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PhD THESIS

**Implications of microRNA modifications
at the ocular level**

ABSTRACT

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ABSTRACT

Introduction

Vision is our most important sense and plays a key role in every stage of our lives. Everyone, if they live long enough, will experience at least one eye disease in their lifetime. While some eye conditions usually do not cause visual disturbances such as blepharitis, conjunctivitis, or dry eye syndrome, others can cause serious visual defects. Among these harmful eye conditions, the main causes of global visual impairment are uncorrected refractive errors, cataracts, age-related macular degeneration (AMD), glaucoma and diabetic retinopathy (DR) .

In its inaugural World Vision 2019 report, the World Health Organization (WHO) , has recommended the inclusion of ophthalmic care in universal health coverage to improve access to improve and strengthen the ophthalmic branch in health systems worldwide.

Globally in 2020, it was estimated that 94 million people had moderate to severe visual impairment or blindness due to cataracts, this treatable disease being the first cause of blindness . In 2021. The World Health Assembly approved a resolution by all countries to increase the effective coverage of cataract operations during this decade, which will require improvements in both access and quality of services .

The general part

The eukaryotic genomes generate more than 100 non-coding RNAs (ncRNAs) .Among these ncRNAs, the microRNAs (miRNAs), which proves to be a key element in the regulation of fundamental cellular processes .MicroRNAs (miRNAs) are small (with an average of 22-25 nucleotides in length), evolutionary conserved non-coding RNA molecules involved in gene regulation. Since the discovery, thousands of miRNAs have been identified in different species including human, and the number of miRNAs continues to increase. The human genome includes approximately 2,600 mature microRNAs that regulate at least 60% of protein-coding genes, which indicates miRNAs' ubiquitous role in fundamental processes such as proliferation, apoptosis, differentiation, and development.

miRNA dysregulation is implicated in many diseases, such as cancers, cardiovascular diseases, neurodegenerative disorders. The miRNAs genetic variation is linked to some inherited diseases, such as hearing loss and growth defects . Given their biological importance, miRNAs are currently recognized as new disease biomarkers and potential therapeutic targets for the development of new interventions.

The main role of miRNA in the human body is gene regulation, by mediating the degradation of mRNA and also by regulating transcription and translation through canonical and non-canonical mechanisms.

Although most miRNAs have been detected in cells, surprisingly, significant numbers of miRNAs, commonly known as extracellular miRNAs or circulating miRNAs, have also been identified in the extracellular environment, including various biological fluids such as serum, plasma, urine, saliva, breast milk, colostrum, seminal fluid, tears, bronchial lavage, peritoneal and cerebrospinal fluid .

Unlike cellular miRNAs, which are rapidly degraded (within seconds) in the extracellular environment by high levels of RNase activity in plasma, circulating miRNAs are extremely stable, being resistant to RNase digestion, storage manipulation, multiple freeze-thaw cycles, or long-term storage at room temperature .

Over the past two decades, numerous studies recognize the clinical value of miRNAs in the diagnosis of virtually all major diseases, including cancers, cardiovascular diseases, and neurodegenerative diseases.

An ideal biomarker should be accessible via non-invasive techniques, be reasonably sensitive to distinguish early clinical symptoms and should be undetectable or low in healthy people. Their non-invasive or minimally invasive accessibility, relative stability, low complexity, and ease of detection via various techniques make circulating miRNAs ideal candidates as biomarkers for several diseases.

The evolving interest in miRNAs has also spread in the ocular field, where numerous miRNAs have been implicated in playing essential roles in human eye disorders, such as glaucoma, keratoconus, or corneal dystrophy.

Despite the recent advancements, there is still a significant lack of understanding regarding the expression of miRNAs in human ocular tissues. This knowledge gap has spurred numerous studies to comprehensively characterize all miRNAs present in both diseased and normal ocular tissue.

In addition to their role during eye development and photoreceptor maturation, microRNAs are essential for eye homeostasis by regulating the physiology of major eye-specific cell types such as photoreceptors, RPE cells, ganglion cells, and keratocytes. The effect of microRNAs on ocular homeostasis is further confirmed by a vast population of microRNAs expressed in the human eye, as revealed by deep sequencing analysis.

As with many other organs, microRNAs are key factors in the normal development and homeostasis of the human eye, but are also associated with several eye pathologies. Each component of the eye undergoes microRNA regulation, which can lead to disease if not properly regulated. The retina, choroid, sclera, cornea, and lens are thus the main components of the

eye that can be affected by microRNA dysregulation, leading to various diseases such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinitis pigmentosa, refractive errors, cataracts, uveitis, glaucoma, and keratitis.

Studying miRNA changes in eye diseases is important because miRNAs have been implicated in a variety of eye pathologies, including diabetic retinopathy, age-related macular degeneration, and cataracts. By understanding the changes of miRNA expression in these diseases, researchers can gain insight into the underlying mechanisms and potentially develop new therapeutic approaches. One of the main reasons for the importance of studying miRNA changes in eye diseases is their potential as biomarkers in disease diagnosis and prognosis. MiRNAs have been shown to be present in a stable form in various body fluids such as tears, aqueous humour and vitreous humour, making them readily available for analysis. Although the respiratory system is the main site of SARS-CoV-2 injury, various reports suggest that the eyes may be affected early or late in the disease process.

Available evidence suggests that SARS-CoV-2 infection may alter miRNA expression in ocular tissues, potentially impacting immune response, apoptosis and fibrosis. These changes in miRNA expression could contribute to the development of diverse ocular manifestations, including conjunctivitis, dry eye, and retinal complications, in COVID-19 patients. Further studies are needed to elucidate the specific miRNAs involved and their role in ocular pathologies associated with SARS-CoV-2 infection.

Potential ocular implications of changes in miRNA expression induced by SARS-COV-2 infection include increased sensitivity to ocular diseases and impaired ocular healing processes. Changes in miRNA expression can also affect the functioning of the ocular immune system, which can lead to the development of ocular inflammation and other immune-related conditions. In addition to these pharmacological treatments, there is also a potential for using miRNA-based therapies to target miRNA changes induced by SARS-CoV-2 in ocular tissues. These miRNAs have been found to be involved in the regulation of immune responses and inflammation, which are important processes in the development of ocular manifestations. Targeting these dysregulated miRNAs with mimic miRNAs or miRNA inhibitors may help modulate the immune response in eye tissues and reduce the severity of eye symptoms in COVID-19 patients.

In recent years, eye pathology has experienced diversification and aggravation, generated, on the one hand, by the aging population and, on the other hand, by the pandemic situation caused by SARS-Cov-2.

Paradoxically, the mechanisms involved in both aging process and the SARS-COV-2 infection, have common key points: mitochondrial dysfunction, oxidative stress, weakening of

defense mechanisms at the cellular and tissue levels, inflammation, and damage to control mechanisms at the genomic and transcriptomic levels.

In these new conditions, it is necessary to develop new procedures, equipment, and innovative molecules to face the new challenges.

Recently, non-RNA-coding microRNAs have emerged, the implications of which have been demonstrated in a large group of pathological conditions. These short RNA molecules therefore prove to be promising candidates for drug development.

Recent studies have focused on studying miRNA expression in all ocular tissues. Transcriptome analyses revealed unique tissue and developmental stage specific miRNA expression patterns in the retina, lens and cornea, suggesting their potential role in development, normal function and disease progression in ocular tissues. However, despite these advances, the specific functions and contribution of miRNAs to ocular tissues remain largely unknown.

The special part

miRNAs play an important role in the eye diseases pathogenesis, and studying their changes can provide valuable information for diagnosing diseases, mechanisms and potential treatment strategies. With technological advances and the development of more specific and sensitive miRNA analysis techniques, further research into miRNA changes in eye diseases is warranted.

The potential of miRNAs as biomarkers and therapeutic targets may have a significant impact on the future, for diagnosis and treatment of eye diseases.

Our study proposed new methods for investigating and treating cataracts, including the use of miRNA as biomarkers for pathogenesis and vitreous management strategies in surgery.

Crossing the pandemic period, SARS-Cov-2 has generated new challenges at the level of ocular pathology, with the eye being both an entrance gate and a connection to the respiratory and gastrointestinal systems. Additional research is required to explore the complex relationship between the SARS-COV-2 virus and the human eye. In this new and challenging context, our study investigated the changes produced by the virus at the level of particular miRNA species at the ocular level.

The present work represents the first contribution, at the national level, related to the mechanism of the involvement of particular species of miRNA in cataracts, simultaneously with the investigation of the molecular mechanism, at the level of these species of miRNA, of the involvement of diabetes in the pathology of diabetic cataracts.

The study aims to identify biomarkers and molecular therapeutic targets, of the miRNA type, in evaluating and treating some eye diseases. MicroRNAs, small non-coding RNA molecules that play a key role in regulating gene expression, are dysregulated in most diseases, including diabetes mellitus and cataracts.

Several studies have shown that there is a strong association between T2 diabetes and cataracts, with miRNAs playing a potential mechanism that connects these 2 diseases.

The miRNA profile in diabetic cataract patients has only been vaguely investigated . Besides, the identification of specific and non-invasive biomarkers (such as plasma miRNAs) would be a valuable asset in achieving early diagnosis and better management of the disease. Hsa-miR-19b, hsa-miR-34a, and hsa-miR-146a are miRNA species that have been previously linked to DM and/or cataract.

However, data on their plasma expression and potential interference with the occurrence of DM in cataract are missing. In this study, we aimed to determine to what extent the levels of has-miR-19b, hsa-miR-34a, and hsa-miR-146a are influenced by the presence of DM and whether this impacts the features of cataract. We used the qRT-PCR technique to investigate the expression profile of the three circulating miRNAs in a group of cataract patients with associated DM (diabetes mellitus cataract group [DMCG]), compared to an on-DM cataract group (cataract group [CG]).

In the present study, we made use of the sensitivity and specificity of the qRT-PCR technique to investigate the plasma expression levels of three miRNAs in patients with DM and cataract, compared to patients with cataract. We show that miR34a and miR-146 are significantly upregulated in the case group, while the miR-19b changes are statistically irrelevant.

Our data on miR-34a confirm previous findings in age-related cataract , conducted, notably, on lens or lens epithelial cells (LECs).

Our study showed a rather expected (given the critical role inflammation plays in the pathogenesis of diabetic complications) increased expression of plasma miR-146a in DMCG patients . In contrast, others found that circulant miR-146a downregulation is associated with DM type 2 susceptibility . However, there is a lack of consistency regarding the direction of miR-146a changes in various tissues from diabetic patients. In diabetic cornea, miR-146a upregulation seems to be associated with delayed wound healing, while in AH of diabetic patients, increased levels of miR-146a are associated with proliferative DR, although not statistically significant .

Although lacking sufficient statistical power, our findings also imply the expression level of miR-34a is higher in patients with damaged retinal tissue.

All three studied species of miRNA are involved in aging processes, processes associated with primary eye diseases such as cataracts. age macular degeneration, diabetic retinopathy. While miR34 is involved in majority of ageing process (apoptosis, altered DNA damage response, loss of telomeres, mitochondrial dysfunction, inflammation, alteration of stress response, vulnerability to oxidative stress) , miR 146 is involved in

inflammation(inflammaging) and mitochondrial dysfunction(mitomiRs) , and miR19 in the process of altered nutrient sensing.

Diabetes is an inflammatory state characterized by dyslipidemia, hyperglycemia, and hypertension, so it is not surprising that in our study, we have a statistically significant increase in the levels of inflammaging miR34 and miR146, in conditions of cataract-related diabetes . Several studies have shown that there is a strong association between T2 diabetes and cataracts, and miRNA changes have been identified as a potential mechanism underlying this association.

Our results show a significant upregulation of circulating miR34a and miR-146 in patients with DM and cataract, compared with non-diabetic cataract controls. This suggests that two miRNAs are involved in distinct pathophysiological mechanisms of cataract formation, depending on the presence or the absence of DM

Our study concluded that hsa-miR-34a and hsa-miR-146a can serve as potential biomarkers for the detection and evolution of cataracts in patients with T2DM, and also as therapeutic targets using antimir antagonists to reduce the upregulated miRNA species. Regarding the contributions to cataract post-therapy management these are included in the framework of the new concept of personalized medicine, a concept that also includes the choice of the therapeutic option depending on the individual and pathological characteristics.

The purpose of the study was to examine the destruction of corneal endothelium cells following cataract surgery between pars plana and conventional anterior vitrectomy, as well as posterior capsule rupture.

The main problems after posterior capsule rupture are the potential loss of corneal endothelial cells and barrier function, leading to corneal decompensation, edema, and vision loss. In this case, it is mandatory to restore the anterior chamber, preferably with dispersive viscoelastic, before removal of the phacoemulsification specimen. However, if vitreous prolapse still occurs, vitrectomy will be required. Because of this main risk of vitrectomy, the evaluation parameter of the two methods was minimal damage to the corneal endothelium. The goals of anterior vitrectomy are to remove the vitreous in the anterior chamber while maintaining lens capsule integrity, minimizing retinal traction and the amount of vitreous removed.

Two variants can be used to manage the posterior capsule rupture associated with vitreous prolapse: classical anterior vitrectomy using corneal incisions or pars plana vitrectomy.

Classic anterior vitrectomy is done using counter incisions, never the main incision. The use of infusion is recommended, while dry vitrectomy may be associated with hypotonia, myosis, and suprachoroidal hemorrhage. The infusion should use a different counterincision than vitrectomy, and a higher cutting rate is recommended to minimize vitreoretinal traction.

Pars plana vitrectomy is performed using an incision of about 3.5 mm from the limbus, usually with the 23 G vitrectomy, with or without trocar. The infusion can be performed through a corneal counterincision. Each of the two vitrectomy techniques has advantages, and the preferential use of one usually depends on the particular condition of the eye. Cataract surgery is invariably associated with a decrease in corneal endothelial cell density. This decrease is significantly more significant in surgery associated with vitrectomy and may be a risk factor for visual prognosis.

Our study was prospective, involving consecutive cases of cataract surgery post associated with posterior capsule rupture. Five patients in group A underwent classical anterior vitrectomy, while five patients underwent pars plana anterior vitrectomy in group B. The Stellaris phacoemulsification device (Bausch & Lomb, tm) and the associated vitrectomy device were used for all cases.

Our results are more relevant when the decrease in endothelial cell was evaluated as counts in absolute value and, respectively, in percentage compared to the initial examination.

The absolute decrease in endothelial cell count was statistically significantly ($p = 0.0038735$) higher in Group A ($314.8 \pm 108.0752/ \text{mm}^2$) than in Group B ($190.2857 \pm 89.1369/ \text{mm}^2$).

The decrease in endothelial cell density, as a percentage compared to preoperative examination, was statistically significantly different ($p = 0.0037455$), with a smaller decrease in Group B ($8.8129 \pm 2.745\%$) compared to Group A ($14.042 \pm 2.6847\%$).

The rate of corneal endothelial damage following pars plana anterior vitrectomy was significantly lower, both as a percentage of starting density and in absolute terms.

The main limitation of this study is the small number of patients enrolled. Even so, pars plana anterior vitrectomy was statistically more effective in protecting the corneal endothelium. For routine cases, with good corneal endothelial cell density, it does not necessarily bring a significant additional benefit. On the other hand, for cases in which the endothelium is damaged it can represent the difference between a good and a significantly worse visual prognosis.

Pars plana anterior surgery is a somewhat unfamiliar technique for anterior pole surgeons. However, it is easy to learn and brings a decrease in the rate of corneal endothelial damage.

The present study is the first national study of host-viral miRNA interaction at the ocular level. In the context of SARS-CoV-2 infection, many more molecular changes at the ocular level need to be elucidated to better understand COVID-19.

The main point of this study is to analyse the transcriptional modifications induced by the viral infection at the ocular level, mediated by miRNAs. We have managed to identify more miRNAs specifically involved in eye disorders that are strongly dysregulated by SARS-CoV-2.

The SARS-COV-2 pandemic worsened existing pathologies. The viral action was focused on weakening or neutralizing the host's defense systems, neutralizing the control systems at the transcriptomic and translational levels, and facilitating intrusion and extrusion at the cellular level. In this context, the virus also intervened by remodeling the normal cellular processes of the host, among which are its viral miRNA molecules. With SARS-COV-2 infection, we have a new and aggressive element in regulating gene expression at the transcriptional level of the host cell, including at the miRNA level. This element is the viral transcriptional regulatory apparatus, which includes miRNAs produced by the virus and will interfere with the host cell's normal gene expression regulatory system based on the host's miRNAs. In addition, more than one hundred small viral RNAs (sRNAs) derived from SARS-CoV-2 have been discovered and are predicted to interact with differentially expressed host mRNAs and miRNAs, playing a role in the propagation of SARS-CoV-2 by modulating post-transcriptional gene regulation. Many studies have used computational approaches to scan the SARS-CoV-2 genome for putative miRNAs and to predict viral miRNA targets on the virus and the human genome, as well as host miRNA targets on the viral genome.

In SARS-CoV-2 infection, there are several mechanisms that would cause miRNAs to affect the virus, such as interfering with viral replication, translating, and even modulating host expression. Recent studies have shown that both virus - and host-derived microRNAs (miRNAs) play a key role in the pathology of virus infection. The gene function analysis tools demonstrated that viral-derived miRNA candidates could target various human genes involved in critical cellular processes, including metabolism, defense system, and multiple signaling pathways involved in inflammation, oxidative stress, angiogenesis, and apoptosis.

Under these conditions, the profile of miRNAs involved in eye diseases changes, and most miRNAs are modified in these new conditions. The previous disease over which the coronavirus infection overlaps leads to a different profile of the miRNAs involved.

In SARS-COV-2 infection, the eye represents an important route of infection, the infection process being frequently associated with a wide variety of symptoms frequently associated with keratoconjunctivitis. The variety of eye symptoms and the abundance of miRNAs that can be found in the eye made us question whether the SARS-CoV-2 has a direct impact on eye miRNAs. Among the miRNAs known to be involved in ocular disorders, we specify the ones that were also found to be associated with COVID-19.

Related to this, we have managed to identify more miRNAs specifically involved in eye disorders that are strongly dysregulated by the SARS-CoV-2. We could identify associations between dysregulated miRNAs and AMD by looking for evidence in the literature, based on in vitro or in vivo studies conducted in animal models or human cells, that miRNAs may also directly contribute to the pathogenesis of AMD.

In the case of age-related macular degeneration (AMD) and COVID-19, among the associated miRNAs we have remark the following: miR-146, miR-125, miR-23, miR-20, miR-17, miR-574 and miR-223 . Most of them are known as inflamming miRNA or inflammamiRs, based on fact that the aging and viral infection have common element as suggestive of a dysregulated immune response to infection which could include exaggerated immunesenescence.

These identified miRNAs are involved in controlling the NF- κ B pathway, mTOR, TNF α , HIF signaling, the expression of the complement factor H (CFH) gene (components which modulates the innate immune), the Fas cell surface death receptor (FAS) gene in RPE cells, oxidative damage prevention, tumorigenesis, mitochondrial gene expression, and angiogenesis.

These miRNAs, especially miR-17 and miR- 146, can be used as potential biomarkers for AMD evaluation or therapeutic targets in ocular diseases, using mimics or antimir for miRNA targets. In the case of miRNA therapeutics, it must be considered that AMD is a multi-factorial disease associated with other comorbidities, which makes it difficult to point out the single miRNA as the therapeutic target.

Generally, every miRNA has multiple mRNA targets, leading to a cascade of events that changes the expression of many genes, increasing the chance of the off-target effect.

The attainment of these objectives required a transdisciplinary effort, leveraging the resources and the expertise of the Ophthalmology Discipline from UMF,, Victor Babeș,, from Timișoara, the Ophthalmology Clinic within the Timișoara Municipal Emergency Clinical Hospital, the Biochemistry Discipline from UMF,, Victor Babeș,, from Timișoara, and last but not least the support and guidance of the Doctoral School of Medicine-Pharmacy from UMF, Victor Babeș, from Timișoara.