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Review article

Challenging corneal diseases and microRNA expression: Focus on rare diseases and new therapeutic frontiers

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ABSTRACT

MicroRNAs (miRNAs) function as posttranscriptional regulators of gene expression by targeting specific messenger RNA (mRNA). This interaction modulates mRNA stability or translational efficiency, ultimately impacting the level of protein production.

Emerging evidence suggests that miRNAs act as critical regulators in corneal diseases. These molecules finetune key processes like cell proliferation, differentiation, inflammation, and wound healing.

We reviewed the literature to understand the role that miRNAs may play in the development of challenging and poorly understood corneal diseases. We focused on vernal keratoconjunctivitis, neurotrophic keratitis, keratoconus, Fuchs endothelial corneal dystrophy, and limbal stem cell deficiency.

Furthermore, we explored currently studied agonists or antagonists of miRNAs that share similar pathways with ocular diseases and could be employed in ophthalmology in the future.

The distinct miRNA expression profiles observed in different ocular surface pathologies, combined with the remarkable stability and relatively easy access of miRNA sampling in biofluids, present possibilities for the development of noninvasive and highly accurate diagnostic tools. Furthermore, comprehending miRNA's pathophysiological role could open new frontiers to a more comprehensive understanding of the pathophysiology underlying ocular surface diseases, thereby paving the way for the creation of novel therapeutic strategies.

1. Introduction

In recent years, microRNAs (miRNAs) have emerged as key players in the pathogenesis of various diseases, including ocular disorders. Due to their stability and tissue-specific expression patterns, miRNAs have garnered considerable interest as potential biomarkers for disease diagnosis, prognosis, and therapeutic targets.[20,44,94]

In corneal diseases, miRNAs play pivotal roles in regulating key biological processes such as cell proliferation, differentiation, inflammation, apoptosis, and wound healing. Dysregulation of miRNAs in the ocular surface tissues can contribute to the development and progression of various ocular diseases, including dry eye disease, allergic conjunctivitis, and meibomian gland dysfunction.[8,65]

The diagnostic potential of miRNAs in corneal diseases lies in their stability and presence in various biofluids and cells, such as tears and conjunctival epithelial cells. Noninvasive collection of these samples makes miRNAs attractive candidates for developing reliable diagnostic tools.[26]

Through *in vitro* and animal model studies, researchers have elucidated the functional roles of specific miRNAs in regulating key cellular

Abbreviations: AEs, adverse events; AGO, argonaute; CEPC, corneal epithelial progenitor cells; CHCECs, cultivating corneal endothelial cells; DNK, diabetic neurotrophic keratitis; DGCR8, DiGeorge syndrome critical region gene 8; ECM, extracellular matrix; FECD, Fuchs endothelial corneal dystrophy; HS, healthy subjects; HSV, herpes simplex virus; HCEC, human corneal epithelial cells; LEC, limbal epithelial stem cells; KC, keratoconus; LSCD, limbal stem cell deficiency; LNA, locked nucleic acid; MRNAs, messenger RNAs; MiRNAs-miR, microRNAs; MI, myocardial infarction; NOX4, NADPH oxidase 4; NK, neurotrophic keratitis; HCEnCs, non-proliferative human corneal endothelial cells; PUK, peripheral ulcerative keratitis; Pri-miRNA, primary miRNA; RTK, receptor tyrosine kinase; RhNGF, recombinant human nerve growth factor; SAEs, serious adverse events; SIRT1, silent mating type information regulation 2 homolog 1; TLR3, toll-like receptor 3; TGF-β1, transforming growth factor beta; VEGF, vascular endothelial growth factor; VKC, vernal keratoconjunctivitis; Wnt, wingless-related integration site.

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processes implicated in corneal diseases pathogenesis. This knowledge not only enhances our understanding of disease mechanisms, but also opens new avenues for the development of targeted therapies aimed at restoring miRNA dysregulation and improving ocular surface homeostasis.[65]

In this review, we examine what is already known about the role of miRNAs at the level of corneal diseases, focusing on rare and orphan conditions. We decided to exclude more common diseases such as dry eye disease or Sjögren syndrome that have been extensively reviewed elsewhere.[3,64,65,91] We aim to stimulate ophthalmologists to explore this new field of research that could potentially improve our management of corneal diseases.

1.1. MiRNA synthesis and silencing mechanisms

MicroRNAs are small, noncoding RNA molecules that regulate gene expression post-transcriptionally by binding to specific messenger RNAs (mRNAs) and modulating their stability or translational efficiency.[6] Since their discovery in 1993, miRNAs have been linked to a wide spectrum of cellular mechanisms and shown a deep involvement in numerous pathologies.[4] The mechanisms underlying the biosynthesis and regulation of miRNAs have been studied in depth. The known biosynthetic mechanisms involve 2 main pathways: canonical and noncanonical.[69]

The canonical pathway is initiated by the transcription of miRNA genes by RNA polymerase II, resulting in the production of a primary miRNA (pri-miRNA).[7] Within the nucleus, the pri-miRNA undergoes cleavage, mediated by a complex known as Microprocessor, to form approximately a 60-nucleotide stem-loop structure known as pre-miRNA. The Microprocessor complex comprises Drosha and DiGeorge syndrome critical region gene 8 (DGCR8). Drosha is a member of the RNase III enzyme family that exhibits double-stranded RNA-specific endoribonuclease activity.[42] The 2 molecules of the partner protein DGCR8 serve as a double-stranded RNA binding protein, functioning as the noncatalytic component of the Microprocessor complex.[56] Subsequently, pre-miRNA is transported to the cytoplasm through the action of exportin-5 and Ras-related nuclear protein, a small GTP-binding protein.[49] In the cytoplasm, the pre-miRNA is further processed by another endonuclease with 2 RNase III domains named Dicer, coupled with its partner protein (TRBP in mammals).[28]

The resulting miRNA duplex (miRNA plus the passenger strand) is then loaded into the Argonaute (AGO) protein that determines the expulsion of the passenger strand and the formation of the mature miRNA.[13] The AGO-miRNA complex, alongside other co-factors, forms the mature silencing complex that can degrade the complementary mRNA by binding its 3'-untraslated region.[72] It's a key notion that each miRNA can bind a wide number of mRNAs because perfect pairing is not needed in mammals. Consequently, a single miRNA can regulate multiple targets participating in analogous cellular processes and pathways, thus magnifying the cellular response. Conversely, a particular mRNA can be subjected to targeting by numerous miRNAs.[63,70]

Several other noncanonical pathways have been described. These pathways are mainly Microprocessor-independent or Dicer-independent. An alternative pathway, initially elucidated in the generation of mirtrons, was identified wherein the usual Drosha-mediated processing step is circumvented, leading to the production of a small RNA precursor via mRNA splicing.[9] Similarly, in instances involving small RNAs derived from endogenous short hairpin RNAs, the Drosha-mediated processing step is also circumvented, with these RNAs being directly generated through transcription.[5] The presence of alternative pathways underscores the evolutionary adaptability of miRNA biogenesis. Nonetheless, it is important to recognize that only a small fraction, approximately 1 %, of conserved miRNAs, are produced through these pathways. Therefore, caution should be exercised when interpreting the functional significance of noncanonical miRNAs.[28] Mature miRNAs' primary function is to modulate gene expression post-transcriptionally by binding to the 3′ untranslated regions of target mRNAs. This binding occurs through partial base pairing between the miRNA's "seed sequence" and complementary sequences in the mRNA. Gene silencing is achieved through two main mechanisms:

1. mRNA degradation: When there is perfect or near-perfect complementarity between the miRNA and the target mRNA, the target mRNA can be cleaved leading to its degradation and reduced protein production.
2. Translation repression: In cases where miRNA-mRNA binding is imperfect, the mature silencing complex can hinder translation initiation and elongation. This results in decreased protein synthesis from the target mRNA without mRNA degradation.[12,57]

1.2. MiRNA expression in human cornea

MiRNA expression and its role in human healthy cornea has been poorly studied, and knowledge is currently based on studies conducted in corneal diseases or in animal models. A deep understanding of the most expressed miRNAs and their pathways in healthy cornea would be necessary to develop a comprehensive model.

A well-functioning limbal/corneal epithelium relies on the orchestrated activity of numerous signaling molecules and pathways inherent to these cells. Recent discoveries concerning the interplay between stem cells and early transient amplifying cells within the limbal epithelium have shed light on emerging molecules such as miRNAs. miRNA expression profiling revealed the preferential expression of the miR-103/107 family in the limbal epithelium, influencing various characteristics of epithelial stem cells such as quiescence, proliferative capacity, cell-cell communication, as well as macropinocytosis and end-stage autophagy.[60] The continuous renewal of the corneal epithelium relies on specialized undifferentiated corneal epithelial progenitor cells (CEPC) found in the basal epithelium of the limbus, a vascularized ring situated between the clear cornea and the bulbar conjunctiva. This mechanism ensures a stable cell population for the regular replacement of corneal epithelial cells and is also activated during wound healing processes. In a previous study, the investigators observed that the miR-143/145 cluster showed the highest expression in the human limbal-peripheral corneal epithelium, which contains CEPC, compared to the central corneal epithelium lacking CEPC.[41] In a follow-up study performed on 9 human corneas, MiR-184 and 638 were the top 2 most expressed microRNAs. When studying the different expressions between limbal epithelium and central corneal epithelium, specific microRNAs (miR-10b, 126, 127, 139, 142–3p, 143, 145, 155, and 338) displayed upregulation. Among these, miR-143 exhibited the most significant upregulation. Following miR-143, miR-10b was the next highly expressed microRNA, along with miR-126 and miR-388, whereas 4 microRNAs were significantly downregulated in limbal epithelium: miR-184, miR-149, miR-193b, and miR-575. The hypothesis is that these miRNAs may play a role in the formation and maintenance of corneal epithelium.[78] Apart from the microRNAs enriched in limbal epithelial cells, several microRNAs in corneal epithelium have been identified and characterized, including miR-31, miR-184, and miR-210. Investigators have demonstrated a distinct distribution of ephrin-A1 ligand in the limbal epithelium and EphA2 receptor in the corneal epithelium.[34] The equilibrium between EphA2 and ephrin-A1 maintains steady-state homeostasis at the limbal-corneal junction, and mir-210 could have the ability to modulate both the expression level and signaling of EphA2, thereby influencing enhanced migration during the healing process of corneal epithelial wounds. This characterization unveils a new mechanism for regulating EphA2 receptor tyrosine kinase (RTK) signaling by miR-210, indicating its potential significance in preserving a dynamic boundary between the limbus and cornea .[35]

Specific miRNAs in the corneal stroma have not yet been characterized, while some information is available on the corneal endothelium. The clarity of human vision relies on the transparency of the cornea,

which is upheld by a single layer of non-proliferative human corneal endothelial cells (HCEnCs). Any malfunction of these cells can lead to permanent visual impairment. It is crucial to pinpoint the pivotal factors that constrain the proliferation of HCEnCs. Studies are mainly based on cultivating corneal endothelial cells (cHCECs) from donor corneal tissue and have identified miR29, miR146a, and miR378 as key players in cHCECs functionality.[81,83]

miR-195–5p could potentially serve as a crucial target for therapeutic strategies aimed at enhancing the proliferation of HCEnCs. Inhibition of miR-195–5p resulted in a decrease in cell area, indicating increased proliferation leading to a more compact cell structure. Moreover, the expression of hallmark biomarkers of HCEnCs, including ZO-1 and Na+/K+-ATPase, was maintained. This suggests that inhibiting miR-195–5p can induce HCEnCs to proliferate while preserving their functional activity.[59]

2. miRNAs expression in challenging corneal diseases

2.1. Vernal keratoconjunctivitis

Vernal keratoconjunctivitis (VKC) is a severe disease from the allergic conjunctivitis spectrum affecting predominantly preadolescent males. When left untreated, the development of corneal scars can lead to severe visual acuity impairment, even though the disorder is usually self-limiting after puberty.[68] Early diagnosis of VKC could be useful for better disease management and prevention of severe corneal damage.

In this context, miRNAs could provide a better understanding of etiopathological mechanisms. This line of research could elicit new cortisone-sparing treatments and allow for fewer side effects in this young population. Some miRNAs, such as miR-19b and miR-146a, play an important role. MiR-146a's downregulation has been shown to enhance inflammation in allergic conjunctivitis.[27,98] miR-628–3p may regulate innate immunity by suppressing pathogen-associated molecular patterns such as toll-like receptor 3 (TLR3). Patients affected by atopic dermatitis and concomitant severe allergic keratoconjunctivitis had significantly higher plasmatic levels of this specific miRNA when compared to atopic dermatitis patients without ocular involvement.[85]

To our knowledge, just a single study has evaluated the miRNA profile in a limited number of VKC patients. This study identified 51 different miRNA expressions in the tears of VKC patients. The expressions of miR-1229–5p, miR-6821–5p, miR-6800–5p, miR-4466, miR-3665, miR-4530, miR-7110–5p, miR-1207–5p, miR-6875–5p, miR-762, miR-4741, miR-6740–5p, and miR-4298 were significantly increased, while the expressions of miR-7975, miR-7977, and miR-1260a were significantly reduced in VKC patients compared to healthy controls (Figure 1). Multiple intersecting target genes were forecasted to modulate inflammatory immune reactions in VKC through diverse pathways, encompassing the NF-kappa pathway, cytokine signaling pathway, and involvement of Treg cells; however, the small sample size (n = 4 per group) may limit the reproducibility of these results.[76]

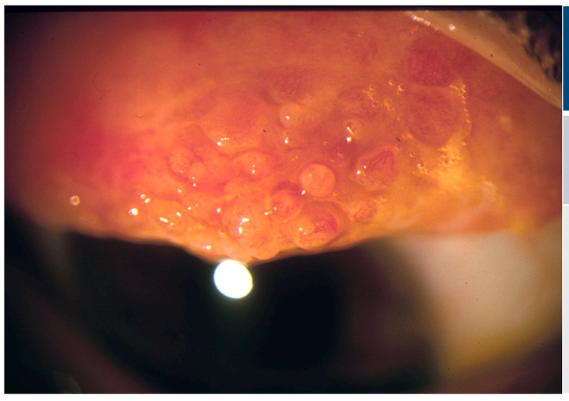
miR-4530 has been associated with the suppression of cell proliferation and regulation of inflammatory processes.[102] The observed increase of this miRNA in the tears of VKC patients could be a response to pro-inflammatory species and stress factors present during the disease. Increased expression of miR-762 has been shown to negatively regulate host defense genes in human corneal epithelial cells exposed to external antigens.[53] This could contribute to an abnormal corneal epithelial layer allowing greater allergen absorption, leading to increased activation of inflammatory responses. The study by Mun and coworkers also found increased expression of miR-1207 in the corneal epithelium in response to bacterial antigens, suggesting the involvement of miR-1207, upregulated in VKC patients, in host defense mechanisms. miR-4466 was found to be overexpressed in the exosomes of human corneal stromal cells from keratoconus patients compared to healthy controls. This increase was also observed in the tears of VKC patients and may indicate the potential involvement of miR-4466 in corneal surface integrity.[46]

2.2. Neurotrophic keratitis

Neurotrophic keratitis (NK) is a rare degenerative disease determined by systemic or local factors that lead to damage to the corneal nerves. The site of the damage can range from the trigeminal ganglion to the terminal nerves.[25] Most of the published studies conducted on human cells and murine models specifically focused on diabetic NK (DNK).

Funari and coworkers demonstrated first the impact of miRNA in impaired wound healing in diabetic corneas. The authors, employing microarrays, identified 29 miRNAs differentially expressed between normal and diabetic autoptic samples. The most expressed miRNAs (miR-146a and miR-424) were further tested by transfection of human corneal epithelial cells (HCEC) *in vitro*. Interestingly, the transfected cells showed a slower wound-healing process when compared to healthy cells, a mechanism reversed by the transfection with the corresponding inhibitor.[23] A further study involving miR-146a was conducted on both limbal epithelial stem cells (LEC) and human cadaver corneas. Results showed that miR-146a is upregulated in the limbal compartment when compared to the central cornea, and the expression of miR-146a is upregulated in diabetic corneas when compared to normal corneas. Moreover, the upregulation of miR-146a in cultured LEC determined a delay in wound healing. The inhibition of miR-146a through the administration of the relative antagomir determined a restoration of wound healing processes in both the cultured corneas and LEC.[92] Interestingly, miR-146a polymorphism rs1188095 shows a potential neuroprotective impact in a young population of type 1 diabetes patients.[62]

Another study conducted on circulating plasmatic miRNAs of type II



Vernal Keratoconjunctivitis

miRNA	Population studied	Reference
miRNAs were miRNA-1229, miRNA-6821, and miRNA-6800↑, miRNA-7975, miRNA-7977, and miRNA-1260a↓ (most significant)	Human tears	[76]

Fig. 1. On the left a representative image of VKC, papillary hypertrophy, cobblestone-like, of the superior tarsal plate; on the right, a table showing up- and down-regulated miRNAs in VKC. Vernal keratoconjunctivitis: VKC.

diabetes patients, demonstrated a downregulation of miR-92b-3p, while miR-22–3p was upregulated in patients with corneal nervous loss. Both miRNAs showed a consistent role in neuroinflammation and axonal regeneration.[37]

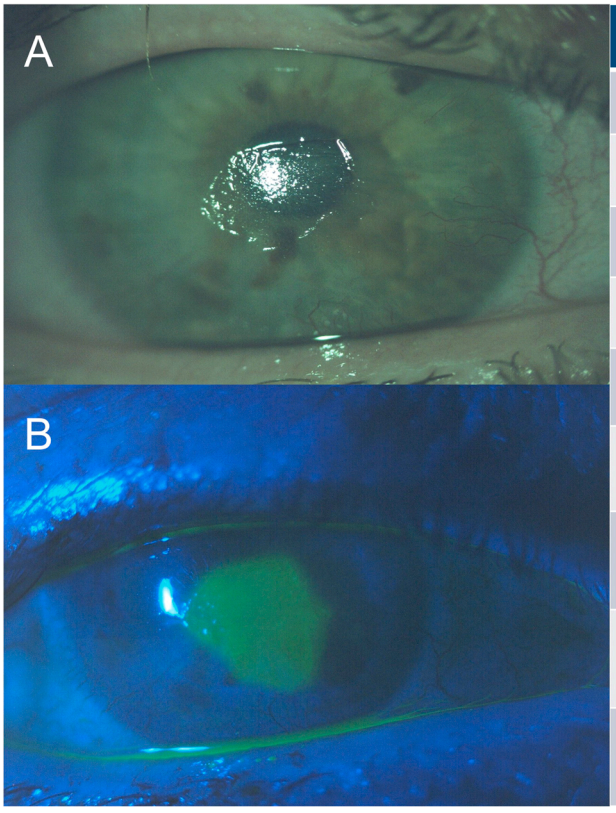
Hu and coworkers conducted a study on streptozotocin-induced diabetes in mice to evaluate the impact of miR-34c on peripheral neuropathy. Subconjunctival injections of the miR-34c antagomir were administered to diabetic mice. This treatment led to a noteworthy improvement in corneal nerve density and the healing of epithelial wounds, as opposed to diabetic mice in the untreated control group. These findings indicate that the miR-34c antagomir triggers the initiation of various signaling pathways essential for nerve regeneration.[31] Similar results were found by Hu and coworkers with miR-181a. They demonstrated that the inhibition of miR-181a, through the relative antagonist, was responsible for the increase in the regeneration of sensory corneal fibers and corneal epithelium. The effect was mediated by the BCL-2 inhibition of apoptosis.[32]

Recent interesting studies suggested a correlation between the activity of the longevity gene Silent mating type information regulation 2 homolog 1 (SIRT1) and the expression of several miRNAs. SIRT1 is a gene strongly downregulated in the diabetic eye, potentially responsible for the development of several key features of diabetic retinopathy and diabetic keratopathy.[55] Gao and coworkers verified that miR-204–5p was able to downregulate, at a post-transcriptional level the expression of SIRT1. They observed an upregulation of SIRT1 mRNA in murine limbal/corneal epithelium-derived progenitor cells (TKE2 cells) cultivated in a high glucose environment transfected with miR-204–5p inhibitor. Moreover, in a diabetic type 1 murine model they observed that the subconjunctival injection of miR-204–5p antagonist promoted wound healing.[24] The interplay between SIRT1 and the miRNA appears to be involved in the direct process of neurodegeneration linked to a high-glucose environment. Wang and coworkers postulated the existence of a SIRT1-miR-182-NADPH oxidase 4 (NOX4) axis responsible for the degeneration-regeneration of corneal nerves in a murine model. The researchers highlighted that the expression of SIRT1 and miR-182 is downregulated in trigeminal sensory neurons in diabetic mice. The administration of SIRT1 activators such as resveratrol was responsible for the replenishment of both SIRT1 and miR-182 expression. The treatment with miR-182 agonist was responsible for the promotion of neurite growth of diabetic trigeminal sensory neurons *in vitro*.

Moreover, when compared to negative-treated controls, the transfection with miR-182 agonist determined a reduction in the dimension of artificially induced defects in corneal epithelium and a consistent increase in corneal nerve density. These mechanisms are at least partially mediated by the downregulation of NOX4 induced by miR-182.[89] Lastly, in a recent study Compagnoni and coworkers evaluated the role of recombinant human nerve growth factor (rhNGF) on the miRNA profile in a human corneal cell line. They found that rhNGf, a currently-approved therapy for NK, is responsible for the regulation of over 20 miRNA (miR-26a-1–3p, miR-30d-3p, miR-27b-5p, miR-146a-5p, miR-362–5p, mir-550a-5p, mir-34a-3p, mir-1227–3p, mir-27a-5p, mir-222–5p, mir-151a-5p, miR-449a, let7c-5p, miR-337–5p, mir-29b-3p, miR-200b-3p, miR-141–3p, miR-671–3p, miR-324–5p, mir-411–3p, and mir-425–3p). Further analysis highlighted around 80 unique target genes, oftentimes of more than a single miRNA, involved in several cell functions such as survival and apoptosis.[15] Main results are summarized in Figure 2. All these pieces of evidence, despite being in early stages, suggest a strong relationship between the miRNA expression and the processes involved in epithelial wound healing and nerve regeneration in NK and DNK.

2.3. *Fuchs dystrophy*

Fuchs endothelial corneal dystrophy (FECD) is a rare, bilateral,



Neurotrophic Keratitis

miRNA	Population studied	Reference
miRNA-146a ↑ miRNA-424 ↑	Human corneal tissues and HCEC	23
miRNA-146 ↑	Human corneal tissues and LEC	92
miRNA-92 ↓ miRNA-22 ↑	Human serum	37
miRNA-34c ↑	STZ treated C57BL/6 mice, mice TG neurons	31
miRNA-181a ↑	Mice TG neurons, mice corneal epithelium	32
miRNA-204 ↑	Mice corneal epithelium, murine limbal/corneal epithelium-derived progenitor cell line	24
miRNA-182 ↓	Mice TG neurons, mice corneal epithelium	89
21 differentially expressed miRNA	rh-NGF treated human corneal epithelial cells	15

Fig. 2. Image on the left shows a central epithelial defect with rolled margins due to NK in green light (A) and with fluorescein staining (B); on the right, a table showing up- and down-regulated miRNAs in NK. human corneal epithelial cells: HCEC; limbal epithelial stem cells: LEC; neurotrophic keratitis: NK; trigeminal ganglion: TG; recombinant human nerve growth factor: h-NGF; streptozotocin: STZ; upregulated: ↑; downregulated: ↓.

progressive disease that determines progressive opacity of the cornea with visual impairment.[2] FECD is caused by the loss of endothelial cell function with stromal edema and corneal scarring.[51] Numerous miRNAs have been reported in the pathogenesis of FECD. Pan and coworkers highlighted that miR-199b is highly hypermethylated in the endothelium of FECD patients. MiR-199b directly downregulates two zinc-fingers transcription factors Snai1 and ZEB1. These transcription factors are responsible for the deposition of extracellular matrix in FECD. The downregulation of miR-199b, determined by hypermethylation, causes an upregulation of Snai1 and ZEB1 with consequent aberrant deposition of the extracellular matrix. Thus miR-199b may represent a potential therapeutic target for FECD.[58]

In a similar experiment, Matthaei and coworkers demonstrated that 87 different miRNAs are downregulated in FECD endothelium when compared to healthy controls. They placed their focus on 3 members of the miR-29 family (namely miR-29a-3p, miR-29b-2–5p, and miR-29c-5p).[50] The miR-29 family is considered a potential key regulator of extracellular matrix (ECM) homeostasis, and it has been reported to have an antifibrotic effect in ocular tissue. Studies have demonstrated that miR-29b suppresses type I collagen in human tenon fibroblasts, and the miR-29 family induces the suppression of ECM proteins, including SPARC, collagen I, and IV, in human trabecular meshwork cells.[43,48] Similarly, the altered regulation of miR-29 appears to have an impact on the transcriptional and translational expression of its targets in the ECM, specifically collagen I, collagen IV, and laminin. This observation suggests a potential influence on the accumulation of subendothelial extracellular matrix in FECD.[50,75] In a follow-up experiment by the same group, the transfection of immortalized endothelial cells of FECD patients, with miR-29b, produced comparable results. They observed a significant reduction in the expression of abnormal ECM components, namely collagen type 1 alpha 1, collagen type 4 alpha 1, and laminin gamma 1. This result suggests that miR-29b may be an effective treatment for FECD in the future.[81] Figure 3 summarizes the main results of the studies mentioned in this chapter.

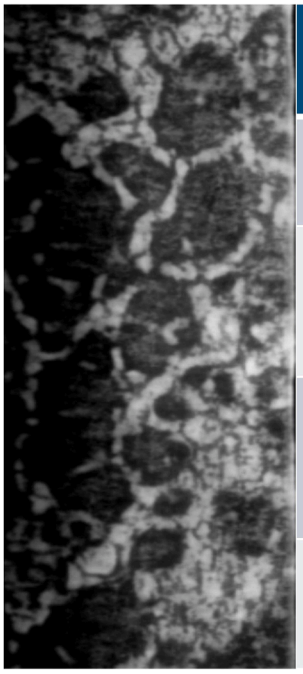
2.4. Limbal stem cell deficiency

Limbal stem cell deficiency (LSCD) is a rare condition in which the damage to limbal stem cells determines the progressive proliferation of the conjunctiva with consequent opacification and vascularization of the cornea.[29] The etiology of LSCD is wide; most commonly LSCD is caused by thermal or chemical burns, aniridia, or inflammatory eye diseases such as ocular mucus membrane pemphigoid.[29,82] Unfortunately, there is a paucity of published papers on this topic. Latta and coworkers evaluated the miRNA expression profile in the conjunctival tissue of patients affected by aniridia. Of the 2549 analyzed miRNAs, mir-204 resulted the most downregulated in corneas with severe neovascularization when compared to healthy subjects.[40] The downregulation of mir-204 plays a key role in angiogenesis. Specifically, it has been demonstrated that mir-204 negatively regulates angiopoietin-1 and the consequent Tie2/PI3K/Akt pathways in different models of corneal trauma.[36,47] Moreover, mir-204 downregulation may also promote migration and proliferation of conjunctival cells over the limbus into the cornea.[40] Other works have investigated the potential role of miRNAs as biomarkers in limbal stem cell transplantations. Ruiz and coworkers demonstrated that the concentrations of miR-6723–5p positively correlate to stem/progenitor cells in cultivated limbal epithelial cells, and therefore may function as a prognostic marker in limbal stem cell transplantations.[67] The main findings of these studies are outlined in Figure 4.

Further studies are needed to evaluate the impact of different miRNA expressions in the wide spectrum of etiologies that present as underlying causes of LSCD.

2.5. Keratoconus

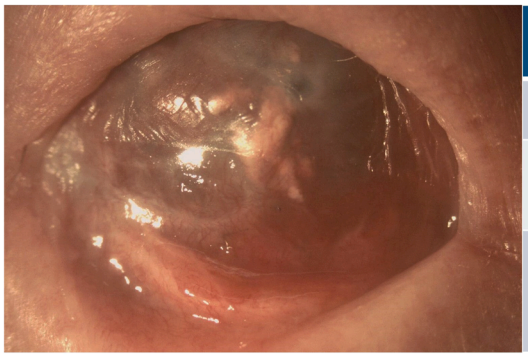
Keratoconus (KC) is a degenerative corneal disease characterized by progressive corneal ectasia. The etiology of KC, despite numerous years of research, is not understood. Nowadays, a thriving field of research is represented by the characterization of the microRNA profile at the level of the ocular surface in patients affected by KC. Few miRNAs have been proposed for a probable role in the etiology of the disease.



Fuchs' Dystrophy

miRNA	Population studied	Reference
miR-199b↓	Human corneal endothelium	58
miR-29 family↓	Human corneal endothelium	50
miR-29b↓	iFECD	81

Fig. 3. Image on the left shows a specular microscopy image with guttae at the level of the endothelial layer; on the right, a table showing up- and down-regulated miRNAs in Fuchs endothelial dystrophy. Immortalized Fuchs human corneal endothelial cell line: iFECD; upregulated: ↑; downregulated: ↓.



Limbal stem cell deficiency

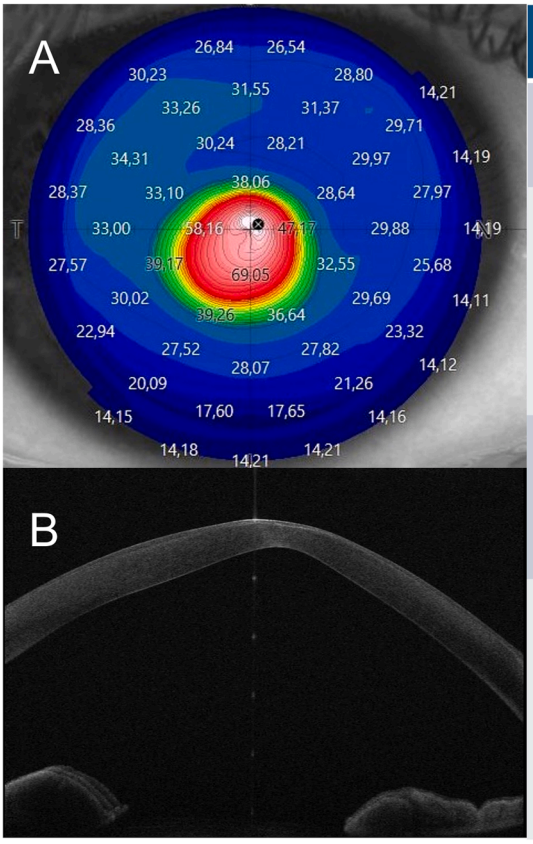
miRNA	Population studied	Reference
miRNA-204 ↓	Human bulbar conjunctival cells	40
miRNA-6723 (potential biomarker of limbal stem cells)	Human sclerocorneal tissues	67

Fig. 4. Image on the left shows the presence of limbal ischemia, conjunctivalization and epithelial defect following severe chemical burn; on the right, a table showing up- and down-regulated miRNAs in limbal stem cell deficiency. Downregulated: ↓.

In 2022, Zhang and coworkers conducted a study in which they analyzed miRNA patterns in the aqueous humor of keratoconic eyes. Four miRNAs (hsa-miR-7–5p, hsa-miR-193b-5p, has-miR-195–3p, and hsa-miR-199a-5p) resulted upregulated, while ten miRNAs (hsa-miR-28–3p, hsa-miR-222–3p, hsa-miR-363–3p, hsa-miR-95–3p, hsa-miR-181a-5p, hsa-miR-novel-chrX_13407, hsa-miR-320a-3p, hsa-miR-22–3p, hsa-miR-423–5p, hsa-miR-185–5p) were downregulated, for a total of 14 differently-expressed miRNAs between KC eyes and normal eyes. The supposed pathways involved include antigen response, endocytosis, and mismatch repair.[101]

Mir-195 has been linked to oxidative stress pathways,[99] which can contribute to corneal stromal degradation. Mir-199 appears to be involved in vascular endothelial growth factor (VEGF) down-regulation,[45] whose role has yet to be investigated. Mir-193 seems to play a role in regulating collagen production in other parts of the body.[33] Mir-181's different expression was described by Tian and coworkers in keratoconic corneas and associated with TGF-beta-induced gene and collagen (collagen type IV) decomposition pathways.[80] The altered expression of mir-181, mir-28 and mir-195 had also been previously reported by Wang and coworkers, together with 7other miRNAs (hsa--miR-151a-3p, hsa-miR-138–5p, hsa-miR-146b-5p, hsa-miR-194–5, hsa-miR-151a-3p, hsa-miR-185–5p, and hsa-miR-194–5p) both from corneal epithelium samples harvested after surgery and from corneal epithelial samples collected with impression cytology.[90]

Finally, it is known that mir-184 is highly expressed in the human cornea, and mutations have been proposed as causative in ectatic corneal diseases.[21]Mutations in a single base of mir-184 are linked to keratoconus, mostly associated with other ocular abnormalities.[74] mir-184 interacts with the wingless-related integration site (Wnt) signaling pathway, important for immune cell maintenance, renewal,



Keratoconus

miRNA	Population studied	Reference
miR-7, miR-193b, miR-195, and miR-199a, ↑ miR-28, miR-222, miR-363, miR-95, miR-181a, miR-novel-chrX_13407, miR-320a, miR-22, miR-423, miR-185 ↓	Human aqueous humor	101
mir-181 ↓	Keratoconus-associated RNA regulatory network.	80
miR-151a, miR-138, miR-146b, miR-194, miR-28, miR-181a ↓ miR-195, miR-151a, miR-185, and miR-194↓	Human corneas Samples of human corneal epithelium obtained from impression cytology	90

Fig. 5. The image shows: on the upper left, a steeper corneal curvature on the anterior tangential map (A) on the lower left, an AS-OCT image with severe thinning at the apex (B); on the right, a table showing up- and down-regulated miRNAs in keratoconus. Upregulated: ↑; downregulated: ↓.

and cell homeostasis. Its increased expression in the healthy human cornea and its link to KC and ocular abnormalities suggests a role of this messenger in corneal disease development.[1] Up and downregulated miRNAs in KC are summarized in Figure 5.

3. Future perspectives and conclusions

miRNAs are small RNA molecules that negatively regulate gene expression at the posttranscriptional level. Alterations in miRNA expression have been observed in various ocular pathologies affecting the cornea, suggesting their utility as diagnostic, prognostic biomarkers, and therapeutic targets.

We reviewed the literature to understand the role that miRNAs may play in the development of numerous ocular pathologies, including VKC, NK, KC, FECD, and LSCD; however, miRNAs involved in challenging diseases such as cicatricial conjunctivitis (e.g. ocular mucous membrane pemphigoid) have not been studied yet. miRNAs represent a new promising field of research that could lead to better understanding and managing these sight-threatening diseases. Ocular fibrosis has been studied in trachoma by Derrick and coworkers in 3 consecutive studies. Although an infectious disease, some pathways may be in common with chronic cicatricial conjunctivitis. In patients affected by severe scarring associated with active inflammation, mir-147b, and mir-1285 levels were significantly elevated.[17] Also, mir-184 was down-regulated[18]: in corneal wound healing and remodeling processes, chronic mir-184 downregulation may predispose infected corneas to uncontrolled scarring. Moreover, after 4 years of follow-up, patients subject to less severe scarring, at the beginning of the disease presented with highly down-regulated mir-184.[19] This confirms the necessity to further analyze the pathways linked to this miRNA.

Xie and coworkers have recently published an article suggesting the role of mir103a-3p in ocular fibroblast activation pathways in thyroid eye disease.[93] More specifically, the inhibition of mir103a-3p acts through the regulation of the transforming growth factor beta (TGF-β) pathway and therefore prevents fibrosis. TGF-β plays a key role in ocular cicatricial diseases as it influences the viability of activated fibroblasts, impacting their ability to deposit collagen lamellae thereafter.[77] Similarly, Ueta and coworkers showed that the upregulation of miRNA-455 in conjunctival tissue of Stevens–Johnson syndrome patients may be responsible for the development of the disease. miRNA-455 shows the capacity to regulate numerous immune related genes such as CXCL1,2 and 8.[84] In addition, the role of TGF-β this key regulator has been studied in glaucoma as well: miR-146 has been shown to modulate fibrosis by inhibiting TGF-β regulated myofibroblast transdifferentiation, while miR-29 family strongly inhibits the TGF-β pathway and therefore the deposition of extracellular matrix both at conjunctival and trabecular levels.[30,73] A large body of evidence have indicated that the TGF-β is also involved in systemic fibrotic diseases and is broadly influenced by miRNAs. miR-21, miR-29 family, miR-101, miR-29, miR-200, miR-942, and miR-193, can impact various fibrotic conditions through the regulation of TGF-β signaling pathway.[95] Peripheral ulcerative keratitis shares the same lack of direct evidence in the literature for miRNA regulation. Further studies will probably show the involvement of these small non-coding nucleotides in these autoimmune diseases as well.

As for the modulations of miRNA expression and activities numerous systems have been developed in recent years. Antisense oligonucleotides, or "antagomirs" act as mRNA binding inhibitors, allowing them to repress miRNA expression.[39,54] Locked nucleic acid constructs are conformationally restricted nucleotides supposed to increase the stability and affinity of the nucleic acids[5,61]. The microRNA sponges are another means to prevent mRNA binding, by sequestering the molecules and depressing their activity.[22] Table 1 represents a synthesis of therapeutic molecules currently in development in the pre-clinical stage mainly for vascular and neurological diseases. Despite notable advancements in pre-clinical investigations, the field of miRNA-based therapeutics remains is still in its early phase, with only a handful progressing to clinical trials.[14,71] Table 2 represents the clinical trials (phase I-II) that have been conducted or are presently ongoing for the development of new therapeutic molecules; however, none have advanced to phase III trials or received approval from the US Food and Drug Administration, and several have been discontinued due to concerns regarding toxicity. Specifically, clinical trial EudraCT 2015–001535–21 was terminated for serious hyperbilirubinemia, while NCT01829971 was terminated for severe immune-mediated adverse events that resulted in deaths. As Tables 1 and 2 show, therapeutic molecules antagonize miRNAs known to play a role in ocular diseases, suggesting a possible use of these new drugs in ophthalmology. For example, the mir-155 inhibitor, developed for hematological disorders,[86] may antagonize mir-155 activity in several infectious keratitis. On the other hand, other developing drugs, such as INT 1B3, a mir-193-a3p mimic, upregulated in keratoconus, may determine ocular side effects in the long term, not evident in phase I clinical trials. Conversely, other developing drugs in this field may determine ocular side effects in the long term: for example, INT 1B3, a mir-193-a3p mimic, which is upregulated in KC, may negatively affect the eye, even though no evident issues were detected during phase I clinical trials.

Table 1
miRNA drugs in development, preclinical stage for other diseases that share similar miRNA pathways alterations with ocular diseases. LNA: locked nucleic acid; MI: Myocardial infarction; ↑: upregulated; ↓: downregulated.

Therapeutic Molecule	Target miRNA	Target Disease	Potential ocular surface involvement	Stage of Development
MGN−1374	miR−15/195	Post-MI remodeling of the heart	Keratoconus↑[101]	Preclinical stage
MGN−2677	miR−143/145	Vascular disease	Pterygium ↓,[79] Corneal fibrosis and scarring ↑[66]	Preclinical stage
MGN−4220	miR−29	Cardiac Fibrosis	Fuchs dystrophy↓[50]	Preclinical stage
MGN−6114	miR−92	Peripheral arterial disease	Corneal nerve loss in diabetic patients, Sjögren's syndrome↓,[96] Herpes epithelial keratitis↑[38]	Preclinical stage
MRG−107	miR−155	Amyotrophic lateral sclerosis	Trachoma ↑[18]	Completed preclinical phase-Discontinued

These challenges underscore the need to address various hurdles for the widespread clinical adoption of miRNA-based therapies. Key obstacles include elucidating the sensitivity, specificity, and selectivity of miRNAs towards their target molecules, minimizing immunogenic reactions and unintended effects, refining delivery methods for precise targeting, and optimizing dosing regimens to achieve therapeutic efficacy while minimizing adverse reactions. In this perspective, the topical route that characterizes most of the ophthalmological therapies may prevent the onset of serious and systemic complications.

Furthermore, the clinical use of miRNAs is hindered due to incomplete knowledge of their multiple functions. In conclusion, the role of miRNAs in diagnosing and studying ocular surface diseases is a rapidly evolving field with immense promise. The unique expression patterns of miRNAs in various ocular surface diseases, coupled with their stability and accessibility in biofluids and cells, provide opportunities for non-invasive and precise diagnostic approaches. Additionally, the functional insights gained from miRNAs studies contribute to a deeper understanding of ocular surface disease pathophysiology and facilitate the development of innovative therapeutic strategies. Future research endeavors focusing on miRNAs profiling and functional investigations will

Table 2
Current and future Clinical trials with miRNA therapeutics for other diseases that share similar miRNA pathway alterations with ocular diseases. AEs: adverse events; SAEs: serious AEs; HS: healthy subjects; HSV: herpes simplex virus; ↑: upregulated; ↓: downregulated.

Clinical trial registration number	Treatment	Study design	Geographic area	N. patients	Primary outcome	Potential ocular surface involvement	Expected end date/last update	Study condition
NCT04675996	INT 1B3 miR-193a-3p mimic	Phase 1/1b, open-label, multiple ascending dose, first-in-human study	Europe/North America	40	Incidence of AEs and SAEs	Keratoconus ↑[101]	2023-24- 03 (terminated)	Advanced solid tumors
NCT03603431	MRG-110 /anti-mir-92a	Phase 1, Randomized, Double-blind, Placebo-controlled	United States	42	Incidence of AEs	Corneal nerve loss in diabetic patients ↓[37]	13-03-2019 (completed)	Skin wounds
NCT03601052	MRG-201/ Remlarsen /mir-29 inhibitor	A Phase 2, Double-blind, Placebo-Controlled Study	United States	14	Percentage of subjects with confirmed keloid formation at treated vs. untreated lesions at 24 weeks	Fuchs dystrophy ↓[81]	24-06-2020 (completed)	Keloid scars
EudraCT 2015-001535-21	RG101/ mir-122 inhibitor	A Phase2, randomized double-blind, placebo-controlled	Europe	32	Safety and efficacy of rg-101 in combination with oral agents in treatment naïve, genotype 1 and 4, chronic hepatitis	Pterygium ↓,[16] corneal transplants ↓[88]	27-10-2014 (terminated)	Chronic C hepatitis
NCT01727934	SPC3649/ Miravirsen/mir-122 inhibitor	A Phase 2, Open-Label	United States	10	Miravirsen study in null responder to Pegylated Interferon Alpha Plus Ribavirin subjects with chronic hepatitis C	Pterygium↓,[16] corneal transplants ↓[88]	01-01-2015 (status unknown)	Chronic C hepatitis
NCT01727934				10			01-01-2014 (status unknown)	
NCT01646489	as above	Phase 1, Open-Label, Drug Interaction Study	as above Europe	5	Safety, tolerability, and pharmacokinetics in HS	as above	30-09-2012 (completed)	Healthy volunteers
NCT00979927				30			30-09-2010 (completed)	
NL-OMON33484				30			15-09-2015 (completed)	
NCT00688012				64			01-05-2009 (completed)	
NL-OMON34513	as above	Phase 2a, Randomized, Double-Blind, Placebo-Controlled	United States/ Europe	16	Safety and tolerability and antiviral activity of miravirsen in subjects with chronic hepatitis C in naive patients	as above	27-01-2011 (completed)	Chronic C hepatitis
NCT01200420				38			01-12-2010 (completed)	
EUCTR2010-019057-17-DE				40			11-05-2010 (not recruiting)	
NCT02855268	Lademirsen/mir-21 inhibitor	Phase 2 Randomized Double-blind placebo-controlled study	United States, China	43	To assess the efficacy of Lademirsen in reducing the decline in renal function in Alport syndrome	neovascularization post-alkali-burn↑ [100]	22-09-2022 (completed)	Alport syndrome
NCT05953831	CDR123L / mir-132 inhibitor	Phase 2 Multicenter Randomized Double-blind placebo-controlled study	Unknown Europe	130	To assess the efficacy and safety of CDR123L in heart failure	corneal neovascularization post HSV infection ↑[52]	September 2025 (not recruiting)	Heart failure
NCT05350969				294			11-03-2025 (not recruiting)	
NCT02580552	Cobomarsen/MRG-106/mir-155 inhibitor	A Phase 1 Dose-ranging Study to Investigate the	United States	66	Safety, tolerability, and pharmacokinetics subjects with various lymphomas and leukemias	Trachoma ↑[18] P. Aeruginosa keratitis ↑[97] HSV-1 stromal keratitis ↑[10] corneal wound healing ↑[87] fungal keratitis↑[11]	06/10/2022 (completed)	Mycosis fungoides, chronic leukemia, diffuse large B-cell lymphoma, adult T-cell leukemia/lymphoma
NCT03837457	Cobomarsen/MRG-106/mir-155 inhibitor	Phase 2 Randomized Double-blind open-label active comparator study	United States, Europe, Australia	8	Safety and efficacy of Cobomarsen in cutaneous T cell lymphoma, mycosis fungoides subtypes	as above	27-07-2020 (terminated)	Mycosis fungoides, chronic leukemia, diffuse large B-cell lymphoma, adult T-cell leukemia/lymphoma
NCT03713320				37			01-12-2020 (terminated)	

undoubtedly pave the way for improved diagnosis, treatment, and management of ocular surface diseases.

4. Method of literature search

An advanced literature search was performed on PubMed central database, using "miRNA" or "microRNA" and a combination of other keywords referring to ocular surface disorders with Boolean operators AND and OR. Specifically, we searched for: “keratitis”, “keratopathy”, “cornea”, “corneal epithelium”, “corneal stroma”, “corneal endothelium”, "vernal keratoconjunctivitis", “neurotrophic keratitis”, “corneal dystrophies”, “Fuchs endothelial dystrophy”, “keratoconus”, “trachoma”, “limbal stem cell deficiency”, “cicatricial ocular diseases”, “Stevens-Johnson syndrome”, “graft-vs-host disease”, “Mooren ulcer”, “peripheral ulcerative keratitis” as keywords. Experimental, clinical studies on human, animal, and *in vitro* models and comprehensive reviews published in English or Italian regarding miRNA profiles on corneal diseases published between January 2000 and January 2024 were included. Information on ongoing and/or terminated clinical trials were obtained from the ClinicalTrials.gov website (https://clinicaltrials.gov) and the EudraCT website (https://eudract.ema.europa.eu)

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Luca Lucchino: Data curation. **Ludovico Alisi:** Writing – review & editing, Writing – original draft. **Marta Armentano:** Data curation. **Francesca Giovannetti:** Writing – review & editing, Writing – original draft. **Alice Bruscolini:** Supervision, Methodology. **Alessandro Lambiase:** Supervision, Project administration, Methodology, Conceptualization.

Declaration of Competing Interest

None

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to ensure good readability and proper English grammar. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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