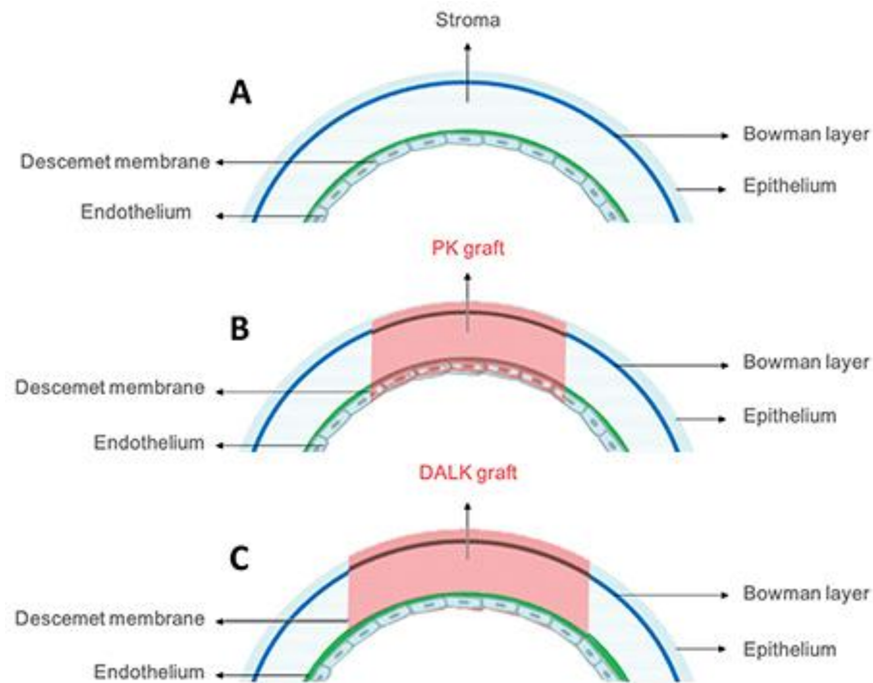

Figure 6: Schematic representation of A) human cornea, B) penetrating keratoplasty (PK) and C) deep anterior lamellar keratoplasty (DALK), source: http://www.clspectrum.com/

1.4.2 Association of KC with other diseases

KC be may presented as an isolated sporadic disorder, or it may be associated with rare genetic disorders, such as Down syndrome, Leber congenital amaurosis or connective tissue disorders (Rabinowitz, 1998). Hard contact lens wear and eye rubbing have been also shown to pose an increased risk for KC development, as well as positive family history for the disease or atopy (Sugar and Macsai, 2012; Rabinowitz, 1998). Associations of rare disorders with KC are important, as shared manifestations of both diseases might provide clues to uncover important features unravelling some pathogenic mechanisms.

Corneal ectasia and thinning, hallmarks of KC, have also been reported in patients with various corneal dystrophies, and the association seems to be higher than it would be expected by chance (Cremona *et al.*, 2009). This has prompted a debate whether there could be common genetic factors involved in the development of these pathologies (Cremona *et al.*, 2009; Lechner *et al.*, 2013).

Regular corneal astigmatism and corneal thinning are characteristic features of macular corneal dystrophy (MCD, OMIM #217800) (Donnenfeld *et al.*, 1986). Corneal astigmatism and corneal thinning are also features of KC, and in this respect the

phenotype of the two conditions is similar. MCD is an autosomal recessive corneal dystrophy caused by mutations in carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6; *CHST6,* OMIM *605294). Mutations in *CHST6* gene result in improper sulfation of keratan sulfate (Hassell *et al.*, 1980). Although fewer or shorter sulfated keratan sulfate chains were found in KC corneas (Sawaguchi *et al.*, 1991), the contribution of possible CHST6 impairment to the development of KC is not known (Burdon and Vincent, 2013).

1.4.3 Genetics of keratoconus

Although most KC cases are sporadic, genetic factors play an important role. Several loci with presumed genetic factors increasing susceptibility to KC have been reported (Figure 7); however, no unequivocal disease-causing gene has been identified (Gajecka *et al.*, 2009; Liskova *et al.*, 2007; Bisceglia *et al.*, 2009; Liskova *et al.*, 2010).

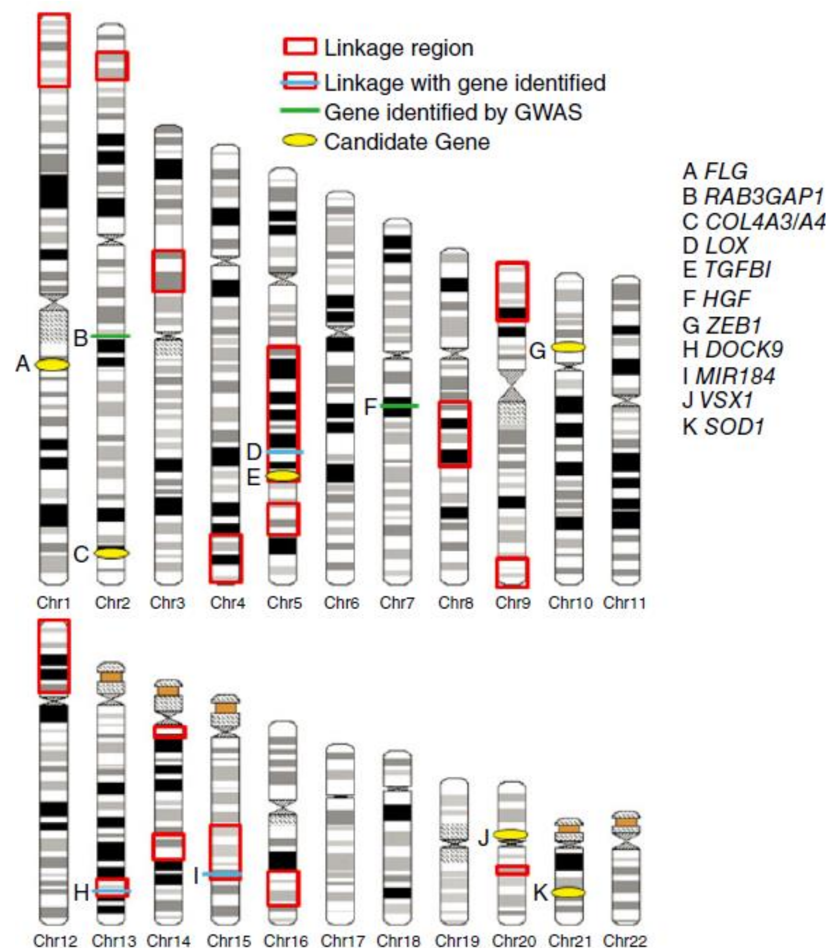

Figure 7: Reported disease-causing and candidate genes for KC and chromosomal loci identified through linkage and genome-wide association studies (Burdon and Vincent, 2013)

Linkage analysis and genome-wide association studies (GWAS) represent two main approaches used to identify genes with variants implicated in disease development. Linkage analysis search for chromosomal region(s) segregating with the disease and evaluates the best positional candidate gene(s) that could be functionally relevant to disease development. In association analysis including GWAS, a relationship between a genetic marker with a particular trait is examined. Until today association studies have posed in KC a less frequently applied methodology than linkage studies (Table 1) (Nowak and Gajecka, 2011).

Table 1: Loci identified by linkage and association studies in KC patients

Locus	Method used	Candidate gene excluded (no mutations found)	Population	Reference
1p36, 8q13-q21	linkage	*ENO1, CTNNBIP1, PLOD1, UBIAD1, SPSB1, TCEB1*	Australian	(Burdon *et al.*, 2008)
2p24	linkage	—	Caucasian and Arab	(Hutchings *et al.*, 2005)
2q13	association	—	Korean	(Kim *et al.*, 2008)
3p14-q13	linkage	*COL8A1*	Italian	(Brancati *et al.*, 2004)
5q14.1-q21.3	linkage	—	Americans	(Tang *et al.*, 2005)
5q21, 5q32-q33, 14q11, 15q15	linkage	—	Italian	(Bisceglia *et al.*, 2009)
9p34	linkage	—	Caucasian and Hispanic	(Li *et al.*, 2006)
13q32	linkage	—	Equadorian	(Gajecka *et al.*, 2009)
14q24.3	linkage	*VSX2*	Mixed	(Liskova *et al.*, 2010)
15q22-q24	linkage	*CTSH, CRABP1, IREB2, RASGRF1*	Nothern Irish	(Hughes *et al.*, 2003)
16q22-q23	linkage	—	Finnish	(Tyynismaa *et al.*, 2002)
17p13	linkage	*RETGC-1, PEDF*	Pakistani	(Hameed *et al.*, 2000)
20q12	linkage	*MMP9*	Australian (UK descent)	(Fullerton *et al.*, 2002)

Although a number of genetic loci have been implicated from linkage studies in extended families (Table 1), only one locus has been independently replicated (5q21.2, previously reported by Tang *et al.* (2005) has been replicated by Bisceglia *et al.* (2009)).

Genome-wide association methods using a case-control cohort increases the analysis power allowing for detection of loci with lower effects (Nowak and Gajecka, 2011).

Only two potential genes have been identified through GWAS (Burdon *et al.*, 2011; Bykhovskaya *et al.*, 2012) leaving the pathogenic role of the variants indicated as disease-causing still debatable. Neither identified loci reached widely accepted statistical

significance threshold and had limited power due to small sample sizes of multi-ethnic provenance where stratification may have been a serious issue (Burdon *et al.*, 2011; Bykhovskaya *et al.*, 2012).

The first performed GWAS by Burdon *et al.* (2011) identified association between KC and three SNPs in the promoter region of the hepatocyte growth factor (*HGF*) gene. The study also showed a relationship between genotype at the associated SNP (rs3735520) and serum HGF levels in normal individuals. In addition, this gene has been associated with refractive error and specifically with myopia in multiple studies (Kok *et al.*, 2012; Veerappan *et al.*, 2010; Yanovitch *et al.*, 2009) making it an attractive candidate for KC.

The second GWAS study for KC described the findings from the USA cohorts that also contributed to the *HGF*-association results. After two-stage genome-wide linkage scan in KC families, locus at chromosome 5q23.2, overlapping *LOX* gene, was identified. Two SNPs (rs10519694 and rs2956540) in *LOX* were associated with KC by family-based association testing and were also found to be significantly associated with KC in case-control cohorts (Bykhovskaya *et al.*, 2012). Two additional SNPs (rs1800449 and rs2288393) were subsequently detected by TaqMan SNP Genotyping Assay. The association of SNPs in *LOX* with KC was then validated in Iranian population confirming minor allele of rs1800449 as a risk factor for KC development (Hasanian-Langroudi *et al.*, 2014).

Details on these association studies (population origin, genotyping methods used, number of KC and control samples and reported p-values) on or concerning the *LOX* and *HGF* locus are shown in Table 2.

Table 2: Studies identifying and validating association of SNPs within the *LOX* and *HGF* loci with KC

Only results provided for independent case-control panels and SNPs discovered by genome-wide association studies are shown. Australian discovery samples were pooled and SNPs shown as significant were validated by individual genotyping, reported p-values are shown from this experiment.

Initial study on the *LOX* locus (Bykhovskaya *et al.*, 2012)					
SNP	**Population origin**	**Genotyping method**	**KC cases**	**Controls**	**Reported p-value**
rs10519694	US Caucasian discovery	**GWAS** - HumanCNV370-Quad BeadChip; (Illumina Inc., San Diego, CA, USA)	222	3324	2.3×10^{-3}
	US Caucasian replication	Infinium iSelect BeadChip (Illumina)	304	518	0.30
rs2956540	US Caucasian discovery	**GWAS** - HumanCNV370-Quad BeadChip (Illumina)	222	3324	7×10^{-3}
	US Caucasian replication	Infinium iSelect BeadChip (Illumina)	304	518	3.5×10^{-3}
rs1800449 [α]	US Caucasian discovery	TaqMan SNP Genotyping Assay (Applied Biosystems, Inc., Foster City, CA, USA)	377	114	0.07
rs2288393 [α]	US Caucasian discovery	TaqMan SNP Genotyping Assay (Applied Biosystems)	377	114	NA [β]
Independent replication of the *LOX* locus (Hasanian-Langroudi *et al.*, 2014)					
SNP	**Population**	**Genotyping method**	**KC cases**	**Controls**	**Reported p-value**
rs1800449	Iranian	Allele-specific PCR	112	150	0.011
rs2288393	Iranian	Allele-specific PCR	112	150	0.404
Initial study on the *HGF* locus (Burdon *et al.*, 2011)					
SNP	**Population**	**Genotyping method**	**KC cases**	**Controls**	**Reported p-value**
rs3735520	Australian Caucasian discovery	iPLEX GOLD chemistry with Sequenom MassArray (Sequenom, San Diego, CA, USA).	97	216	0.002 [ζ]
	Australian replication cohort 1 [δ]	iPLEX GOLD chemistry with Sequenom MassArray (Sequenom)	96	72	
	Australian replication cohort 2 [ε]	TaqMan SNP Genotyping Assay (Applied Biosystems)	215	112	0.664
	US Caucasian discovery	**GWAS** - HumanCNV370-Quad BeadChip (Illumina)	222	3324	6.1×10^{-7}
	US replication cohort	Infinium iSelect BeadChip (Illumina)	304	518	0.655
rs1014091 [γ]	Australian Caucasian discovery	**GWAS** - HumanHap 1M (Illumina) followed by individual genotyping with iPLEX GOLD chemistry (Sequenom)	97	216	0.0084
	Australian replication cohort 1 [δ]	iPLEX GOLD chemistry with Sequenom MassArray (Sequenom)	96	72	0.0083
	US Caucasian discovery	**GWAS** - HumanCNV370-Quad BeadChip (Illumina)	222	3324	0.018
	US replication cohort	Infinium iSelect BeadChip (Illumina)	304	518	0.658
rs17501108 [γ]	Australian Caucasian discovery	**GWAS** - HumanHap 1M (Illumina)	97	216	0.0002 [ζ]
	Australian replication cohort 1 [δ]	iPLEX GOLD chemistry with Sequenom MassArray (Sequenom)	96	72	
	Australian replication cohort 2 [ε]	TaqMan SNP Genotyping Assay (Applied Biosystems)	215	112	0.006

Abbreviations: **GWAS**, genome-wide association study; **HGF**, hepatocyte growth factor; **KC**, keratoconus; **LOX**, lysyl oxidase; **NA**, not available; **SNP**, single nucleotide polymorphism

α Genotyped to validate the *LOX* locus after the discovery of association of rs10519694 and rs2956540 with KC

β Only p-value (0.02) after meta-analysis of case-control and family samples was provided

ɣ rs17501108 in complete linkage disequilibrium (r^2 =1.0) with rs1014091 in International HapMap Project.

δ composed of 39 Australian cases, 57 cases from Northern Ireland (no further details on ethnicity provided).

ε composed of 186 Australian cases, 29 cases from Northern Ireland (no further details on ethnicity provided) and 112 control genotype were data downloaded CEU samples from International HapMap Project

ζ Study provided only p-value for combination of the pooled Australian discovery and Australian replication cohort 1 samples

1.4.4 Histological and biochemical signs of KC

Typical histopathological features of KC corneas include stromal thinning, iron deposition in the epithelial basement membrane, and breaks in Bowman layer and epithelial basement membrane (Rabinowitz, 1998). The change in corneal geometry, such as thickness and curvature, associated with KC is in fact a manifestation of the changes in structure (collagen fibre organization) and composition (amount of proteoglycans, collagen, and keratocytes) that further affects the mechanical and optical properties of the cornea (Ambekar *et al.*, 2011).

It is widely accepted that oxidative stress plays a critical role in the development and progression of KC (Kenney *et al.*, 2005; Chwa *et al.*, 2008). The impairment of antioxidant enzymes induces the accumulation of cytotoxic reactive oxygen and nitrogen species (Buddi *et al.*, 2002) and leads to the activation of degradative enzymes, a decrease of their inhibitors (Matthews *et al.*, 2007) as well as the accumulation of mitochondrial DNA mutations (Atilano *et al.*, 2005). The imbalance of ECM remodelling enzymes and their inhibitors plays a role in the stromal thinning and Bowman layer and/or basement membrane breaks that are characteristic of KC corneas (Cristina Kenney and Brown, 2003).

Stromal thinning may be caused as well by a reduction in the number of lamellae within the affected region (Patey *et al.*, 1984), but the mechanism is uncertain. It has been proposed that collagen fibres are not lost but simply redistributed within the cornea by slippage between the lamellae (Polack, 1976).

The expression of a range of proteins, including cytokines and enzymes, is altered in KC when compared with normal controls, suggesting the involvement of apoptosis and scarring in the disease process. However, it is unclear whether the pathways are modified as a primary or secondary phenomenon (Davidson *et al.*, 2014).

2. Hypotheses and aims of work

KC is characterized by the progressive corneal thinning and ectasia that leads to significant refractive error and visual impairment that negatively affects patients' life. Although it has been recognized for more than 150 years (Rabinowitz, 1998), despite extensive research, the exact etiopathogenesis of KC remains unknown.

Hypothesis 1: Impairment of LOX enzymes in KC corneas

LOX enzyme family is the crucial enzyme involved in the formation of links between collagens and is considered to be one of the candidate genes for KC development. The upregulation of LOX expression was found in the epithelium of KC corneas compared to control tissue (Nielsen *et al.*, 2003), but until now, there have been no reports on the distribution and activity of LOX enzyme in the human cornea.

We hypothesize that improper cross-linking in KC cornea is caused by an impairment of LOX activation and we expect to find a lower LOX activity compared to healthy cornea. This hypothesis is supported by the fact that increasing the amount of cross-links in corneal tissue induced by CXL is able to stop KC progression.

Aims:

- To detect the presence, distribution of LOX and lysyl oxidase propeptide (LOPP) in control corneas and KC explants and to investigate whether there are differences between healthy and diseased tissues (**Paper 1, Dudakova *et al.*, 2012**).
- To compare the total LOX activity (LOX and LOX-like enzymes) in media of cultured stromal cells (keratocytes) derived from control and KC corneas (**Paper 1, Dudakova *et al.*, 2012**).
- To detect the presence and distribution of LOXL1-4 enzymes in control and KC explants and to investigate whether there are differences between healthy and diseased tissues (**Paper 2, Dudakova *et al.*, 2015a**)

Hypothesis 2: Association of KC with other diseases

Association of KC with other diseases has been shown in numerous publications. Concurrent manifestation of rare diseases and KC might provide clues to discover unknown processes important in disease development.

Aims:

- To find out whether any of KC-associated diseases exhibits signs that may reflect LOX impairment (**Paper 3, Dudakova *et al.*, 2013**).
- To identify the molecular genetic cause of MCD in four Czech probands, characterize phenotypic similarities between MCD and KC and thus to contribute in elucidation of the *CHST6* role to KC development (**Paper 4, Dudakova *et al.*, 2014**).

Hypothesis 3: The role of SNPs in *LOX* and *HGF* in KC

SNPs in these two genes identified by GWAS have been reported to increase susceptibility to KC development.

Aim:

- To validate the effect of these SNPs in Czech patients with KC - the first Caucasian cohort of non-Anglo-Saxon patients (**Paper 5, Dudakova *et al*., 2015b**).

Hypothesis 4: Copper as unrecognized environmental factor in KC pathogenesis

The pathogenesis of KC remains unclear, but current theories are based on the observed alterations in collagen fibrils organization and structure (Meek *et al.*, 2005), oxidative stress (Chwa *et al.*, 2008; Cristina Kenney and Brown, 2003) or an increase of degradation enzymes (Balasubramanian *et al.*, 2012). These hypotheses concern individual processes which have not been linked together.

Alterations of many Cu-dependent enzymes have been connected with KC (Atilano *et al.*, 2005; Udar *et al.*, 2006; Dudakova *et al.*, 2012), and the decrease of Cu levels in KC corneas has been reported (Avetisov *et al.*, 2011). Nonetheless, a systemic insight into the role of Cu in the pathogenesis of corneal ectasias has not been provided.

Aim:

- To focus on the current pathways of KC development in the context of Cu imbalance and connect them into one common pathway (**Paper 6, Dudakova *et al*., 2015c**).

3. Material and Methods

To achieve the aims of our work, a wide spectrum of methods was used. Individual detailed protocols are described in the appended publications. Partial steps mentioned below were managed by the author of this Doctoral Thesis.

Paper 1

- Indirect fluorescent immunohistochemistry of LOX and LOPP on cryosections of control and KC corneas and slides examination.
- Establishment of primary cell lines from control and KC corneas and cultivation of corneal cells; identification of cell types using indirect fluorescent immunocytochemistry (antibodies against α-smooth muscle actin (α-SMA) cells to exclude myofibroblast phenotype and cytokeratin 3/12 (CK3/12) to exclude the presence of epithelial cells).
- LOX activity measurement in media of cultured cells using fluorometric assay (implementation and optimization of the method).
- Statistical analysis (One-way ANOVA).

Paper 2

- Indirect fluorescent immunohistochemistry of LOXL1-4 on cryosections of control and KC corneas and slides examination.
- Western blot analysis of LOXL1-4 in KC and control corneas.

Paper 3

- Review of current knowledge about diseases associated with KC in respect to LOX impairment.

Paper 4

- Isolation of DNA from blood or saliva samples of MCD probands and family members
- Sanger sequencing of the entire coding region of *CHST6* gene – primer pair optimization, PCR reactions, sequence data analysis, analysis of potentially pathogenic variants – database search, bioinformatical predictions of pathogenicity (PolyPhen2, MutPred, SNP&GO).
- Preparation of tissue sections for histology and their examination.

Paper 5

- Isolation of DNA from blood or saliva samples of KC patients.
- Qualitative aliquoting of DNA for external analysis (concentration and purity measurements, diluting), Sanger sequencing (verification of genotyping data).
- Involvement in statistical data analysis.

Paper 6

- Studying of metabolic pathways affected in KC in relation to Cu imbalance.

4. Results

The most important results of the work performed are summarized below. For more detailed results, please see the appended publications.

Impairment of LOX enzymes in KC corneas

In the control tissue, the most intense signal for LOX was present in the corneal epithelium; less intense staining was present in keratocytes, the extracellular matrix and in the corneal endothelium. The distribution of LOX was clearly decreased in at least five of the eight KC specimens. The most marked signal reduction was observed in the stromal matrix and in keratocytes. Moreover, the signal in pathological specimens revealed a more irregular pattern, including the presence of intra- and extracellular clumps in the epithelium. Interestingly, endothelial cells showed no or very weak staining in areas just beneath the negative stromal tissue (Figure 8).

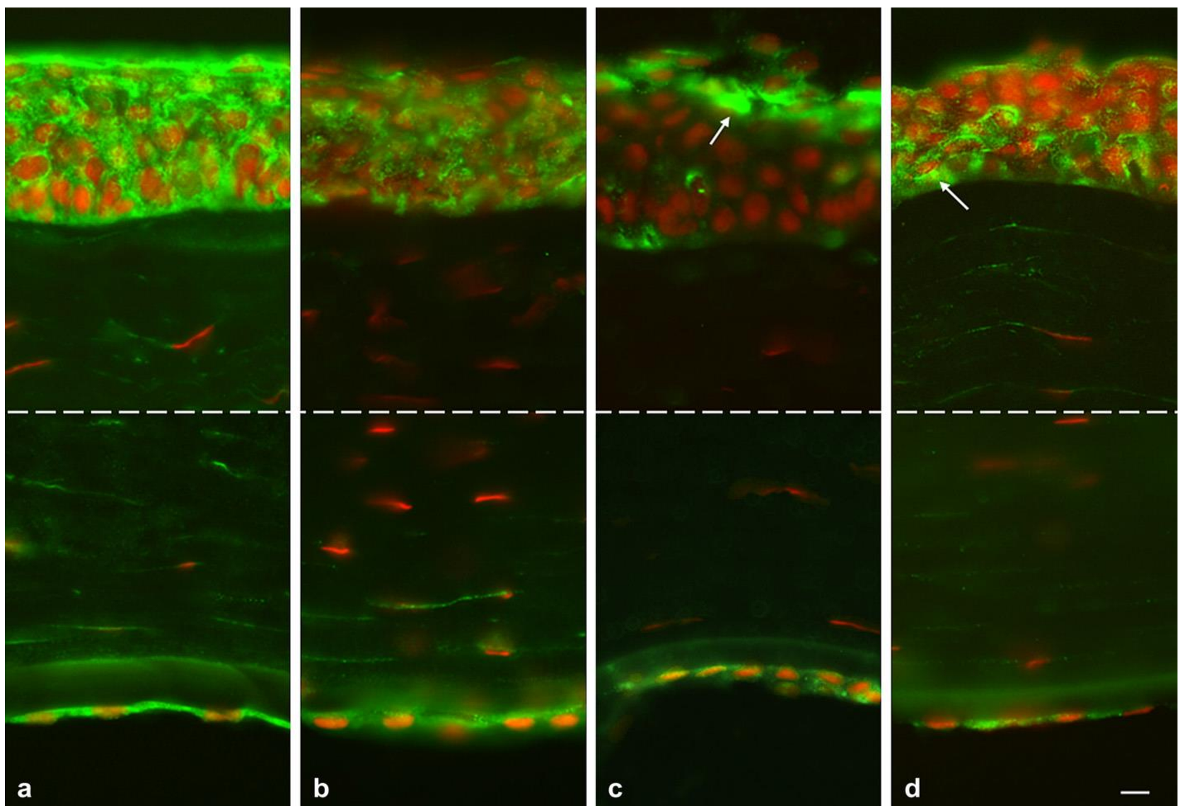

Figure 8: Immunohistochemistry localization of LOX in control (a) and KC corneas (b, c, d)
We detected active form of LOX (green). In KC corneas, the signal is less intense and the epithelium exhibits some staining irregularities expressed as a clump-like pattern in the cytoplasm (arrows). The nuclei were counterstained with propidium iodide (red). Scale bar, 10 μm (Dudakova *et al.*, 2012).

The staining of LOPP in the majority of the control epithelial cells revealed intense diffuse positivity. In all control specimens, weak to moderate staining was diffusely present in the ECM. Moderate to very intense staining was observed in keratocytes and endothelial cells. The epithelium of three corneas exhibited a clumplike staining pattern (Figure 9). A markedly diminished signal in the ECM and keratocytes was found in seven out of eight KC specimens compared to the control ones. The punctuate-like pattern present in all control specimens in the ECM was absent in three and diminished in four KC specimens. The signal in the endothelium was diminished in all KC specimens, and interestingly, the endothelial cells showed no or very weak staining in the vicinity of the ECM areas where the signal was absent or very low.

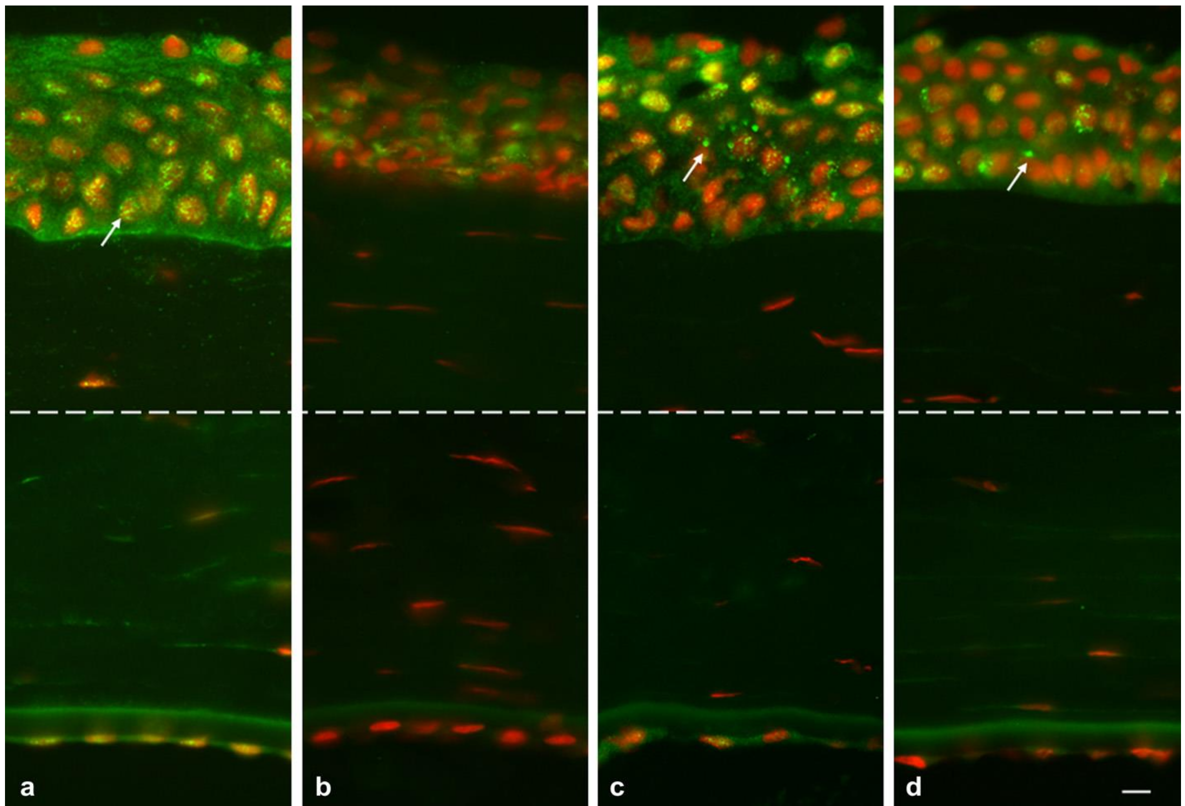

Figure 9: LOPP localization in control (a) and KC corneas (b, c, d)
Using indirect immunohistochemistry we detected N-glycosylated form of LOPP protein (green). Perinuclear staining is present in control as well as in KC epithelium, where it exhibits a more clump-like pattern (arrows). A markedly diminished signal is seen in the stroma and in endothelial cells in the vicinity of the extracellular matrix areas where the signal is absent or very low (b). The nuclei were counterstained with propidium iodide (red). Scale bar 10 μm (Dudakova *et al.*, 2012).

The migration of KC stromal cells began earlier than that of control cells. In the second passage about 80% of KC cells from all patients showed moderate to intense cytoplasmic positivity for α-SMA. At the fifth passage, the α-SMA signal diminished. Using phase contrast microscopy, almost all cells showed the phenotype of corneal fibroblasts.

Only a few cells in all cultures and passages were positive for CK3/12 indicating epithelial phenotype (Moll *et al.*, 1982).

The mean total LOX activity in the KC samples (2.60 ± 2.23 nM H_2O_2/mg of total protein) was more than 2.5-fold lower than in the control tissue (6.83 ± 2.53 nM H_2O_2/mg of total protein), and the decrease was statistically significant ($p = 0.0178$).

Moderate to intense staining was detected for LOXL1 in all parts of the cornea in the control as well as in the KC samples. Using LOXL2 antibody, moderate to intense staining was observed in the epithelium, stroma and endothelium of all control corneas. Staining irregularities in the epithelium (a decrease of staining and a clump-like pattern) and a gradual anterior-posterior weakening of the signal from moderate to weak were observed in the stroma of KC corneas (Figure 10).

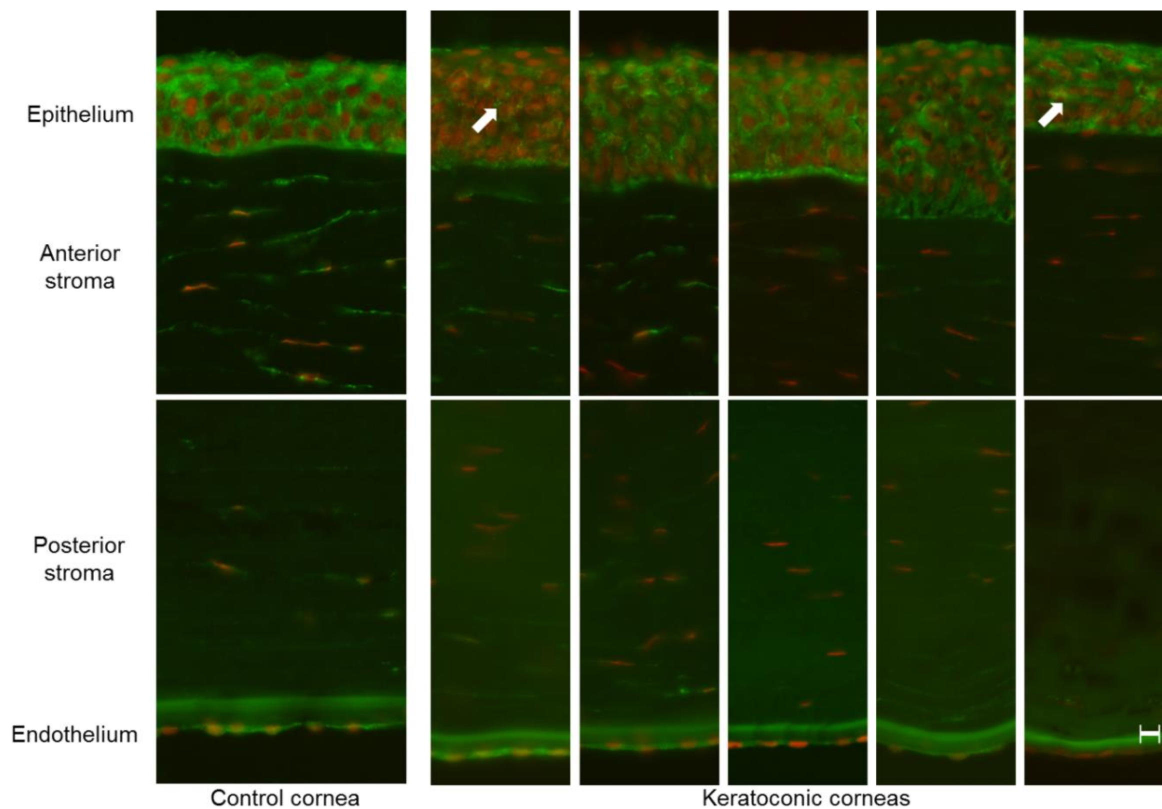

Figure 10: LOXL2 staining in control and KC corneas
Note the decrease of the signal and irregularities in the epithelium of keratoconic corneas (arrows). Magnification 20x (Dudakova *et al.*, 2015a).

LOXL3 antibody revealed moderate to intense staining in the epithelium and endothelium of both control and KC samples. In KC samples, we observed a local increase of staining. The staining of the ECM was moderate and intense for keratocytes in controls but weak or almost absent in most of the KC specimens (Figure 11).

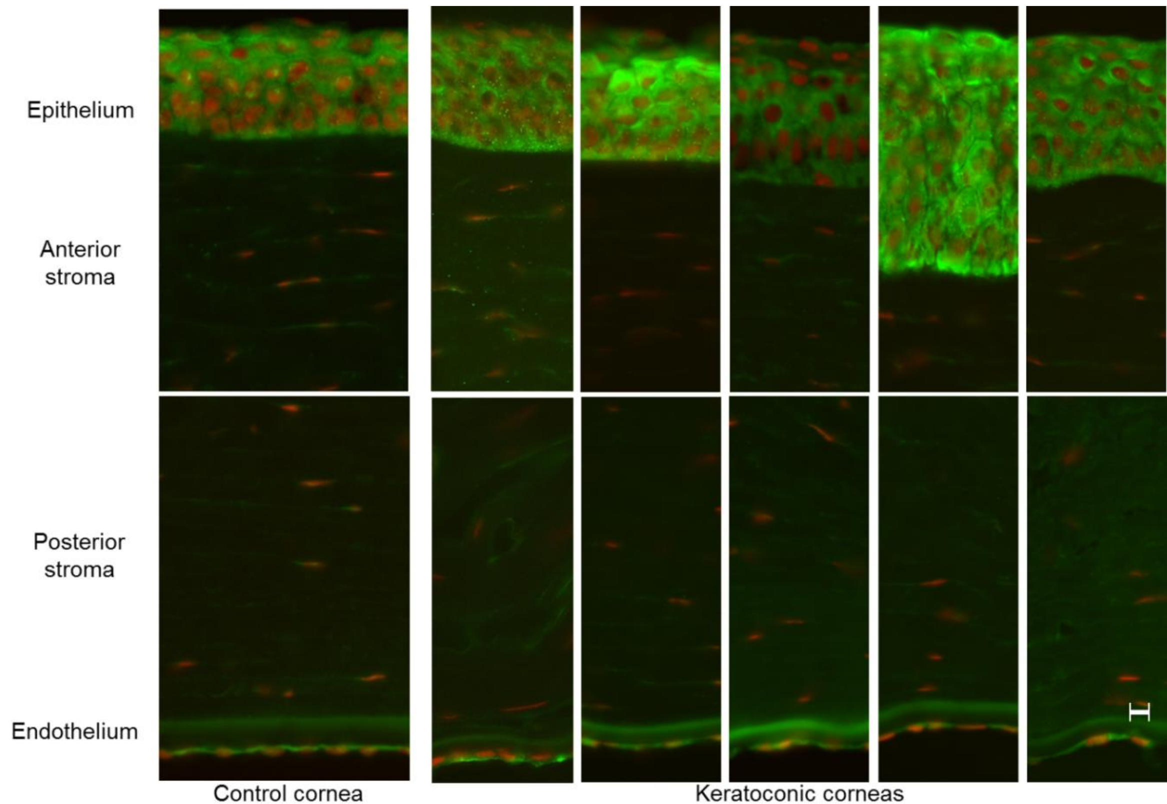

Figure 11: LOXL3 staining in control and KC corneas
Note the decreased staining in the stroma of KC corneas. Magnification 20x (Dudakova *et al.*, 2015a).

Using LOXL4 antibody, the epithelium and endothelium of both control and KC samples showed a moderate to intense signal, while a moderate signal with a punctate-like pattern was observed in the stroma.

All LOX-like enzymes were present in the limbus and conjunctiva of control samples. For LOXL1 we observed intense and for LOXL2, -3 and -4 moderate to intense staining. LOXL1 and LOXL3 exhibited staining heterogeneities – cells adjacent to the superficial layer of the epithelium showed higher positivity compared to cells located in the deeper layers.

Using Western blot analysis we did not find differences between control and KC samples when staining with anti-LOXL1 antibody. We observed a decrease of LOXL2 and 4 in KC samples compared to controls (Figure 12), while a slight increase of LOXL3 was observed in KC corneas.

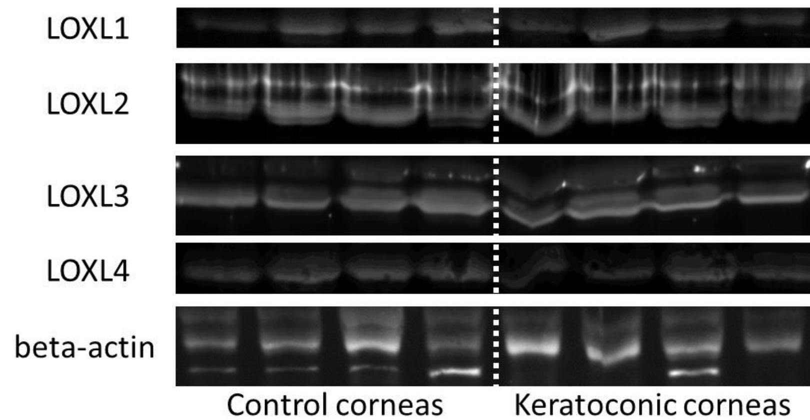

Figure 12: Western blot analysis of control and KC corneas (Dudakova *et al.*, 2015a)

Association of KC with other diseases

Nearly 70 systemic disorders have been reported in association with KC, most of them affecting the ECM. We found similar changes, particularly at the level of collagen metabolism, including LOX impairment in mitral leaflets, which may reflect a reported association between KC and mitral valve prolapse. Among other disorders that have been found to coincide with KC, we did not find any in which the LOX enzyme may be directly or indirectly impaired.

Homozygous or compound heterozygous *CHST6* mutations were identified in all cases of MCD patients, including two novel mutations, c.13C>T; p.(Arg5Cys) and c.289C>T; p.(Arg97Cys). Histopathological examination of all available corneas confirmed the presence of corneal thinning together with characteristic glycosaminoglycan deposits that stained positive with Alcian blue, in keratocytes, endothelial cells, and extracellularly in the stroma and Descemet membrane. Pentacam measurements were taken in six eyes of three probands with MCD. All of them showed diffuse corneal thinning with paracentral steepening of the anterior corneal surface that was graded as KC by the integrated software, but without associated ectasia of the posterior corneal surface or regional thinning.

The role of SNPs in *LOX* and *HGF* in KC

Two out of the seven SNPs analysed showed significant association with KC in a co-dominant allelic test. The rs2956540-C, located within in the fourth intron of the *LOX* gene (OR=0.69; 95% CI, 0.50–0.96 for allele C; P=0.024) and rs3735520-A located in genomic region upstream of the *HGF* transcription initiation site (OR=1.45; 95% CI, 1.06–

1.98 for allele A; P=0.018). Explorations of alternative models of inheritance changed little the association significance for both loci, although genotypes homozygous for alleles increasing susceptibility to KC were associated with higher ORs than under the allelic (co-dominant) model, suggesting (although not proving) a possible recessive effect in both SNPs.

Copper as unrecognized environmental factor

For the first time, we connected all the known phenomena involved in the pathogenesis of KC (corneal thinning, apoptosis, changes in expression, breaks in mitochondrial DNA, iron deposition, increased activity of proteinases and decreased amount of their inhibitors) in to one common pathway. Partial steps in this model are working hypotheses, which will be hopefully verified in the future. Summarized concept of all affected metabolic pathways could be found in Figure 10.

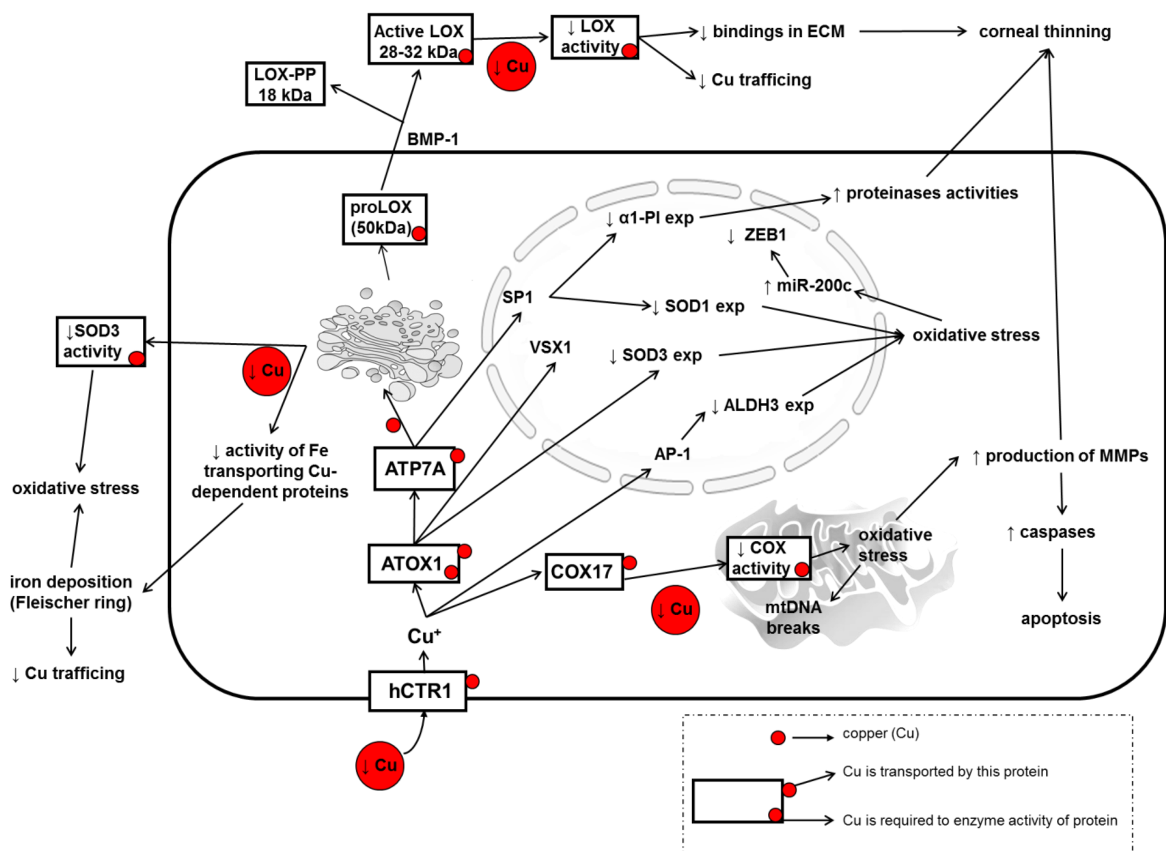

Figure 13: Schematic representation of our current understanding of the pathophysiology of KC in respect to Cu imbalance (Dudakova *et al.*, 2015c)

5. Discussion and Conclusions

Impairment of LOX enzymes in KC corneas

Although LOX is the key enzyme for collagen cross-linking, we were the first who evaluated its distribution and activity in the human cornea. As based on the function of this amino oxidase, it was hypothesized that LOX impairment may be involved in KC pathogenesis therefore we have localized LOX and determined the total LOX activity in corneas from patients with KC and compared these parameters to healthy tissue.

The staining pattern in KC sections was markedly irregular compared to control samples. We hypothesize that the upregulation of LOX expression in the epithelium of KC corneas compared to normal tissue (Nielsen *et al.*, 2003) may be consistent with the increased amount of inactive protein represented by the LOX-positive clumps that we found in the KC epithelium. The most obvious differences between normal and KC corneas were observed in the stroma – markedly decreased signal in ECM and keratocytes.

Besides *in situ* changes in LOX distribution, we detected decreased total LOX activity in cultured KC fibroblasts compared to fibroblasts derived from control corneas. We suggest that lower LOX activity leads to impaired cross-linking and thus to a loss of cohesion between collagen fibrils, promoting corneal ectasia by collagen lamellae slippage, and a decrease in their number in the area of thinning, as was previously described (Daxer and Fratzl, 1997; Hayes *et al.*, 2007; Meek *et al.*, 2005). We did not find any correlation between disease severity and immunohistochemistry changes or the results of LOX activity measurements.

The decreased LOX activity has been found in the vitreous of patients with proliferative diabetic retinopathy and rhegmatogenous retinal detachment (Coral *et al.*, 2008), similarly as in other pathologies in which inadequate collagen cross-linking may cause changes in the ECM of the connective tissue (Kuivaniemi *et al.*, 1982).

As we detected a decrease in the presence of LOX in the KC stroma, as well as a decline in its activity in corneal fibroblasts derived from KC corneas compared to control ones, **we suggest that LOX enzyme may play an important role in KC pathogenesis.**

LOXL1 is important for elastic fibre formation (Liu *et al*., 2004). Elastin fibres are present mostly in the mid-posterior part of the peripheral human cornea (Kamma-Lorger *et al*., 2010), while the corneal thinning in KC occurs in the central part. Due to these facts and according to our results, **we hypothesize that LOXL1 is not directly involved in the disease pathogenesis**.

Corneal keratocytes normally remain quiescent but during corneal wound healing they are activated and undergo transformation into corneal fibroblasts and myofibroblasts (West-Mays and Dwivedi, 2006). LOXL2 is abundantly expressed in senescent fibroblasts, cells with limited proliferation (Saito *et al*., 1997). The decrease of LOXL2 staining in KC corneas compared to controls could be caused by the transformation of keratocytes into myofibroblasts in KC corneas (Maatta *et al*., 2006; Bystrom *et al*., 2009; Dudakova *et al*., 2012). Additionally, we have observed a gradual anterior-posterior weakening of the LOXL2 signal which may be attributed to the fact that keratocytes in the posterior stroma are more likely to be activated (Hindman *et al*., 2010).

After transformation of keratocytes into myofibroblasts, these cells migrate to the wound site where they increase the synthesis of ECM components, proliferate and acquire contractile properties (West-Mays and Dwivedi, 2006). The increased expression of LOXL2 has been shown in several adherent tumour cell lines, while down-regulation has been observed in several non-adherent tumour cell lines. This suggests that LOXL2 may be involved in cell adhesion and that a loss of this protein may be associated with the loss of cell adhesion (Saito *et al*., 1997). The observed decrease of LOXL2 staining in KC corneas could enable the migration of activated corneal cells. Barker *et al*. (2013) have shown that cancer-associated fibroblasts express more LOXL2, further enhancing cancer progression. Treatment with LOXL2-specific inhibitors inhibits cell invasion and metastasis. In tumour cells, deregulation of LOXL2 expression may occur and an increased amount of this protein may lead to the persistent activation of cells in contrast to corneal cells, where after activation, LOXL2 presence diminished.

We presume that the decrease of LOXL2 in KC corneas is more likely a consequence of the associated pathological processes (activation of stromal cells due to tissue weakening and consequent structural changes) **than a direct cause leading to KC development.**

At this time, we are unable to provide a coherent explanation of the observed changes in LOXL3 and LOXL4 expression in KC corneas compared to the control tissue. The increase of the LOXL3 signal in the Western blot experiment could be caused by the local intensity increase observed in epithelial cells in the IHC experiments. Since LOXL enzymes are expressed in many tissues, it is difficult to investigate the functions and interpret the roles of individual LOXL enzymes in cellular processes in these tissues (Molnar *et al*., 2003). Different expression regulators, alternative splicing, structural and substrate specificities; all of these could contribute to their varied functions and their location in the ECM. **A more detailed characterization of LOXL proteins will be necessary in the future to understand the diverse functions of this group of enzymes.**

The therapeutic targeting of extracellular proteins is becoming hugely attractive in light of the evidence implicating the tumour microenvironment as pivotal in all aspects of cancer initiation and progression. Secretion of the LOX family members by tumours and their roles in tumorigenesis have been a subject of intense research (Barker *et al.*, 2012). Much attention is focused on LOX and LOXL2 as their increased expression has been observed in aggressive cancers and has shown significant correlation with decreased survival in a number of clinical cancer studies (Barker *et al.*, 2012). Both of these enzymes were found to be decreased in KC corneas (Dudakova *et al*., 2012; Dudakova *et al*., 2015a). Therefore, **studying the involvement of these enzymes in corneal pathology will help to understand their role in ECM remodelling in a broad context.**

<u>**Association of KC with other diseases**</u>

KC is associated with many disorders linked with a spectrum of biochemical alterations affecting collagen and elastin cross-link formation. We have suggested that there is a **similar origin of KC and mitral valve prolapse**, with respect to the alterations in LOX and higher presence of the Down syndrome in both diseases. The cases in which an association of KC with other connective tissue disorders occurs (Marfan syndrome, Ehlers–Danlos syndrome and others) support the suggestion that **KC may not arise as a localized manifestation, but instead may be induced as the result of a more complex connective tissue disorder**.

Given our current lack of knowledge on the cause of KC, it is important to determine whether there is any potential involvement of the *CHST6* gene or associated

pathways. Interestingly, concurrent KC and MCD has been previously described in five cases (Javadi *et al.*, 2004; Balestrazzi *et al.*, 2006; Mohammad-Rabei *et al.*, 2012; Al-Hamdan *et al.*, 2009) and one of the linked loci for KC (16q22.3-q23.1) contains the *CHST6* gene (Tyynismaa *et al.*, 2002). When we used the Pentacam Scheimpflug system to evaluate anterior corneal surface parameters in cases with MCD, there was a pattern suggestive of KC in all six eyes that were examined. However, there was no elevation of the posterior corneal surface.

Importantly, in contrast to the changes that characterize KC in which stromal thinning is localized, corneas with MCD showed diffuse thinning that involved the whole diameter of the cornea. Diffuse corneal thinning was also present on histopathological examination of corneas with MCD compared with control corneal specimens. Therefore evaluation of the anterior corneal surface in isolation can give indices that spuriously suggest the presence of KC, and correlation with posterior corneal elevation maps and regional pachymetry are required (Tomidokoro *et al.*, 2000; Schlegel *et al.*, 2008). The origin of the apparent anterior corneal elevation is uncertain, but the stromal deposits of MCD probably affect the quality of data capture.

In conclusion, our results suggest that the change in anterior corneal curvature and diffuse corneal thinning in MCD patients is a phenocopy of the changes that occur in KC. The apparent ectasia in this cohort of patients with MCD differs in several important aspects from the changes that define KC. Thinning and corneal distortion is to be expected if there is dysregulation of keratan sulfate proteoglycan synthesis or catabolism that influences corneal structure (Akhtar *et al.*, 2011).

The role of SNPs in *LOX* and *HGF* in KC

Our study provides the first independent validation of rs2956540-C (minor allele serving as a protective factor) **and rs3735520-A** (minor allele serving as a risk factor) **associations with KC in a population of European descent**, **further confirming that *LOX* and *HGF* genes have a role in the aetiology of the disease** (Burdon *et al.*, 2011; Bykhovskaya *et al.*, 2012). Recently, association of the *HGF* locus was also achieved in an independent study comprising a population of European descent from Australia (Sahebjada *et al.*, 2014). However, alternative SNPs to those shown statistically significant associations with KC in GWAS were tested (Burdon *et al.*, 2011).

Although the underlying mechanism of common variants contributing to the disease development remains unknown, the potential effect could lie in affecting the biologic activity of LOX via tissue specific alternative splicing or regulation of expression (Dudakova *et al.*, 2012). Mechanisms of how common variants within the *HGF* gene alter susceptibility to KC are yet to be determined, but involvement of inflammatory pathways has been previously suggested (Burdon *et al.*, 2011).

The main statistics used (allelic test) implicitly assumes codominance, and is similar to the additive models assumed in the previous reports (Burdon *et al.*, 2011; Bykhovskaya *et al.*, 2012; Sahebjada *et al.*, 2014; Hasanian-Langroudi *et al.*, 2014). Although some caution is invited in the interpretation of our findings because of the relatively small sample size, the validation of rs2956540-C and rs3735520-A showing the same effect directions as previous studies in populations of European ancestry adds weight to the existing evidence (Burdon *et al.*, 2011; Bykhovskaya *et al.*, 2012). Although not reaching a statistically significant threshold, the higher minor allele frequency (MAF) of rs1800449-T and rs10519694-T in controls compared with the KC cases in our study, was consistent with protective effects of these alleles reported in another study using Caucasian case-control panels (Table 2) (Bykhovskaya *et al.*, 2012).

rs1014091-A and rs17501108-T were previously also shown to have a protective effect (Burdon *et al.*, 2011), whereas in our study their MAF was higher in KC cases that, albeit not statistically significant, indicated a tendency towards the opposite direction of the effect. The failure to replicate the effect direction may be caused by differences in linkage disequilibrium patterns (Table 3) between these markers and the causative variants within the same gene in the Czech population.

Table 3: Linkage disequilibrium of SNPs genotyped in a Czech case-control panel
SNPs shown to be associated with KC in our study are indicated in bold. Numbers represent r^2 value.

	rsID	rs1014091	rs17501108	**rs3735520**
HGF	rs1014091		0.9783	0.1037
	rs17501108	0.,9783		0.1116
	rs3735520	0.1037	0.1116	

	rsID	rs1800449	rs2288393	**rs2956540**	rs10519694
LOX	rs1800449		0.9079	0.2598	0.0652
	rs2288393	0.9079		0.2988	0.0505
	rs2956540	0.2598	0.2988		0.4071
	rs10519694	0.0652	0.0505	0.4071	

Copper as unrecognized environmental factor

Our hypothesis suggests that Cu availability contributes to the development of KC by influencing the activity, biogenesis and stability of cuproproteins. In the absence of Cu or its low concentrations, these enzymes are prone to aggregation, misfolding and/or degradation by the proteasome pathway (Nittis and Gitlin, 2004).

While the total absence of Cu would lead to complete inactivity of Cu-dependent enzymes such as in Menkes disease, the precise effects of a mild Cu deficiency are very difficult to be predicted. No distinct symptoms that could be clearly attributed to Cu deficiency in populations with Cu consumption below the recommended dietary allowance have been identified (Hambidge, 2003; Griffith *et al.*, 2009).

Current evidence suggests that the development of KC in the great majority of patients depends on the interplay between genetic and environmental factors. Cu deficiency may be an unrecognized factor increasing susceptibility to the disease. The higher prevalence of KC in males could be explained by lower plasma levels of Cu compared to females (Johnson *et al.*, 1992). However, as in some families where KC follows a Mendelian mode of inheritance, other mechanisms independent of Cu involvement are likely to also be involved.

Similarly to other disorders associated with a deficiency of trace elements, an individual's genetic makeup may increase his or her susceptibility towards the disease development (Jellen *et al.*, 2009).

Our hypothesis that Cu deficiency may act as an independent environmental factor in KC development is supported by the X-ray structural analysis performed by Avetisov et al. (Avetisov *et al.*, 2011). They explained the diminished Cu levels in KC corneas by higher pH of KC patients' tears, leading to the oxidization of dichlorocuprate (Cu^{+}) into cupric oxide (Cu^{2+}), which cannot be utilized by cells. They also suggested that this may be associated with a decrease of LOX activity (Avetisov *et al.*, 2011).

As the cornea is composed mainly of collagen fibrils, which form many tissues, and part of the current cancer research is focused on Cu chelation as a new anti-cancer therapy (Yoshii *et al.*, 2001) with expected outcomes (to increase oxidative stress in cancer cells, induce apoptosis, activate proteinases, decrease LOX activity, etc.) that evoke signs described in relation to KC (Antoniades *et al.*, 2013), studying the metabolic pathways involved in KC is highly relevant in a broader context.

Our findings have implications for CXL therapy that has been shown to halt the progression of KC. This treatment has the potential to significantly reduce the number of corneal transplants Testing for *LOX* polymorphisms in patients with KC may further improve the effectiveness and safety of CXL treatment, for example by identifying nonresponders prior to the treatment.

In summary the research undertaken has helped to elucidate some aspects involved in KC pathogenesis, which may become soon important for patient counselling, lead to improvements in current disease management and to the development of novel therapies in the future.

6. Future research

The causes of LOX impairment

We should focus on potential malfunction in processing, activation or assembly of LOX and LOXL.

Potential role of LOX in mitral valve prolapse

Detecting possible changes on RNA and DNA levels – expression changes, presence of SNPs; and on protein levels - distribution and activity; could help to better understanding of pathogenesis of both diseases.

Evaluation of the direct influence of SNPs in *LOX* and *HGF* associated to KC development

Detected SNPs could influence the expression or alternate the transcription of the genes, RNA stability and translation, protein assembly or activity of these enzymes.

Other SNPs as factors associated with KC development

Influence of tens SNPs have been implicated as risk alleles for KC development, these are estimated to explain however a very small part of KC heritability. It is likely that numerous SNPs with a small effect on KC development remain to be discovered.

Evaluation role of Cu in KC development

Proving our hypothesis on a histochemical level will be difficult. Although there are multiple methods available for visualisation of Cu presence in tissue (staining with orcein, rhodanine, rubeanic acid or Timm's sulfide-silver staining method (Nemolato *et al.*, 2008)), in clinical practice the occurrence of negative staining in samples from patients affected by Wilson's disease (Mahjoub *et al.*, 2012) is very frequent, even in cases in which high Cu levels have been demonstrated by atomic absorption spectroscopy (Pilloni *et al.*, 1998). The reason for the failure of Cu histochemical demonstration remains unknown (Nemolato *et al.*, 2008).

Serum or plasma Cu and ceruloplasmin concentrations are not very widely used laboratory indicators of Cu level status as they are only decreased in moderate or severe Cu deficiency. In addition, both indicators of Cu levels show significant variations depending on age, sex, and pregnancy status and may be increased in the presence of other conditions not related to Cu status (inflammatory or infectious processes, neoplasm and estrogen therapy) (Milne, 1998).

The activity of several cuproenzymes is decreased in cases of mild Cu deficiency. However, their utility is limited by the lack of standardized assays and high interindividual variability and because some of these indicators are affected by other conditions as well (Olivares *et al.*, 2008).

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8. List of publication and poster presentations

All of the author’s publications, i. e. related and unrelated to the thesis subject, listed below, are sorted in order mentioned in Chapter 2. Impact Factor (IF) values and citation reports correspond to the ISI Web of Science (accessed 5/2015).

Publications related to the Thesis

Dudakova L., Liskova P., Trojek T., Palos M., Kalasova S., Jirsova K. Changes in lysyl oxidase (LOX) distribution and its decreased activity in keratoconus corneas. *Experimental Eye Research*. 2012;104:74-81 ***IF = 3.017; citations: 10***

Dudakova L., Sasaki T., Liskova P., Palos M., Jirsova K. The presence of lysyl oxidase-like enzymes in human control and keratoconic corneas. *Histology and Histopathology*. 2015a, accepted ***IF = 2,236***

Dudakova L., Jirsova K. The impairment of lysyl oxidase in keratoconus and in keratoconus-associated disorders. *Journal of Neural Transmission*. 2013;120(6):977-82 ***IF = 2.871; citations: 4***

Dudakova L., Palos M., Svobodova M., Bydzovsky J., Huna L., Jirsova K, Hardcastle A, Tuft S., Liskova P. Macular corneal dystrophy and associated corneal thinning. *Eye (Lond)*. 2014;28(10):1201-5 ***IF = 1.897***

Dudakova L., Palos M., Jirsova K., Stranecky V., Krepelova A., Hysi PG., Liskova P. Validation of rs2956540:G>C and rs3735520:G>A association with keratoconus in a population of European descent. *European Journal of Human Genetics*. 2015b; [Epub ahead of print] ***IF = 4.225***

Dudakova L., Liskova P., Jirsova K. Is copper imbalance an environmental factor influencing keratoconus development? *Journal of Medical Hypotheses*. 2015c;84(5):518-24 ***IF = 1.152***

Conference abstracts related to Thesis published in Journals with IF

Dudakova L., Kalasova S., Jirsova K. Lyzyl oxidáza v tkanivovej kultúre kontrolnej a keratokonickej rohovky. *Chemické listy*. 2011;105(5):394 ***IF = 0.453***

Dudakova L., Trojek T., Liskova P., Kalasova S., Jirsova K. Potencionálna úloha Cu a aktivity lyzyl oxidázy v patogenéze keratokonu. *Chemické listy*. 2012;106(5):420 ***IF = 0.453***

Dudakova L., Kalasova S., Jirsova K. Porovnanie výskytu „lysyl oxidáza-like“ enzýmov v kontrolnej a akeratokonickej rohovke. *Chemické listy*. 2013;107(5):410 ***IF = 0.453***

Dudakova L., Kalasova S., Jirsova K. Presence of lysyl oxidase-like enzymes in human control and keratoconic corneas. *Investigative Ophthalmology and Visual Science.* 2013;54:E-Abstract 5293. ***IF = 3.661***

Dudakova L., Klema J., Jirsova K., Liskova P. Analýza vplyvu rs2956540:G>C a rs3735520:G>A na fenotyp pacientov s keratokónusom. *Chemicke listy.* 2015;109:in press ***IF = 0.453***

Publications not related to the Thesis

Dudakova L., Palos M., Hardcastle AJ., Liskova P. Corneal Endothelial Findings in a Czech Patient with a Compound Heterozygous Mutation in *KERA*. *Ophthalmic Genetics*. 2014;35(4):252-4 ***IF = 1.233, citation: 1***

Jirsova K, **Dudakova L**, Kalasova S, Vesela V, Merjava S. The OV-TL 12/30 clone of anti-cytokeratin 7 antibody as a new marker of corneal conjunctivalization in patients with limbal stem cell deficiency. *Investigative Ophthalmology and Visual Science*. 2011;52(8):5892-8 ***IF = 3.661; citations: 5***

Liskova P., **Dudakova L**., Palos M., Tesar V., Bednarova V., Kidorova J., Jirsova K., Davidson AE., Hardcastle AJ. Detailed assessment of renal functions in a proband with Harboyan syndrome caused by a novel nonsense homozygous *SLC4A11* mutation. *Ophthalmic Research*. 2015;53(1):30-5 ***IF = 1.376***

Evans CJ, Liskova P, **Dudakova L**, Hrabcikova P, Horinek A, Jirsova K, Filipec M, Hardcastle AJ, Davidson AE, Tuft SJ. Identification of Six Novel Mutations in *ZEB1* and Description of the Associated Phenotypes in Patients with Posterior Polymorphous Corneal Dystrophy 3. *Annals of Human Genetics*. 2015;79(1):1-9 ***IF = 1.926***

Selected presentations at meetings

The partial results were presented in 10 oral and 6 poster presentations. Three oral and one poster contribution were presented in English on conferences in Slovakia, France, Australia and USA. Awarded presentations are mentioned above.

Dudakova L., Stranecky V., Kalasova S., Jirsova K., Liskova P. Analýza jednonukleotidových polymorfizmov v génoch pre lyzyl oxidázu a hepatocytárny rastový faktor u pacientov s keratokonem (Oral presentation). 15th Student Scientific konference, First Medical Faculty, Charles University in Prague, Czech Republic, 2014

*** Special award of GRADA Publisher**

Dudakova L., Kalasova S., Liskova P., Jirsova K. Úloha lyzyl oxidázy a jej izoenzýmov v patogenéze keratokonu (Oral presentation). 14th Student Scientific konference, First Medical Faculty, Charles University in Prague , Czech Republic, 2013

*** 3rd place**

Dudakova L., Kalasova S., Jirsova K. Porovnanie výskytu „lysyl oxidáza-like“ enzýmov v kontrolnej a keratokonickej rohovke (Poster). XIII. Interdisciplinary meeting of young biologists, biochemists and chemists from the Czech Republic and Slovakia, 2013

*** Best poster award**

Dudakova L., Jirsova K. Úloha medi v patogenéze keratokonu (Oral presentation). 13th Student Scientific konference, First Medical Faculty, Charles University in Prague, 2012

*** Best presentation award**

Dudakova L., Kalasova S., Jirsova K. Detekcia LOX v tkanivovej kultúre zdravej a keratokonickej rohovky (Oral presentation). Student Scientific conference, Faculty of Natural Sciences, Comenius University in Bratislava, Slovakia, 2011

* **Sigma Aldrich Award, Best contribution in Biology**

Appendix 1

Dudakova L., Liskova P., Trojek T., Palos M., Kalasova S., Jirsova K. Changes in lysyl oxidase (LOX) distribution and its decreased activity in keratoconus corneas. *Experimental Eye Research*. 2012;104:74-81

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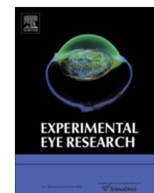

Changes in lysyl oxidase (LOX) distribution and its decreased activity in keratoconus corneas

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ABSTRACT

Inadequate cross-linking between collagen lamellae is a characteristic feature of keratoconus corneas. The formation of covalent bonds between collagen and elastin fibrils, which maintain the biomechanical properties of the cornea, is mediated by the cuproenzyme lysyl oxidase and four lysyl oxidase-like enzymes. The aim of this study was to determine the distribution of lysyl oxidase and the total lysyl oxidase activity (lysyl oxidase and the four lysyl oxidase-like enzymes) in control and keratoconic corneas. Seven control and eight keratoconic corneas were used for the imunohistochemical detection of lysyl oxidase in corneal cryosections using two different antibodies. The total lysyl oxidase activity in the culture medium of corneal fibroblasts from six explanted keratoconic and four control corneas was measured using a fluorometric assay in the presence and absence of the lysyl oxidase inhibitor beta-aminopropionitrile and determined as the production of H_2O_2 in nM per μg of total protein. In the control tissue, the most intense signal for lysyl oxidase was present in the corneal epithelium, in which perinuclear dots brightly projecting from more or less homogenous cytoplasmic staining may represent the lysyl oxidase propeptide. Less intense staining was present in keratocytes, the extracellular matrix and in the corneal endothelium. The epithelium of the limbus and the perilimbal conjunctiva showed intense to very intense staining. The distribution of lysyl oxidase was clearly decreased in at least five of the eight keratoconic specimens. The most marked signal reduction was observed in the stromal matrix and in keratocytes. Moreover, the signal in pathological specimens revealed a more irregular pattern, including the presence of intra- and extracellular clumps in the epithelium. Interestingly, endothelial cells showed no or very weak staining in areas just beneath negative stromal tissue. The mean activity of total lysyl oxidase in the keratoconic samples (2.60 ± 2.23 nM H_2O_2/μg of total protein) was more than 2.5-fold lower than in control tissue (6.83 ± 2.53 nM H_2O_2/μg of total protein), and the decrease was statistically significant ($p = 0.0178$). The location of lysyl oxidase in the healthy cornea, limbus and perilimbal conjunctiva was described. We hypothesize that the restricted lysyl oxidase distribution in keratoconic corneas, and particularly the decrease of total lysyl oxidase activity in cultured keratoconic fibroblasts, is one potential reason for the inadequate collagen cross-linking that is a hallmark of this disease.

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1. Introduction

Keratoconus (KC) is a non-inflammatory disease characterized by progressive corneal thinning and ectasia manifesting as myopia and irregular astigmatism (Krachmer et al., 1984). It is one of the leading causes of corneal transplantation in developed countries. The prevalence of KC in the Caucasian populations has been estimated at approximately 1:2000 (Kennedy et al., 1986) (Rabinowitz, 1998). Despite extensive research and attempts to discover the etiopathogenesis of KC (Cristina Kenney and Brown, 2003), the mechanism underlying this disease remains unknown.

Recently, corneal collagen cross-linking has become a standard method used to stabilize the progression of corneal ectasia in patients with keratoconus. The approach is based on the formation of covalent bonds between collagen fibrils, thus enhancing corneal rigidity (Wollensak, 2006) (Kanellopoulos, 2012).

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Under physiological conditions, the final enzymatic step required for collagen and elastin cross-linking in the extracellular matrix is catalyzed by the copper-dependent enzyme lysyl oxidase (LOX) (Mizobe et al., 2008) (Kagan et al., 1986). This amine oxidase is synthesized as an inactive 50 kDa proenzyme that is N-glycosylated in the endoplasmic reticulum and the Golgi complex and then secreted into the extracellular environment, where it is processed by pro-collagen C-proteinases – mammalian Tolloids and particularly by bone morphogenetic protein-1 (BMP-1) – into an active enzyme (28–32 kDa) and an 18 kDa propeptide (LOPP) that can be N-glycosylated intracellularly into a ~35 kDa form (Trackman et al., 1992) (Uzel et al., 2001) (Guo et al., 2007). In addition to LOX, four LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4) catalyze the oxidative deamination of lysine residues in collagen and elastin, but their specific mechanism of action remains unknown (Molnar et al., 2003) (Csiszar, 2001).

LOX enzymes are critical for the stability of connective and vascular tissues; they are highly expressed in the skin and play an important role in promoting tumor cell invasion and the metastasis of many cancers (Nishioka et al., 2012) (Kuivaniemi, 1985) (Rodriguez et al., 2008). In the eye, the LOX enzyme have been detected in the trabecular meshwork, ciliary body, lens and retina (Coral et al., 2008) (Sethi et al., 2011). Due to its function, *LOX* has been regarded as a candidate gene for KC (Nielsen et al., 2003). Despite a suggestive linkage in a familial KC panel (Bisceglia et al., 2009) to the 5q23.2 region where *LOX* is located, no pathogenic sequence variants have so far been identified (Bykhovskaya et al., 2012) (De Bonis et al., 2011).

However, the upregulation of *LOX* expression was found in the epithelium of KC corneas compared to normal ones (Nielsen et al., 2003). Until now, there have been no reports on the distribution and activity of LOX enzyme in the human cornea. The purpose of this study was to characterize the localization of LOX in control and KC corneas and to investigate differences in total LOX activity (the activity of LOX and LOX-like proteins) in the media of cultured KC fibroblasts compared to normal ones and thus to contribute to the understanding of KC pathogenesis.

2. Materials and methods

2.1. Specimen preparation

The study followed the ethical standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki.

Control specimens obtained from the Institute of Forensic Medicine and Toxicology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, were processed within 24 h after collection. The integrity of the tissue and the absence of pathological signs were checked using phase contrast microscopy (Olympus OPTICAL CO., Japan). None of the donors had a history of ocular disease, diabetes, hypertension, carcinoma, or sepsis. The diameter of the control corneas was standardized to the diameter of the explants (8.0–8.25 mm). Pathological explants obtained from the Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General Teaching Hospital in Prague, were processed within 4 h after penetrating keratoplasty. The clinical diagnosis of KC was based on the presence of typical corneal signs such as thinning, protrusion, Vogt striae, Fleischer ring and scarring. The assessment of the anterior and posterior corneal surfaces and pachymetry were performed by Scheimpflug imaging (Pentacam, Oculus, Germany).

All KC corneas included in the study were stage III to IV based on the Amsler–Krumeich classification, grading severity from mild (I) to severe (III/IV) (Choi and Kim, 2012). None of the patients had worn contact lens for at least 6 months prior to the keratoplasty surgery. Corneas with hydrops and any other previous treatment for keratoconus except for contact lenses were excluded. Further exclusion criteria were pregnancy, glaucoma, acquired pseudokeratoconus, corneal ulceration and signs of ocular inflammatory disease including atopic keratoconjunctivitis.

Seven control corneas from four male (in one case both eyes) and two female donors (34–72 years; mean age 49.2 ± 18.9 years) and eight keratoconic corneas (4 male and 4 female; 23–29 years; mean age 26.3 ± 2.0 years) were dissected and snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at −70 °C. Four 7 μm thick cryosections were placed per slide and used for indirect fluorescent immunohistochemistry.

Six KC explants (18–47 years; mean age 32.5 ± 14.5) from five Czech male and one female patients and four unaffected donor corneal buttons (59–76 years; 67.5 ± 8.5; two male, two female) serving as controls were used for cultivation.

2.2. Indirect fluorescent immunohistochemistry

Control and KC cryosections 7 μm thick were used for indirect immunofluorescence as described previously (Merjava et al., 2009). Briefly, three cryosections on each slide were stained with a single antibody. The fourth section (primary antibody omitted) served as a negative control. At least two independent experiments were performed. The tissue was fixed with cold acetone for 10 min, rinsed in PBS and incubated with the primary antibody diluted in 1% bovine serum albumin (BSA, Sigma–Aldrich Corporation, St. Louis, MO, USA) in PBS for 1 h at room temperature. A rabbit polyclonal antibody (ab31238, 1:350, Abcam, Cambridge, UK) detecting a ~35 kDa protein corresponding to the N-glycosylated form of LOPP protein and a rabbit polyclonal antibody (NB-59729, 1:100, Novus Biologicals, Cambridge, UK) detecting the active form of LOX (31 kDa) were used. Then the specimens were washed three times in PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG, 1:400, Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 h at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide (Vector Laboratories, Inc., Burlingame, USA) to counterstain nuclear DNA. Cryosections of placenta obtained from the Ocular Tissue Bank Prague served as a positive control (Hein et al., 2001).

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200–1000×. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany) and JENOPTIK ProgRes C12plus (Jenoptik, Laser, Optik, Systeme GmbH, Jena, Germany). At least two hundred epithelial and stromal cells (600/slide) and one hundred endothelial cells (300/slide), if available, were examined, and the percentage of positive cells was calculated separately in the different corneal layers, also in the limbus and the perilimbal conjunctiva. The intensity of cell staining was graded using a 0–4 scale according to Diebold et al. (1997): 0: no discernible staining, 1: weak, 2: moderate, 3: intense, and 4: very intense staining.

2.3. Corneal fibroblast cultures

The epithelium and the endothelium of all corneas intended for cultivation were mechanically removed, and the absence of surface cells was checked using phase contrast microscopy. Then the corneas were rinsed in sterile phosphate buffered saline (PBS) and cultured as described previously (Berulava and Horsthemke, 2010) (Buss et al., 2010). Briefly, each sample of tissue was placed in 1 ml of AmnioMAX C-100 Basal media with AmnioMAX Supplement

(Invitrogen, Carlsbad, CA, USA) in a Petri dish, cut into approximately 1 mm blocks and seeded into 25 cm^2 tissue culture flasks (TPP, Switzerland). After 24 h at 37 °C, 5% CO_2 in a humidified incubator, 2 ml of Amniomax were added to the flasks. As soon as the first cells migrated from the explants, the culture medium was renewed. On reaching confluence the cells were passaged using 0.05% Trypsin–EDTA (Invitrogen) for 15 min at 37 °C. From the second passage phenol red-free Dulbecco's Modified Eagle Medium F12 (DMEM/F12) with 10% new born calf serum (NBCS), a 1% solution of Penicilline/Streptomycine and a 0.1% solution of Fungizone (Invitrogen) was used. The medium was replaced twice a week.

For indirect fluorescent immunocytochemistry, cells were seeded 24 h before examination in 4-well chamber slides (Lab Tek, Brendale, Australia). Cells from the second and the fifth passages were used. Besides phase contrast microscopy (Olympus, CK40), the cell phenotype was examined using antibodies against α-smooth muscle actin (α-SMA, 1:300, Sigma–Aldrich Corporation) to detect myofibroblasts (Jester et al., 1996) and cytokeratin 3/12 (CK3/12, 1:300, RDI, Acton, MA, USA) to detect possible epithelial contamination (Moll et al., 1982). The cells were rinsed with PBS and fixed in cold acetone for 1 min. After permeabilisation with 0.33% Triton X100 for 10 min, the sections were blocked in 2.5% BSA. The primary antibody (all monoclonal mouse anti-human) was applied overnight at 4 °C, followed by rinsing in PBS and incubation for 1 h with the secondary antibody (FITC anti-mouse IgG, 1:350, Jackson ImmunoResearch, Newmarket, Suffolk, UK) diluted in 0.1% BSA. Then the slides were rinsed in PBS and mounted with propidium iodide (Vectashield; Vector Laboratories, Inc. Burlingame, CA, USA). The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200–400×. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany).

2.4. Measurements of total LOX activity

Whole samples of media (2 ml) in which corneal fibroblasts were cultured were centrifuged; the supernatant was collected and frozen at −70 °C and analyzed within one month. The total LOX activity (LOX and LOX-like enzymes) was measured by a fluorimetric assay as described previously in the presence and the absence of beta-aminoproprionitrile (BAPN) (Palamakumbura and Trackman, 2002), an irreversible inhibitor of LOX and LOX isoenzymes that binds covalently to its active site (Tang et al., 1983). Each specimen was measured in duplicate. Briefly, the samples were prepared in a final volume of 0.5 ml containing 1 unit/ml horseradish peroxidase (HRP, Invitrogen), 10 μM Amplex Red (Invitrogen), 10 mM diaminopentane (Sigma–Aldrich Corporation), and 1.2 M urea and were incubated at 37 °C for 30 min. The samples were placed on ice and the fluorescence was measured using an Infinite M200 fluorescence spectrophotometer (Tecan, Männedorf, Switzerland) with excitation and emission wavelengths at 315 and 425 nm. Parallel assays were prepared with 500 mM BAPN and the difference in emission intensity was recorded. The amount of hydrogen peroxide (H_2O_2) produced by the action of total LOX was determined by comparing the amount of fluorescence to a standard plot relating the fluorescence change to nM of H_2O_2 added to assays lacking LOX. A standard curve was prepared by dissolving H_2O_2 (0–10 μM H_2O_2, Sigma–Aldrich Corporation) in culture medium. Enzyme activity was calculated as the production in nM of H_2O_2 per μg of total protein. The total protein concentration was determined using a BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA). Total LOX activity was measured after 5 passages, when the expression of α-SMA in KC fibroblasts was negligible, and both control and pathological cells revealed a similar phenotype (44–61 days, mean 52.5 ± 8.5).

2.5. Statistical analysis

One-way ANOVA (Analysis of Variance) was used to determine if there was a difference between the total LOX activity in the media of KC and control corneal fibroblasts. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Indirect fluorescent immunohistochemistry

3.1.1. Novus antibody (detecting 31 kDa active LOX)

The staining intensity and pattern of all control corneas were homogenous (Table 1). Most of the control epithelial cells revealed very intense diffuse homogenous intracellular positivity (Fig. 1a). The stromal ECM of control corneas was moderately positive, and all keratocytes exhibited an intense signal. Moderate to very intense staining was present in the endothelial cells, while an intense signal was present in the control limbal epithelium

Table 1
The immunohistochemical localization of LOX in the different corneal layers of control (Co) and keratoconus (KC) specimens. Co3 and Co4 represent the left and right eyes of the same donor; the dot-like peri-nuclear staining of the epithelium is shown in parentheses for the Abcam antibody, while the percentage of the ECM area exhibiting a punctate-like staining pattern is presented in (*).

Co/KC	Age/gender	Corneal epithelium		Stromal ECM		Keratocytes		Endothelium	
		Novus	Abcam	Novus	Abcam	Novus	Abcam	Novus	Abcam
		% of positive cells/intensity							
Co1	25/M	100/4	100/3 (100/4)	100/2	100/2 (100*)	100/3	100/3	100/4	100/3
Co2	33/M	100/4	100/3 (100/4)	100/3	100/2 (100*)	100/4	100/3	100/4	100/3
Co3	34/M	100/4	95/2 (50/3)	100/2	100/1 (100*)	100/3	100/2	100/3	100/2
Co4	34/M	100/4	100/3 (20/4)	100/2	100/1 (100*)	100/3	100/3	100/4	30/2
Co5	63/M	100/4	100/3 (30/4)	100/2	100/2 (100*)	100/3	100/3	100/3	100/2
Co6	68/F	100/4	100/3 (80/4)	100/2	100/2 (100*)	100/3	100/3	100/3	100/3
Co7	72/F	100/4	100/2 (100/4)	100/2	100/2 (100*)	100/3	100/3	100/3	100/3
KC1	28/F	90/3	50/2 (20/4)	30/2	50/2 (−)	30/3	50/2	50/3	50/1
KC2	29/F	70/4	100/3 (75/4)	100/0	100/1 (5*)	10/1	100/1	40/1	30/1
KC3	25/F	100/4	100/3 (90/4)	100/2	100/1 (100*)	100/3	100/2	100/3	100/2
KC4	27/M	100/4	100/3 (95/4)	100/1	100/1 (50*)	100/3	100/2	100/2	100/1
KC5	25/M	100/4	100/3 (10/3)	100/2	100/1 (50*)	100/3	100/2	100/2	100/1
KC6	23/M	50/3	100/2 (50/4)	80/1	50/1 (−)	80/2	50/1	60/2	100/1
KC7	34/F	100/4	100/2 (10/3)	100/1	100/1 (20*)	100/2	100/1	100/2	100/1
KC8	27/M	100/4	80/2 (40/3)	100/2	100/1 (−)	70/1	100/1	100/2	100/1

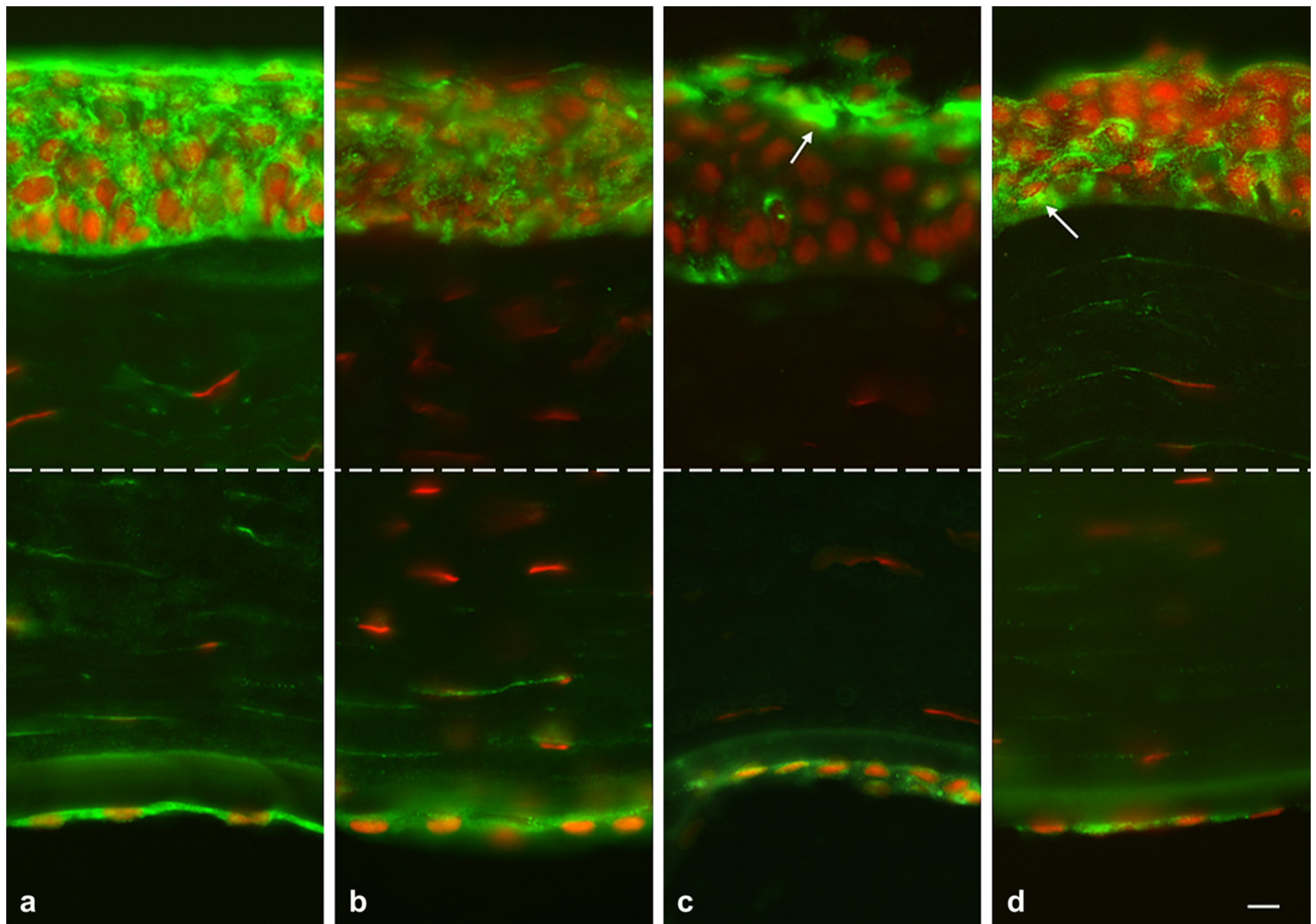

Fig. 1. LOX localization in control (a) and keratoconus (KC) corneas (b, c, d) using immunohistochemistry with a Novus Biological antibody detecting a ~31 kDa protein (FITC, green). In KC corneas, the signal is less intense and the epithelium exhibits some staining irregularities expressed as a clump-like pattern in the cytoplasm (arrows). The nuclei were counterstained with propidium iodide (red). Scale bar, 10 μm.

(Fig. 3a). The basal cells show greater positivity than the upper suprabasal and superfacial cells. The epithelium of the peri-limbal conjuctiva was very intensely positive (Fig. 3b).

The intensity of staining and the signal distribution in the epithelium of five out of eight KC corneas was similar to those in controls. In three explants, the signal was less intense and was distributed in a lower number of cells. The epithelium exhibited some staining irregularities expressed as a clump-like pattern in the cytoplasm (Fig. 1b, c, d). A less intense signal compared to that of the controls was observed in keratocytes and the stromal ECM in five out of eight KC explants (Table 1). The signal in the endothelium was diminished in almost all KC specimens. The endothelial cells showed no or very weak staining just in the vicinity of the ECM areas where the signal was absent or very low.

No signal was found in the negative controls (Fig. 3c) Most of the cells in the positive control tissue (placenta) showed a very intense intracellular signal (Fig. 3d).

3.1.2. Abcam antibody (detecting ~35 kDa LOPP)

All control corneas were homogenous in the intensity and pattern of their staining (Table 1). The majority of the control epithelial cells revealed intense diffuse positivity. Intensely stained peri-nuclear granules were present in 69% of cells. In all specimens weak to moderate staining was diffusely present in the ECM, together with very weak punctate-like positivity. Moderate to very intense staining was observed in keratocytes and those endothelial cells in which perinuclear granules were present (Fig. 2a). A very intense signal with projecting peri-nuclear granules was found in the epithelium of the limbus (Fig. 3e) and peri-limbal conjunctiva (Fig. 3f).

The epithelium of five out of eight KC corneas exhibited the same signal distribution and intensity as controls. The mean percentage of cells with peri-nuclear staining reached 49% in KC specimens. The epithelium of three corneas exhibited a clump-like staining pattern (Fig. 2c, d). A markedly diminished signal in the ECM and keratocytes was found in seven out of eight KC specimens compared to control ones (Table 1). The punctuate-like pattern present in all control specimens in the ECM was absent in three and diminished in four KC specimens. The signal in the endothelium was diminished in all KC specimens, and interestingly, the endothelial cells showed no or very weak staining in the vicinity of the ECM areas where the signal was absent or very low (Fig. 2b).

No signal was found in the negative controls (Fig. 3g). Approximately 30% of the cells in the positive controls (placenta) showed a very intense intracellular signal, while the remaining cells showed diminished or no positivity with a clump-like pattern. The ECM was almost negative (Fig. 3h).

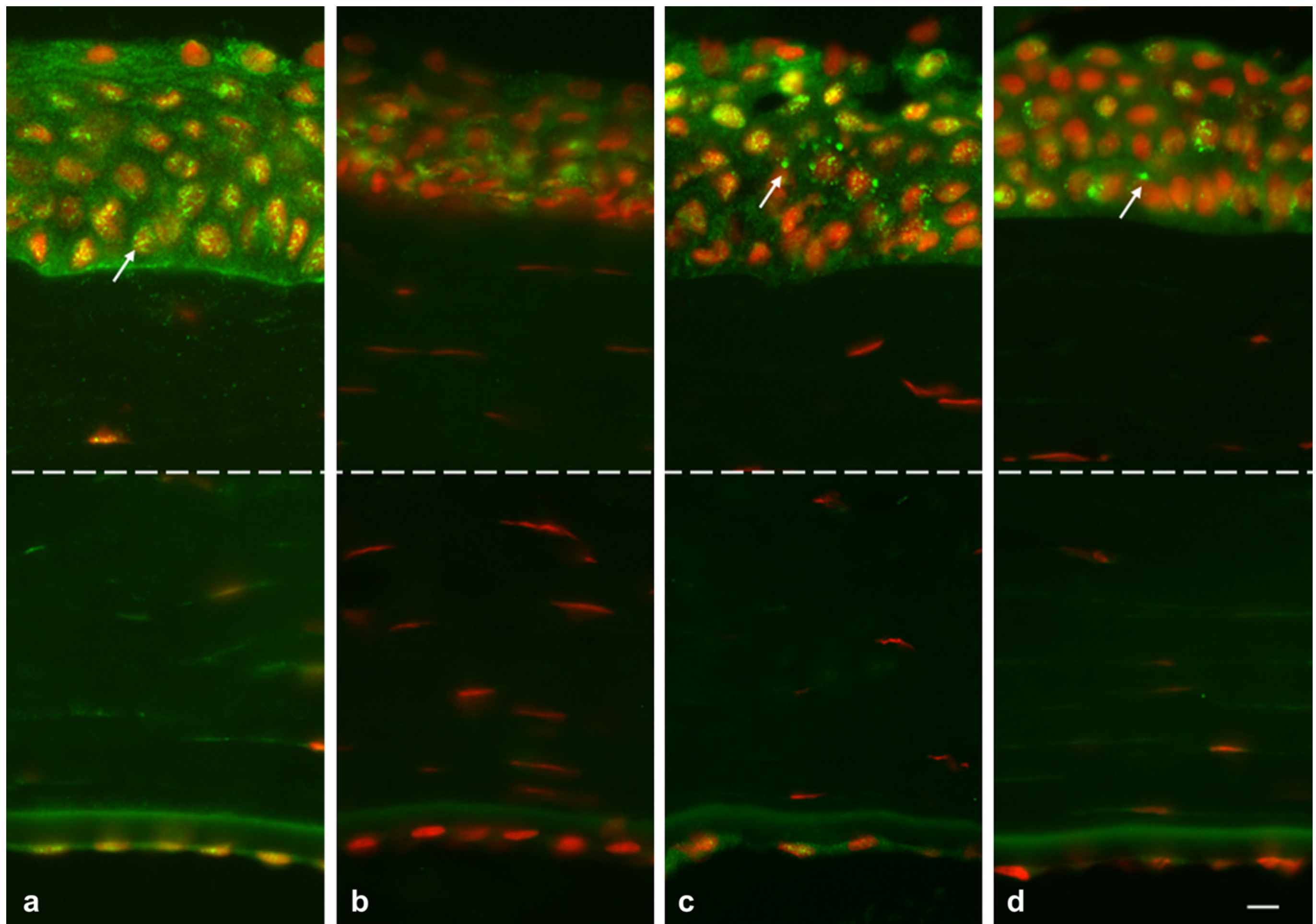

Fig. 2. LOX localization in control (a) and keratoconus (KC) corneas (b, c, d) using immunohistochemistry with an Abcam antibody detecting a ~36 kDa protein (FITC, green). Perinuclear staining is present in control as well as in KC epithelium, where it exhibits a more clump-like pattern (arrows). A markedly diminished signal is seen in the stroma and in endothelial cells in the vicinity of the extracellular matrix areas where the signal is absent or very low (b). The nuclei were counterstained with propidium iodide (red). Scale bar, 10 μm.

3.2. Corneal fibroblast cultures

The migration of KC stromal cells began within 6–10 days (8 ± 2 days), whereas control cells migrated within 9–14 days (11.5 ± 2.5) of cultivation. In the second passage about 80% of KC cells from all patients showed moderate to intense cytoplasmic positivity for α-SMA, excluding one patient cornea with staining present in the nucleus only. A very weak signal was present in one of four control corneas, approximately in 1% of cells. At the fifth passage, two KC and one control cultures were weakly positive for α-SMA, in less then 1% of cells (Fig. 4). Using phase contrast microscopy, almost all cells showed the phenotype of corneal fibroblasts. Only a few cells in all cultures and passages were positive for CK3/12. A signal for β-actin was homogenously present in all cultivated cells and passages.

3.3. Total LOX activity

In control specimens the mean specific total LOX activity was found to be 6.8 ± 2.5 nM H_2O_2/μg of total protein, ranging from 4.2 to 9.4. Relative to the controls, the specific activity of the KC specimens showed a more than 2.5-fold decrease to 2.6 ± 2.2 nM H_2O_2/μg of total protein, ranging from 1.1 to 6.5. In four KC samples out of six, the LOX activity was extremely low compared to the control samples (Fig. 5). The difference was statistically significant (p value $= 0.0320$).

4. Discussion

Although LOX is the key enzyme for collagen crosslinking, until now no study has evaluated its distribution and activity in the human cornea. As the function of this amino oxidase indicates that LOX impairment may be involved in keratoconus pathogenesis, we have localized LOX and determined the total LOX activity in corneas from patients with this common disorder in comparison to healthy tissue.

The most intense signal for both detected LOX forms (the active 31 kDa protein detected by Novus Biologicals and the 36 kDa protein detected by the Abcam antibody) was found in the corneal epithelium with the difference that the 36 kDa N-glycosylated form of LOPP protein revealed characteristic peri-nuclear dots that may correspond to its location in the Golgi apparatus and endoplasmic reticulum (Guo et al., 2007), while the 31 kDa mature protein was more-or-less homogenously present throughout the cytoplasm. Similar staining was present in the limbal and perilimbal conjuntival epithelium. The staining pattern in KC sections was not so homogenous, and the peri-nuclear signal formed more clumps than individual dots. We hypothesize that the upregulation of LOX expression in the epithelium of KC corneas compared to normal tissue (Nielsen et al., 2003) may be consistent with the increased amount of inactive protein, which can be represented by the LOX-positive clumps that we

Fig. 3. Indirect fluorescent immunocytochemistry using the Novus Biologicals (a–d) and Abcam (e–h) antibodies in the limbus (a, e), conjuctiva (b, f), corneal epithelium – negative control (c, g) of control corneoscleral buttons and placenta – positive control (d, h), (FITC, green). The nuclei were counterstained with propidium iodide (red). Scale bar, 10 μm.

found in the KC epithelium. The most obvious differences between normal and KC corneas were observed in the stroma. Both intracellular and extracellular staining were markedly diminished in 63% of KC specimens.

Besides *in situ* changes in LOX distribution, we have detected decreased total LOX activity in cultured KC fibroblasts compared to fibroblasts derived from control corneas. A significant decrease was found in cells from the fifth passage in which both pathological and

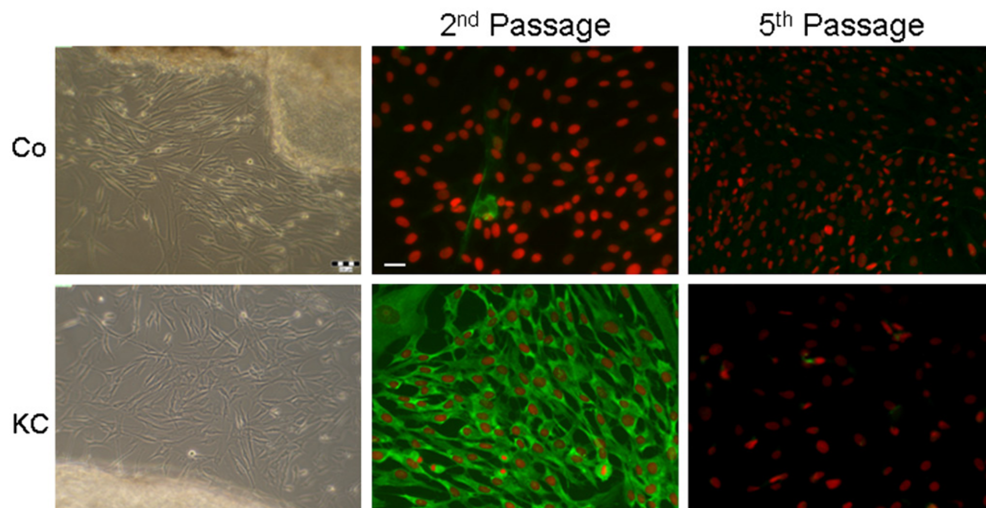

Fig. 4. The differences between control (Co) and keratoconic (KC) cells – phase contrast microscopy of expanding keratocytes (scale bar, 100 μm) and α-SMA presence in cells from the second and fifth passages (scale bar, 10 μm).

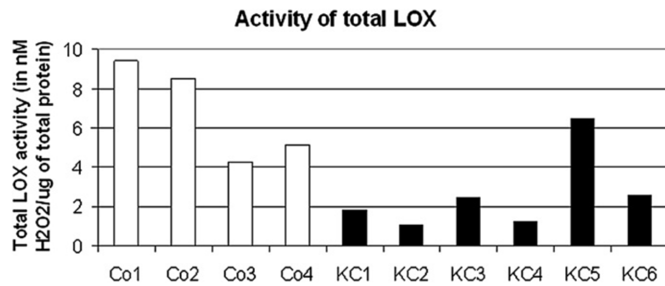

Fig. 5. Total LOX activity of control (Co) and keratoconus (KC) corneal fibroblasts (in nM H_2O_2/μg of total protein).

control stromal cells revealed a corneal fibroblast (but not myofibroblast) phenotype. We suggest that lower LOX activity leads to impaired cross-linking and thus to a loss of cohesion between collagen fibrils, promoting corneal ectasia by collagen lamellae slippage, and a decrease in their number in the area of thinning, as was previously described (Daxer and Fratzl, 1997) (Hayes et al., 2007) (Meek et al., 2005). We did not find any correlation between disease severity and immunohistochemistry changes or the results of activity measurements.

Decreased LOX activity has been found in the vitreous of patients with proliferative diabetic retinopathy and rhegmatogenous retinal detachment (Coral et al., 2008), similarly as in other pathologies in which inadequate collagen cross-linking may cause changes in the ECM of the connective tissue (Kuivaniemi et al., 1982). Moreover, changes in the expression of LOX-like proteins, particularly LOXL1, may play a role in the pathogenesis of pseudoexfoliation syndrome and glaucoma (Schlotzer-Schrehardt, 2011) (Sethi et al., 2011).

It has been suggested that the lower LOX activity may by a consequence of the copper (Cu) imbalance recently described in KC corneas (Avetisov et al., 2011), but experiments confirming this hypothesis have not been performed. On the other hand, a decrease of LOX activity associated with Cu deficiency induced by starvation was shown (Schoster et al., 1995) (Rucker et al., 1996) (Werman et al., 1997). Because Cu serves as a cofactor for many anti-oxidative enzymes (cytochrome c oxidase, superoxide dismutase 1 and 3), and because signs of oxidative stress in KC corneas have been shown in several studies (Kenney et al., 2005) (Udar et al., 2006), we hypothesize that changes in Cu bioavailability in KC corneal tissue are reflected not only by decreased LOX activity, but also by the disruption of the metabolic pathways of other Cu-dependent proteins.

It has been shown that keratocytes derived from human donor corneas may grow as keratocytes (serum-free conditions), as corneal fibroblasts (in the presence of serum) or as myofibroblasts (cultivation with TGF-β) (Espana et al., 2004) (Jester et al., 2003). The presence of myofibroblasts in scarred areas of pathological corneas, including KC tissue, has been shown consistently (Maatta et al., 2006) (Bystrom et al., 2009) (Kenney et al., 2001). Here, we have shown that under our cultivation conditions, stromal cells derived from KC corneas changed from primarily a myofibroblastic phenotype (80% of all cells) at the beginning of culturing (second passage) to corneal fibroblasts at the fifth passage, while stromal cells from control corneas expressed a corneal fibroblastic phenotype from the first passage. In addition, the morphology of all cells at the fifth passage was consistent with corneal fibroblasts but not keratocytes or myofibroblasts.

We have localized the distribution of LOX, recently shown to be an important enzyme responsible for collagen cross-linking in the cornea (Sethi et al., 2012), throughout normal and KC corneas. As we have detected a decrease in the presence of LOX in the KC stroma, as well as a decline in its activity in corneal fibroblasts derived from KC corneas compared to control ones, we suggest that LOX enzyme may play an important role in KC pathogenesis.

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Appendix 2

Dudakova L., Sasaki T., Liskova P., Palos M., Jirsova K. The presence of lysyl oxidase-like enzymes in human control and keratoconic corneas. *Histology and Histopathology*. 2015a, accepted

The presence of lysyl oxidase-like enzymes in human control and keratoconic corneas

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Running title: Lysyl oxidase enzymes in human cornea

Abstract

Purpose: Lysyl oxidases, a family comprising lysyl oxidase (LOX) and four LOX-like enzymes (LOXL1-4), catalyse the cross-linking of elastin and collagen fibrils. Keratoconus (KC) is characterized by progressive thinning leading to irregular astigmatism, resulting in significant visual impairment. Although the pathogenesis of KC remains unclear, one of the current hypotheses is based on alterations in the organization and structure of collagen fibrils. To extend existing general knowledge about cross-linking enzymes in the human cornea, in the present study we have focused on the detection of LOXL enzymes.

Method: The localization and distribution of LOXL1-4 were assessed in cryosections of 7 control donors (three males and three females; 25-68 years; mean age 46 ± 17.6 years) and 8 KC corneas (5 males and 3 females; 25-46 years; mean age 31.3 ± 7.5 years) using indirect fluorescent immunohistochemistry (IHC). The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200-1000x. Western blot analysis of 4 control and 4 KC corneas was performed for all tested enzymes.

Results: All four LOX-like enzymes were present in all layers of control corneas as well as in the limbus and conjunctiva. Almost no differences between control and pathological specimens were found for LOXL1. A lower staining intensity of LOXL2 was found using IHC and Western blot analysis in KC specimens. Decreases of the signal and small irregularities in the staining were found in the epithelium, keratocytes and extracellular matrix, where a gradual anterior-posterior weakening of the signal was observed. LOXL3 IHC staining was lower in the corneal stromal extracellular matrix and keratocytes of KC samples. No prominent differences were detected using IHC for LOXL4, but a slight decrease was observed in KC corneas using Western blot analysis.

Conclusion: We presume that the decrease of LOXL2 in KC corneas is more likely a consequence of the associated pathological processes (activation of stromal cells due to tissue weakening and consequent structural changes) than a direct cause leading to KC development. At this time, we are unable to provide a coherent explanation for the observed decrease of LOXL3 and LOXL4 in KC corneas.

Keywords: cornea, lysyl oxidase-like enzymes, keratoconus, immunohistochemistry

Introduction

Lysyl oxidases are extracellular copper-dependent enzymes that catalyse the formation of lysine- and hydroxylysine-derived cross-links in collagens and lysine-derived cross-links in elastin (Kagan and Li, 2003). These cross-links are essential for the tensile strength of collagens and the rubber-like properties of elastin; both are abundant extracellular matrix proteins necessary for the structural integrity and function of connective tissues (Kagan and Li, 2003; Myllyharju and Kivirikko, 2004). In addition to lysyl oxidase (LOX) (Trackman *et al.*, 1990; Hamalainen *et al.*, 1991), four LOX-like (LOXL) proteins are currently known: LOXL1, LOXL2, LOXL3, and LOXL4 (Kenyon *et al.*, 1993; Saito *et al.*, 1997; Jourdan-Le Saux *et al.*, 2001; Maki and Kivirikko, 2001). Up till now only LOX has been detected in healthy human corneas (Dudakova *et al.*, 2012).

The molecular functions of LOX and the LOXLs and the degree to which their functions overlap remain up till now unsatisfactorily understood. The LOX-like enzymes are thought to act in the same way as the LOX enzyme, which is highly likely given the close homology of their catalytic domains at the C-terminal (Kagan and Li, 2003; Molnar *et al.*, 2003). The primary structures of the LOX and LOXL1 polypeptides at the N-terminal of the protein differ dramatically from those of LOXL2, LOXL3, and LOXL4, suggesting that these structural differences may impact their functions or their location in the extracellular matrix (Table 1). LOXL1 is specifically required for tropoelastin cross-linking and for elastic fibre formation (Liu *et al.*, 2004). The existence of a putative signal sequence and four scavenger receptor cysteine-rich region (SRCR) domains suggests that the LOXL2, LOXL3 and LOXL4 isoenzymes are extracellular proteins (Csiszar, 2001; Maki, 2009). The SRCR protein super family is involved in quite different functions, such as pathogen recognition, modulation of the immune response, epithelial homeostasis, stem cell biology and tumour development (Resnick *et al.*, 1994; Yamada *et al.*, 1998).

Table 1. A comparison of LOX family members (adapted from Hornstra *et al.*, 2003)

Family member	Human chr.	mRNA and protein size	Greatest mRNA tissue distribution	Protein domains
LOX	5	4.4/5.2 kb; 187/417 AA	Lung, skeletal muscle, kidney, heart	AO
LOXL1	15	2.4 kb; 574 AA	Lung, heart, spleen, skeletal muscle, pancreas	AO
LOXL2	8	3.8 kb; 774 AA	Lung, thymus, skin, testis, ovary	4 SRCR, AO
LOXL3	2	3.0/3.3/3.7 kb; 392/608/753 AA	Heart, uterus, testis, ovary	4 SRCR, AO
LOXL4	10	3.7 kb; 756 AA	Skeletal muscle, testis, pancreas	4 SRCR, AO

AA – aminoacid; AO - Amine oxidase; chr – chromosome; SRCR - Scavenger Receptor Cysteine-Rich;

Keratoconus (KC) is a corneal thinning disorder, typically diagnosed in the patient's adolescent years, usually affecting both eyes. It is one of the leading causes of corneal transplantation in young adults in the Western world (Rabinowitz, 1998). KC corneas display both compositional and structural changes. A decrease in the number and undulating shape of collagen lamellae; reduced diameter and interfibrillar spacing of collagen fibrils together with an increase of proteoglycans density can be observed in the KC corneal stroma (Sawaguchi *et al.*, 1991; Akhtar *et al.*, 2008). Other structural stromal changes include a reduced number of keratocytes and a disorganization of the collagen fibre network (Radner *et al.*, 1998; Meek *et al.*, 2005).

We have previously demonstrated an irregular staining pattern of LOX in KC corneas compared to healthy controls and a lower total LOX activity (encompassing LOX and the LOXL enzymes) in the media of cultivated KC keratocytes (Dudakova *et al.*, 2012). To date no report on the presence of LOXL enzymes in the human cornea has been published. The aim of the current study was to determine whether LOXL enzymes distributions are changed in KC corneas compared to control corneas.

Materials and Methods

Specimen preparation

The study followed the standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki.

Seven control corneas from three male (in one case both eyes) and three female donors (25-68 years; mean age 46 ± 17.6 years) and eight KC corneas (5 males and 3 females; 25-46 years; mean age 31.3 ± 7.5 years) were dissected and snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at -70° C. Four 7 µm thick cryosections were placed per slide and used for indirect fluorescent immunohistochemistry.

The clinical diagnosis of KC was based on the presence of typical corneal signs such as thinning, protrusion, Vogt striae, Fleischer ring and scarring. Corneal topography to validate the diagnosis was performed using an EyeMap EH-290 (Alcon Laboratories, Inc., Fort Worth, TX, USA) in three patients. In four patients the assessment of the anterior and posterior corneal surfaces and pachymetry were performed by Scheimpflug imaging (Pentacam, Oculus, Germany). None of the patients had any previous corneal surgery and their characteristics are shown in Table 2.

Control specimens, also without a history of previous corneal surgery, were obtained from the Bank of Biological Material (First Faculty of Medicine, Charles University in Prague and General Teaching Hospital in Prague) and processed within 4 hours after enucleation; pathological explants were obtained from the Department of Ophthalmology (First Faculty of Medicine, Charles University in Prague and General Teaching Hospital in Prague).

Table 2: Clinical characteristics of the keratoconic samples used in this study

Sample No.	Age at surgery	Gender	TKC	Contact lens wear	Histopathological findings (Bowman breaks, scarring, hydrops etc.)
Kc1	28	F	2	Y Since 24 years	no scarring
Kc2*	27	M	4	N	thinning in centre of cornea, scarring, Vogt striae
Kc3	25	F	3	N	no scarring
Kc4	27	M	4	N	scarring, Vogt striae
Kc5	25	M	4	N	thinning in centre of cornea, scarring, Vogt striae
Kc6*	35	M	2	N	no scarring, Vogt striae
Kc7*	37	M	4	N	scarring
Kc8*	46	F	4	N	thinning in centre of cornea, scarring, Vogt striae

* samples used in Western blot analysis; TKC - topographic keratoconus classification

Indirect fluorescent immunohistochemistry

Three cryosections on each slide were stained with a single antibody. The fourth section (primary antibody omitted) served as a negative control. Three independent experiments were performed. The tissue was fixed with cold acetone for 10 min, rinsed in PBS and incubated with the primary antibody diluted in 1% bovine serum albumin (Sigma Aldrich Corporation, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 1 h at room temperature. The expression of LOX-like enzymes was detected using the following antibodies: rabbit polyclonal anti-LOXL1, anti-LOXL3 (sc-68939, Santa Cruz Biotechnology, Heidelberg, Germany), and anti-LOXL4 (ab88186, Abcam, Cambridge, UK) and mouse monoclonal anti-LOXL2 (ab60753, Abcam). Anti-LOXL1 was raised against recombinant human LOXL1 propeptide expressed in *E. coli* (Sasaki, unpublished data). Following incubation with the primary antibody, the specimens were washed three times in PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide (Vector Laboratories, Inc., Burlingame, CA, USA) to counterstain nuclear DNA.

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200-1000x. Images were taken using a Vosskühler VDS

CCD-1300 camera (VDS Vosskühler GmbH, Germany) and JENOPTIK ProgRes C12plus camera (Jenoptik, Laser, Optik, Systeme GmbH, Jena, Germany). The cells in the epithelium, stroma and endothelium of KC corneas as well in the limbus and conjunctiva in the case of control corneas were examined. At least two hundred epithelial cells and keratocytes per section (600/slide) and one hundred endothelial cells per section (300/slide), directly connected to Descemet's membrane, were examined, and the percentage of positive cells was calculated. The intensity of cell staining was graded using a 0-4 scale as follows: 0: no discernible staining, 1: weak, 2: moderate, 3: intense, and 4: very intense staining.

Western blot analysis

Samples were thawed and homogenized in a lysis buffer (0.2% Triton X-100, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and protease inhibitors in PBS). Subsequently, all samples underwent protein extraction for two days at 4 °C, followed by centrifugation for 30 minutes at 10,000× g. The supernatants were removed and frozen at -20 °C.

The protein concentration was determined with a commercial kit (BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL, USA). Equal volumes of protein extract and sample buffer were mixed, reduced by 5% β-mercaptoethanol, and fractionated on 10% SDS polyacrylamide gels. After electrophoresis was completed, the proteins were transferred to nitrocellulose membranes (Serva Electroforesis GmbH, Heidelberg, Germany) and blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) at 4°C overnight.

After washing in PBS-T, membranes were probed with rabbit and mouse antibodies against LOXL1-4 (1:1,000, same as used in immunohistochemistry experiments) and β-actin (1:2,000; ab8226, Abcam) for 2 hours at room temperature. After another wash in PBS-T, the membranes were incubated with the appropriate peroxidase conjugated anti-mouse (P0260, DakoCytomation, Glostrup, Denmark) and anti-rabbit antibodies (P0399, Dako) diluted 1:8,000 in 1% BSA for 45 minutes at room temperature and washed with PBS-T.

Positive reactions were visualized with an enhanced chemiluminescence technique (SuperSignalWest Femto Maximum Sensitivity Substrate kit; Pierce Biotechnology) for 5 minutes and examined with a membrane documentation system (Syngene Chemigenius-Q and the GeneSnap program; Synoptics Ltd., Cambridge, UK).

Results

Moderate to intense staining was detected for LOXL1 in all parts of the cornea in the control as well as in the KC samples (Fig. 1).

Using LOXL2 antibody, moderate to intense staining was observed in the epithelium, stroma and endothelium of all control corneas. Staining irregularities in the epithelium (a decrease of staining and a clump-like pattern) and a gradual anterior-posterior weakening of the signal from moderate to weak were observed in the stroma of KC corneas (Fig. 2).

LOXL3 antibody revealed moderate to intense staining in the epithelium and endothelium of both control and KC samples. In KC samples, we observed a local increase of staining (Fig. 3). The staining of the ECM was moderate and intense for keratocytes in controls but weak or almost absent in most of the KC specimens (Fig. 3).

Using LOXL4 antibody, the epithelium and endothelium of both control and KC samples showed a moderate to intense signal, while a moderate signal with a punctate-like pattern was observed in the stroma (Fig. 4).

All LOX-like enzymes were present in the limbus and conjunctiva of control samples. For LOXL1 we observed intense and for LOXL2, -3 and -4 moderate to intense staining (Fig. 5). LOXL1 and LOXL3 exhibited staining heterogeneities – cells adjacent to the superficial layer of the epithelium showed higher positivity compared to cells located in the deeper layers.

Using Western blot analysis we did not find differences between control and KC samples when staining with anti-LOXL1 antibody. We observed a decrease of LOXL2 and 4 in KC samples compared to controls (Fig. 6), while a slight increase of LOXL3 was observed in KC corneas.

Discussion

In this study we show for the first time the distribution of LOXL1-4 in the normal human cornea, limbus and conjunctiva as well as in corneal buttons obtained from patients with KC.

Previous studies indicated a disorganized collagen fibre network in KC corneas (Radner *et al.*, 1998; Meek *et al.*, 2005) suggesting that these abnormalities may contribute to the mechanical weakness of KC corneas, hence leading to their conical shape. Thinning of the

collagen lamellae has been attributed to a decrease in the number of cross-links (bonds between and within collagen fibrils) (Meek *et al.*, 2005).

LOXL1 is important for elastic fibre formation (Liu *et al.*, 2004). Elastin fibres are present mostly in the mid-posterior part of the peripheral human cornea (Kamma-Lorger *et al.*, 2010), while the corneal thinning in KC occurs in the central part. Due to these facts and according to our results, we hypothesize that this enzyme is not directly involved in the disease pathogenesis.

Corneal keratocytes normally remain quiescent but during corneal wound healing they are activated and undergo transformation into corneal fibroblasts and myofibroblasts (West-Mays & Dwivedi, 2006). LOXL2 is abundantly expressed in senescent fibroblasts, cells with limited proliferation (Saito et al., 1997). The decrease of LOXL2 staining in KC corneas compared to controls could be caused by the transformation of keratocytes into myofibroblasts in KC corneas (Maatta *et al.*, 2006; Bystrom *et al.*, 2009; Dudakova et al., 2012). Additionally, we have observed a gradual anterior-posterior weakening of the LOXL2 signal which may be attributed to the fact that keratocytes in the posterior stroma are more likely to be activated (Hindman *et al.*, 2010).

After transformation of keratocytes into myofibroblasts, these cells migrate to the wound site where they increase the synthesis of ECM components, proliferate and acquire contractile properties (West-Mays & Dwivedi, 2006). The increased expression of *LOXL2* has been shown in several adherent tumour cell lines, while down-regulation has been observed in several non-adherent tumour cell lines. This suggests that LOXL2 may be involved in cell adhesion and that a loss of this protein may be associated with the loss of cell adhesion (Saito *et al.*, 1997). The observed decrease of LOXL2 staining in KC corneas could enable the migration of activated corneal cells. Barker *et al.* (2013) have shown that cancer-associated fibroblasts express more LOXL2, further enhancing cancer progression. Treatment with LOXL2-specific inhibitors inhibits cell invasion and metastasis. In tumour cells, deregulation of *LOXL2* expression may occur and an increased amount of this protein may lead to the persistent activation of cells in contrast to corneal cells, where after activation, LOXL2 presence diminished.

We presume that the decrease of LOXL2 in KC corneas is more likely a consequence of the associated pathological processes (activation of stromal cells due to tissue weakening and consequent structural changes) than a direct cause leading to KC development.

At this time, we are unable to provide a coherent explanation of the observed changes in LOXL3 and LOXL4 expression in KC corneas compared to the control tissue. The increase

of the LOXL3 signal in the Western blot experiment could be caused by the local intensity increase observed in epithelial cells in the IHC experiments. Since LOXL enzymes are expressed in many tissues, it is difficult to investigate the functions and interpret the roles of individual LOXs in cellular processes in these tissues (Molnar et al., 2003). Different expression regulators, alternative splicing, structural and substrate specificities; all of these could contribute to their varied functions and their location in the ECM. A more detailed characterization of LOXL proteins will be necessary in the future to understand the diverse functions of this group of enzymes.

The therapeutic targeting of extracellular proteins is becoming hugely attractive in light of the evidence implicating the tumour microenvironment as pivotal in all aspects of cancer initiation and progression. Secretion of the LOX family members by tumours and their roles in tumorigenesis have been a subject of intense research (Barker *et al.*, 2012). Much attention is focused on LOX and LOXL2 as their increased expression has been observed in aggressive cancers and has shown significant correlation with decreased survival in a number of clinical cancer studies (Barker *et al.*, 2012). Both of these enzymes were found to be decreased in KC corneas (Dudakova et al., 2012 and this study). Therefore, studying the involvement of these enzymes in corneal pathology will help to understand their role in ECM remodelling in a broad context.

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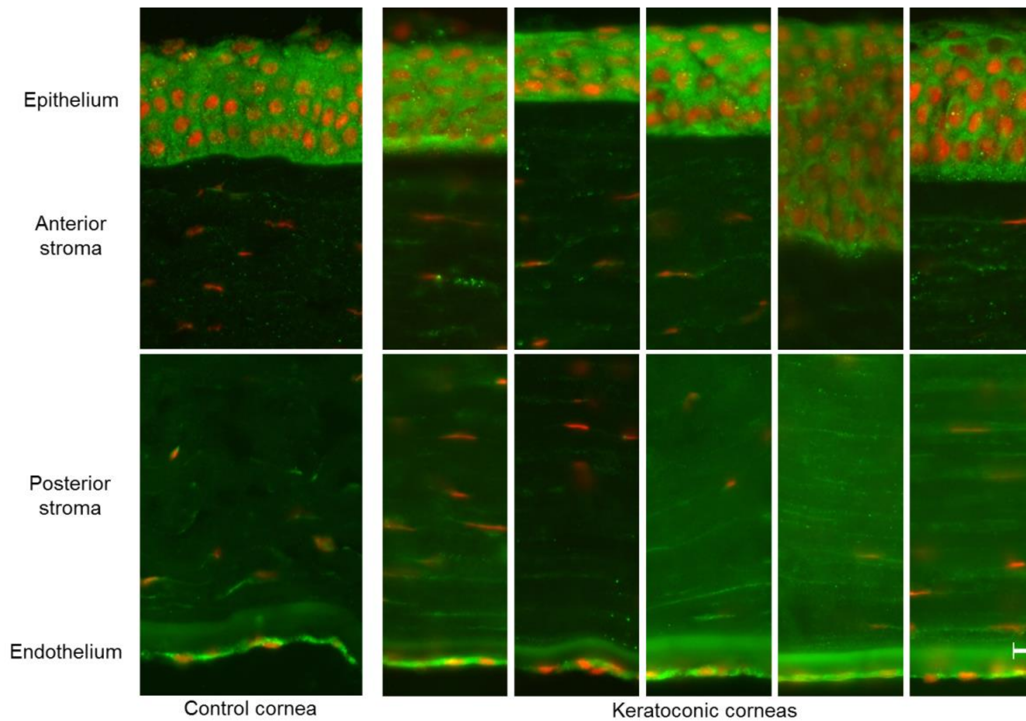

Figure 1: LOXL1 staining in control and keratoconic (KC1-5) corneas

No differences were observed between control and keratoconic specimens. Scale bar represents 10 µm.

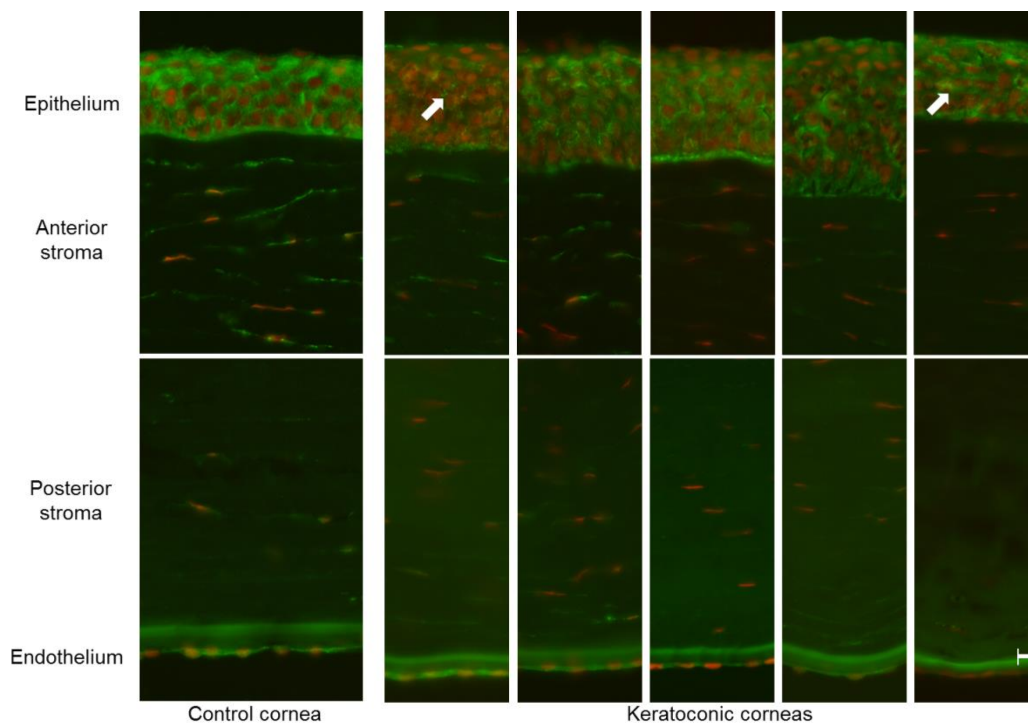

Figure 2: LOXL2 staining in control and keratoconic (KC1-5) corneas

Note the decrease of the signal and the staining irregularities in the epithelium of keratoconic corneas (arrows). Scale bar represents 10 µm.

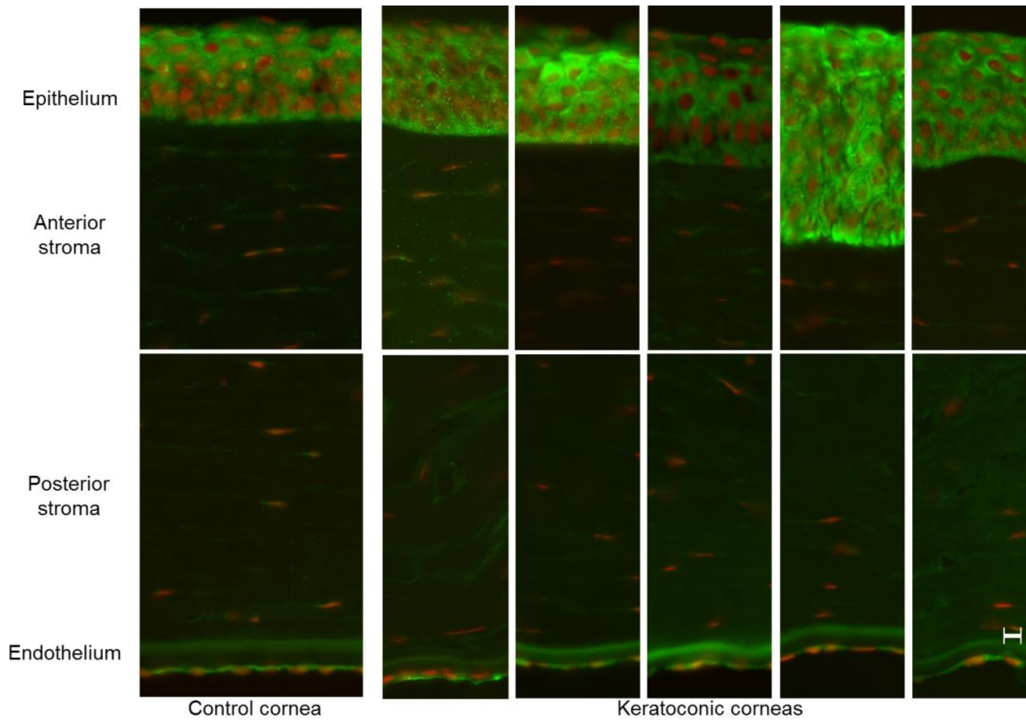

Figure 3: LOXL3 staining in control and keratoconic (KC1-5) corneas

Note the decreased staining in the stroma and local increase of signal in epithelium of keratoconic corneas. Scale bar represents 10 µm.

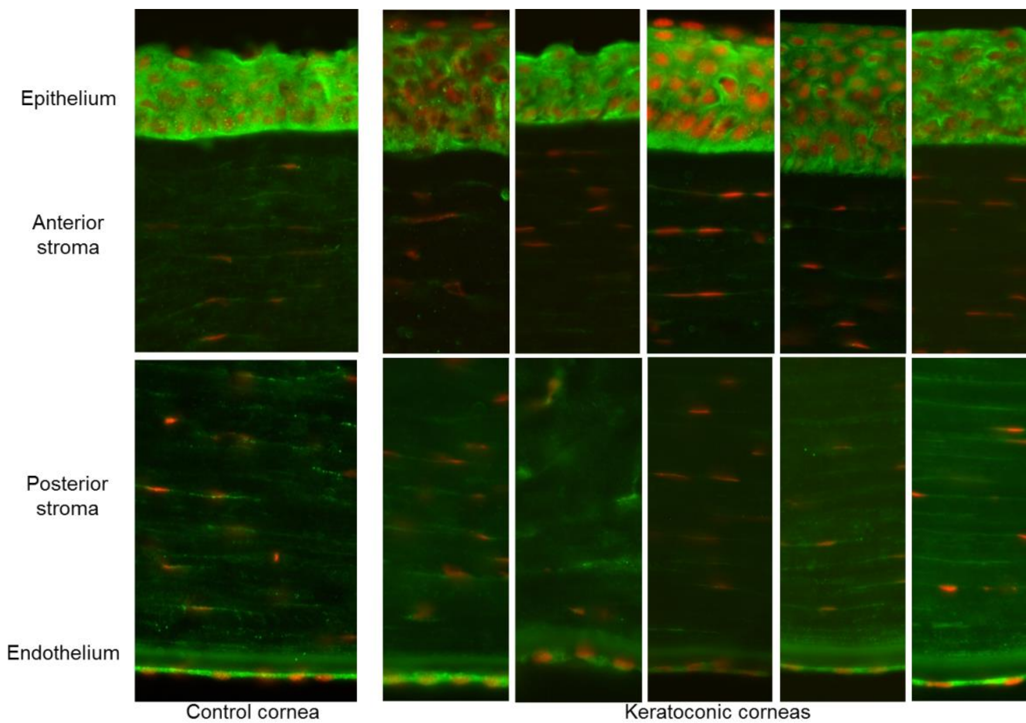

Figure 4: LOXL4 staining in control and keratoconic (KC1-5) corneas

No prominent differences were observed between control and keratoconic specimens. Scale bar represents 10 µm.

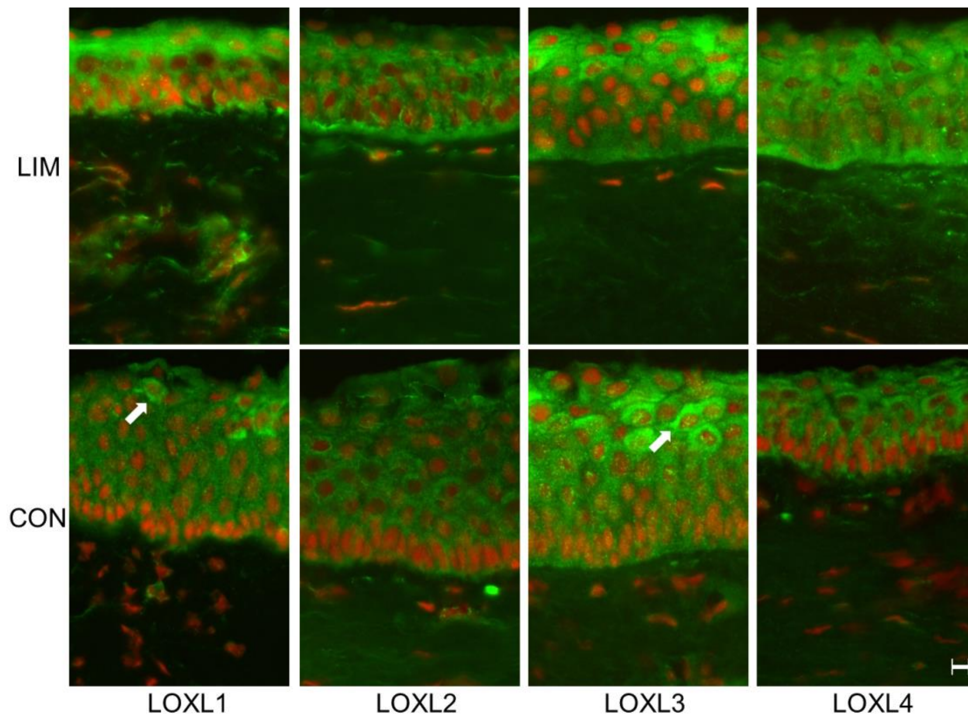

Figure 5: Indirect fluorescent immunohistochemistry using antibodies against LOXL1-4 in the limbus (LIM) and conjunctiva (CON) of control samples

Note the increased staining of cells in the superficial epithelial layer (arrows). Scale bar represents 10 µm.

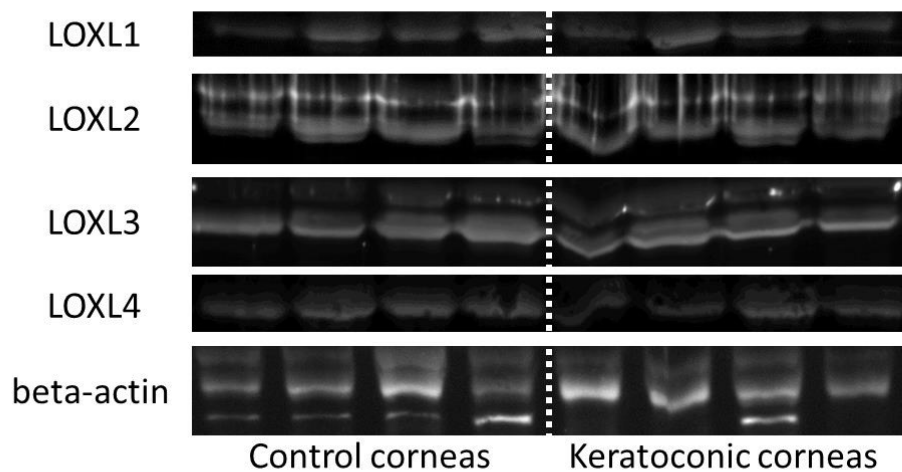

Figure 6: Western blot analysis of control and keratoconic corneas

Note the slight decrease of the signal for LOXL2 and -4 and increase of LOXL3 in the keratoconic samples.

Appendix 3

Dudakova L., Jirsova K. The impairment of lysyl oxidase in keratoconus and in keratoconus-associated disorders. *Journal of Neural Transmission*. 2013;120(6):977-82

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NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - REVIEW ARTICLE

The impairment of lysyl oxidase in keratoconus and in keratoconus-associated disorders

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Abstract Keratoconus (KC) is an eye disease characterized by the progressive thinning and protrusion of the cornea, which results in the loss of visual acuity. This disorder remains poorly understood, although recent studies indicate the involvement of genetic and environmental factors. Recently, we have found that the distribution of the cross-linking enzyme lysyl oxidase (LOX) is markedly decreased in about 63 % of keratoconic specimens. Similarly, LOX activity is significantly reduced by 38 % compared to control tissue. Nearly 70 systemic disorders have been reported in association with KC, most of them affecting the extracellular matrix. In this review we attempted to ascertain whether any KC-associated diseases exhibit signs that may reflect LOX impairment. We hypothesized that very similar changes in the extracellular matrix, particularly at the level of collagen metabolism, including LOX impairment in mitral leaflets, may reflect an association between KC and mitral valve prolapse. Moreover, this putative association is supported by the high frequency of Down syndrome in both diseases. Among other disorders that have been found to coincide with KC, we did not find any in which the LOX enzyme may be directly or indirectly impaired. On the other hand, in cases where KC is present along with other connective tissue disorders (Marfan syndrome, Ehlers–Danlos syndrome and others), KC may not arise as a localized manifestation, but rather may be induced as the result of a more complex connective tissue disorder.

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Keratoconus

Keratoconus (KC) is a heterogeneous disorder that usually manifests itself at puberty and that is almost always bilateral. The cornea assumes a conical shape as a consequence of a gradually progressive thinning of the corneal stroma. This leads to significant visual impairment, irregular astigmatism and high myopia; as a result, KC is the leading cause of corneal transplantation in developed countries (Rabinowitz 1998). The prevalence of KC lies between 8.8 and 54.5 per 100,000 citizens (Ihalainen 1986; Kennedy et al. 1986). Despite extensive research, the etiopathogenesis of KC is still unknown, but it is generally accepted that KC is a multifactorial condition or that it represents the final stage of a variety of different pathological processes. Although most KC cases are sporadic, the participation of genetic factors in its etiology is evident due to proven familial inheritance, discordance between dizygotic twins, and association with other known genetic disorders (Edwards et al. 2001; Nowak and Gajecka 2011). The analysis of gene expression in KC epithelium indicates massive changes in the cytoskeleton, reduced extracellular matrix remodeling, altered transmembrane signaling, and modified cell-to-cell and cell-to-matrix interactions (Nielsen et al. 2003). Besides corneal thinning, the following phenomena have been reported to be involved in KC pathogenesis: oxidative stress (Kenney et al. 2005), keratocyte apoptosis (Kim et al. 1999), the activation of proteases (Zhou et al. 1998; Balasubramanian et al. 2012), the accumulation of mtDNA mutations (Atilano et al. 2005), abnormalities in lysosomal enzymes (Sawaguchi et al.

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1989), changes in the expression of transcriptional factors (Mace et al. 2011) and iron deposition (Loh et al. 2009).

More than 70 % of the stroma, the thickest part of the cornea, is comprised of collagen (Newsome et al. 1981), fibrils of which form about 300–500 lamellae parallel to the corneal surface (Hamada et al. 1972). Besides collagen, elastin fibrils are also present, particularly in the mid-posterior part of the human peripheral cornea (Alexander and Garner 1983; Kamma-Lorger et al. 2010). Under physiological conditions the final enzymatic step required for collagen and elastin cross-linking in the corneal stroma is catalyzed by lysyl oxidase (LOX) (Mizobe et al. 2008; Kagan et al. 1986). Recently, artificial corneal collagen cross-linking method has become regularly used for the KC stabilization. This approach is based on the formation of covalent bonds between collagen fibrils, mimicking the physiological function of LOX and, thus, enhancing corneal rigidity and preventing further corneal protrusion (Wollensak 2006; Kanellopoulos 2012).

Lysyl oxidase in keratoconus

Lysyl oxidase is a copper-dependent amine oxidase that is synthesized as an inactive 50 kDa pro-enzyme and then processed by pro-collagen C-proteinases—mammalian Tolloids and particularly by bone morphogenetic protein-1 (BMP-1)—into the active enzyme (28–32 kDa) (Trackman et al. 1992; Uzel et al. 2001; Guo et al. 2007; Chen and Greenaway 2011).

Beside LOX, four LOX-like proteins (LOXL1–LOXL4) catalyze the oxidative deamination of lysine residues in collagen and elastin, although their specific mechanism of action remains unknown (Molnar et al. 2003; Csiszar 2001). LOX enzymes are critical for the stability of connective and vascular tissues, play an important role in promoting tumor cell invasion (Nishioka et al. 2012; Kuivaniemi 1985), and are potential chemokines inducing the migration of different cell types (Lucero et al. 2011). In the eye, the LOX enzyme has been detected in the trabecular meshwork, ciliary body, lens and retina (Coral et al. 2008; Sethi et al. 2011). Recently, we have found LOX in the normal human cornea, where it is expressed in the epithelium, stroma and endothelium (Dudakova et al. 2012).

Due to its function in cross-linking the extracellular matrix, LOX has been considered as a candidate gene for KC (Nielsen et al. 2003). Despite a suggestive linkage in a familial KC panel to the 5q23.2 region where *LOX* is located (Bisceglia et al. 2009), no pathogenic sequence variants have so far been identified (Bykhovskaya et al. 2012; De Bonis et al. 2011). Although *LOX* expression was found to be higher in KC corneas compared to normal ones (Nielsen et al. 2003), we have recently found lower LOX activity in tissue culture medium in which KC corneal fibroblasts were cultured. In addition, we have observed changes in LOX distribution in KC corneas: the presence of the active LOX protein was decreased in all corneal layers, particularly in the extracellular matrix of the stroma. The epithelium exhibited some staining irregularities expressed as a clump-like pattern in the cytoplasm compared to the homogenous staining observed in control tissue (Dudakova et al. 2012).

This finding led us to consider how LOX may by included in KC pathogenesis and to review whether LOX impairment may play a role in the pathogenesis of KC-associated diseases.

Relationship between keratoconus and connective tissue diseases

KC occurs mostly as an isolated condition, but has been reported to be associated with almost forty systemic conditions, many of which are of genetic origin and many of which affect the ophthalmic system. In addition, another 30 corneal or extracorneal ocular diseases have been found in KC patients (Rabinowitz 1998; Sugar and Macsai 2012; Edwards et al. 2001). Most KC-related disorders may be sorted into four main groups: connective tissue disorders, diseases with abnormal retinal function with oculodigital stimulation (pressing on the eye), diseases associated with atopy or eczema and eye rubbing or diseases with low mental function (Sugar and Macsai 2012).

KC-linked connective tissue diseases in which collagen or elastin abnormalities frequently occur include Ehlers–Danlos syndrome, osteogenesis imperfecta, Marfan syndrome, joint hypermobility, mitral valve prolapse (MVP), nail patella syndrome, GAPO syndrome and Williams–Beuren syndrome (Rabinowitz 1998; Sugar and Macsai 2012; Pinsard et al. 2010; Greenfield et al. 1973; Wajntal et al. 1990). In these diseases, impaired corneal extracellular matrix formation frequently results in features that overlap with features of KC such as astigmatism, decreased corneal thickness and changes in corneal curvature (Liu et al. 2011; Konradsen et al. 2012; Evereklioglu et al. 2002). This connection led to the suggestion that KC may not be a distinct eye disease, but rather a nonspecific sign representing a more generalized systemic disorder, possibly a mild collagen tissue abnormality (Street et al. 1991).

Mitral valve prolapse, frequently associated with myxomatous degeneration (pathological weakening of the connective tissue), is the most common cause of mitral regurgitation and affects 2–22 % of the general population (Chapman 1994; Guy and Hill 2012; Levine and Slaugenhaupt 2007; Scordo 2005).

The first study showing an association between KC and MVP was published in 1982 by Beardsley and Foulks (1982). Since that time, at least three other studies have confirmed a statistically significant higher occurrence of MVP in KC patients with a reported prevalence of 23–66 % compared to the 7–13 % prevalence of MVP in the normal population (Rabbanikhah et al. 2011; Javadi et al. 2007; Sharif et al. 1992). Taken together, these studies were performed on more than 176 KC patients and 368 control subjects. Similar results were found when the opposite correlation was studied (i.e., KC was detected in MVP patients) (Lichter et al. 2000). In contrast to the above-described results, no statistically significant difference in MVP prevalence was seen in 95 KC patients compared to that found in 96 matched controls in a study by Street et al. (1991).

A significantly higher occurrence of MVP has been found in severe forms of KC compared to others, varying severities of KC, but no differences were seen in the prevalence of MVP among different age groups or between men and women (Rabbanikhah et al. 2011; Sharif et al. 1992).

The etiology of MVP, similarly as that of KC, is only poorly understood (Devereux et al. 1976; Towbin 1999; Nowak and Gajecka 2011). Both disorders have been associated with systemic collagen diseases such as pseudoxanthoma elasticum, Marfan syndrome, Ehlers–Danlos syndrome, osteogenesis imperfecta and joint hypermobility (Rabinowitz 1998; Devereux et al. 1976; Cavenaghi et al. 2009; Lebwohl et al. 1982; Jaffe et al. 1981).

Both human heart valves and the human cornea are composed of collagen types I, III and V (Bashey et al. 1978; Hammer et al. 1979; Newsome et al. 1981; Marshall et al. 1991). Collagen I and III abnormalities have been demonstrated in patients suffering from severe MVP with ruptured chordae tendenae (Hammer et al. 1979; Nasuti et al. 2004; Purushothaman et al. 2012), but the underlying mechanism has not yet been discovered. Similarly, histopathological changes underlying myxomatous degeneration in MVP consist of the replacement of the normal collagen matrix of the fibrosa layer with acid mucopolysaccharides (Marshall and Shappell 1974; Sherman et al. 1970). It was shown that myxomatous degeneration affecting the cornea shares some features with KC: the damage is present particularly in the anterior part of the stroma, Bowman layer is disrupted and stromal keratocytes are transformed into cells with myofibroblastic differentiation (Belliveau et al. 2012).

Sharif et al. (1992), suggest that the histopathological and biochemical similarities between KC and MVP are not coincidental and may represent different clinical manifestations of a subtle defect in the mesenchymal system. Moreover, they attribute this association to the alteration of collagen during embryogenesis, affecting at the same time both the corneal stroma and the mitral valves, both of which are formed during the sixth and seventh weeks of fetal life.

Electron microscopic studies have shown changes in the ratio of collagen to ground substance and the breakage of collagen crosslinks (Beardsley and Foulks 1982). Similarly, the alterations observed in the elastic fibers of the perforated floppy mitral valve resembled those of the elastic fibers in animals treated with beta-aminopropionitrile, an inhibitor of LOX (Tamura et al. 1998). Finally, immunohistochemistry revealed a fivefold decreased expression of LOX in prolapsed mitral valve leaflets compared to the healthy ones (Purushothaman et al. 2012). These findings may indicate a very similar origin of KC and MVP, spotentially including LOX damage.

The most frequently KC-associated disease is Down syndrome. The reported prevalence of KC in Down syndrome patients ranges from 5.5 to 30 %, which is 10–300-fold higher than in the normal population (Cullen and Butler 1963; Hestnes et al. 1991; Rabinowitz 1998). This strong association may reflect the massive, 50 % prevalence of MVP occurring in Down syndrome patients (Barnett et al. 1988).

The other connective diseases accompanying KC occur with much lower frequencies compared to those of MVP or Down syndrome. On the other hand, some of these diseases have an already characterized etiopathogenesis that is connected with the collagen and elastin metabolism. One of these disorders is Williams–Beuren syndrome, caused by microdeletions at chromosome 7q11.23, which includes the elastin gene (Pinsard et al. 2010). Occasionally, KC is accompanied by Ehlers–Danlos syndrome type VI, associated with the deficient activity of lysyl hydroxylase, which is responsible for post-translational collagen modification (Ha et al. 1994; Cameron 1993). The relationship of KC with subtypes II and IV of Ehlers–Danlos syndrome, both of which are characterized by deficient collagen I, III or V synthesis, has been described as well (Robertson 1975; Kuming and Joffe 1977). KC is also associated with joint hypermobility for which mutations of collagen I and III are typical (Robertson 1975; Cavenaghi et al. 2009). It was found that KC patients are five times more likely to show hypermobility of the metacarpophalyngeal and wrist joints (Woodward and Morris 1990). Vice versa, the characteristics of this multisystematic disorder coincide with the features of osteogenesis imperfecta, Ehlers–Danlos and Marfan syndrome, all of which have been described as being associated with KC (Cavenaghi et al. 2009). Among other disorders that have been found to coincide with KC, we did not find any in which the LOX protein would be directly or indirectly impaired.

Conclusion

KC is associated with many disorders linked with a spectrum of biochemical alterations affecting collagen and elastin cross-link formation. Here, we present the hypothesis of similar origin of KC and MVP, with respect to the alterations in LOX and elevated presence of the Down syndrome in both diseases. Cases in which an association of KC with other connective tissue disorders occurs (Marfan syndrome, Ehlers–Danlos syndrome and others) support the suggestion that KC may not arise as a localized manifestation, but instead may be induced as the result of a more complex connective tissue disorder.

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Appendix 4

Dudakova L., Palos M., Svobodova M., Bydzovsky J., Huna L., Jirsova K, Hardcastle A, Tuft S., Liskova P. Macular corneal dystrophy and associated corneal thinning. *Eye (Lond)*. 2014;28(10):1201-5

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CLINICAL STUDY

Macular corneal dystrophy and associated corneal thinning

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Abstract

Purpose **To identify the molecular genetic cause of macular corneal dystrophy (MCD) in four probands, and characterize phenotypic similarities between MCD and keratoconus.**
Methods **We performed ophthalmological examination, Scheimpflug imaging (Pentacam, Oculus Inc.), histopathological examination of excised corneal buttons, and direct sequencing of the *CHST6* coding region.**
Results **Pentacam measurements were taken in six eyes of three probands. All showed diffuse corneal thinning with paracentral steepening of the anterior corneal surface that was graded as keratoconus by the integrated software, but without associated ectasia of the posterior corneal surface or regional thinning. Homozygous or compound heterozygous *CHST6* mutations were identified in all cases, including two novel mutations, c.13C > T; p.(Arg5Cys) and c.289C > T; p.(Arg97Cys).**
Discussion **Localized elevation of the anterior corneal curvature can occur in MCD in the absence of other features of keratoconus. The identification of a further two Czech probands with the compound allele c.[484C > G; 599T > G] supports the enrichment of this allele in the study population.**
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Introduction

Macular corneal dystrophy (MCD, OMIM #217800) is an autosomal recessive condition characterized by gray–white stromal opacities with indistinct borders, and haze of the intervening stroma.[1] Corneal thinning is an important clinical feature of MCD.[2] MCD is caused by mutations within the *CHST6* gene and is a rare disorder.[1] *CHST6* encodes the enzyme carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6 that catalyzes the transfer of a sulfate group to the GlcNAc residues of keratan sulfate, and disease-causing mutations result in abnormal deposition of glycosaminoglycans in corneal stroma, keratocytes, Descemet membrane, and the corneal endothelium.[1,3]

Keratoconus is a complex disorder characterized by bilateral thinning and ectasia of the cornea. It has a strong genetic component and the prevalence is estimated to be between 29 and 229 per 100 000 depending on the population studied.[4,5] Corneal ectasia and thinning, hallmarks of keratoconus, have also been reported in patients with other corneal dystrophies, which has prompted a debate as to whether there could be a common etiology in some affected individuals.[6,7]

We therefore screened *CHST6* for mutations in patients diagnosed with MCD and used Scheimpflug imaging to look for features of keratoconus. Excised corneal buttons were examined when available.

Materials and methods

The research was conducted in accordance with the Declaration of Helsinki and institutional ethical committee approval was obtained. We examined four previously unreported probands (three females and one male) diagnosed with MCD as well as one previously genotyped individual.[8] Both parents of probands 1, 3, 4, 5 were of white Czech origin, while proband 2 was the child of Moroccan and white Czech parents. Pentacam (Oculus Inc., Wetzlar, Germany) analysis with the automated keratoconus detection software was performed in individuals 1, 2, and 5. Individual 4 had bilateral grafts and individual 3 had such advanced disease that image capture was impossible. Only the anterior surface of the

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right cornea of individuals 1 and 5 could be reliably evaluated. Measurements were compared with an individual with keratoconus without any other corneal disorder.

DNA of the cases and available first degree relatives was extracted from venous blood samples using Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Coding exons of *CHST6* were amplified and sequenced as previously described.[8,9] NCBI NM_021615.4 was used as the reference sequence. Mutation description followed current recommendations of Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/).[10] The 1000 Genomes dataset (http://browser.1000genomes.org/index.html) and the Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/) were examined to determine the frequency of previously unreported sequence variants identified. Potential pathogenicity of missense variants was evaluated using PolyPhen2,[11] MutPred,[12] and SNPs&GO algorithms.[13]

Corneal buttons obtained after right penetrating keratoplasties of probands 2, 3, and 4 were dissected, snap frozen in liquid nitrogen, and embedded in Optimal Cutting Temperature Compound. Tissue was cryosectioned at a thickness of 7 μm and stained with haematoxylin–eosin and Alcian blue for morphological assessment by light microscopy.

Results

Clinical characteristics and observed genotypes are shown in Table 1. Segregation of the identified mutations in available first degree relatives of probands 1, 2, and 4 confirmed that each mutation has been located on a separate chromosome. Proband 3 was the only person studied within the family. The topography data is summarized in Table 2. The family history for MCD was negative in all cases, which is consistent with an autosomal recessive mode of inheritance. Slit-lamp biomicroscopy showed typical changes of MCD, gray–white stromal opacities with indistinct borders and haze of the intervening stroma, without features of keratoconus in all cases.

In all of the six eyes analyzed, a keratoconus pattern could be distinguished on anterior sagittal curvature maps. Examination of the posterior elevation maps using best fit sphere, posterior sagittal curvature, pachymetry maps, and corneal thickness spatial profiles showed diffuse thinning without focal ectasia (Figures 1a and b), which was supported by manual inspection of the

Table 1 Clinical data and genotypes of probands with macular corneal dystrophy

Case no/gender	*Subjective age of onset (y)*	*Age at examination (y)*	*BCVA prior to PK*		*Age at PK*		*CHST6 genotypes*	*Mutations at protein level*
			RE	*LE*	*RE*	*LE*		
1/F	15	18	0.33	0.66	19	NP	c.[484C>G; 599T>G];[484C>G; 599T>G]	p.[(Arg162Gly; Leu200Arg)];[(Arg162Gly; Leu200Arg)]
2/F	15	37	0.25	0.5	37	NP	c.[1A>T];[484C>G; 599T>G]	p.[Met1?];[(Arg162Gly; Leu200Arg)]
3/F	26	32	0.33	0.33	32	33	c.[289C>T(;)1046G>A]	p.[(Arg97Cys(;)Cys349Tyr)]
4/M	26	30	0.33	0.5	30	33	c.[13C>T];[1046G>A]	p.[(Arg5Cys)];[(Cys349Tyr)]
5/F[a]	16	18	0.66	0.66	NP	NP	c.[484C>G; 599T>G];[?]	p.[(Arg162Gly; Leu200Arg)];[?]

Abbreviations: BCVA, best corrected visual acuity; LE, left eye; NP, not performed; PK, penetrating keratoplasty; RE, right eye; y, year.
[a]Molecular genetic findings in case 5 have been previously reported.[8]
In case 3 segregation analysis of mutations in the family was not performed therefore a possibility remains that there is a deletion of the second allele as delineated by the HGVS nomenclature with (;). In case 5, screened previously for *CHST6* coding sequence variants, mutation in the second allele has not been identified as delineated by the HGVS nomenclature with [?].

Table 2 Pentacam measurements of six corneas with macular corneal dystrophy

Case no/eye	*K1 (D)*	*K2 (D)*	*TKC*[a]	*CCT (μm)*	*Thinnest (μm)*	*Apex (μm)*	*Pattern and localization of steepening on anterior sagittal maps*
1/RE	50.8	55.4	2–3	420	371	422	Central and inferotemporal
1/LE	46.5	52.9	2	375	335	374	Inferior (asymmetric bow tie with skewed radial axes)
2/RE	42.1	46.2	1–2	438	428	436	Inferotemporal
2/LE	43.4	45.2	1	427	416	425	Inferotemporal
5/RE	42.3	43.8	2	409	396	399	Inferotemporal
5/LE	45.6	47.7	2-3	418	382	419	Inferotemporal

Abbreviations: CCT, central corneal thickness; D, diopter; K1, flat keratometry; K2, steep keratometry; LE, left eye; RE, right eye; TKC, topographic keratoconus classification.
[a]As evaluated by keratoconus software module.

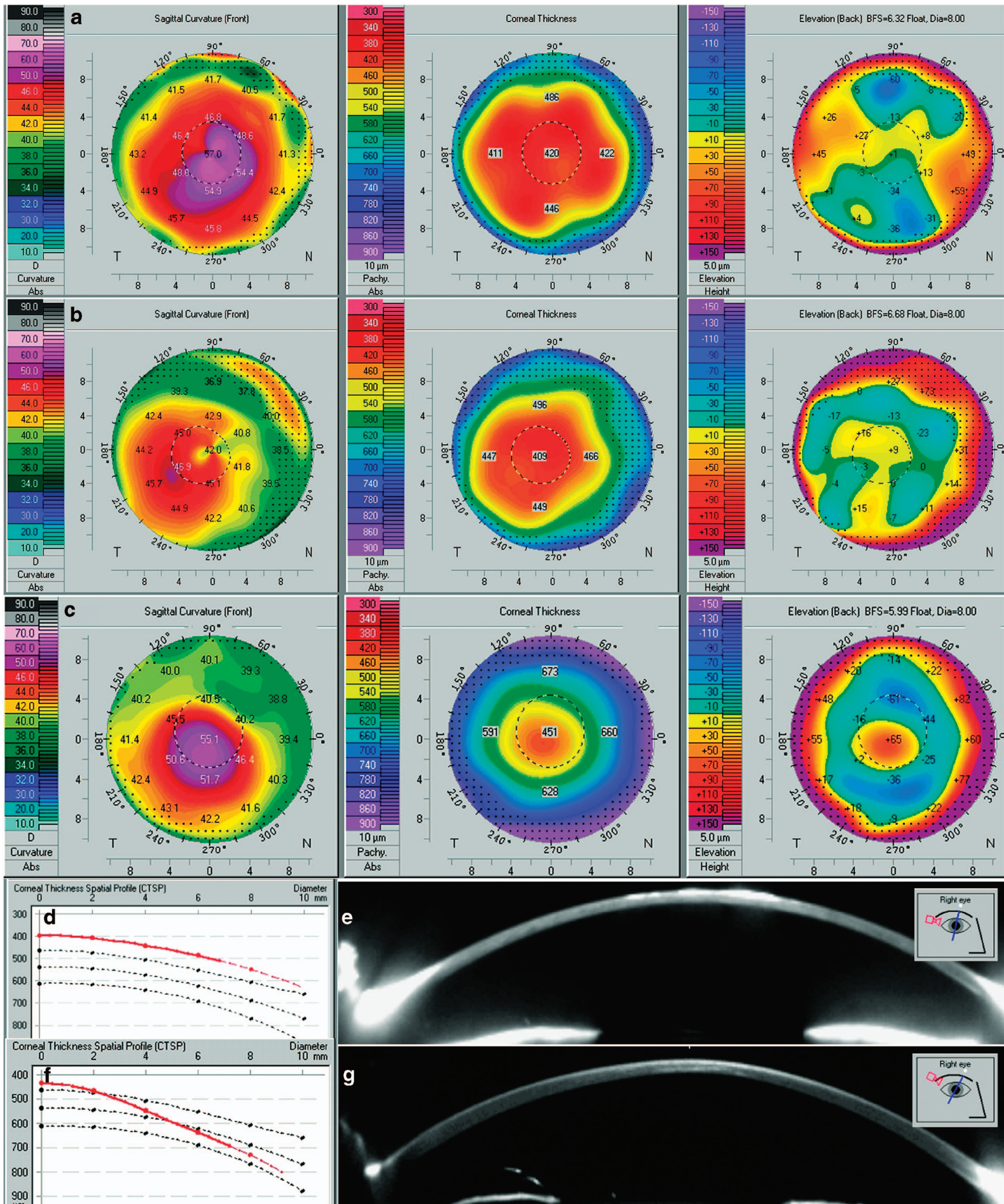

Figure 1 Scheimpflug imaging of macular corneal dystrophy and keratoconus corneas. Anterior sagittal curvature, pachymetry, and posterior elevation maps of the right cornea of proband 1 (a) and the right cornea of proband 5 (b), note diffuse thinning on pachymetry map and lack of posterior elevation in both a and b. Right cornea of a patient with keratoconus, note localized ectasia observed on all three maps (c). Corneal thickness spatial profile of the right cornea of proband 5, note uniformly thin cornea shown by a red line (d). Single Scheimpflug image of the right cornea of proband 1 further documenting diffuse thinning; imbedded image shows meridian of capture (e). Corneal thickness spatial profile of keratoconic cornea, note progressive thinning towards the corneal center (f). Single Scheimpflug image of the right cornea of a keratoconus patient documenting localized paracentral thinning, imbedded image shows meridian of capture (g).

individual Scheimpflug images (Figure 1e). These observations are in contrast to the focal thinning and posterior corneal elevation seen in the patient with keratoconus (Figures 1c, f and g). Histopathological examination of all three corneas from probands 2–4 confirmed the presence of corneal thinning together with characteristic glycosaminoglycan deposits that stained positive with Alcian blue, in keratocytes, endothelial cells, and extracellularly in the stroma and Descemet membrane (data not shown).

Two novel *CHST6* mutations, c.13C > T; p.(Arg5Cys) and c.289C > T; p.(Arg97Cys), and four previously reported disease-causing mutations were identified (Table 1, Supplementary Material). The novel missense mutations were not found in sequence variant databases. MutPred indicated p.(Arg5Cys) to be possibly harmful with an overall score of $g = 0.556$ and Polyphen2 as damaging scoring 1.000, whereas SNPs&GO evaluated the mutation as neutral with a reliability index of 8. All three tools predicted p.(Arg97Cys) to be deleterious (PolyPhen2 scored it 1.000, MutPred 0.789, and SNPs&GO as disease related with a reliability index of 9). Within the compound allele c.[484C > G; 599T > G], the variant at position 484 is a common polymorphism in linkage disequilibrium with the disease-causing mutation at position 599.[8]

Discussion

It has been suggested that the concomitant occurrence of keratoconus and various other corneal dystrophies may be higher than would be expected by chance.[6] Regular corneal astigmatism and corneal thinning are characteristic features of MCD.[2] Corneal astigmatism and corneal thinning are also features of keratoconus, and in this respect the phenotype of the two conditions is similar. MCD is an autosomal recessive corneal dystrophy with an identified genetic cause; however, the contribution of genetic factors to the development of keratoconus is not known.[14] Given our current lack of knowledge of the cause of keratoconus, it is important to determine whether there is any potential involvement of the *CHST6* gene or associated pathways. Interestingly, concurrent keratoconus and MCD has been previously described in five cases,[15–18] and one of the linked loci for keratoconus (16q22.3-q23.1) contains the *CHST6* gene.[19]

When we used the Pentacam Scheimpflug system to evaluate anterior corneal surface parameters in cases with MCD, there was a pattern suggestive of keratoconus in all six eyes that were examined. However, there was not an associated elevation of the posterior corneal surface. Importantly, in contrast to the changes that characterize keratoconus in which there is localized stromal thinning, corneas with MCD showed diffuse thinning that involved the whole diameter of the cornea. Diffuse corneal thinning was also present on histopathological examination of corneas with MCD compared with control corneal specimens. Therefore evaluation of the anterior corneal surface in isolation can give indices that spuriously suggest the presence of keratoconus, and correlation with posterior corneal elevation maps and regional pachymetry is required.[20,21] The origin of the apparent anterior corneal elevation is uncertain, but the stromal deposits of MCD probably affect the quality of data capture.

Pathogenic mutations within *CHST6* were found in all four probands with MCD, including two novel missense mutations; c.13C > T; p.(Arg5Cys) and c.289C > T; p.(Arg97Cys) were considered disease-causing based on their absence in sequence variant databases and bioinformatic analysis. Over the last 10 years we have examined 17 affected individuals from 12 families with MCD at the Department of Ophthalmology in Prague. The complex allele with a polymorphic missense variant in linkage disequilibrium with the causative mutation c.[484C > G; 599T > G]; p.[Arg162Gly; Leu200Arg] was found in 8 of the 12 families of this Czech MCD cohort, with two homozygous probands and a further six probands who were heterozygous.[8]

In conclusion, our results suggest that the change in anterior corneal curvature and diffuse corneal thinning is a phenocopy of the changes that occur in keratoconus. The apparent ectasia in this cohort of patients with MCD differs in several important aspects from the changes that define keratoconus. Thinning and corneal distortion is to be expected if there is dysregulation of keratan sulfate proteoglycan synthesis or catabolism that influences corneal structure.[22]

Summary

What was known before

- Corneal thinning is an important clinical feature of macular corneal dystrophy.
- Corneal astigmatism and corneal thinning are also features of keratoconus, and in this respect the phenotype of the two conditions is similar.

What this study adds

- Localized elevation of the anterior corneal curvature can occur in macular corneal dystrophy in the absence of other features that would define a diagnosis of keratoconus.
- The identification of a further two Czech probands with the compound allele c.[484C > G; 599 T > G] supports the enrichment of this allele in the study population.
- Two novel *CHST6* mutations were also found.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Eye website (http://www.nature.com/eye)

Appendix 5

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SHORT REPORT

Validation of rs2956540:G > C and rs3735520:G > A association with keratoconus in a population of European descent

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Corneal ectasias, among which keratoconus (KC) is the single most common entity, are one of the most frequent reasons for corneal grafting in developed countries and a threatening complication of laser *in situ* keratomileusis. Genome-wide association studies have previously found lysyl oxidase (*LOX)* and hepatocyte growth factor (*HGF*) associated with susceptibility to KC development. The aim of our study was to validate the effects of seven single-nucleotide polymorphisms (SNPs) within *LOX* and *HGF* over KC. Unrelated Czech cases with KC of European descent (108 males and 57 females, 165 cases in total) and 193 population and gender-matched controls were genotyped using Kompetitive Allele Specific PCR assays. Fisher's exact tests were used to assess the strength of associations. Evidence for association was found for both of the tested loci. It was strongest for rs3735520:G > A near *HGF* (allelic test odds ratio (OR) = 1.45; 95% confidence interval (CI), 1.06–1.98; *P* = 0.018) with A allele being a risk factor and rs2956540:G > C (OR = 0.69; 95% CI, 0.50–0.96; *P* = 0.024) within *LOX* with C allele having a protective effect. This first independent association validation of rs2956540:G > C and rs3735520:G > A suggests that these SNPs may serve as genetic risk markers for KC in individuals of European descent.
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INTRODUCTION

Keratoconus (KC) is characterised by progressive corneal thinning and ectasia, leading to significant refractive error and potential scarring.[1] KC prevalence in populations of European descent varies from 5.5 to 8.6 per 10 000 inhabitants[2,3] and is more common in males.[4,5]

Although most KC cases are sporadic, familial aggregations and twin studies suggest a strong genetic component in the aetiopathogenesis of the disease.[6,7]

Single-nucleotide polymorphisms (SNPs) in two genes, lysyl oxidase (*LOX*; OMIM *153455) and hepatocyte growth factor (*HGF*; OMIM *142409), identified by a genome-wide association study (GWAS) have been reported to increase susceptibility to KC.[8,9] In this study we attempted replication for seven SNPs in *LOX* and *HGF* in a Czech case-control cohort.

MATERIALS AND METHODS

The study followed the ethical standards of the Ethics Committee of the General Teaching Hospital in Prague and Charles University in Prague, and adhered to the tenets set out in the Declaration of Helsinki.

A total of 165 unrelated cases with KC were included in the study: 57 females and 108 males, average age at enrolment was 37.2 ± 13.3 years (range 15–69 years). The diagnosis of KC was made in agreement by two cornea specialists. All cases exhibited KC pattern on anterior sagittal maps together with localised corneal thinning in at least one eye.[1] Only patients with KC grade 1 or higher according to the Oculus topographic KC classification based on data obtained by Pentacam (Oculus Optikgeräte GmbH, Wetzlar, Germany) were included.[10] Some eyes had advanced disease with typical signs such as Vogt striae, Fleischer ring and stromal scarring, but this was not an inclusion requirement for the purposes of this study. Bilaterally grafted patients for KC were also considered affected and thus included in the study.

193 unrelated Czech Caucasian individuals (79 females, 114 males average age of 39.5 ± 13.7 years, range 20–81 years) were recruited as controls. They self-reported absence of any ocular conditions impairing vision, other than age-related cataract.

DNA was extracted from venous blood by conventional methods, in some patients Oragene saliva kit was used (Oragene OG-300, DNA Genotek, Ottawa, ON, Canada). SNPs reported to show the strongest associations, although not reaching genome-wide significance, in GWAS[8,9] were tested (Supplementary Table 1). Genotyping was performed using custom designed Kompetitive Allele Specific PCR (KASP) assays at LGC Genomics (LGC Genomics, Hoddesdon, UK). KASP enables bi-allelic discrimination through a competitive PCR harnessing the fluorescence resonance energy transfer principle and it has been shown to have high reliability (98.4–99.3%).[11] For the purpose of this study KASP genotyping assays were designed towards sequences shown in Supplementary Table 1. For internal quality control 10 samples were randomly selected and PCR fragment containing rs1800449: C > T was Sanger sequenced.[12] The concordance rate was 100%.

Fisher's exact tests were used to calculate odds ratios (ORs) and probabilities of association. The primary model was the allelic test, which assumes co-dominance. Secondary analyses of association

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Table 1 Analysed SNPs in genomic areas encompassing *HGF* and *LOX* and results of their association testing with keratoconus in Czech cases of European descendent

			Allelic test				Dominant model			Recessive model		
SNP	*Minor allele*	*MAF cases*	*MAF controls*	*OR*	*95% CI*	P-*value*	*OR*	*95% CI*	P-*value*	*OR*	*95% CI*	P-*value*
rs1800449:C>T	T	0.133	0.176	0.72	0.47–1.10	0.12	0.69	0.43–1.14	0.16	0.58	0.09–2.76	0.52
rs2288393:C>G	G	0.118	0.166	0.67	0.43–1.06	0.087	0.63	0.38–1.05	0.07	0.69	0.11–3.64	0.73
rs2956540:G>C	C	0.318	0.402	0.69	0.50–0.96	**0.024**	0.55	0.35–0.87	**0.0069**	0.78	0.39–1.56	0.52
rs10519694:C>T	T	0.200	0.233	0.82	0.56–1.19	0.32	0.70	0.45–1.10	0.13	1.59	0.47–5.67	0.43
rs1014091:G>A	A	0.152	0.143	1.08	0.69–1.66	0.75	1.08	0.66–1.76	0.81	1.17	0.21–6.41	1
rs17501108:G>T	T	0.149	0.145	1.03	0.66–1.59	0.92	1.04	0.64–1.71	0.90	0.93	0.18–4.42	1
rs3735520:G>A	A	0.458	0.368	1.45	1.06–1.98	**0.018**	1.48	0.94–2.36	0.097	1.86	1.03–3.37	**0.036**

Abbreviations: CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.
The effect sizes are reported with reference to the minor allele. The *P*-values are rounded up to the second non-zero decimal and are in bold wherever significant (<0.05). Alleles are shown aligned to the forward strand.

under assumptions of dominant and recessive models were also carried out for each SNP. All statistical analyses were done using STATA 12 (STATA Corp., College Station, TX, USA). An $\alpha<0.05$ was chosen as the statistical significance threshold for validation, because the analysed variants were pre-selected on the basis of strong prior probabilities of association. Methodology used as well as results of our study were submitted to the GWAS Central database (http://www.gwascentral.org/study/HGVST1826).

RESULTS

The genotype call rate for each SNP was >99.3%. All SNPs were in Hardy–Weinberg equilibrium ($P>0.05$) in both the controls and in the combined case-control panel. Allele frequencies in the control group were largely in line with those reported in panels of other European ethnic groups by the HapMap project (http://hapmap.ncbi.nlm.nih.gov/; Table 1).

Two out of the seven SNPs analysed showed significant association with KC in a co-dominant allelic test (Table 1). The rs2956540:G>C, located within in the fourth intron of the *LOX* gene (OR=0.69; 95% CI, 0.50–0.96 for allele C; $P=0.024$) and rs3735520:G>A located in genomic region upstream of the *HGF* transcription initiation site (OR=1.45; 95% CI, 1.06–1.98 for allele A; $P=0.018$). Explorations of alternative models of inheritance changed little the association significance for both loci, although genotypes homozygous for alleles increasing susceptibility to KC were associated with higher ORs than under the allelic (co-dominant) model, suggesting (although not proving) a possible recessive effect in both SNPs.

DISCUSSION

Our study provides the first independent validation of rs2956540:G>C (minor allele serving as a protective factor) and rs3735520:G>A (minor allele serving as a risk factor) associations with KC in a population of European descent, further confirming that *LOX* and *HGF* genes have a role in the aetiology of the disease.[8,9]

Details on previous association studies (population origin, genotyping methods used, number of KC and control samples and reported *P*-values) on the *LOX* and *HGF* locus are shown in Supplementary Table 2.

Recently, association of the *HGF* locus was also achieved in an independent study comprising population of European descent from Australia.[13] However, alternative SNPs to those shown statistically significant associations with KC in GWAS were tested.[8,13]

LOX enzyme catalyses the final enzymatic reaction required for cross-linking of two basic components of extracellular matrix, collagens and elastin. Although the underlying mechanism of common variants contributing to the disease development remains unknown, the potential effect could lie in affecting the biologic activity of *LOX* via tissue specific alternative splicing or regulation of expression.[14] Mechanisms of how common variants within the *HGF* gene alter susceptibility to KC are yet to be determined, but involvement of inflammatory pathways has been previously suggested.[8]

The main statistics used here (allelic test) implicitly assume co-dominance, and is similar to the additive models assumed in the previous reports.[8,9,13,15] Although some caution is invited in the interpretation of our findings because of the relatively small sample size, the validation of rs2956540:G>C and rs3735520:G>A showing the same effect directions as previous studies in populations of European ancestry adds weight to the existing evidence.[8,9] Although not reaching a statistically significant threshold, the higher MAF of rs1800449:C>T and rs10519694:C>T in controls compared with the KC cases in our study, was consistent with protective effects of these alleles reported in another study using Caucasian case-control panels (Supplementary Table 2).[9]

rs1014091:G>A and rs17501108:G>T were previously also shown to have a protective effect,[8] whereas in our study their MAF was higher in KC cases that, albeit not statistically significant, indicated a tendency towards the opposite direction of the effect. The failure to replicate the effect direction may be caused by differences in linkage disequilibrium patterns (Supplementary Table 3) between these markers and the causative variants within the same gene in the Czech population.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appendix 6

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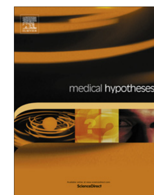

Is copper imbalance an environmental factor influencing keratoconus development? ☆

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ABSTRACT

Keratoconus is a bilateral disease characterized by progressive corneal thinning leading to irregular astigmatism that results in significant visual impairment. Despite extensive research, the exact etiopathogenesis of keratoconus remains unknown.

Many copper-dependent enzymes such as superoxide dismutases, cytochrome c oxidase and lysyl oxidase have been shown to be altered in keratoconic corneas, and a decrease of copper levels in the diseased tissue has been reported as well.

We propose a hypothesis linking all the putative pathways of keratoconus development and suggest that copper imbalance in corneal tissue may be an independent risk factor for the disease. The assessment of copper levels and its distribution in keratoconic corneas warrants further investigation.

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Introduction

Keratoconus (KC) is characterized by progressive corneal thinning and ectasia, typically affecting young people in their second or third decade of life [1]. This disease is one of the leading causes of corneal transplantation in developed countries with a prevalence in populations of European descent ranging between 5.5 and 8.6 per 100,000 [2,3]. However, despite extensive research the exact etiopathogenesis of KC remains unknown [4]. Although most KC cases are sporadic, genetic factors play an important role. Several genetic susceptibility loci have been identified, but no gene has been unequivocally proven to be disease causing, suggesting a multifactorial etiology with the interaction of genetic and environmental factors [4–6].

Oxidative stress [7], keratocyte apoptosis [8,9], activation of proteinases resulting in the degradation of the extracellular matrix [10,11], accumulation of mtDNA mutations [12], abnormalities in lysosomal enzymes [13], changes in the expression of transcription factors [14], increased expression of inflammatory molecules [15,16] and iron (Fe) deposition [17] have all been shown to be associated with the disease.

Copper (Cu) is an essential element necessary for the proper function of more than 30 proteins [18]. After its active transport into the cell, Cu is bound by chaperones delivering it to the mitochondria (cytochrome c oxidase copper chaperone; COX17) [19], to the trans-Golgi network (antioxidant 1 copper chaperone; ATOX1) [20], and to the cytosol (copper chaperone for superoxide dismutase; CCS) [21]. Apart from being a chaperone, ATOX1 is also a Cu-activated transcription factor involved in cell cycle control [22,23].

Cu serves as a cofactor for enzymes that generate cellular energy such as cytochrome c oxidase (COX) and sulfhydryl oxidase, detoxify oxygen-derived radicals such as superoxide dismutases (SODs) (intracellular encoded by *SOD1* and extracellular by *SOD3*), mobilize iron (Fe) such as ceruloplasmin and hephaestin, influence the expression of various proteins through the modulation of their transcription factors such as activator protein 1 (AP-1) and specificity protein 1 (SP1), and cross-link connective tissue such as lysyl oxidase (LOX) and LOX-like enzymes [24–26].

The hypothesis

The pathogenesis of KC remains unclear, but current theories are based on alterations in the organization and structure of collagen fibrils [27], oxidative stress [28,29] or an increase of degradation enzymes [11]. These hypotheses concern individual processes which are not linked.

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Alterations of many Cu-dependent enzymes have been connected with KC [12,30,31], and the decrease of Cu levels in KC corneas has been reported [32]. Nonetheless, a systemic insight into the role of Cu in the pathogenesis of corneal ectasias has not been provided. In the following text we will focus on the current hypothetical pathways of KC development in the context of Cu imbalance and connect them into one common pathway.

Evaluation of the hypothesis

Corneal thinning

It has been shown that LOX catalyzes the final enzymatic step required for collagen and elastin cross-linking in the extracellular matrix (ECM) [33,34]. LOX has a high avidity for Cu; only 1 mol of tightly bound Cu/mol enzyme is a prerequisite for its functional activity; however, it also loosely binds a further 5–9 Cu/mol enzyme [35]. A relation between LOX and Cu levels *in vivo* has been previously demonstrated in the skin of healthy men, who exhibited a decrease of its activity of 24% following a dietary-induced decline of Cu plasma levels of 9% [36]. Due to its function, *LOX* has been regarded as a candidate gene for KC [37], and although a suggestive linkage to the 5q23.2 region where *LOX* is located has been found [38,39], no sequence variants that could be regarded as clearly pathogenic have been identified [40,41].

The failure to detect deleterious *LOX* mutations in the genomic DNA of KC patients, the observation of the elevated expression of LOX in the epithelial cells of KC corneas [37], changes in LOX distribution (the formation of a clump-like pattern) in KC corneas and lower LOX activity in the medium of cultured KC fibroblasts [31] lead us to the hypothesis that lower Cu levels in KC corneas may lead to insufficient collagen cross-linking followed by a loss of cohesion between collagen fibrils, thus promoting corneal ectasia by lamellae slippage and a reduction of their number in the area of thinning [27,42,43].

It has been suggested that LOX may also function as a transporting enzyme for Cu from cells [44], therefore decreased LOX activity may also lead to impaired Cu trafficking in the ECM. Consequently, Cu impairment could affect the biosynthesis and activity of other Cu-dependent enzymes. The biosynthesis of LOX under physiological conditions and hypothetical changes occurring under lower Cu concentrations are shown in Fig. 1.

Oxidative stress

It is widely accepted that oxidative stress plays a critical role in the development and progression of KC [7,28]. The impairment of antioxidant enzymes induces the accumulation of cytotoxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) [45] and leads to the activation of proteinases such as matrix metalloproteinases (MMP1, −3, −7 and −9) and other degradative enzymes (for example, cathepsin B and cathepsin G), a decrease of tissue inhibitors of MMPs (TIMPs) [46] as well as the accumulation of mitochondrial DNA (mtDNA) mutations. All of these processes have been found in KC corneas [12]. The consequences of this cascade in KC corneas include ECM degradation, keratocyte apoptosis [8,9] and an increase in the amount of lysosomal enzymes and proteinases [10,11,13,47].

Cu is an integral part of important antioxidant enzymes (e.g., COX, ATOX1, SOD1 and SOD3), and its deficiency increases cellular susceptibility to oxidative damage [48].

A mitochondrial COX is the final electron acceptor in the mitochondrial electron transport chain. Cu is not only required for COX catalytic function, but also for its biogenesis, assembly and stability [49,50]. The higher COX mRNA levels found in KC corneas [28] and decreased levels of COX protein in the areas of corneal thinning

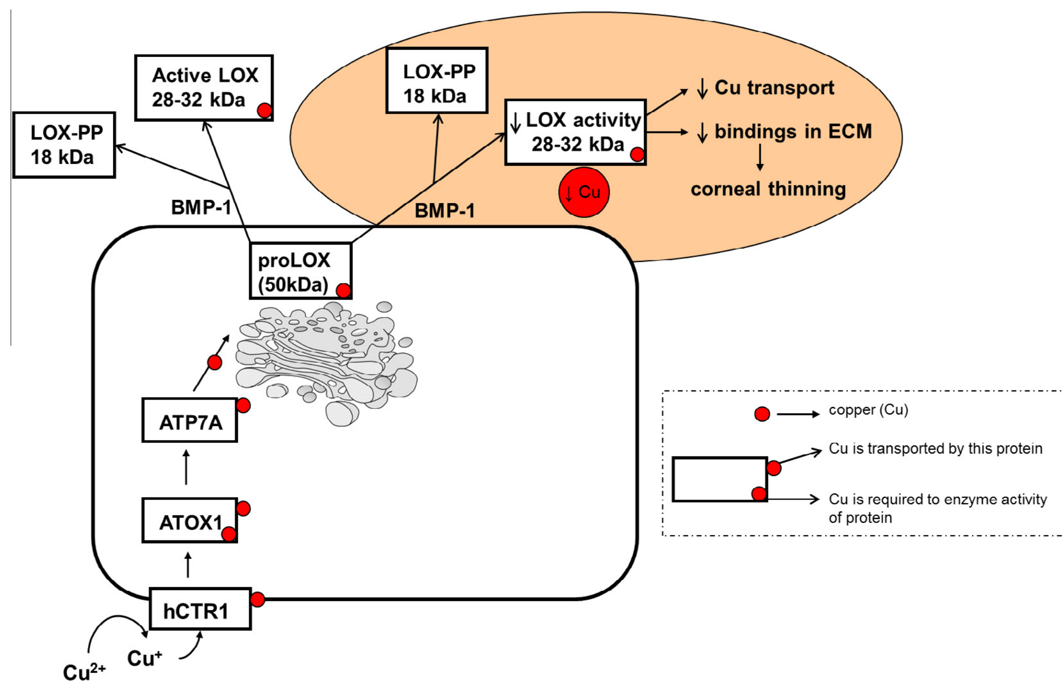

Fig. 1. LOX biosynthesis and activity under normal and low Cu concentrations. Cu is transported into the cell in a reduced form (Cu^{+}) via human copper transporter (hCTR1). Intracellularly it binds to antioxidant 1 copper chaperone (ATOX1), delivering Cu via its interaction with the Cu^{2+} transporting alpha polypeptide (ATP7A) to the Golgi apparatus where it is incorporated into secretory cuproenzymes. Lysyl oxidase (LOX) is secreted as a proenzyme (proLOX) and transported into the extracellular space. Outside the cell, proLOX is cleaved by bone morphogenic protein 1 (BMP-1) into 28–32 kDa catalytically mature LOX and an 18 kDa propeptide (LOX-PP). We propose that under conditions of Cu deficiency (in the orange ellipse), lower LOX activity may lead to decreased Cu trafficking into the extracellular matrix, impaired LOX activity and a decrease of collagen and elastin links in the extracellular matrix, potentially leading to corneal thinning. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

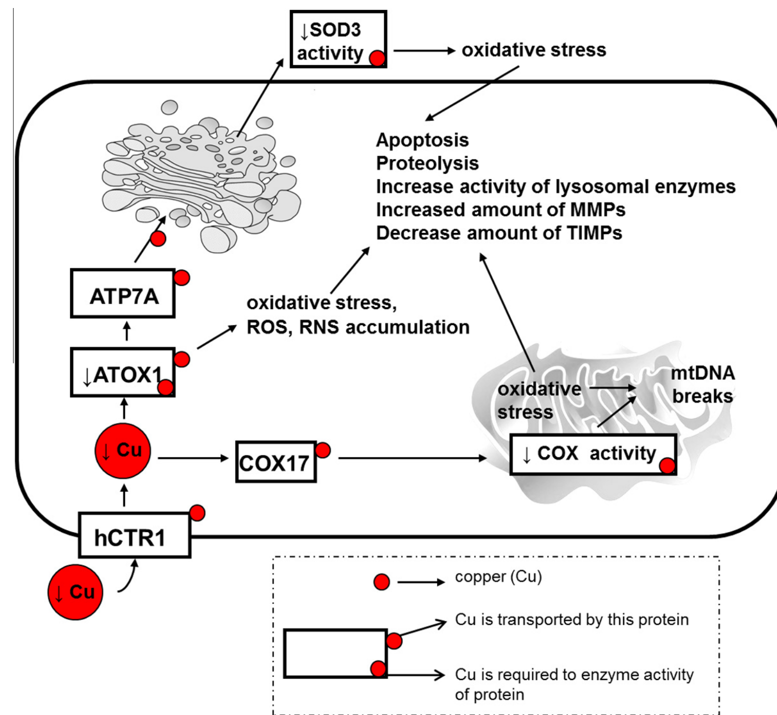

Fig. 2. Influence of Cu impairment on antioxidant enzymes and the consequences of their impairment in the keratoconic cornea. A decrease of Cu may lead to the reduced activity of extracellular SOD3. ATOX1 functions as an antioxidant enzyme sensitive to Cu levels. Oxidative stress in mitochondria may be induced by the lower activity of COX (Cu incorporated by cytochrome c oxidase copper chaperone; COX17). The alteration of antioxidant enzymes in KC corneas may lead to the accumulations of cytotoxic ROS and RNS, the accumulation of mitochondrial DNA (mtDNA) mutations, apoptosis, an increased amount of lysosomal enzymes and proteolysis.

[12] may indicate a lack of available Cu for proper COX biogenesis and activity.

Intracellular SOD1 and extracellular SOD3, physiologically found in a 1:1 ratio, are two of the most important antioxidative enzymes protecting the cornea against oxidative damage [51,52]. A decrease of 50% in the activity of SOD3 in the corneal centre compared to the periphery has been detected in KC corneas, while no difference in the activity of SOD1 was found [53,54] and no deleterious changes have been identified in the *SOD1* coding sequence in patients with KC [30,41,55–58].

LOX differs from other cuproenzymes by being secreted; therefore it may be located in specific cell compartments that are largely inaccessible for Cu transporting enzymes [59] (as well as extracellular SOD3 and COX located in mitochondria). In contrast, cuproenzymes located in the cytoplasm, such as SOD1, may have better access to Cu.

We hypothesize that inadequate Cu availability may lead to the decreased activity of many antioxidative enzymes in KC corneas (SOD3, ATOX1 and COX). The decreased activity of aldehyde dehydrogenase 3 (ALDH3) found in KC corneas [54], an enzyme that detoxifies aldehydes produced by UV-induced lipid peroxidation, may be also related to changes in Cu availability as its expression is controlled by the Cu-inducible transcription factors AP-1 and SP1 [60].

The possible influence of Cu deficiency on antioxidant enzymes and the consequences of oxidative stress in KC corneas are shown in Fig. 2.

The alterations of transcription factors

Transcriptome analysis of KC corneas revealed that 69 of 87 genes with reduced expression compared to control tissue are particularly regulated by a Cu-inducible transcriptional factor known as AP-1 [14,61].

Another transcription factor influenced by Cu metabolism, ATOX1, is proposed to bind to DNA repeats after Cu-dependent homodimerization [23,62,63]. ATOX1 is known to regulate visual system homeobox 1 (*VSX1*); found to be enriched by rare coding variants in some KC patient populations [64–67], ATOX1 also serves as a positive regulator for the Cu-induced increase of *SOD3* transcription [22].

Another transcription factor suggested to be involved in KC pathogenesis is zinc finger E-box binding homeobox 1 (ZEB1) [68]. ZEB1 plays a key role in the epithelial-mesenchymal transition; however, it also has many other functions including the regulation of proteins involved in alterations of the extracellular matrix. In particular, ZEB1 has been found to repress the expression of E-cadherin, *COL4A2* and other genes of basement membrane-forming proteins [69]. Under oxidative stress, upregulation of miR-200c leads to *ZEB1* inhibition and ROS-induced apoptosis [70].

A study on zebrafish has shown that the expression of *SOD1* and *SP1* (transcriptional regulator of SOD1) are modulated by ATP7A in response to intracellular Cu status [71]. Studies on mammalian cells have further demonstrated that SP1 downregulates α1-proteinase inhibitor (α_1-*PI*) expression [72] and induces the expression of human copper transporter (*hCTR1*) [73]. Upregulation of SP1 as well as the reduced expression of α_1-*PI* to one-fourth of normal levels in KC corneas have been documented [74–76].

We hypothesize that an imbalance between proteinases and their inhibitors together with the increased expression of degradative enzymes, both previously shown to be involved in KC pathogenesis [10,77,78], may be influenced not only by oxidative stress (described above), but also by changes in transcription factor activities related to a Cu tissue imbalance (Fig. 3).

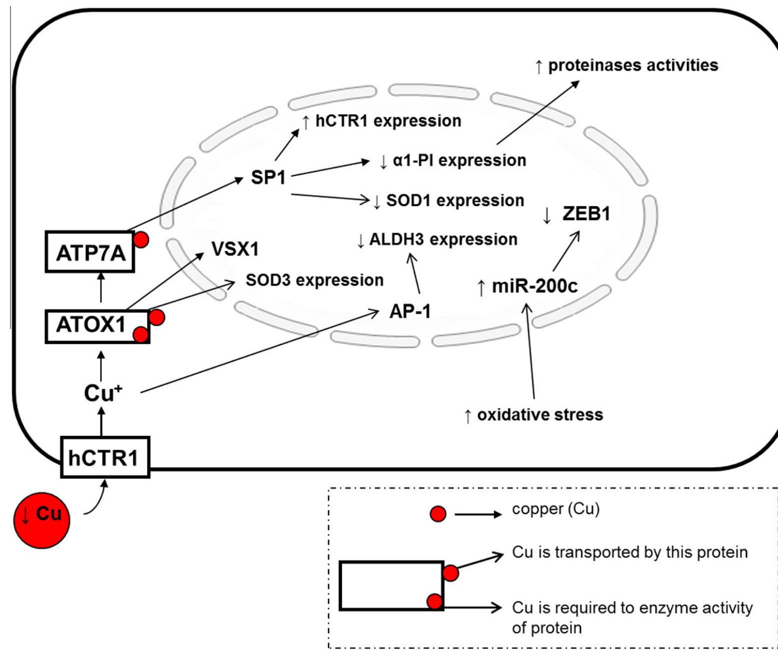

Fig. 3. Alterations of transcription factors in Cu deprived cells. A low level of Cu may lead via ATOX1 to the downregulation of *VSX1* and *SOD3*. Similarly, ATPA7 may increase the expression of *hCTR1* and downregulate α_1-*PI* expression and *SOD1* via SP1 with a consequent increase of proteinases activities. AP-1 is a Cu-inducible transcription factor controlling *ALDH3* expression. Under oxidative stress, an increased production of miR-200 was found, leading to ZEB1 inhibition.

Iron and copper deposition

The linkage between Cu and Fe metabolism is well documented [18]. Fe transport into and out of the cell is Cu-dependent [79]. One of the main histopathological features of KC is Fe deposition in the epithelial cells and basement membrane at the base of the conus called the Fleischer ring [80] (Fig. 4A), which is adjacent to the area of thinning and bowing of the KC cornea (Fig. 4B). The interplay between Fe and Cu homeostasis in healthy and diseased corneal tissue remains largely unknown as well as the reason for Fe deposition in KC [81].

Interestingly, recent measurements using X-ray structural analysis on two control and two KC corneas showed that the Fleisher ring is composed preferentially by Cu compared to Fe [32]. In addition, Cu concentration in the stroma under the Fleisher ring is 500–600 times higher compared to that in control tissue. Inside the area delineated by the ring, the Cu concentration is under detection limits both in the epithelial basement membrane and the stroma. At the same time, there is no difference in Cu concentration between the central and peripheral parts of control corneas. Avetisov et al. hypothesized that Fe blocks Cu trafficking, leading to a localized Cu deficiency centripetally from the Fleischer ring [32].

As Fe trafficking in and outside the cell is dependent on Cu transporting enzymes [82], it is unknown whether Cu deficiency in the area of the actual KC cone and its abundance in the Fleischer ring is a consequence of Fe deposition or if it is the reason for Fe deposition. The impairment of Fe in KC corneas can also

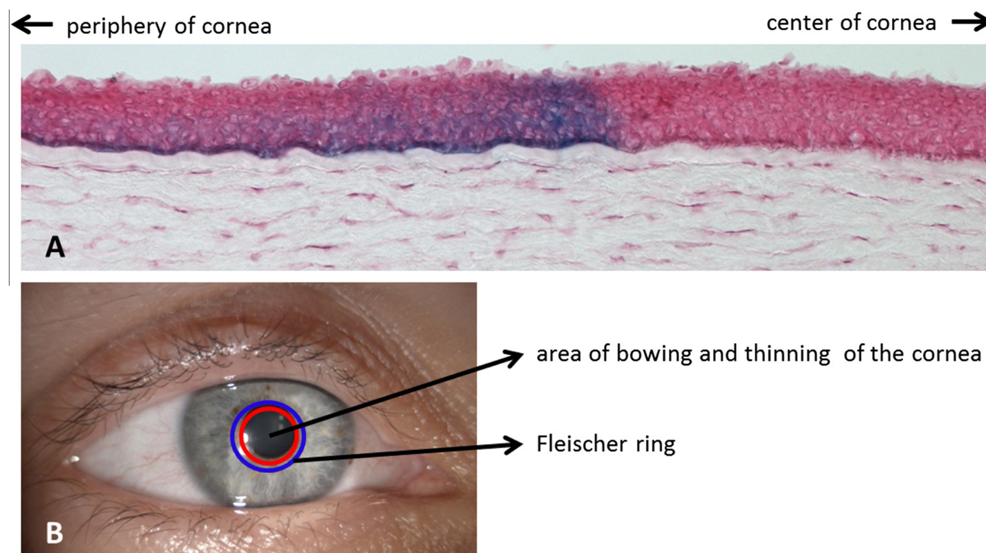

Fig. 4. Fleischer ring. Prussian blue staining of a keratoconic cornea to detect iron deposition (blue color) (A) and a schematic drawing of Fleischer ring localization (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contribute to disease pathogenesis as it has been shown that Fe deposition leads to an increase of oxidative stress and free radical production [81]. Fe deficiency results in a decrease of Fe-requiring proteins involved in collagen synthesis; for example, levels of lysyl hydroxylase, which affects the diameter of collagen fibers in the cornea, have been reported to be lower in patients with KC compared to controls [83].

Immune response

Increased levels of proinflammatory molecules in KC corneas [15,16], strong associations between KC and several autoimmune and allergic disorders point to the involvement of the immune system in the pathogenesis of this corneal disease [84].

Cu is known to have an immunomodulatory effect, but the exact mechanism of this action is not known [85,86]. The importance of Cu for the immune system can be observed in children with Menkes disease (*OMIM.* 309400; a disease associated with Cu malabsorption), who usually die due to infections [87].

Consequences of the hypothesis and discussion

We hypothesize that Cu deficiency in KC corneal tissue is reflected not only by lower LOX activity, but also by the disruption of the metabolic pathways of other Cu-dependent proteins. For a better understanding of our hypothesis, we have summarized the current concepts of all the affected metabolic pathways in Fig. 5. Partial steps in this model are working hypotheses, which will hopefully be verified in the future.

Our hypothesis suggests that Cu availability contributes to the development of KC by influencing the activity, biogenesis and stability of cuproproteins sensitive to Cu levels. In the absence of or low concentrations of Cu, these enzymes are prone to aggregation, misfolding and/or degradation by the proteasome pathway [88].

To prove our hypothesis on a histochemical level will be difficult. Although there are multiple methods available for tissue Cu histochemistry (staining with orcein, rhodanine, rubeanic acid or Timm's sulfide-silver staining method [89]), in clinical practice the occurrence of negative staining in samples from patients affected by Wilson's disease [90] is very frequent, even in cases in which high Cu levels have been demonstrated by atomic absorption spectroscopy [91]. The reason for the failure of the histochemical demonstration of Cu remains unknown in a large number of cases [89].

Serum or plasma Cu and ceruloplasmin concentrations are the most widely used laboratory indicators to evaluate Cu status as these indicators are tightly controlled: they are only decreased in the case of a moderate or severe Cu deficiency. Changes in both indicators can be related to age, sex, and pregnancy and are increased by other conditions not related to Cu status (inflammatory or infectious processes, neoplasm and estrogen therapy) [92], so their reliability is compromised. The activity of several cuproenzymes is decreased in cases of mild Cu deficiency. However, their utility is limited by the lack of standardized assays and high interindividual variability and because some of these indicators are affected by other conditions as well [93].

While the total absence of Cu would lead to the complete inactivity of Cu-dependent enzymes such as in Menkes disease, the precise effects of a mild Cu deficiency are very difficult to predict. No distinct symptoms that could be clearly attributed to Cu deficiency in populations with Cu consumption below the recommended dietary allowance have been identified [94,95].

Current evidence suggests that the development of KC depends in the great majority of patients on the interplay between genetic and environmental factors. Cu deficiency may be an unrecognized

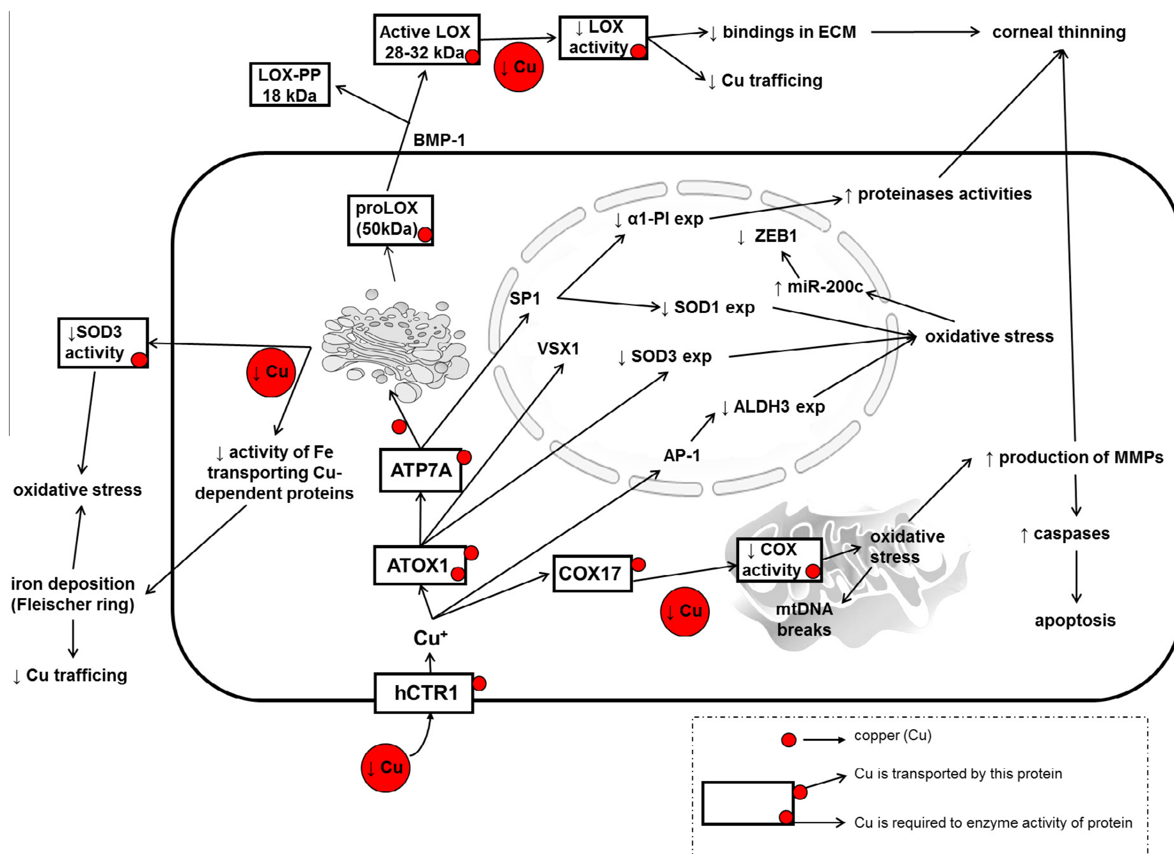

Fig. 5. Schematic representation of our current understanding of the pathophysiology of keratoconus in respect to Cu imbalance. *Abbreviation* – exp – expression.

factor increasing susceptibility to the disease. The higher prevalence of KC in males could be explained by lower plasma levels of Cu compared to females [96]. However, as in some families where KC follows a Mendelian mode of inheritance, other mechanisms independent of Cu involvement are likely to also be involved. Similarly to other disorders associated with a deficiency of trace elements, an individual's genetic makeup may increase his or her susceptibility towards disease development [97].

Our hypothesis that Cu deficiency may act as an independent environmental factor in KC development is supported by X-ray structural analysis performed by Avetisov et al. [32]. They explained the diminished Cu levels in KC corneas by the higher pH of KC patients' tears, leading to the oxidization of dichlorocuprate (Cu^{+}) into cupric oxide (Cu^{2+}), which cannot be utilized by cells. They also suggested that this may be associated with a decrease of LOX activity [32].

As the cornea is composed mainly of collagen fibrils, which form many tissues, and since current cancer research is focused on Cu chelation as a new anti-cancer therapy [98] with expected outcomes (to increase oxidative stress in cancer cells, induce apoptosis, activate proteinases, decrease LOX activity, etc.) that evoke signs described in relation to KC [99], studying the metabolic pathways involved in KC is highly relevant in a broad context.

Our hypothesis connects for the first time all the known phenomena involved in the pathogenesis of KC while not excluding the influence of genetic factors.

Conflict of interest statement

None.

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