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Teaser The lack of ultrasensitive diagnostic strategies and the identification of definitive diagnostic markers are major challenges in keratoconus. management. However, understanding the etiopathology and insights into crosstalk between genetic, epigenetic, and hereditary factors could help to develop new strategies for drug discovery.

Interplay between hereditary and environmental factors to establish an *in vitro* disease model of keratoconus

Subhadeep Roy[1], Saumya Yadav[2], Tanushree Dasgupta[1], Shikha Chawla[1], Radhika Tandon[2] and Sourabh Ghosh[1]

[1] Regenerative Engineering Laboratory, Department of Textile Technology, Indian Institute of Technology, New Delhi, India
[2] Cornea & Refractive Surgery Services, Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India

Keratoconus (KC) is a bilateral corneal dystrophy and a multifactorial, multigenic disorder with an etiology involving a strong environmental component and complex inheritance patterns. The underlying pathophysiology of KC is poorly understood because of potential crosstalk between genetic–epigenetic variants possibly triggered by the environmental factors. Here, we decode the etiopathological basis of KC using genomic, transcriptomic, proteomic and metabolic approaches. The lack of relevant models that accurately imitate this condition has been particularly limiting in terms of the effective management of KC. Tissue-engineered *in vitro* models of KC could address this need and generate valuable insights into its etiopathology for the establishment of disease models to accelerate drug discovery.

Introduction

The cornea is the principal refractive element of the human eye and is engineered to gather light and focus vision [1,2]. However, the refractive competence of the cornea is affected by changes in the surface topography, radius of curvature, and transparency or matrix composition. KC is associated with visual impairment resulting from the increased thinning of the cornea and ectasia, leading to curvature myopia in the initial stages of the disease [3]. With progression, increased irregularity in shape results in irregular astigmatism. In addition to the shape-dependent changes in refractive status, the cornea also tends to develop scarring and opacification [4,5].

KC usually manifests during puberty, affecting adolescents and young adults and shows a relatively slower advancement thereafter [2]. It affects individuals from diverse ethnic groups, although with a predominant predilection towards the Asian population compared with Caucasians [6,7]. Its incidence of occurrence exhibits geographical asymmetry, possibly because of the presence of asymptomatic or subclinical forms of the disease, variability in diagnostic measures and/or criteria, and inherent genetic heterogeneity among populations [8,9]. Based

Corresponding author: Ghosh, S. (sghosh08@textile.iitd.ac.in)

Subhadeep Roy is a postdoctoral research associate at the Department of Textile Technology at the Indian Institute of Technology, Delhi, India. His current research focuses on the modulation of biomaterial-mediated immune responses, macrophage polarization, decellularized corneas, and *in vitro* disease models. He received his PhD from Babasaheb Bhimrao Ambedkar University, Lucknow, where he investigated targeting estrogen-dependent breast cancer metastasis using *in vitro* and *in vivo* rodent models. He has also worked as a visiting senior research fellow at the Center of Biodesign (CBD), Translational Health Science and Technology Institute (THSTI), Faridabad, researching targeted drug delivery in human pulmonary macrophages.

Radhika Tandon is a Professor of Ophthalmology at Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India. She is the head of a unit that specializes in cornea and external diseases, cataracts, and refractive ocular oncology, and low vision. She is also the co-chairperson of the National Eye Bank and President Eye Bank Association of India.

Sourabh Ghosh is a Professor of Textile Technology at the Indian Institute of Technology Delhi, India, where he is also the head of the Regenerative Engineering laboratory. His research focuses at the interface between fundamental and applied research by combining the principles of Textile Technology and Tissue engineering, using silk and collagen proteins, bioprinting of different 3D tissue, and establishment of *in vitro* disease models for drug screening.

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on results from studies targeted at investigating the genetic-genomic [10], transcriptomic-proteomic [11], and inflammatory signatures [12] of KC, it is becoming increasingly clear that KC is likely to be a multifarious disease that is potentially governed by complex interplay between the genetic-nongenetic 'epigenetic' variants and environmental stimuli [13].

Here, we critically review the pathophysiology of KC, highlighting the missing links. Updates on the latest clinical management modalities and their current limitations are required to facilitate drug discovery and to develop novel therapeutic strategies.

Disruption of corneal anatomy and homeostasis in KC

KC is associated with the progressive thinning and ectasia (Fig. 1a) of paracentral and central cornea, most commonly in a inferotemporal paracentral location [14]. Thinning of cornea can precede ectasia. Progressive thinning and ectasia lead to increasing irregular astigmatism. As the disease progresses, corrected visual acuity of the patient declines. In advanced cases, patients become intolerant to rigid contact lenses [2]. Severe thinning can lead to rupture of Descemet's membrane and egress of aqueous humor into corneal stroma, resulting in acute corneal hydrops, which is a painful condition [15].

A hemosiderin full circle/arc, known as Fleischer's ring (Fig. 1b), can be seen in moderate to advanced KC [16]. It is usually seen around the base of the cone of protrusion. Fleischer's ring is suggested to occur as a result of severe changes in corneal curvature and/or alteration of the normal epithelial sliding process, which leads to iron deposition from the tear film onto the cornea [16]. It is brown in color and is best visualized with the cobalt blue filter of a slit lamp biomicroscope. Superficial corneal opacities (Fig. 1b) are common with long-term contact lens use in patients with KC [17]. Chronic hypoxia and tear film changes resulting from prolonged usage of rigid gas permeable lenses lead to scarring. Deep scars can also be seen in patients with KC with healed hydrops [15]. Corneal tomography with a rotating Scheimpflug camera (Fig. 2a,b) is useful in diagnosing even the earliest stages of KC because it gives a detailed quantitative estimation of both anterior and posterior surfaces of the cornea. In patients with KC, a Four Map Refractive

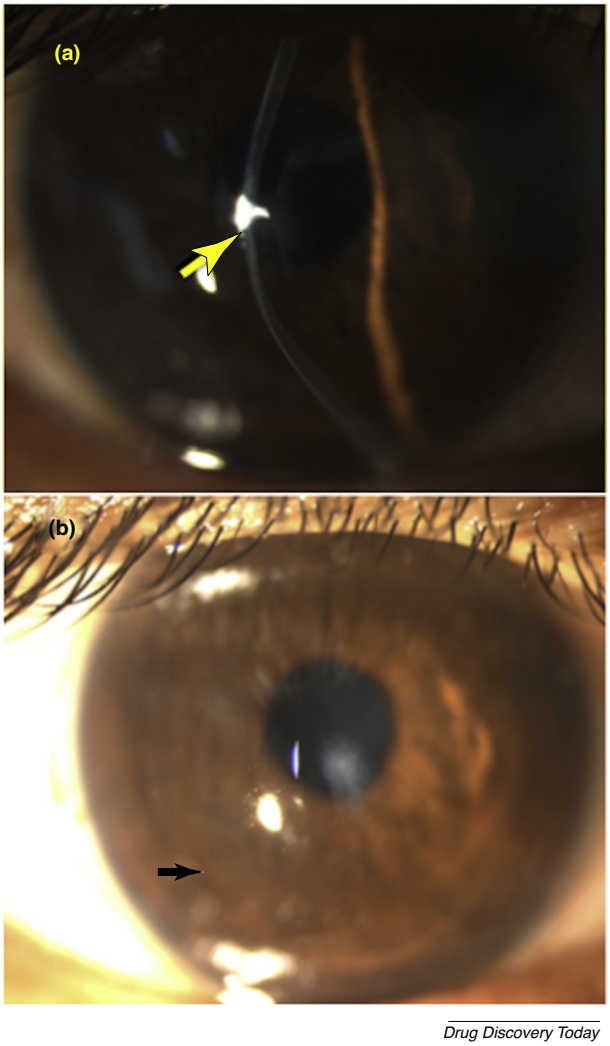

FIGURE 1

Morphological changes. (a) Thinning of central cornea as seen in a patient with keratoconus (KC). (b) Fleischer ring in a patient with KC. Contact lens-induced apical scarring can also be seen.

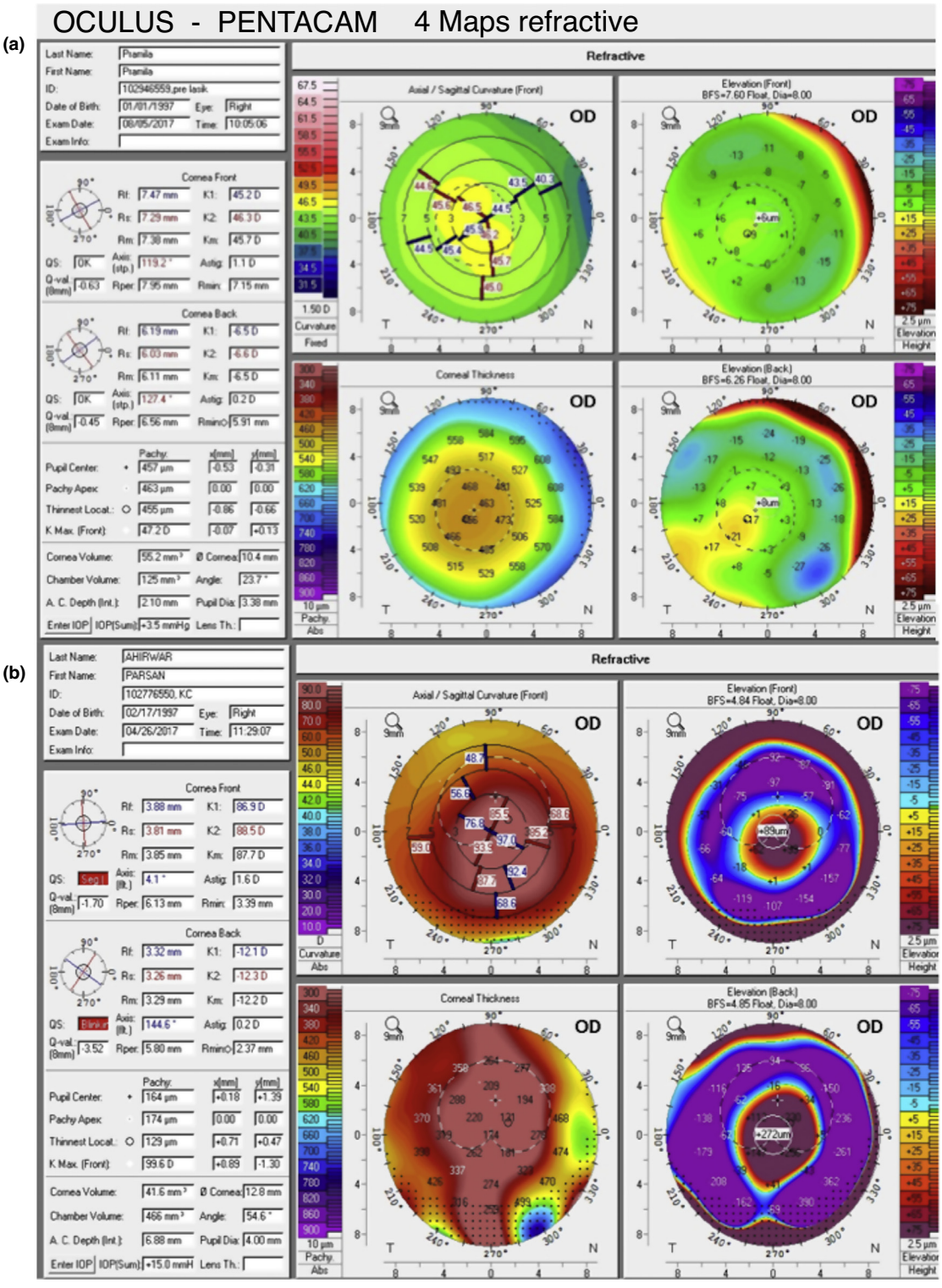

FIGURE 2

Corneal tomography in early stage and late stage KC. (a) Scheimpflug imaging in patient with early-stage keratoconus (KC) (K_{max} = 47.0 D, thinnest pachmetry = 455 Mm, posterior elevation = 121 Mm). (b) Scheimpflug imaging showing severe thinning in a patient with advanced KC (K_{max} = 99.6 D, thinnest pachmetry = 129 Mm, posterior elevation = 1272 Mm).

Display provides a color-coded representation of increased anterior curvature, increased anterior and posterior elevation, and cornea thinning [18,19]. Steep areas are displayed in hot colors (red and orange) and flat areas in cooler colors (blue and green). The pachymetric map in the Four Map Refractive Display not only shows corneal thinning in KC, but also the distribution of corneal thickness across the entire corneal diameter. This can be useful in patients with subclinical KC to determine whether the thinnest

point corresponds to the corneal apex. Examination of the posterior surface of the cornea is essential to rule out any elevation, steeping, and stromal thinning, which might be present in patients with subclinical KC without any obvious anterior surface changes [20].

Histopathologically, the three hallmark signs of KC are: stromal corneal thinning; rupture in Bowman's layer (Fig. 3a2); and iron deposits within the basal layer of corneal epithelium [2,21]. Central epithelial thinning, with irregular cells, more significantly at the apex of the cone, has also been noted (Fig. 3c4,e7) [22]. The earliest changes in KC occur in the corneal epithelium basement membrane and Bowman's membrane. The basement membrane is disrupted and epithelial basal cells degenerate and grow towards Bowman's layer (Fig. 3b3), leading to the deposition of ferritin particles into and between epithelial cells, which clinically appear as Fleisher's ring [23]. Basal cell density in KC is also reduced compared with normal corneas [24] (Fig. 3c4). TUNEL-positive apoptotic cells extend up to the basal layer of epithelium in KC [25]. Varying degrees of rupture of Bowman's membrane (Fig. 3a2) are often accompanied by distortion of underlying stroma. The defects of Bowman's membrane can be filled with collagen from the stroma with accompanying apoptotic cells and fibrotic changes [26]. In the stroma, although the thickness of collagen lamellae is unchanged, a decrease in the number of lamellae, alteration in their gross organization, and an uneven distribution of the inter- and intralamellae collagen fibrillar mass (Fig. 3d5) more significantly around the apex of the cone have also been noted [27]. Reduction in keratocyte density and changes in their morphology have also been observed in KC. Keratocyte density is lowest in the most anterior part of stroma, the reduction being greater in more advanced disease [28]. Descemet's membrane is usually unaffected in KC, although it can rupture in some cases because of eye rubbing or in advanced KC [29] (Fig. 3f8), leading to egress of aqueous into stroma and the development of hydrops [2]. Endothelium is also generally normal in the disease but can demonstrate intracellular dark bodies, pleomorphism, and elongation of endothelial cells pointing towards the cone (Fig. 3g9) [2]. Clinically prominent corneal nerve fibers can be seen by slit lamp biomicroscopy. Corneal nerves in keratoconics have thicker fiber bundles, reduced density, and subepithelial plexus compared with normal nerves. Keratocytes wrap around the nerves as they pass through Bowman's layer from the stroma into the epithelium at the sites of breaks in Bowman's membrane, giving rise to localized nerve thickenings in the epithelium (Fig. 3h10) [30].

Current therapeutic options and their limitations

Therapeutic options for the restoration of sight in patients with KC are gradually increasing with the advent of new technologies and innovations in surgical techniques. During the initial stages, refraction and prescription of spectacles is adequate to restore eyesight. However, further deterioration (with an increase in astigmatism) requires the use of rigid contact lenses or specialized lenses, such as Super Cone, Rose K, Hybrid lenses, Piggyback or Scleral lenses [31] to overcome the optical distortion associated with KC. In patients with progressive KC, those with clear central corneas and thinner central corneal thickness (CCT <400 μm), collagen crosslinking (CXL) treatment helps to stabilize the cornea. CXL induces physical crosslinking of collagen and proteoglycans predominantly at the collagen fibril surface, halting progression by improving the biomechanical strength of the cornea [32]. Long-term follow-up studies on CXL reported improvement in visual acuity alongside an improvement in the topographic parameters associated with KC [33]. Modified CXL procedures, such as accelerated CXL (ACXL), hypo-osmolar CXL [34], and trans-epithelial CXL (TECXL), are also being performed. Topography guided-photorefractive keratectomy (T-PRK) along with CXL, rather than CXL alone, is considered in patients with progressive KC intolerant to contact lens wear, which reduces disease progression by regularizing the cornea, thus improving contact lens fit and allowing better quality of vision with spectacle correction [35].

Combined intrastromal corneal ring segment (ICRS) implantation with CXL is a feasible treatment option for patients with moderate to advanced KC (CCT <450 μm) [36]. An ICRS can reduce corneal steepening and decrease irregular astigmatism, leading to improved visual acuity [37], while the simultaneous crosslinking restores the biomechanical strength of the cornea. To this end, INTACS (a type of ICRS) has been shown to improve both spherical and cylindrical refractive errors along with a reduction in the average keratometry readings [38].

Advanced cases of KC with stromal scarring, such as those involving the visual axis, require a lamellar or full-thickness penetrating keratoplasty (PK) depending upon the depth of corneal opacity, which involves removal of the entire thickness of the cornea followed by replacement with a transparent corneal tissue. However, the associated risk factors are presence of corneal scarring, meager visual acuity despite contact lens correction, and higher corneal astigmatism [39].

KC predominantly affects the corneal stroma, often sparing the endothelial layer underneath. However, endothelial injury can occur in advanced stages of the disease. Unless severe apical thinning, Descement's membrane (DM) scarring, or acute corneal hydrops are present, deep anterior lamellar keratoplasty (DALK) is an excellent surgical alternative.

DALK allows for replacement of the corneal stroma excluding the host endothelium, reducing the risk of endothelial rejection and also curtailing endothelial cell attrition [40]. Big bubble is the most commonly used surgical technique to obtain a DALK [41]. There are two variations available for DALK: Predescemetic (PD-) DALK and Descemetic (D-) DALK. In PD-DALK, after stromal dissection, a slender section of the remaining stroma is left on the recipient bed adherent to DM, which can lead to interface opacification and stromal scarring; in D-DALK, exhaustive stromal excision is carried out with complete exposure of DM on which the donor button is directly placed. Studies have shown that, although D-DALK leads to a faster visual recovery, eventual long-term visual outcome is comparable in both PD-DALK and D-DALK [42].

In terms of the relatively uncommon condition acute corneal hydrops associated with KC, corneal imaging systems, such as anterior segment optical coherence tomography (AS-OCT) and intraoperative-OCT, which can identify DM tears, have greatly improved the management of this condition. Other surgical techniques used for the effective management of acute corneal hydrops include compressive sutures along with gas injection [43], cyanoacrylate tissue adhesive with a bandage

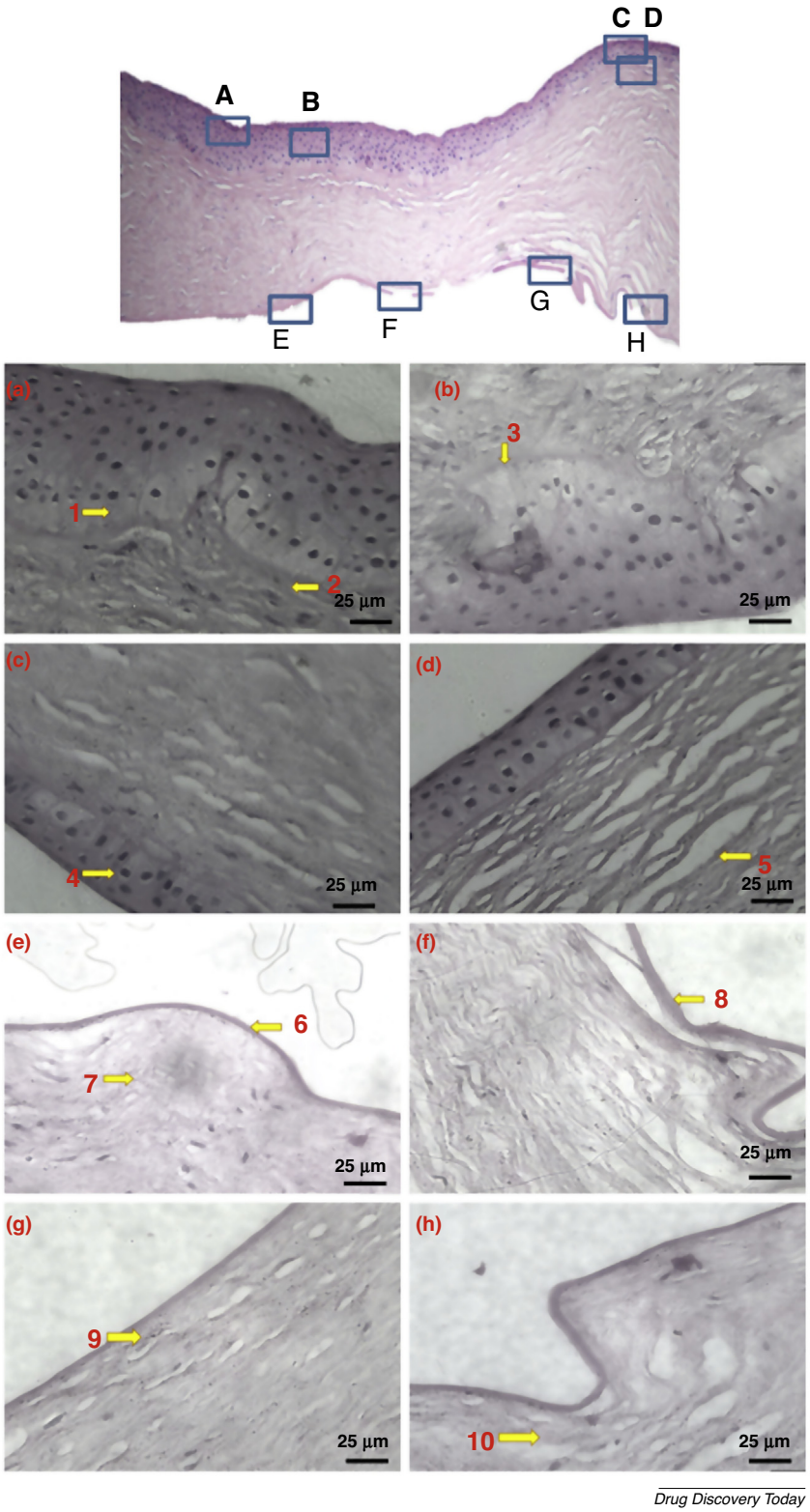

FIGURE 3

Hematoxylin and eosin (H&E) staining to identify different histological features of keratoconus (KC). Crinkling and breaks in Bowman's layer with accumulation of fibrocytes in the stroma (a1). Presence of irregular and apoptotic cells in fibrotic region (a2). Downgrowth of epithelial cells into the gap with uneven integrin (b3). Thinned epithelium with low basal integrity (c4). Altered fibril orientation and loss of lamellae (d5). Anterior cornea with distinct irregular epithelial (e6). Increased number of cells in mid and anterior stroma (e7). Rupture folds of Descemet's membrane (f8). Pleomorphic disruption of endothelial mosaic stability (g9). Stromal terminal nerve fiber-containing electron dense body (h10).

contact lens [44], and amniotic membrane transplantation with cauterization [45].

Although all treatment options discussed earlier have been proven safe in the management of KC, none of the treatment methods provide a definitive cure. Even though CXL prevents the progression of KC, cases of post-CXL ectasia are not uncommon. Even with keratoplasties, there are issues with suture-related complications, graft rejection, graft infection, and recurrence of KC in many cases. Thus, in-depth investigation of pathogenic factors involved in KC alongside a better understanding of the regulation of the underlying signaling pathways is crucial to develop more innovative and effective therapeutic strategies.

The role of hereditary factors

Genetics

KC has long been considered to have a genetic component central to its etiopathology, given its association with other genetic conditions, such as Down's, Crouzon's, Angelman's, Noonan's, or Apert syndromes [46]; its prevalence in first-degree relatives [47]; and concurrence among monozygotic twins [48]. Consanguinity has also been documented to be a risk factor for KC, providing compelling evidence for the hypothesis that KC is, in part, a genetic disease [49]. In certain families, KC has been found to follow an apparent autosomal dominant/recessive mode of inheritance [50]. Nevertheless, sporadic cases show no Mendelian patterns of inheritance [51]. Thus, a segregation analysis of unrelated sporadic cases of KC [51] suggested that, although genetics is a component of this condition, it might not be the only governing factor. One school of thought believes that susceptibility towards KC development is provoked by an environmental insult in genetically predisposed individuals [52]. To characterize the genetic framework of KC, researchers have used comprehensive genetic tools, such as genome-wide linkage studies (GWLS), genome-wide association studies (GWAS), meta-analyses, and next-generation sequencing (NGS) technologies [11,53], to identify marker genes and mutations and/or single nucleotide polymorphisms (SNPs) implicated in the etiopathogenesis of KC that could aid in devising gene-based screening tests and personalized therapies for KC diagnosis and management in the future.

Rather than exhibiting a classical Mendelian mode of inheritance (relying on a single gene eliciting the disease phenotype), KC is suggested to be polygenic [10]. To this end, mapping of several distinct chromosomal loci (17 till date) for KC explains its genetic heterogeneity [54]. Some of the candidate genes with reported mutations and/or SNPs associated with KC across multiple ethnic groups include the genes encoding: visual system homeobox 1 (*VSX1*), which regulates the expression of photoreceptor cells [55]. It is also the most studied gene in KC; *miR184*, which is robustly expressed in the cornea/lens epithelia. Mutation in its seed region potentially alters its function [56]; and transforming growth factor beta induced (*TGFbI*), which interacts with extracellular matrix (ECM) proteins and has roles in tissue injury and repair [10,55]. Other genes include: dedicator of cytokinesis 9 (*DOCK9*), which regulates the expression of CDC42, a G protein [55,57]; *SOD1*, which encodes for and regulates the expression of superoxide dismutase [58]; and hepatocyte growth factor (*HGF*), SNPs in which have been found to associate with KC [59,60]. Activity of the gene encoding the collagen and elastin cross-linking enzyme lysyl oxidase, *LOX*, is noticeably reduced in KC [61]. Other key candidate genes identified in multiethnic KC cohorts include: *CAST*, which encodes calpastatin, an endogenous inhibitor of calpains [62]; interleukin 1β (*IL1B*), polymorphisms in which have been linked with risk conferment for KC across different geographical groups [63,64]; *COL4A3* and *COL4A4* collagen type IV α3/4, (*COL4A3* and COL4A4), which are major structural proteins of the cornea. Mutations and/or polymorphisms in these genes in developing KC risk are population specific [63].

In addition to these genes, genes associated with two markedly heritable human traits, central corneal thickness (CCT) and corneal curvature (CC), were identified to confer susceptibility to KC. Across a large cohort involving European and Asian subjects (>20 000), 16 genomic loci associated with CCT were identified, two of which were shown to pose a relatively large risk for KC development [64]. Six SNPs within or close to the genes *FOXO1, RXRA-COL5A1, COL5A1, BANP-ZNF469, MPDZ-NFIB*, and *FNDC3B* were also associated with risk conferment for KC. Taking CC into consideration, two studies, one in an Asian population [65] and the other in Australians of Northern European ancestry [66], confirmed the involvement of *PDGFRA* (encoding platelet-derived growth factor receptor isoform-α, which has intracellular tyrosine kinase activity) in CC. The kinase activity of *PDGFRA* via its impact on corneal epithelial cells and collagen fibrils also affects CC. Thus, based on these studies, it appears that, apart from environmental causes of KC, the genetic predisposition to developing KC could partly result from genes underlying CCT and CC, given the phenotypic features of KC (i.e., progressive corneal thinning and disproportionate corneal curvature).

Although multiple genes and risk-associated chromosomal loci have been mapped in families with KC, it remains to be confirmed which ones are causative or confer susceptibility to the disease. Another important concern in terms of identifying pathogenic genetic variants and/or susceptibility loci influencing KC is the lack of unanimity and/or replication across subpopulations (those originating from different geographical regions), because many of these variants are representative of specific populations only, which further complicates determination of an actual causal relationship.

Epigenetics

Despite efforts to identify candidate susceptibility loci and/or genes implicated in familial KC, genetics is still unequipped to explain most of the sporadic cases of KC and some familial cases. These strongly hint at the influence of either external stimuli (environmental factors) or mechanisms that are not explained by alterations in the underlying DNA. These are evident through DNA (cytosine) methylation and/or covalent modifications of the histone (proteins that bind DNA) tails, histone modifications suggesting 'trans-generational epigenetic inheritance', which was initially postulated by Jean-Baptiste Lamarck during the 19th century [67].

The inherent environmental associations [eye rubbing, contact lens wear trauma, and/or ultraviolet (UV) exposure] of KC make it plausible that a proportion of KC cases could have an epigenetic basis that we are currently unaware of, and that a certain degree of phenotypic disparity could be attributed to intraindividual epigenetic variation. Given that the contribution of epigenetic net-

works in the disease biology of KC is largely unknown, there is scope to tease out the consequences of gene–environment interactions through genome-wide epigenetic studies. Unraveling this epigenetic association could also explain some of the disparity observed in multiethnic cohorts of patients with KC, as seen in the genetic and/or genomic studies.

Epigenetic marks help to create cellular identities and are often retained post cell division. Therefore, failure in preservation of these marks could build up over time and initiate inappropriate activation or inhibition of cellular signaling circuitries, giving rise to abnormal phenotypes in diseased conditions. Thus, deciphering the epigenetic landscape of KC could be a major imperative for KC research. Only the methylation status of the tissue inhibitor of metalloproteinase 3 (*TIMP3*) promoter [68] has been investigated. Overexpression of *TIMP3* induced apoptosis in corneal stromal cell cultures, which could lead to KC [69]. However, there was no evidence of any causative mutations or promoter methylation of *TIMP3* (at least in the small cohort of patients tested, with three healthy versus three KC corneas) [68].

Unlike genetic abnormalities, epigenetic aberrations are reversible (owing to the retention of normal DNA sequences), suggesting that it might be possible to recover the wild-type function of the affected genes. Therefore, epigenetic interventions could pave the way for reversing the effects of pathogenic genes and detrimental environmental effects as part of the personalized therapeutic regimen for KC diagnosis and management.

Oxidative stress and its effect on mitochondria

KC corneal fibroblasts (hKFs) exhibit increased levels of free radicals, such as reactive oxygen and nitrogen species (ROS/RNS) and are also more prone to oxidative challenges compared with wild-type fibroblasts [70]. For ROS/RNS scavenging, the normal human cornea uses antioxidant defense mechanisms comprising antioxidant enzymes, such as SOD, glutathione peroxidase, catalase, aldehyde dehydrogenase class 3 (ALDH3) [71], and other nonenzymatic antioxidants, such as ferritin, ascorbic acid, and glutathione [72]. However, in KC corneas, malfunctioning antioxidant enzymes (with altered and/or reduced antioxidant capacity) are unable to scavenge these free radicals [73]. Consequentially, protein denaturation and lipid peroxidation are triggered (as evidenced by the elevated levels of reactive aldehydes in KC corneas) [74], which leads to the further generation of ROS/RNS. The exhausted ROS/RNS levels induce oxidative damage to the mitochondrial DNA (mtDNA) [75], affecting the mitochondrial respiratory chain [76] and triggering apoptotic pathways, resulting in oxidative stress in corneal cells [53]. Reduced antioxidant defenses coupled with increased ROS/RNS in KC corneas modify the tissue components and progressively degrade the ECM of the corneal stroma, thus making them susceptible to gradual thinning [74]. Lysosomal proteolytic enzymes (including cathepsins) released in response to elevated levels of reactive aldehydes (by-products of accumulated ROS that disrupt the lysosomal membranes) [77,78] can further add to stromal thinning via their degradative activities against ECM components of the corneal stroma, including apoptosis of keratocytes [79], which have a pivotal role in the production and maintenance of collagen.

DNA damage introduced by oxidative stress could negatively influence the protein-coding region of mtDNA and have a detrimental impact on the oxidative phosphorylation functionality of mitochondria [80]. From a morphological perspective, the mitochondria in KC corneal tissues are swollen [81]. In addition to extensive genetic analyses using genomic DNA of patients with KC, few studies have attempted to unravel the genetic aspects of KC taking into consideration the mitochondrial DNA genome. One such study focusing on the mitochondrial complex I genes found novel genetic variants in genes such as *ND1-ND6* in an Indian cohort [82]. These mutations were found to harbor the ability to impact transcription or translation or have collegial effects with other variants giving rise to KC. The authors concluded that an association exists between the observed mutations and subjacent ATP levels, and increased ROS and malondialdehyde levels, which could lead to the alteration of protein function and induction of apoptosis, inflicting subsequent injury to corneal tissues [82]. Another recent study also confirmed mtDNA mutations in patients with KC in a Saudi Arabian cohort [83]. The same group was successful in showing that the average relative mtDNA content in patients with KC was higher than in control cases [84]. Intriguingly, contradictory to this study, the mtDNA content in KC corneas was found to be significantly lower in another study using an Asian cohort [85], although the transcript level of the mtDNA was increased in KC corneas [85]. Results from this study also showed that KC corneas exhibited higher mtDNA damage, elevated ROS levels, and lower mitochondrial membrane potential and ATP levels [85]. Despite the mtDNA copy number variation in these two studies [85], which might be attributed in part to the cohorts belonging to different ethnic populations and, thus, responding differently to oxidative stress cues because of their inherent genetic heterogeneity, these studies have nevertheless added the important association of mitochondrial dysfunction-oxidative stress to the pathophysiology of KC. This is consistent with association of the mtDNA content with other ocular diseases, such as diabetic retinopathy, Leber's hereditary optic neuropathy, and dominant optic atrophy [86]. However, given the disparity in results across different ethnic groups, the explicit causative mechanisms remain unclear. Therefore, determining the causal relationship between the mitochondrial genome and oxidative stress affecting the disease biology of KC warrants large-scale studies potentially involving multiple ethnic subclasses. If confirmed and validated, mtDNA content could be a genetic risk factor contributing discursively to the pathogenesis of KC.

Multifactorial role of hormone in KC

Decades of research established the presence of estrogen receptors, progesterone receptors, and androgen receptors in the nuclei of human epithelial, stromal, and endothelial cells of the cornea. This observation raised concern around the role of hormones and regulation of diseased state in KC. During menopause, female patients with KC can be classified as hormone active versus hormone inactive depending on the loss of ovarian follicular function [87]. Previously, it was postulated that refraction, corneal thickness and curvature in KC were correlated with hormonal changes during puberty, pregnancy, or menopause; however, clinical information related to sex hormones in patients with KC is rather limited, presenting a significant gap in understanding [88]. Several groups have studied the effects of gender and hormone status on the severity and progression of KC in both men and women, but clinical subjects of hormone-

active and hormone-inactive groups during menopausal transition failed to generate any significant advance in our understanding of KC progression [89]. However, cohort clinical cases of pregnant woman and gestational period revealed the role of hormonal changes during pregnancy in corneal biomechanics, progression of post-laser *in situ* keratomileusis, and significant progression in KC [90]. Involvement of collagenolytic and gelatinolytic activities ultimately regulate the synthesis of collagen-degrading enzymes and/or matrix metalloproteinases (MMP-1, MMP-2, and MMP-9) and tissue inhibitors of MMPs (TIMPs), which have pivotal roles in the progression of KC during pregnancy by disruption of collagen networks [91]. Recent studies suggested that changes in estrogen and progesterone levels have a role in developing KC-like symptoms during pregnancy. Estrogen-dependent changes in corneal thickness and curvature affecting the corneal hysteresis and rigidness are common during pregnancy, which reflect a high level of progesterone [92]. *The protective role of high progesterone levels during the last trimester of pregnancy with reduced estrogen levels proven by inhibitory action on prostaglandins that stimulate collagenases and consistent modulation of relax in due to their ability tostimulate the breakdown of collagen* [93]. Thus, cumulative data suggest that, in pregnancy, hormonal changes with interlinking prostaglandin might be unrecognized and multivariable risk factors for progression of KC; however, further studies are needed with large cohorts of patients from various ethnic backgrounds to reach a definitive conclusion regarding the roles of hormones in KC [94].

Inflammatory involvement in KC

Although KC has been classically described as a non-inflammatory disorder, recent studies have suggested an inflammatory etiology [95]. KC has associated with allergic history, ocular trauma, atopy, eye rubbing, and rigid contact lens use [96]. Classic signs of inflammation (heat, redness, swelling, and pain), except for the loss of function, are not present in KC. However, at the molecular level, the presence of inflammatory cytokines, proteins, and transcription factors have been documented [97]. Various studies have found increased inflammatory mediators, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and MMP-9 in tear film of patients with KC [98–100]. Hence, emerging evidences are indicating that chronic inflammation might be involved in the pathogenesis or progression of KC.

Eye rubbing also increases tear levels of MMP-13, IL-6, and TNF-α. In a study by Balasubramanian *et al*. increases in inflammatory factors, including IL-6 and MMPs, was noted in tear film after only 60 s of eye rubbing [101]. The authors proposed that prolonged periods of habitual eye rubbing can aggravate the expression of these inflammatory markers and contribute to the progression of KC. Eye rubbing leads to increased shear stress and hydrostatic pressure within the cornea, which can also trigger inflammation.

MMPs are overexpressed in KC,whereas TIMPs are downregulated, leading to excessive tissue destruction [102]. MMP activity is also modulated by proinflammatory cytokines and cell adhesion molecules expressed in keratoconic eyes. Modulation of MMP expression has been documented in every layer of the KC cornea [98]. Thus, a role of inflammatory factors in the progression of KC cannot be ruled out. Further studies to establish the definitive effect of inflammatory factors, including cytokines, chemokines, cell adhesion molecules, and certain growth factors, on extracellular remodeling are required to determine whether they have potential as anti-inflammatory treatments in the management of KC. Cyclosporine A has been shown to reduce tear levels of MMPs and other inflammatory factors [103]. Newer drugs targeting other inflammatory markers or signaling pathways could serve as new treatment modalities for halting the progression of KC.

Development of *in vivo* and *in vitro* models

Understanding of the corneal stroma

Corneal stroma, which is representative of nearly 85% of the corneal thickness [104], mainly comprises a collagen-rich ECM interspersed with keratocytes [104]. The stroma principally comprises type-I collagen, alongside other collagen types, such as III and V, which are homogeneously distributed (as fibrils 25–30 nm in diameter) and are regularly interwoven as orthogonal layers or lamellae [105]. The organized assembly of collagen fibrils within the stroma is pivotal for maintaining cornea transparency [106]. The presence of proteoglycans, such as decorin, lumican, keratocan, and osteoglycin, regulates collagen fibril growth and serves to stabilize the fibrils thereafter [107]. Stromal keratocytes residing between the collagen lamellae participate in ECM synthesis and, thus, are involved in the overall structural and functional maintenance of the cornea.

LOX has an important role in the formation of cornea ECM by oxidative linkage of collagen [108], and its activity is reduced in keratoconic eyes. Dudakova *et al*. observed LOX activity to be more than 2.5 times lower in KC corneas compared with normal corneas [109]. Shetty *et al*. reported similar results in a larger cohort and showed that the level of LOX decreased with increasing severity of KC [110].

COLIA1 and COLIVA1 are two major types of collagen required to maintain the normal corneal architecture. COLIA1 is found across the corneal stroma [111], whereas COLIVA1 is found predominantly in the epithelial basement membrane. COLIA1 is reduced during the early stages of KC and COLIVA1 is decreased in the advanced stages [110]. This could explain the stromal thinning seen during early KC and the involvement of Bowman's membrane in advanced KC [112].

In KC, the corneal stromal composition is altered, with a marked reduction in the amount of collagen types I, III, V, and XII, along with keratocan and lumican [105]. Declines in the interfibrillar distance of the collagen sheets and increase in abnormally configured proteoglycans have also been reported with progression of the condition. These changes result in increased contact between the collagen sheets and the proteoglycans, thus modifying the stromal architecture, where variations in interlamellar proteoglycans could contribute to slippage of the lamellae [113]. Massive realignment of the horizontal and vertical collagen lamellae has been encountered in KC, suggesting that both slippage and remodeling function in the redistribution of collagen fibrils [27]. In addition, KC corneas have decreased lamellar interweaving and reduced and/or lost lamellar insertion into the Bowman's layer [114]. The presence of agranular nonkeratocytic cells has also been demonstrated in KC corneas, and are suspected to have roles in the breakdown and phagocytosis of corneal tissues [115]. Two ECM glycoproteins, fibronectin and tenascin, are also present in the anterior section of KC corneas; however, these were not detected in the anterior region of normal or scarred corneas [116]. Given that the anterior section of the cornea is

engaged in the pathogenesis of KC [116], the expression of fibronectin and tenascin in this area is suggestive of roles in eliciting a repair response to keratoconic damage, given their key roles in corneal wound repair and healing.

Existing in vivo *models and their limitations*

In an effort to establish an animal model for hereditary KC, an inbred line of spontaneous mutant mice (SKC mice) with KC-afflicted corneas was developed that phenotypically mimic human KC corneas to a certain extent (conical cornea exhibiting apoptosis and elevated expression of c-fos protein in keratocytes) [117]. A striking observation was that the SKC mouse phenotype was exclusively observed in males but only in females injected with testosterone, demonstrating androgen dependency [117]. Linkage analysis revealed the predisposition locus for this androgen-reliant KC phenotype to be on mouse chromosome 17, mapped to a MHC region comprising the gene encoding sex-limited protein (*Slp*). The same group identified another animal model for hereditary KC (JKC mice), wherein the KC phenotype was seen in both males and females, unlike SKC mice, and displayed features reminiscent of human KC, such as conical corneas with some evidence of CCT [118]. The genomic locus responsible for the JKC phenotype was mapped to Chromosome 13. Despite resemblance to human KC corneas, both these murine models of KC showed some dissimilarities with human KC, such as the appearance of a red punctum at the apex in the SKC mice and the corneas in some JKC mice being thick and infiltrated with capillaries and hemocytes. Although both these putative murine models of hereditary KC have been valuable in generating insights into KC pathophysiology, given these discrepancies with human KC, there is a need to develop more relevant models recapitulating the *in vivo* KC corneal environment.

Strategies to establish relevant in vitro *models*

The first step towards establishing an *in vitro* model of KC was reported by Karamichos *et al.* [119] using hCFs and hKFs cultured in 3D Transwell, to examine differential ECM synthesis over 4 weeks of stimulation with vitamin C derivative with/without TGF-β1 or β3. The study showed increased endogenous matrix production (type I and V collagens), as well as a significant reduction in the expression of corneal scarring markers (collagen III and α-smooth muscle actin) when hKFs were cultured with TGF-β. Based on these results, *in vitro* models have been used to identify oxidative stress levels as the potential mechanism of KC progression [120] (Fig. 4). hCFs and hKFs exposed to TGF-β displayed low levels of keratocyte markers (collagens I, V, and keratocan), as well as increased fibrosis markers (collagen III), suggesting myofibroblastic differentiation. Furthermore, hCFs exhibited an increase in oxidative stress compared with keratocytes.

Adding further progress towards the development of *in vitro* corneal models was the establishment of a 2D corneal scar model by using primary corneal keratocytes exogenously stimulated with optimized concentrations of proinflammatory cytokines (IL-6, IL-8, and TGF-β1) [121]. This *in vitro* tissue model reflected phenotypic features akin to *in vivo* corneal scar with excessive deposition of fibrotic ECM (collagen type 1, fibronectin, tenascin, secreted protein acidic and rich in cysteine (SPARC), and glycosaminoglycan). Despite this *in vitro* corneal scar model being developed in a 2D microenvironment, it marks an important first attempt to develop an *in vitro* model of corneal scarring using a cocktail of proinflammatory cytokines. To further extend our knowledge in this direction, a 3D-bioprinted *in vitro* inflammation model has been developed [122,123]. Attempts are underway to target three significant aspects of scar formation: (i) targeting the noncovalent crosslinking of cytokines with silk, given that, *in vivo*, these cytokines form ternary complexes between the ECM-GAG-cytokines/growth factor (GF) receptors for cytokines and GFs; (ii) exploring the role of silk-gelatin bioink in improving inflammatory features of the developed model [123]. The cytokine crosslinked silk-gelatin bioink would help to provide sustained release of these cytokines in the *in vivo* scar and/or inflammatory microenvironment; and (iii) the role of 3D bioprinting technology in guiding and controlling the cell orientation and corresponding ECM deposition, simulating the precise architecture of the scar tissue [124] (Fig. 5). These *in vitro* models would help to identify novel biomarkers. Despite significant efforts to identify sensitive and specific biomarkers for KC, it has not been possible to correlate these to a definitive clinical outcome. Early detection of patients who are at risk of KC would be helpful to address future problem. Oxidative stress, antioxidant dysregulation, lipid peroxidation, NO signaling, damaged mitochondrial membrane potential, and altered biomechanics and collagen structure could be used as KC biomarkers.

Prolactin-induced protein (PIP), lipophilin-A, immunoglobulin J chain, cystatin S, secreted frizzle-related protein, lactotransferrin, zinc-alpha-2-glycoprotein (AZGP1), and TGF-β undergo crucial crosstalk in the prevention and prognosis of KC [125]. The *N*-linked glycosylation pattern of Gross cystic disease fluid protein (GCDFP-15; also known as PIP) in pathological as well as physiological conditions suggests that this protein differently binds to various targeted binding molecules and accordingly varies its mechanism in cellular microenvironments, depending upon whether it is glycosylated or unglycosylated [13]. We have conducted a study using the STRING database to determine the predicted protein–protein interactions in *in vitro* KC model using functional proteomic clustering to identify several crucial proteins related to protein binding, peptidase activity, and metallo-endopeptidase activity, including RUNX3, CST4, IL-6, AZGP1, ESR1, SMAD3, and SMAD2. These proteomic interlinks highlighted the strong correlation between PIP, hormones, and above mentioned proteins in KC, which may help to identify novel markers or association between various proteins in diseased condition (Fig. 6) [126].

Furthermore, a previous study successfully demonstrated that induced pluripotent stem cells (iPSCs) derived from healthy human keratocytes can be used for the treatment of corneal injuries when combined with a carboxymethyl-hexanoyl chitosan hydrogel [127]. Exploiting the potential of iPSCs generated from hKFs appears to be an interesting regenerative medicine approach for the modeling of KC. Instead of using a synthetic hydrogel-based construct, another interesting approach for developing a corneal disease model would be to use decellularized cornea. To address this, a comparative analysis of various decellularization techniques was performed to determine the most efficient strategy for decellularizing a cornea [128] to obtain corneal matrix that is devoid of all resident cell populations but retains its native chemical composition, ECM ultrastructure, and mechanical properties. Such decellularized matrices would enable researchers to induce the complex site-specific conjugation of biochemical motifs to accurately replicate the pathophysiological features of KC [129]. The modulation of secondary conformation of the collagen fibrils was assessed by using Raman spectroscopy. By using different

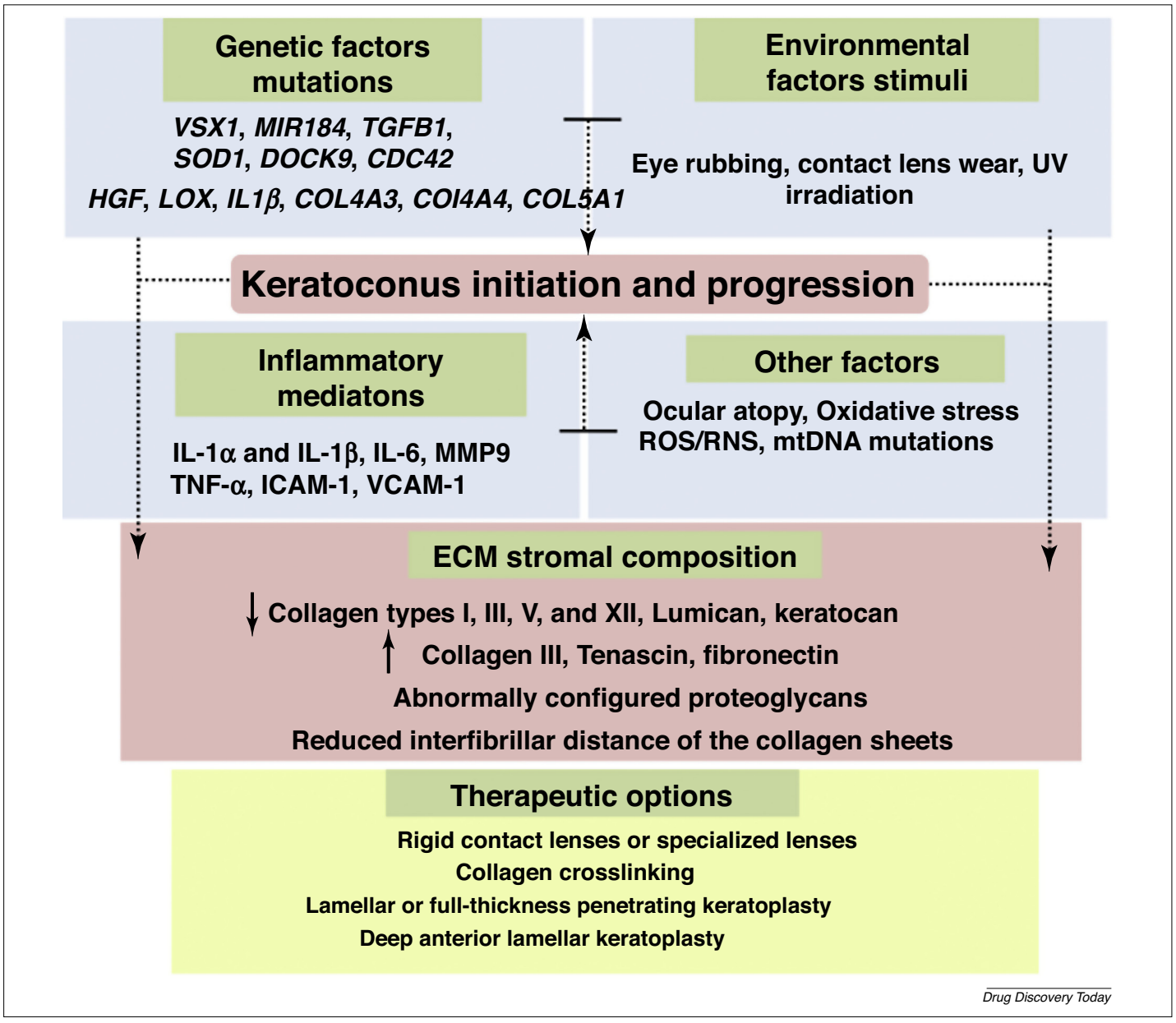

FIGURE 4

Schematic representing factors responsible for keratoconus (KC) initiation and progression, changes in the stromal extracellular matrix composition in KC and therapeutic options available for the treatment of KC. For definitions of abbreviations, please see main text.

concentrations of dextran and glycerol, it was possible to only partially restore the collagen conformational changes of the decellularized corneas [130]. Hence, further research is needed to generate insights into such decellularized corneal matrix characterization to develop relevant *in vitro* disease models of KC.

Concluding remarks and perspectives

Although the number of treatment options that help restore sight in patients with KC has increased, one of the major challenges hindering breakthroughs in the optimal management of this disease is the lack of ultrasensitive diagnostic strategies for the early detection of patients with subclinical asymptomatic KC. Diagnostic tools that clinicians currently rely on include computerized video keratography (several clinical indices have been introduced to increase the sensitivity of these tools) and eye imaging surveyors and/or instruments, such as Pentacam and the Orbscan I and II [131]. Although these tools are sensitive and specific in differentiating between KC, subclinical KC, and normal cornea to a reasonable extent, they are hampered by occasional false-positive results. This emphasizes the need for more accurate and ultrasensitive diagnostic marker tools for early screening of the asymptomatic cases, for effective management of this pathology.

There are enough data to support an inherent underlying genetic cause of KC. Adding further to this insight, improved understanding of the contribution of other environmental and lifestyle factors, which has led to epigenomics, should be at the forefront of our understanding of this pathology. Hence, there is a need to accurately understand the influence of and/or control

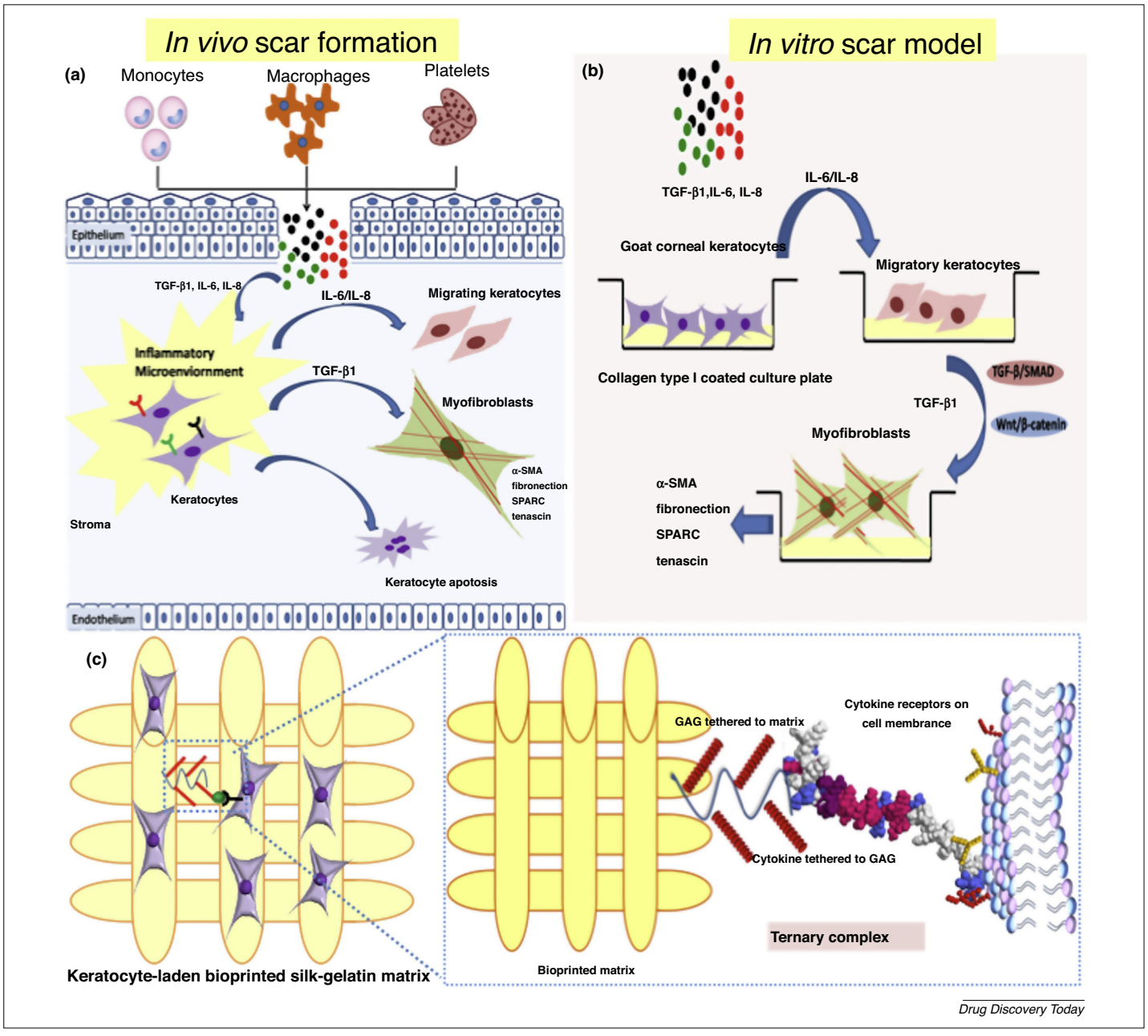

FIGURE 5

Strategies to develop inflammation mediated corneal scar tissue model. (a) *In vivo* corneal scar formation and role of inflammatory cytokines in creating an inflammatory microenvironment and differentiation of keratocytes to myofibroblasts. (b) Establishment of *in vitro* corneal scar model using a set of optimized proinflammatory cytokines. (c) Replication of the multimeric complex formation using silk-gelatin hydrogel and noncovalently attached cytokines in a 3D bioprinted corneal scar model. For definitions of abbreviations, please see main text.

exhibited by epigenetic variants in guiding KC disease biology. Given that genetic and epigenetic abnormalities usually work in tandem in reprogramming events underlying a pathology (as seen in cancer), there could well be an unidentified crosstalk between the genetic and epigenetic regulatory layers underlying KC. Elucidating this multilayered relationship is an immediate research challenge, which could be key for unraveling the etiological determinants of this complex multifactorial disease. Only upon untangling this crosstalk could epigenetic therapy can be incorporated in the clinic, a concept that we have begun to appreciate only recently.

Despite KC being a relatively common corneal dystrophy, its etiopathogenesis remains poorly understood. One of the significant rate-limiting factors is the scarcity of corneal tissues available for research, owing to tissues being obtainable only during corneal transplantation surgery or postmortem. A good model to consider for KC research is a human cell-based 3D *in vitro* tissue-engineered model of KC that could closely reflect the features of *in vivo* diseased tissue. Although the establishment of hereditary animal models of KC has been attempted [117,118,132], the resulting phenotypes are not completely reminiscent of the human condition. Moreover, because not all KC cases are caused by hereditary

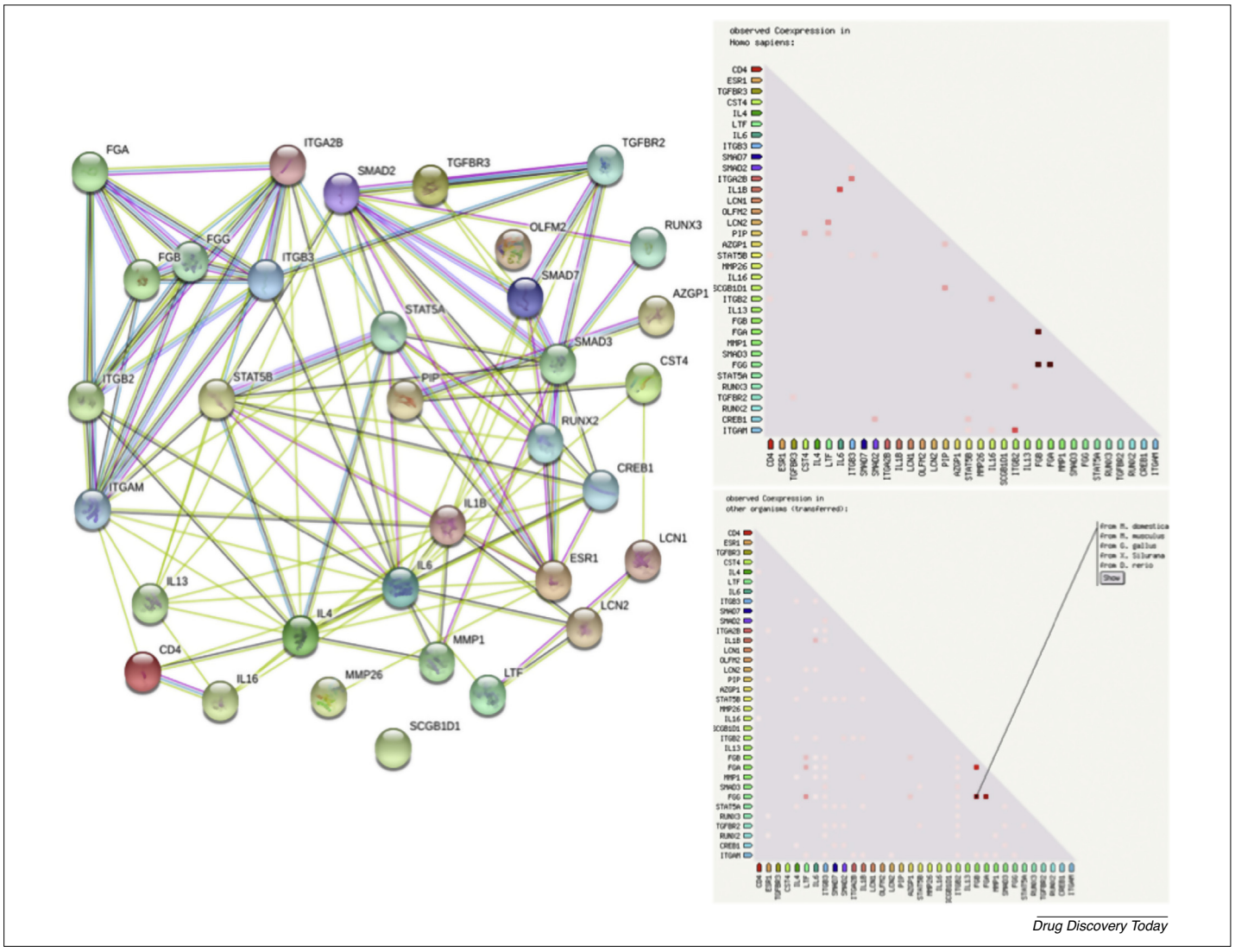

FIGURE 6

Protein–protein interaction and co-expression analysis performed using STRING V 10.0 with respect to differential proteins involved in keratoconus (KC).

factors (e.g., sporadic KC and some familial KC), 3D tissue-engineered models could be relevant alternative models to investigate the role of hereditary as well as other factors governing this disease, or to screen the efficacy of new drug molecules.

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