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The role and function of stromal enzymes in keratoconus pathogenesis

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ABSTRAKT

Keratokonus (KC) je multifaktoriální nezánětlivé onemocnění, charakteristické ztenčováním a vyklenováním rohovky pravděpodobně v důsledku poruch ve vazbách kolagenních vláken. Je jednou z nejčastějších indikací k transplantaci rohovky. Příčina onemocnění ani přesný 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 na onemocnění asociované s výskytem KC a pokusili se najít jejich společné znaky. Replikovali jsme studii s cílem prokázat asociaci jednonukleotidových záměn (SNPs) v genech pro LOX a hepatocytární růstový faktor (*HGF*) s výskytem KC. Pokusili jsme se najít společnou příčinu dříve popsaných projevů KC. Byly použity metody buněčné a molekulární biologie (tkáňové kultury, imunohisto- a imunocytochemie, mikroskopie, měření aktivity enzymů pomocí fluorometrie, genotypování, přímé sekvenování, Western Blot) a statistické analýzy.

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 (MVP), co naznačuje podobný mechanizmus vzniku obou onemocnění. Popsali jsme rozdíly mezi KC a makulární dystrofii rohovky (MCD), i když v minulosti byla popsána asociace těchto dvou onemocnění. Prokázali jsme též asociaci rs2956540-C v *LOX* s protektivním účinkem a rs3735520-A v *HGF* jako rizikový faktor pro rozvoj KC. Publikovali jsme teorii o vlivu mědi na rozvoj KC, v které jsme spojili všechny známé znaky onemocnění do jedné společné dráhy. Přesný způsob jakými se asociované SNPs a deficience mědi podílejí na vzniku KC, zůstávají nejasné.

Potvrdili jsme předpoklad, že u KC dochází k poruchám enzymů tvořících vazby mezi kolageny a podobný mechanismus se pravděpodobně uplatňuje i při vzniku MVP. U našich pacientů jsme vyloučili asociaci MCD a KC.

Klíčová slova: lysyl oxidáza, keratokonus, rohovka, aktivita enzymů, imunohistochemie, 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 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 wide spectrum of methods (cell culturing, immunohisto- and immunocytochemistry, microscopy, fluorimetric enzyme activity measurement, genotyping and direct sequencing, Western blot, statistical analysis).

We demonstrated the presence of the entire family of LOX enzymes in control and in KC corneas, with decreases 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 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* as a risk factor for the development of KC. A theory about the 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 such 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, enzyme activity, immunohistochemistry, association study, 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.

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).

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). 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 1). 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 1):

- 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.

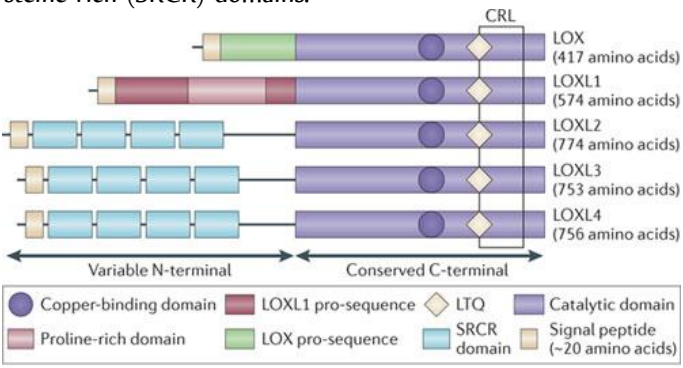

Figure 1: Structure of LOX and LOXL enzymes (Barker *et al.*, 2012)
SRCR - scavenger receptor cysteine-rich domain; **CRL** - cytokine receptor-like domain; **LTQ** - lysyl-tyrosyl quinone residue

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).

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 1). 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).

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). 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).

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. 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).

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; 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).

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 (Nowak and Gajecka, 2011).

Although a number of genetic loci have been implicated from linkage studies in extended families, 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).

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. 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 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.

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. 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.

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.

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.

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, while a slight increase of LOXL3 was observed in KC corneas.

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 2.

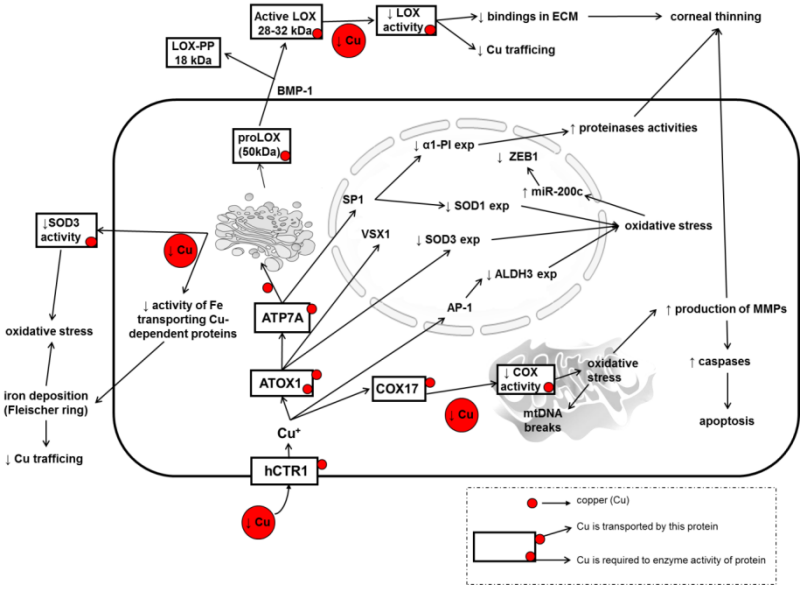

Figure 2: Schematic representation of our current understanding of the pathophysiology of KC in respect to Cu imbalance (Dudakova *et al.*, 2015c)

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 (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 between these markers and the causative variants within the same gene in the Czech population.

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**