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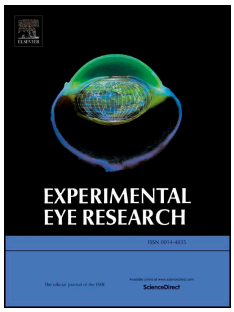

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Matrix metalloproteinases in keratoconus – too much of a good thing?

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Abstract

Keratoconus (KC) is a progressive, early onset, and often bilateral eye condition, in which the cornea gradually weakens and bulges out, and in advanced cases may eventually become cone shaped. The available evidence suggests that it is a multifactorial disease with environmental and genetic contributions. Matrix Metalloproteinases (MMPs) are a family of 24 zinc-dependent proteases with the ability to degrade collagen and other extracellular matrix (ECM) proteins, which are important components of the cornea. During the past two decades a growing body of evidence has accumulated suggesting a link between MMPs and keratoconus. This article aims to summarize the published literature on the role of MMPs in the pathogenesis of KC. MMP-driven ECM remodelling is thought to be a necessary step for cornea healing, but a fine balance in the expression of MMPs is essential in maintaining the integrity and transparency of the cornea and for its correct healing, and an imbalance in this tightly regulated process may, in the long term, result in the progressive weakening of the cornea. There is extensive evidence that MMPs are upregulated in the corneal tissue and tears of KC patients, implicating dysregulated proteolysis in KC, with an increase in the level of some MMPs, particularly MMP-1 and MMP-9, confirmed in multiple independent studies. There is also evidence for a causative link between inflammation, which could result from the mechanical trauma due to contact lens wearing or/and eye rubbing, and the increased MMP production observed in KC. However, the precise role of specific MMPs in the cornea is still unclear and the mechanisms causing their upregulation are mostly undiscovered. Further studies are required to verify the functional role of specific MMPs in KC development and assess the genetic association between common MMP variants and risk of KC. As MMP inhibitors are in development, this information could potentially drive the discovery of new treatments for KC.

1. Introduction

Keratoconus (KC; from the Greek words kerato meaning cornea and conus meaning cone) is an early onset, progressive and often bilateral eye condition in which the cornea, the clear dome-shaped external layer of the eye, thins and weakens, gradually bulging out and eventually becoming cone-shaped in advanced cases [1,2]. As the curvature of the cornea has a key role in determining the optical power of the eye, the disease results in blurred vision due to irregular astigmatism and sometimes monocular diplopia and excessive sensitivity to light [1]. Mild cases can be corrected with rigid gas-permeable contact lenses and progression can be slowed with collagen crosslinking (CXL) [3]. However, some patients eventually require a corneal transplant [4] and KC is still one of the most common indications for keratoplasty worldwide [5-7], although the frequency of corneal transplantations for KC patients is declining in some countries [8]. As the average age at onset of the disease is in early adulthood [9], KC has a long term impact on the quality of life of patients. KC is relatively common, but there is great variation between different countries and ethnicities [10]. Prevalence is approximately 1:2000 in the US population [11], but a frequency as high as 1:25 has been reported in Middle Eastern populations [10]. In the UK, the disease is 4- to 7-fold more common in south Asians than in Caucasians living in the same geographical area [12,13]. The observed variation in prevalence between different ethnicities, combined with evidence that up to one fifth of KC cases are familial, with multiple generations and members of the same family affected [1,14,15], suggests a genetic aetiology for the condition in at least a subset of patients. In an attempt to unravel the genetic causes of KC, a number of candidate-gene, linkage and genome-wide association (GWAS) studies have been performed [16-18]. Several genetic variants have been identified [15,17], but together these account only for a small increment in disease risk, and explain only a proportion of instances of familial clustering. A number of environmental factors have also been associated with KC risk, including contact lens wearing [2,19,20], eye rubbing [21,22], hay fever and other forms of atopy [23,24]. Exposure to UV has also been hypothesized to contribute to the disease [25], and could contribute to the higher prevalence of KC in South Asian and Middle Eastern populations [10]. Overall, these findings suggest that KC is likely to be a complex disease with a multifactorial aetiology.

Matrix Metalloproteinases (MMPs) are a family of 24 zinc-dependent proteases, involved in physiological processes such as tissue remodeling, embryonic development, wound healing, angiogenesis and inflammation [26]. Their function is to break down extracellular matrix (ECM) components, thereby modifying tissue architecture, creating spaces for cell migration, and

influencing cell adhesion [26,27]. They also regulate cell signaling by releasing ECM-bound growth factors, cleaving signaling molecules or modifying interaction between ECM molecules and their receptors on the cell surface [26,27]. Each MMP has specificity for different and often overlapping substrates [28]. Historically they have tended to be divided into four general groups, collagenases, gelatinases, stromelysins and membrane-type metalloproteinases, though these groupings are increasingly thought to be overly simplistic [27]. MMP activity is carefully regulated with both transcriptional and post-transcriptional mechanisms [28]. Firstly, their expression is generally limited in healthy tissue and transcriptionally upregulated only in response to specific cues [28]. Secondly, the enzymes are transcribed as inactive zymogens (pro-MMPs) which require post-translational conversion to their catalytically active form [28]. Finally, the activity of mature MMPs is kept in check by four inhibitory proteins, called Tissue Inhibitors of Metalloproteinases (TIMPs) [28]. Disruption of these tight regulatory mechanisms results in over-activation of MMPs and pathological ECM degradation, which have been linked to a number of diseases[29] including arthritis, cardiovascular disease, periodontal disease, neurodegenerative conditions and cancer. This article reviews the evidence that an imbalance in the activity of MMPs in the cornea may contribute to the development of keratoconus.

2. ECM and MMP alterations in keratoconus

The cornea is composed of five layers (Figure 1): the outer squamous non-keratinized epithelium including the basement membrane, the acellular Bowman's layer, the stroma, the Descemet's membrane, and the endothelium [1,30]. The stroma normally accounts for around 80-90% of corneal thickness. Its main constituents are highly ordered and organized collagenous lamellae and specialized cells called keratocytes, which secrete the stromal extracellular matrix (ECM) components. The correct composition and strict organization of stromal ECM is essential for corneal refractivity, mechanical strength and stability, including the ability to maintain shape and curvature [31]. Besides collagens, ECM components of the cornea include laminins, fibronectins, thrombospondins, proteoglycans and matrilins [30,32].

In KC, a progressive localized thinning of the stromal compartment is observed, often with concomitant variable thinning of the epithelium [1]. Examination of KC corneas reveals a reduction in the density of stromal keratocytes and in the thickness of the collagenous lamellae, along with changes in their orientation relative to the Bowman's layer [1]. In addition, altered levels of ECM components have been observed. For example, a reduction in expression of collagen types I, III, V

and XII proteins has been found in KC[33]. Changes in ECM structure and composition cause a localized thinning and weakening of the cornea, eventually leading to the protrusion and change in shape observed in the disease [1]. Collagen crosslinking by treatment with riboflavin and ultraviolet A light can slow down KC progression by improving corneal biomechanical stiffness [3].

As proteolytic enzymes can degrade ECM, their potential contribution to KC pathogenesis has been examined in several investigations, discussed in detail in the following paragraphs, using three main approaches: biochemical analysis of conditioned media from keratocyte cultures, immunohistochemical analysis of corneal tissue, and proteomics analysis of tear fluids. Most recent studies have focussed on overall MMP protein level, without differentiating between the active forms and the inactive zymogens. However, earlier biochemical studies have looked at enzymatic activity, showing a general increase of gelatinolysis and collagenolysis [34,35] in KC, accompanied by raised levels of the active forms of some MMPs [36]. This is consistent with the increase in collagen degradation products measured in the tears of KC patients [37], pointing to an overall imbalance of proteolysis in KC. Nevertheless, as detailed below, studies on specific MMPs have shown contrasting results, possibly due to the different methodologies used and the small sample sizes in some of the investigations.

MMP-9 (also known as gelatinase B) is one of the most investigated MMPs in relation to KC. Studies of tear composition have shown that levels of MMP-9 protein are raised in KC, with up to 10-fold upregulation in patients compared to controls [38-40]. One study also reported correlation of MMP-9 protein level with severity of the disease [38]. Interestingly, in a patient with asymmetrical KC affecting only one eye, MMP-9 was upregulated only in tears from the affected eye [39]. In another study, elevated tear levels of MMP-9 protein were detected in 90% of eyes with confirmed KC but also in 83% of eyes with subclinical disease, suggesting that MMP-9 in tear fluid may be a useful pre-symptomatic diagnostic marker in at-risk individuals [41]. Pahuja *et al.* found that MMP-9 mRNA levels in KC patients were significantly higher in cells from the cone apex than in those from the corneal periphery, which may be contributing to a focal structural weakness of the cornea [42]. Shetty and colleagues confirmed that the upregulation of MMP-9 in tears of KC patients is accompanied by a significant upregulation of MMP-9 mRNA in their corneal epithelium [40]. In another study, an increase in MMP-9 protein level was also detected in the blood of KC patients compared with controls [43]. Higher levels of MMP-9 were found in tears of KC patients with allergies compared with KC patients without allergies, with the disease significantly progressing only in the first group of patients at a 12 month follow up [44]. However, other studies found no significant difference in the

level of MMP-9 in the tears of patients with sub-clinical [39] or diagnosed [35,45] KC compared to controls, or in KC corneas compared with normal corneal tissue [46,47].

MMP-2 (also known as gelatinase A) has also been intensively studied in KC, but with more conflicting results. Experiments using keratocytes in culture have shown an increase in MMP-2 activity in keratoconic compared with control corneal keratocytes in one study [48] but not in another [34]. Upregulation of the MMP-2 activator, MMP-14 (also called MT1-MMP), but not MMP-2 itself, was also reported in KC corneas compared with normal corneas in one study [49]. However, another investigation on corneal tissue detected no upregulation of MMP-2 and MMP-14 proteins in KC corneas [50]. Two studies comparing tears of KC patients and controls found no significant change in the level of MMP-2 [35,45], consistent with results from other groups showing no difference in protein expression between KC and normal corneal tissue [46,47,51]. However, Ortak and colleagues [52] found that plasma MMP-2 levels were lower in KC patients than controls.

Several studies suggest an increase in expression of MMP-1 in KC [35,45,47,50,53]. Pannebaker *et al.* (2010) found no expression of MMP-1 in tears of normal controls and a significant upregulation in patients with KC [45]. The increase in MMP-1 levels in tears from KC patients was independently verified by Balasubramanian *et al*. [35]. Interestingly, in their investigation the levels of MMP-1 in KC patients who had received CXL treatment were intermediate between levels in control and KC groups [35]. A slight upregulation of MMP-1 protein in KC corneal tissue was also detected in two other studies [50,53], but in contrast one earlier investigation found no overexpression [47].

Other MMPs have also been investigated in relation to KC, with contrasting outcomes. Balasubramanian et al. found upregulation of MMP-3, -7, and -13 proteins in tears from KC patients [35], in conflict with an earlier study, which did not detect any significant difference in the tear expression of MMP-3 and -13 in KC eyes [45]. Another investigation also found no difference in MMP-3 protein expression between KC and normal corneal tissue [47]. Furthermore, no increase in MMP-3 expression was measured in cultures of keratoconic compared with normal keratocytes [34]. However, a moderate increase in MMP-13 protein was also reported in KC corneal tissue [50].

A number of mRNA expression [54,55] and RNAseq [56-58] studies have recently been published comparing RNA profiles in cornea from KC and control patients. These studies have some significant limitations as KC tissue is usually collected fresh from patients undergoing keratoplasty for KC, whilst control tissue is generally collected post-mortem or from patients undergoing corneal surgery for other eye diseases. Thus gene expression in controls may not be representative of fresh or healthy

cornea. In addition, control patients in these studies are generally significantly older than KC patients. Nevertheless, results of three of these studies confirm a dysregulation of the corneal proteolytic balance in KC, as they detected a downregulation of TIMP-1 [55-57], TIMP-2[55,57] and TIMP-3 [56,57] . The other two studies [54,58], however, found no difference in TIMPs expression between tissue of KC and control myopia patients, with the caveat that only the epithelial layer of the cornea was used in one of them [58]. No changes were observed in MMPs expression levels in these investigations, except for downregulation of MMP-9 in a small study [55].

Overall these data suggest a general dysregulation of proteolysis in KC patients, with an increase in level of some MMPs, particularly MMP-1 and MMP-9, confirmed in multiple independent studies. However, findings regarding the upregulation of specific MMPs were not consistently replicated across all studies.

3. MMP upregulation, corneal damage and keratoconus

MMPs play a key role in corneal repair [27]. Corneal healing involves several coordinated steps, including migration to close the wound, proliferation to replace the lost cells, re-stratification and differentiation, and stromal remodelling to return corneal clarity [30,59]. Upregulation of MMP-1, -2, -3, -7, -9, -12, -13 and -14 is detected during the healing of rabbit and rat corneas [60-63], with most studies focussing on MMP-1, MMP-2 and MMP-9. After induction of a superficial wound in rabbit eyes, strong MMP-1 protein expression is detected at the leading edge of migrating epithelial cells, suggesting a function in cell motility [60]. Indeed, inhibition of MMP-1 in *in-vitro* and *ex-vivo* human models of corneal healing significantly halts wound closure [64]. In animal models, a low basal expression of MMP-2 protein is observed in normal undamaged corneas, but its level increases in the stroma during healing and persists above baseline for several months after wounding, suggesting a long-term role in the stromal collagen fibril remodelling that follows wound closure [46]. In similar animal experiments, expression of MMP-9 protein is undetectable in undamaged cornea but observed in both the stromal and epithelial layers soon after injury [46,62]. However MMP-9 expression only persists for a few weeks after wound closure [46]. Blocking MMP-9 activity results in increased corneal wound closure in a human *in-vitro* scratch-wound assay [64] and MMP-9 deficient mice have a faster rate of corneal re-epithelialisation [65], possibly due to a dual inhibitory effect of MMP-9 on corneal cell proliferation and migration. On the other hand, remodelling of the provisional ECM deposited during corneal wound healing is defective in MMP-9 deficient mice, resulting in prolonged cornea clouding [65]. In human eye diseases, over-expression of MMPs, particularly the active forms of

MMP-2 and MMP-9, has been linked to disruption of the basement membrane and the pathogenesis of corneal ulcerations and erosions [66,67]. Therefore, a fine balance in the expression of MMPs appears necessary for maintaining the integrity and transparency of the cornea and for its correct healing.

Several studies indicate a link between contact lens wearing or eye rubbing and altered MMP expression in KC patients. For example, changes in MMP tear protein profiles have been shown in KC patients wearing contact lenses [68,69]. In one study, temporary MMP-9 upregulation in tears was associated with contact lens wear in subjects who had not worn contact lenses before, but this increase was measured only after very extensive wearing which included nights, and it was reversed after a month of wearing [70]. In another investigation in 26 KC patients, a significant increase in MMP-9 level in tears was measured after wearing scleral lenses for an average of 8 hrs [71]. A further study showed an increase in MMP-13 in tears from healthy volunteers after 60 seconds of eye-rubbing [72].

Overall, this evidence provides support for the hypothesis that upregulation of MMPs in KC patients could be triggered by the corneal damage resulting from contact lens wearing or eye rubbing, two of the risk factors for KC [19,20,22]. A certain degree of MMP-driven ECM remodelling may be a necessary step for cornea healing, but an imbalance in this tightly regulated process may, in the long term, result in the progressive weakening of the cornea (Figure 2).

4. MMP upregulation, inflammation and keratoconus

Eye rubbing and contact lens wearing have a clear link with ocular surface inflammation [73,74]. Although KC is generally considered a non-inflammatory eye condition [2], this is still under debate [75-77]. Elevated MMP expression in tears or corneal cell cultures is observed in other corneal diseases associated with auto-immunity, infection and inflammation, such as rheumatoid arthritis with active ocular disease, herpetic eye disease, fungal keratitis and dry eye disease [78,79], raising the possibility that inflammation caused by eye rubbing and/or contact lens wearing may be driving the increased proteolysis observed in KC. Indeed, the upregulation of MMP-1 and MMP-9 in KC corneas, and the increase in MMP-13 observed in tears of healthy volunteers after eye-rubbing are accompanied by an increase in the level of inflammatory markers, such as IL-6 and TNF-α [42,72]. Plasma levels of IL-1β, IL-6 and TNF-α are also increased in KC patients compared with controls [43]. TNF stimulation of both normal and KC corneal fibroblasts was found to upregulate IL-6, which in turn led to an increase in

MMP-1 mRNA and protein expression in these cells [80]. MMP-9 mRNA, protein and overall activity levels in corneal epithelial cells *in-vitro* is stimulated by treatment with TGF-β, IL-1β and TNF-α [36,40,81], and induction of both MMP-9 and MMP-1 mRNAs can be initiated by platelet-activating factor (PAF), an important inflammatory mediator which accumulates in the cornea following an injury [82].

Based on this evidence, a causative link can be hypothesized between inflammation due to mechanical trauma resulting from contact lens wearing or/and eye rubbing and the increased MMP production observed in KC (Figure 2), although this area needs further investigation.

5. Genetic associations between MMP and KC

As KC is thought to have a genetic component, mutations in the coding region of MMPs or TIMPs or promoter variants altering their expression could contribute to disease risk.

An association between KC and a region on chromosome 20q12, to which the *MMP-9* gene maps, was found in a small study in an Australian population. However, linkage with the nearby *MMP-9* gene was excluded in that study [83]. In contrast, a case-control study in an Iranian population found a significant association between KC and the *MMP-9* polymorphism rs17576 [84]. Interestingly, the rs17576 *MMP-9* polymorphism has also been associated with risk of glaucoma[85,86], with the A allele protective in both conditions. This common polymorphism (NM_004994.2, c.855A->G) causes a p.Gln279Arg change in the catalytic domain of the enzyme, which may alter its ability to bind to substrate [87]. The frequency of the G allele varies from 0.74 to 0.22 depending on the ethnicity, with the highest frequencies in East and South Asians, the lowest in Latinos and an intermediate frequency in Europeans (http://exac.broadinstitute.org/, https://www.ncbi.nlm.nih.gov/projects/SNP/), which is consistent with the different risk of KC in these populations. This association therefore warrants further confirmation in other KC patient cohorts. The same investigation also found an association between the *TIMP-1* rs6609533 polymorphism and KC risk. This common change (XM_017029766.1, c.1030A->G) causes a p.Thr219Ala substitution, and the AA genotype was associated with increased KC risk in female patients only (OR = 2.27, 95% CI = 1.06–4.76, P = 0.036).

Overall, genetic association studies in KC have to date found some weak evidence for the involvement of *MMP* and *TIMP* genes. Only a handful of reports on the contribution of *MMP* and *TIMP* polymorphisms to KC risk have been published, and this is therefore another area deserving further investigation.

6. Clinical implications

The emerging role of MMPs in corneal healing and degradation could have important clinical implications for the treatment of KC. Contact and scleral lenses are the first choice treatment in the initial stages of KC, but they have been shown to alter the MMP composition of tear fluids [70,71], raising the possibility that they may also contribute to the progression of the disease. In a recent study, maintaining 3D cultures of KC-derived corneal fibroblasts in hypoxic conditions to simulate the low-oxygen environment associated with contact lens wearing, resulted in a decrease of collagen I secretion and ECM thickness, accompanied by upregulation of MMP-1 and -2 [88]. No change in MMP-3, -9, or -13 was observed. Interestingly, in two independent studies CXL treatment of keratocytes and corneal fibroblasts *in vitro* was associated with a decrease in the expression of inflammatory biomarkers and MMP-1, MMP-2, MMP-3 and MMP-9 [89,90], with MMP-9 downregulation reported by both studies. Cross-linked collagen was also found to be resistant to degradation by MMP-1, -2, -9, and -13 in an *ex-vivo* model using bovine corneas [91]. These findings suggest possible mechanisms by which CXL slows KC progression. In view of the accumulating evidence for a role of MMP-9 in KC, the safety of current medications for other eye conditions, such as Latanoprost, used for glaucoma, and doxycycline, used for ocular diseases involving infection and inflammation, may need to be reviewed in keratoconus patients, as they have been shown to respectively increase and decrease MMP-9 expression [36,92]. Interestingly, Cyclosporine A, which has been approved for the treatment of dry eye disease, another ocular condition linked to raised MMP-9 [93], was found to reduce the expression of inflammatory markers and MMP-9 in corneal epithelial cells *in-vitro* [40]. In a pilot study, 20 KC patients using regular cyclosporine eye drops for 6 months showed a significant reduction of MMP-9 levels in their tears and a trend towards a reduced progression of the disease [40]. Based on these promising results, a larger clinical trial is currently under way (NCT01746823). A point-of-care test is available to quickly measure MMP-9 level in tears [94] and this would facilitate quick identification of patients who could benefit from this treatment.

7. Conclusion and perspectives

During the past decades evidence of a role for ECM degrading enzymes in KC has arisen in the literature. A number of MMPs are upregulated in KC tissue and tears, possibly due to inflammation triggered by repeated physical trauma, with the strongest evidence for a role in KC development implicating MMP-9. As specific MMP inhibitors are in development [95], this information could

potentially drive the discovery of new treatments for KC. Future investigations should confirm the functional role of specific MMPs in KC and explore genetic association between common *MMP* variants and risk of the disease.

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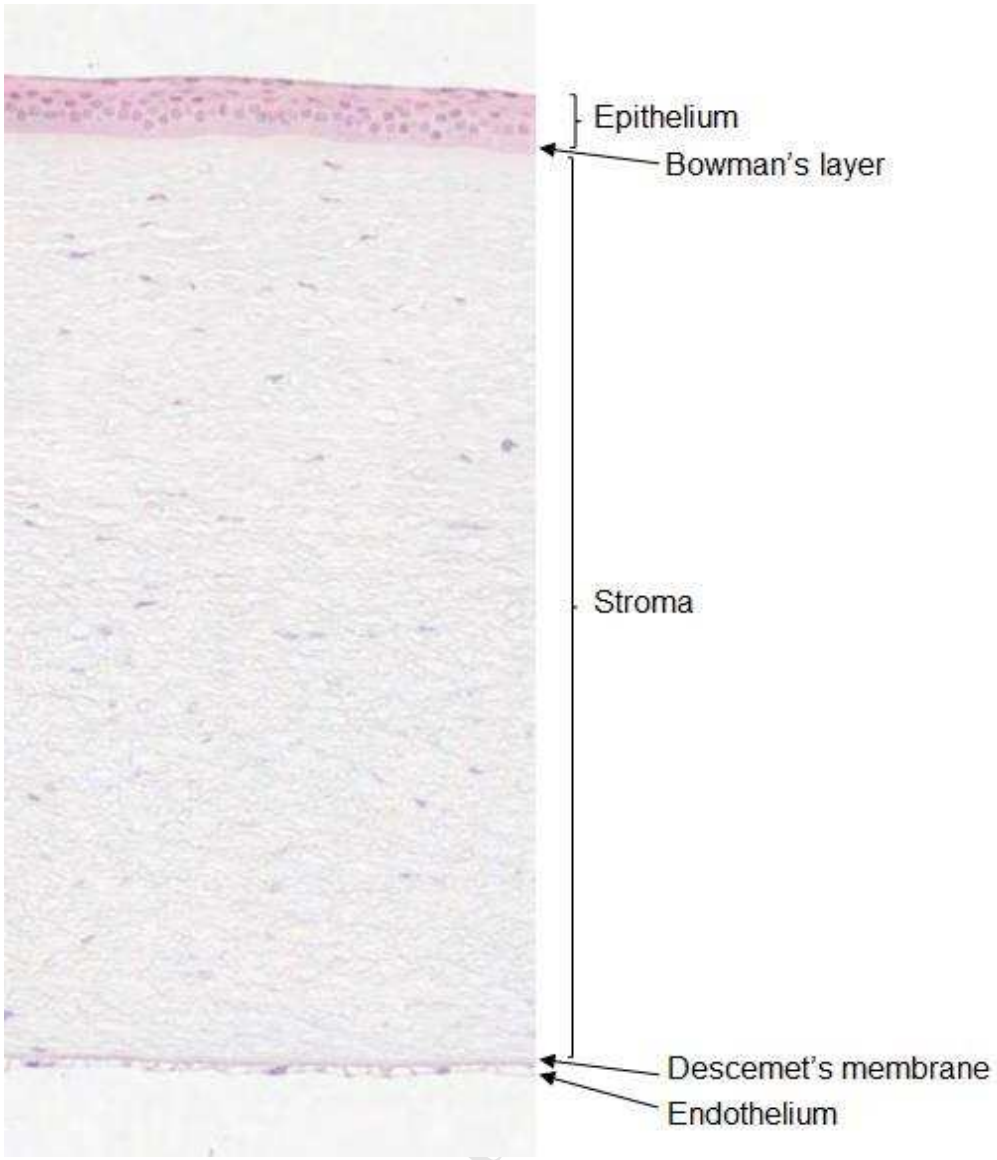
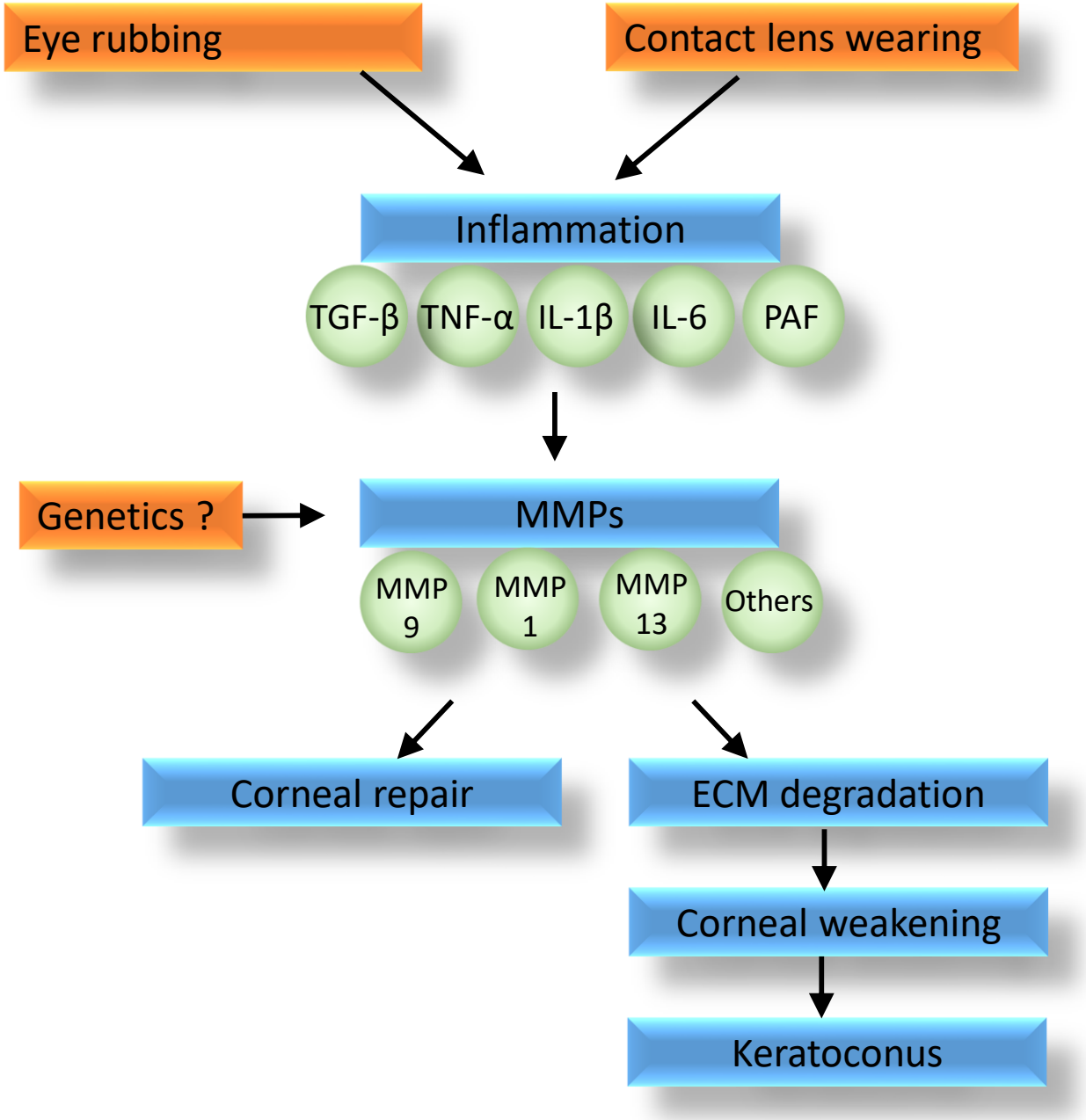
Figure Legends

Figure 1. Sagittal cross-section of the human cornea after staining with haematoxylin and eosin. The epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium are shown (Image courtesy of Mr Mike Shires, Leeds Institute of Cancer & Pathology, University of Leeds).

Figure 2. Potential mechanistic links between KC risk factors (orange) and MMP-mediated corneal weakening. Specific inflammatory molecules and specific MMPs are indicated in green.




Eye rubbing
Contact lens wearing
Inflammation
TGF-β
TNF-α
IL-1β
IL-6
PAF
Genetics ?
MMPs
MMP 9
MMP 1
MMP 13
Others
Corneal repair
ECM degradation
Corneal weakening
Keratoconus

- Keratoconus (KC) is a corneal disease with environmental and genetic causes
- Matrix Metalloproteinases (MMPs) contribute to corneal integrity and healing
- Increased levels of some MMPs, particularly MMP-1 and MMP-9, are found in KC
- Dysregulation of MMPs may contribute to corneal weakening in KC
- Further studies in this area may drive the discovery of new treatments for KC