International PhD Program in Neuroscience
XXXII Cycle
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miRNA expression profiles in vitreoretinal diseases

PhD Thesis

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AA. 2019

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Acknoledgements

I would like to thank prof. Claudio Bucolo and Prof. Michele Reibaldi, who have fully and patiently supported me during the years of my PhD studies. With their great experience and knowledge, they taught me how to approach the research in the field of ophthalmology.

My biggest thanks go to prof. Teresio Avitabile and prof. Robert Rejdak, their leaderships and their precious suggestions have been crucial in my professional and human training.

At least, my thanks go to my beloved parents and my whole family, for their efforts in supporting me during university studies, till the highest degree.

ABSTRACT

MicroRNAs (miRNAs) are non-coding small RNAs, which have been found to regulate gene expression at the post-transcriptional and translational levels. A lot of studies demonstrated that miRNAs regulate various cellular processes, including differentiation, development, aging, apoptosis, oncogenesis and metabolism. Moreover, dysregulation of specific miRNAs is associated with a variety of diseases, including fibrotic disorders. Identification of differenzial pattern expression of miRNAs could be useful for development of novel biomarkers and discovery of new pharmacological targets for human diseases.

The aim of our research was to investigate miRNAs regulation in vitreoretinal diseases, as the Macular Hole (MH), Epiretinal Membrane (ERM) and the Proliferative Vitreoretinopathy (PVR).

MHs can be seen in highly myopic eyes, or following ocular trauma, but the great majority are idiopathic. Full thickness MHs must be differentiated from pseudoholes caused by ERMs. This is important as ERMs are found in approximately two-thirds of eyes with MH. The macular ERM is a pathology caused by a fibrocellular proliferation on the inner limiting membrane (ILM) followed by cellular contraction. ERM can be either idiopathic or secondary to vitreoretinal diseases, such as proliferative vitreoretinopathy (PVR), diabetic retinopathy, and intraocular inflammation.

PVR is a clinical syndrome that occurs after rhegmatogenous retinal detachment (RRD) and its surgical repair, and despite technological advances in vitreoretinal surgery, it is the most common cause of failure in RRD surgery.

Identification of deregulated miRNA and associated pathways common to MH, ERM and PVR might help in the challenging search of biomarkers and novel therapeutic strategies.

Through our first profiling, we identified 5 miRNAs differentially expressed in patients affected by MH and ERM with respect to controls. More specifically, four were downregulated (miR-19b, miR-24, miR-155, miR-451) and 1 was upregulated (miR-29a); TaqMan® assays of the VH of patients affected by MH and ERM, with respect to controls, showed that the most differentially expressed were miR-19b, mir-24 and miR-142-3p. Our network data showed that deregulation of differentially expressed miRNAs induces an alteration of several pathways associated with genes involved in both MH and ERM. These results would suggest the possibility to exploit a possible ocular pharmaceutical RNA-based treatment against these differentially expressed miRNAs that might be administered to the patients affected by these slow-developing alterations, reducing invasive therapeutic approaches, such as vitrectomy.

Later, we identified an altered expression of 20 miRNAs in one or more pathological groups of VH of patients affected by primary RD and a different grading of PVR. The analysis has shown that the expression of 6 miRNAs (miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a) increased with the worsening of the disease and, according to the gene ontology analysis, they participate in biological processes of epithelial–mesenchymal transition (EMT) (such as cell cycle regulation, adhesion to extracellular matrix and regulation of actin cytoskeleton), in which differentiated RPE-derived cell help induces PVR.

To conclude, dysregulated miRNAs in MH, ERM and PVR patients, can target genes regulating pathways linked to mechanisms of fibrosis.

Our findings support the assessment of specific miRNAs as potential biomarkers and therapeutic targets in vitreoretinal diseases by means of further preclinical and clinical studies.

CHAPTER 1.

GENERAL INTRODUCTION

1. IDIOPATHIC MACULAR HOLE

Idiopathic macular hole (MH), as a kind of common macular diseases, involves tissue defects including the retinal internal limiting membrane (ILM) and even the photoreceptor (PR) layer [1], and it is known that vitreous traction on local retina, posterior vitreous detachment (PVD) around the macular fovea, and continuous adhesion are major etiological factors in most idiopathic MH cases [1–4]. These typically affects elderly patients, and approximately two-thirds of patients are females [5]. The main clinical manifestations of idiopathic MH include decreased vision, metamorphopsia, and central dark spots, which can occur suddenly or gradually. The incidence of MH among the common population has been reported to range from 0.2% to 0.8% [4, 6].

1.1.Vitreous Macular Traction in the Pathogenesis of IMH

Among the hypotheses concerning the pathogenesis of IMH, the most extensively accepted one is the exertion of direct A-P (anterior-posterior) traction by the posterior vitreous cortex on the macular fovea [1–3, 7, 8]. In healthy human eyes, the vitreous humor of the posterior vitreous cortex slowly passes through the optic disc, which realizes the separation of the posterior vitreous cortex and the posterior pole. In contrast, in human eyes at the risk of IMH formation, abnormal vitreous macular adhesions produce dynamic traction, and the contraction of collagen fibers in the longitudinal direction causes progressive anterior traction until avulsion of the Müller cap occurs [9–11]. According to recent research on the early stage of IMH formation, the strength of A-P traction was higher when the area of perifoveal vitreous detachment was smaller. However, tangential traction could not have an effect until the IMH had formed. Tangential traction is formed when the residual vitreous remains on the fovea after the PVD contracts [12], during which Müller cells proliferate and invade the ILM. When the proliferating glial cells on the ILM shrinks, the hole is enlarged [13–17].

Studies on the visual distortion of macular holes emphasize the role of the photoreceptor cell layer, which is the origin of the initial stage of the signal transduction pathway. The above exposition on the pathogenesis of the macular hole is from only the macroscopic level. Although this perspective emphasizes the role of the vitreous traction in the fovea, it does not allow for studies of occurrence and development of the macular hole at the cellular and molecular levels [18].

1.2.Surgical treatment

Modern macular hole surgery results in high closure rates of over 90% and good functional results especially in macular holes up to 400 μm in diameter. The standard of care in most of these cases consists of transconjunctival sutureless pars plana vitrectomy, peeling of the inner limiting membrane (ILM) around the hole, followed by gas tamponade and face-down positioning of the patient [19].

Inner limiting membrane is considered to improve anatomical closure rate; however, it is still questionable if peeling is necessary in holes less than 250 μm.

As closure rates and functional results decrease with larger macular hole diameters over approximately 400 μm, alternative surgical techniques have been introduced to improve anatomical and functional results in these cases. These techniques include the positioning of tissue within the macular hole to improve hole closure. This can be performed using an ILM flap or free flap technique and the transplantation of autologous retinal tissue, lens capsule or homologous amniotic tissue in or under the defect. An alternative promising approach is the attenuation of the rim of the hole by

induction of a localized retinal detachment at the posterior pole which is achieved by subretinal injection of balanced salt solution (BSS) using a 41 gauge needle. The operation is completed by an endotamponade using gas or silicone oil [19].

Moreover there are plenty of publications indicating that in the management of small and medium size hole (less than 400 μm), use of long-lasting gas and face-down position is not always required [20]. Ocriplasmin and expansile gas had been reported to be successful for management of small- and medium-sized holes and vitreomacular attachment [20].

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2. IDIOPATHIC EPIRETINAL MEMBRANE

An epiretinal membrane (ERM), also known as a macular pucker, is a condition affecting the avascular fibrocellular membrane over the central macular area between the vitreous and internal limiting membrane (ILM). Its pathogenic mechanism has an unknown etiology and can be idiopathic or secondary to other ocular diseases, trauma, or previous intraocular operation. The incidence of idiopathic ERM reportedly ranges from 2% in patients younger than 60 years to 12%–20% in those older than 70 years [1]. It may reduce visual acuity (VA) and cause micropsia, macropsia, monocular diplopia, metamorphopsia, or even progressive vision loss [2].

2.1.Pathogenesis

Some hypotheses of the pathogenesis of ERM have involved postulating the proliferation of fibroblasts, glial cells, and astrocytes after ILM disruption, following posterior vitreous detachment [3, 4]. Sebag et al. involve speculating that a residual posterior vitreous cortex (vitreouschisis), attached to the macula during the liquefying process of the vitreous body, may play a role in ERM development [5]. Kishi and Shimizu reported that premacular vitreous cortex, which forms the posterior wall of the premacular liquefied pocket, plays a key role in the development of idiopathic preretinal macular fibrosis in eyes with or without posterior vitreous detachment [6].

2.2.Surgical treatment

Pars plana vitrectomy with membrane peeling has been effectively used for the surgical treatment

of ERM since 1978 [7]. A high visual improvement rate, up to 90%, and a recurrence rate of 1%–16% have been reported after successful surgery [3, 8–11]. Currently, the surgical methods for membrane peeling have evolved because of the use of dyes. Triamcinolone stained the cortical vitreous and ERM although not the ILM [12], whereas indocyanine green (ICG), trypan blue, and brilliant blue G (BBG) were used to stain the ILM [13]. Some ILM peeling reports have revealed results such as improved VA outcomes, lower recurrence rates, and reduced retinal striae [14, 15]. The ILM peeling procedure is increasingly being used by retinal surgeons from 25% in 2008 to 44% in 2010 [16]. Although an increasing number of vitrectomy with ILM peeling has been reported, ILM peeling is believed to cause functional and mechanical damage to the Muller cells because the ILM is the basal lamina connected to the end feet of the Muller cells [17–19]. Whether to consider peeling of the ILM a surgical method for treating idiopathic ERM disease continues to be debated among vitreoretinal surgeons.

However a recent meta-analysis has shown that vitrectomy with ILM peeling results in better visual improvement in long-term follow-ups and lower ERM recurrence rates, and vitrectomy with only ERM peeling is more efficacious in reduction of CRT than is vitrectomy with ILM peeling [20].

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3. PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) is a clinical syndrome that occurs after rhegmatogenous retinal detachment (RRD) and its surgical repair [1], and despite technological advances in vitreoretinal surgery, it is the most common cause of failure in RRD surgery [2].

PVR can occur in eyes with RRD untreated, or after all types of retinal procedures, including retinal cryopexy, laser retinopexy, pneumatic retinopexy, scleral buckle and/or pars plana vitrectomy [3,4]. The prevalence of PVR varies among different studies, ranging from 5 to 10% [5, 6], and it has been estimated to account for about 75% of all primary surgical failures [7].

3.1. Classification of PVR

After the classification of the Retina Society Terminology Committee (1983) (Grades A-D) [8], and that of the Silicone Study Group (anterior and posterior forms of PVR) [9], Machemer et al.

[10] developed a new classification (1991) that includes informations about the location, extent, and severity of anterior and posterior PVR.

Three grades have been described:

Grade A: vitreous haze and/or pigment clumps in the vitreous cavity or on the inferior retina.

Grade B: wrinkling of the inner surface of the retina, retinal stiffness, vessel tortuosity, and/or rolled edges of retinal breaks.

Grade C: full-thickness retinal folds and/or subretinal bands; this grade has been further divided into 5 types, in relation to location of contraction (posterior or anterior in relation to the equator of the eye) [posterior (types 1 and 2), subretinal (type 3) or anterior (types 4 and 5)], and to the type of

contraction (1 focal, 2 diffuse, 3 subretinal, 4 circumferential, 5 anterior displacement), and namely:

- type 1, posterior, focal contraction, (starfold);

- type 2, posterior, diffuse contraction, (confluent starfolds, until to closed-funnel configuration);

- type 3, subretinal bands, posterior or anterior, (annular strand near disc; pigmented or nonpigmented linear strands; moth-eatenappearing sheets);

- type 4, anterior, with circumferential contraction (contraction along posterior margin of vitreous base with central displacement of the retina; peripheral retina stretched; posterior retina in radial folds);

- type 5 anterior, with anterior contraction, (vitreous base pulled anteriorly; peripheral retinal trough of varying width: ± stretching of ciliary processes; ± obscuration of ciliary processes by membranes; ± iris retraction) [10].

3.2. Risk factors

The most common preoperative, intraoperative, or postoperative risk factors for PVR development have been identified. Preoperative factors include trauma [5, 11], a history of prolonged intraocular inflammation (uveitis) or infectious retinitis, extensive detachments, large retinal breaks or giant retinal tears [11], multiple retinal breaks, retinal detachment associated with vitreous hemorrhage [12], choroidal detachment, chronic retinal detachment, grade A or B preoperative presence and extension of PVR, the duration of RD before corrective surgery, high levels of vitreal proteins, retinal detachment involving > 2 quadrants and duration, perforating injury and aphakia [13], and retinal detachment associated with abnormal vitreoretinal conditions (such as Stickler syndrome). Intraoperative factors include vitreous or subretinal bleeding, inability to fully close retinal tears,

intraoperative choroidal detachment [14], excessive cryotherapy [14, 15], pigment release during endodrainage [16], episcleral surgery or vitrectomy, caliper of vitrectomy, the use of intraocular gas and silicone [17]. Postoperative factors include prolonged inflammation or uveitis, new or persistent vitreous hemorrhage, choroidal detachment, and persistent traction or breaks [18].

Large areas of exposed retinal pigment epithelium (RPE) and breakdown of the blood-retinal barrier (during RRD or the surgery) are the key factors leading to PVR development [1].

Identification of pre-operative risk factors, recognition of the early signs of PVR, use of adequate surgical techniques and of pharmacological therapy can reduce the PRV incidence [1].

3.3. Pathogenesis

Although several aspects of the pathogenesis of PVR have been elucidated, many mechanisms leading to PVR development are complex and only in part known.

Histologically, PVR is characterized by the presence of contracting cellular or fibrocellular membranes with a progressive contraction [19].

Main types of cells found in epiretinal and subretinal membranes are RPE cells, Muller and glial cells, macrophages, fibrocytes, and myofibroblast-like cells: epiretinal membranes of PVR have a high number of proliferating cells, in particular glial and immune cells, whereas in membranes formed after successful surgery no glial cells and few immune cells can be seen [20].

In experimental models, proliferation during the first days of detachment involves Muller cells, but in the whole process, RPE cells have a central role: they de-differentiate into fibroblast- or macrophage-like morphology cells [21-23]. Three main (overlapping) biological process have been identified in the PVR development:

- cell migration (RPE cells migrate through a retinal break into the vitreal cavity, and glial cells migrate onto the retinal surface);
- cell proliferation (blood-retinal barrier damage leads to progressive exudation of blood components, such as fibrin, elastin, fibronectin, growth factors, and cytokines);
- contraction (collagen synthesis is evidenced by the presence of clearly demarcated membranes, which exert traction on the retina) [21-23].

Following a retinal break, cells within the retina and the underlying RPE are exposed to vitreous, which contains many growth factors and cytokines, including TGF ◎ (that experimentally stimulates the contraction of cells in a collagen matrix [24], the production of extracellular matrix proteins [25], and the transformation of RPE cells into fibroblasts/myo-fibroblasts [24]), Connective Tissue Growth Factor (CTGF) (that promotes migration and proliferation of cells [26], increases production of many extracellular matrix proteins [27], and increases fibrosis of epiretinal membranes [28]), Platelet-Derived Growth Factor (PDGF) (PDGF Receptor ◎ increases the ability of fibroblasts [29], RPE cells and Muller/glial cells to induce experimental PVR [30]), Vascular Endothelial Growth Factor (VEGF) (that determines the mode of PDGF Receptor◎ activation [31]), and many others [31]. This biological phenomenon is referred to as epithelial–mesenchymaltransition (EMT), in which differentiated RPE-derived cell help induces PVR [Figure 1] [32].

Also, the breakdown of the blood-retina barrier allows to serum to enter in the vitreous, with several growth factors [PDGF, [29] TGF-◎ [32], Tumor Necrosis Factor-◎ (TNF-◎) [34]], components of inflammation [complement, immunoglobulins, interleukin (IL) (IL-1 [35], IL-6, [34] IL-8, [34] IL-10

[34]), interferon ◎, [34] monocyte chemotactic protein [36], macrophage-colony stimulating factor, [36] granulocyte colony-stimulating factor [34]], cells (macrophages, granulocites, limphocytes) and other molecules (e.g., fibronectin [37]) [1, 20, 38].

All these factors stimulate cellular responses (migration, proliferation, survival and deposition of ECM) and lead to membrane formation and contraction (growth factor hypothesis) [18, 20, 38- 42].

The cellular composition of PVR membranes can also be modified by surgical intervention: silicone oil and heavy liquids may attract macrophages that produce cytokines and growth factors promoting PVR [43, 44]. PVR membranes can include also fragments of the retinal inner limiting membrane strongly adherent to retina [44], and areas of intraretinal fibrosis [45-47].

3.4. Therapeutic Approaches

Despite the progress in vitreoretinal surgery techniques, preventing and treating proliferative vitreoretinopathy (PVR) remain a serious challenge for vitreoretinal surgeons [48].

Several drugs have been used in order to prevent or reduce the development of PVR during or after retinal detachment surgery, with two main targets: the inflammatory cascade, or the cellular proliferation, including heparin and low molecular weight heparin in combination with steroids or 5-fluorouracil (5-FU), daunorubicin, isotretinoin, systemic or intravitreal steroids aand intravitreal methotrexate

However to date, there is no effective treatment targeting this condition and avoiding PVR; thus, an additional approach is required urgently [49].

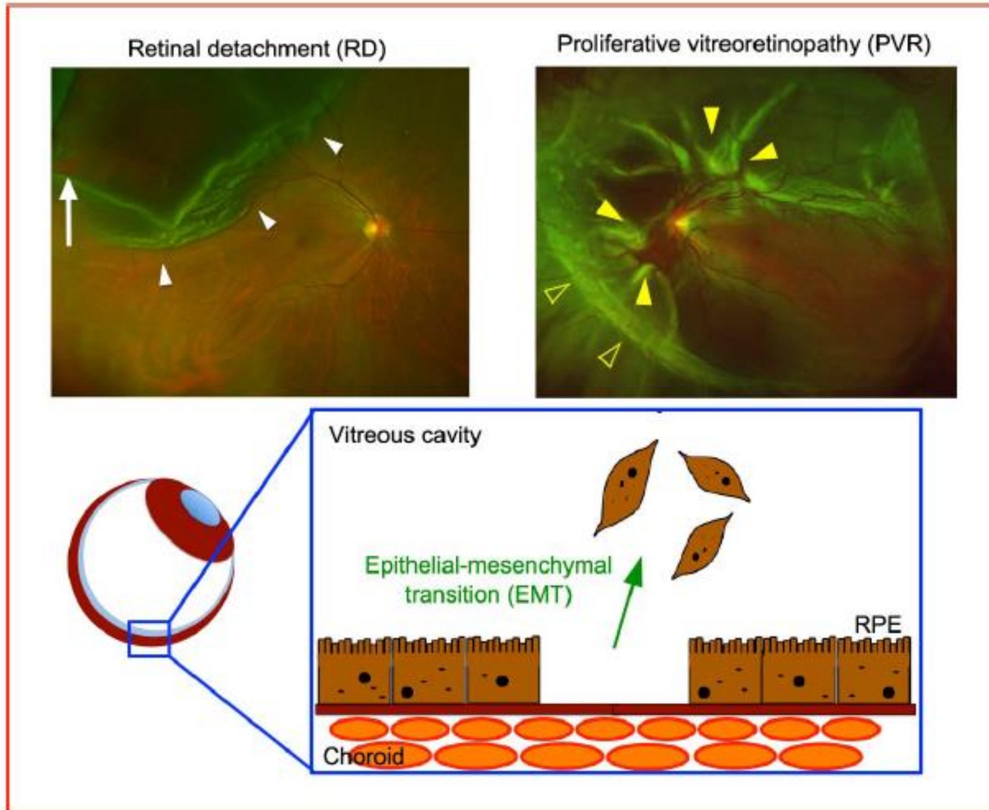

Figure 1. Illustration of pathogenesis of PVR. In eyes with RD (*left*), floating RPE cells receive certain biological signals, induce epithelial–mesenchymal transition, and RPE-derived fibrotic cells migrate on the surface of the retina. Color fundus image of PVR (*right*) showing wrinkling of the retinal surface, retinal stiffness, vessel tortuosity, and subretinal strands. The *white arrow* indicates a retinal break, and the *white arrowheads* indicate detached retina. The *yellow filled arrowheads* indicate wrinkling of the retinal surface, and *yellow open arrowheads* indicate epiretinal fibrotic membranes.

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4. MicroRNA and ocular diseases

Even after robust breakthroughs enabled by the whole human genome project, dozens of diseases remain of which the pathogeneses have not been elucidated perfectly. Unexpectedly, only 2% of the human genome is responsible for coding proteins [1].

Scientific approaches increasingly have begun to use transcriptome analysis. Scientists have recognized that thousands of noncoding RNAs are transcribed in the human body. One of the important but underestimated noncoding RNAs is microRNA.

MicroRNA is an extensive class of endogenous, noncoding, single-strand RNAs with 18 to 24 nucleotides that negatively regulate gene expression by interacting with the 30-untranslated regions (30UTR) of their target mRNAs. By modulating the expression of their target genes, microRNAs have essential roles in homeostasis and pathogenesis [2].

MicroRNAs have been detected in body fluids such as blood, saliva or urine. Moreover circulating microRNAs, found in plasma or serum, could be involved in cell-cell signaling [3-5].

In the human body, more than 2000 microRNAs reportedly are involved in cell proliferation, differentiation, and signaling. These microRNAs regulate cellular processes, including tumor formation, and have been linked to a number of human diseases; thus, the role of microRNA as a therapeutic target or a disease marker has been an active area of research [6-9].

In the eye, various microRNAs are thought to act on the retina or on RPE cells and to have important roles in neuroprotection and angiogenesis [10-13].

The number of publications that have described microRNA experiments and ocular diseases has been increasing (Fig. 1) [14] in the last years. Soon after the first use of the term ''microRNA'' in the literature in 2001 [15-17], articles in PubMed referencing the keywords ''microRNA'' AND ''eye'' appeared in 2001.

In 2006, articles in PubMed referencing the keywords ''microRNA'' AND ''retina'' appeared [Figure 2] [14]. As scientific technology and knowledge of microRNA has increased, the number of microRNA-related publications in ophthalmic research also has increased each year.

Since they can be easily evaluated through a blood draw, they could represent potential minimally invasive biomarkers used for screening and/or monitoring diseases [14].

Moreover, novel classes of chemically engineered oligonucleotides, termed ''antagomirs'' or ''antimiRs'', have been developed and proved to be efficient in the modulation of miRNAs levels, representing potentially future treatment options.

Therefore, identification of different pattern of miRNAs expression could be a potential approach in order to develop novel biomarkers and to discover pharmacological targets in human diseases, such as age-related neurodegenerative diseases.

Finally, once their role in the pathogenesis of human diseases will be definitely clarified, miRNAs could represent novel targets for drug development.

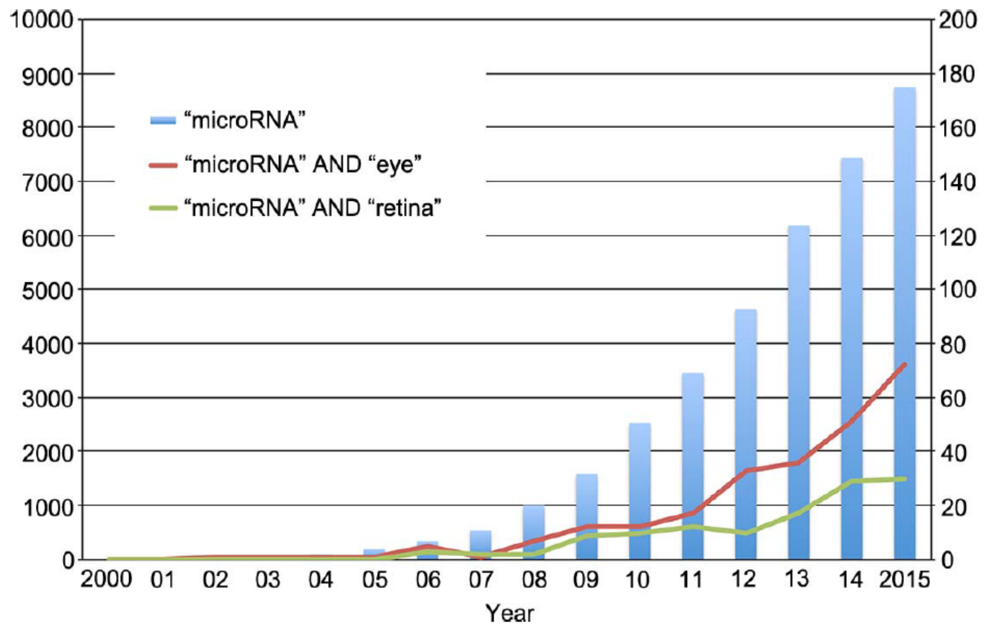

Figure 2. Growth of the number of microRNA-related publications. PubMed entries that reference the term "microRNA" are represented by the *blue bars*, those that reference "microRNA" AND "eye" are represented by the *red line*, and those that reference "microRNA" AND "retina" are represented by the *green line*.

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CHAPTER 2.

MICRORNAS IN THE VITREOUS HUMOR OF PATIENTS AFFECTED BY IDIOPATHIC EPIRETINAL MEMBRANE AND MACULAR HOLE

PLOS ONE

RESEARCH ARTICLE

miRNAs in the vitreous humor of patients affected by idiopathic epiretinal membrane and macular hole

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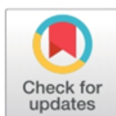

OPEN ACCESS

Citation: Russo A, Ragusa M, Barbagallo C, Longo A, Avitabile T, Uva MG, et al. (2017) miRNAs in the vitreous humor of patients affected by idiopathic epiretinal membrane and macular hole. PLoS ONE 12(3): e0174297. https://doi.org/10.1371/journal.pone.0174297

Editor: Manlio Vinciguerra, University College London, UNITED KINGDOM

Received: December 6, 2016

Accepted: March 7, 2017

Published: March 22, 2017

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authos received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Purpose

The aim of the present study was to assess the expression of miRNAs in the Vitreous Humor (VH) of patients with Macular Hole (MH) and Epiretinal Membrane (ERM) compared to a control group.

Methods

In this prospective, comparative study, 2-ml of VH was extracted from the core of the vitreous chamber in consecutive patients who underwent standard vitrectomy for ERM and MH. RNA was extracted and TaqMan® Low Density Arrays (TLDAs) were used to profile the transcriptome of 754 miRNAs. Results were validated by single TaqMan® assays. Finally, we created a biological network of differentially expressed miRNA targets and their nearest neighbors.

Results

Overall 10 eyes with MH, 16 eyes with idiopathic ERM and 6 controls were enrolled in the study. Profiling data identified 5 miRNAs differentially expressed in patients affected by MH and ERM with respect to controls. Four were downregulated (miR-19b, miR-24, miR-155, miR-451) and 1 was downregulated (miR-29a); TaqMan® assays of the VH of patients affected by MH and ERM, with respect to controls, showed that the most differentially expressed were miR-19b (FC -9.13, p:<0.00004), mir-24 (FC -7.52, p:<0.004) and miR-142-3p (FC -5.32, p:<0.011). Our network data showed that deregulation of differentially expressed miRNAs induces an alteration of several pathways associated with genes involved in both MH and ERM.

Conclusion

The present study suggests that disregulation of miR-19b, miR-24 and miR-142-3p, might be related to the alterations that characterize patients affected by MH and ERM.

Introduction

Vitreo maculopathies are characterized by traction exerted on the macula generated by the vitreous and the inner limiting membrane of the retina. Traction arising from vitreomacular adhesions can be tangential or perpendicular to the retinal surface. Both conditions might determine features of clinical pathologies including epiretinal membrane (ERM) and macular hole (MH); in these diseases epiretinal cell proliferations and fibrosis are essential parts of the pathogenesis [1–2].

Most MHs are idiopathic, however, they can also be found in highly myopic eyes, or after a ocular trauma [3]. Pseudoholes, secondary to ERMs, should be differentiated from full thickness MHs [4]. ERMs are detected in about two-thirds of eyes affected by MHs [5–6].

ERM can be idiopathic or secondary to several vitreoretinal diseases and is characterized by cellular contraction after fibrocellular proliferation on the inner limiting membrane. Posterior vitreous detachment (PVD) can injure the internal limiting membrane, allowing movement of glial cells to the retinal surface. Furthermore, an incomplete PVD might provide appropriate conditions for fibrocellular proliferation in the area between the vitreous and the retina [7]. In the process of ERM formation, extracellular matrix, cytokines and growth factors are involved in cellular signal transmission and in tissue changes [8].

Some studies have shown that a number of regulatory factors have also significant effects on fibrosis and may be related to its inter-organ variability [9].

miRNAs are small, non-coding RNAs with a strictly regulated biogenesis. This is combined with an extremely flexible and sophisticated regulatory function, allowing simultaneous targeting of multiple mRNAs coding for proteins involved in different, crucial biological pathways of specific cell types and tissues [10].

miRNAs exert control over cellular processes such as differentiation and proliferation acting on various targets [11], and may play the role of conductors in the pathogenesis of fibrosis [12].

miRNA alterations are common in different fibrotic disorders such as systemic sclerosis [13], liver cirrhosis [14], cardiac fibrosis [15–16], chronic kidney disease [17], and idiopathic pulmonary fibrosis [18].

The discovery, in 2008, of miRNAs circulating in human blood opened new intriguing perspectives in molecular diagnosis [19,20]. Circulating miRNAs have been shown to be present in several biological fluids (e.g., serum, plasma, cerebrospinal fluid, vitreous humor) in a stable form that prevents their digestion by RNases. However, little is known about the origin and function of circulating miRNAs. One of the most fascinating hypotheses is that extracellular miRNAs may work as mediators of cell-cell communication: specific miRNAs are selectively secreted by donor cells to be functionally transferred to recipient cells [21,22]. Currently, two major release mechanisms of circulating miRNAs have been proposed: (i) secretion of miRNAs stored inside microvesicles or exosomes [23]; (ii) secretion of miRNAs complexed to ribonucleoproteins [24]. Since concentration of circulating miRNAs is related to the physiological and pathological condition of patients, it is not surprising that they have already been exploited as molecular biomarkers for neoplastic and degenerative diseases.

Our group previously showed the presence of miRNAs in the Vitreous Humor (VH) and that the expression of circulating miRNAs in VH is altered in different eye diseases [25,26]. Recent reviews have addressed the role of miRNAs in fibrosis with a focus on organ-specific miRNA alteration [27–30] and both pathologies are caused by mechanisms related to fibrosis.

The aim of the present study was to assess the expression of miRNAs in the VH of patients with MH and ERM compared to a control group.

Methods

This prospective, comparative study included all consecutive eyes of patients who underwent vitrectomy at the Ophthalmic Clinic of the University of Catania, for MH and ERM, between September 2015 and April 2016. Controls were all consecutive eyes of patients, matched by age and sex, who underwent vitrectomy for primary symptomatic idiopathic floaters in the same period.

Floaters represent the least compromised condition for eyes to undergo vitreous surgery, since it is not possible to remove the vitreous from living healthy subjects.

The study adhered to the tenets of the Declaration of Helsinki and was approved by the Local Ethics Research Committee ("Comitato Etico Catania1"). Before the procedures, written informed consent was obtained from all participants in the study.

All eyes had idiopathic MHs with a minimum size > 250 μm [31] and idiopathic fovea-involving ERM, with prominent thickening of the inner retinal layer [32]. Both MHs and ERM were diagnosed ophthalmoscopically and with a Spectralis Optical Coherence Tomography (OCT) examination (Heidelberg Engineering, Heidelberg, Germany).

We excluded from our study patients with diabetes, cardiovascular failure, autoimmune diseases, renal or hepatic failure, Alzheimer's, and Parkinson's disease. We also excluded patients who had undergone previous ocular surgical procedures, affected by glaucoma, uveitis, diabetic retinopathy and other retinopathies, ocular trauma, and any ocular tumor, as the amount of vitreal miRNAs could be modified, depending on the diseases of the eye [25].

All patients underwent a 3-port 25-gauge vitrectomy performed by the same surgeon (M. R.) under local anesthesia. The Resight 700 (Carl Zeiss Meditec AG, Jena, Germany) wide-angle viewing system or the Binocular Indirect Ophthalmol Microscope wide-angle viewing system (BIOM; Oculus Inc, Wetzlar, Germany) were used. Sclerotomies were placed at 3.5 mm to the limbus and performed in a 30° fashion, parallel to the limbus [33]. With closed infusion, a 2-ml vitreous specimen was extracted from the core of the vitreous cavity into a syringe using a three-way tap. The extracted vitreous was then placed in a sterile container, and the vitrectomy continued as normal. Samples were centrifuged at 700 ×g for 10' to pellet and eliminate any circulating cells and finally stored at -80°C until analysis.

RNA extraction

Using a Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), RNA was extracted from 500-μl vitreous samples. RNA was eluted in a 30 μl volume of elution buffer with two repeated steps in the same collection tube. RNAs were quantified by fluorometery (Qubit, Invitrogen) and spectrophotometery (GeneQuant Pro, BioChrom Ltd, Cambridge, UK).

miRNA expression analysis

According to the manufacturer's instructions, 4.5 μl of vitreal RNAs were retrotranscribed and preamplified to profile the transcriptome of 754 miRNAs, and then loaded on TaqMan® Low Density Arrays (TLDAs) TaqMan® Human MicroRNA Array v3.0 A and B (Applied Biosystems, Foster City, CA, USA). PCRs on TLDAs were conducted on a 7900HT Fast Real Time

PCR System (Applied Biosystems). Results were validated by single TaqMan® assays and TaqMan® Universal Master mix II (Life Technologies, Italy) using 20 ng of vitreal RNA, according to the manufacturer's instructions.

Statistical analysis

To obtain an accurate miRNA profiling, we used the global median normalization method, as previously reported for the same kind of analysis [26]. By this approach, we identified small RNAs that presented an expression profile near to the median of TLDAs, i.e. snRNA U6 and miR-223. Accordingly, they were then used as reference genes for analysis of TLDAs. By Significance Analysis of Microarrays (SAM), differentially expressed miRNAs were identified, computed by Multi experiment viewer v4.8.1, by applying a two-class unpaired test among ΔCts and using a p-value based on 100 permutations; imputation engine: K-nearest neighbors (10 neighbors); false discovery rate < 0.05 was used as correction for multiple comparisons. The 2−ΔΔCT method was used to calculate the Expression fold changes (FC). SnRNA U6 was used as reference gene for single TaqMan® validation assays. The unpaired t-test ($p < 0.05$) was applied to statistically evaluate the expression differences between patients and healthy controls by single TaqMan® validation assays. Statistical significance was established at a p-value > 0.05. ΔCts for differentially expressed miRNAs with respect to endogenous control snRNA U6 were used to generate a receiver operating characteristic (ROC) curve (MedCalc 15.11.4). The area under the curve (AUC) and 95% confidence intervals were calculated to assess the accuracy of each parameter (sensitivity and specificity) and to find an appropriate cut-off point. Statistical significance of ROC curves was established at a p-value > 0.05.

Network construction and analysis

To evaluate the biological meaning of differentially expressed miRNAs, we retrieved their experimentally validated targets from miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). To statistically enrich the gene signaling regulated by differentially expressed miRNAs, we built a network based on interactions between differentially expressed miRNA targets and their nearest neighbors. This network was generated using Cytoscape v2.8.3 (www.cytoscape.org/) and MiMI plugin (http://mimiplugin.ncibi.org/). We determined statistical over-representation of pathways by using the FatiGO tool (http://babelomics3.bioinfo.cipf.es) on the genes from the previously generated network that screened Gene Ontology (GO), KEGG and Reactome databases. Statistical over-representation was calculated by using Fisher's exact test; Benjamini & Hochberg FDR Correction; $p \leq 0.005$. The over-represented pathways in this analysis were associated with dysregulated genes involved in ERM and MHs, as reported in the literature (https://www.ncbi.nlm.nih.gov/pubmed/).

Results

Comparison of vitreal miRNA profiles from patients affected by MHs and ERM with those of controls was performed.

VH samples were extracted from 32 eyes after surgery: 10 eyes with MHs) (mean age 60±6), 16 eyes affected by ERM (mean age 59±5) and 6 controls (mean age 60±7). Eighteen (56%) were male and 14 (44%) female.

Using TLDA technology, we determined the profiles of 754 miRNAs in the VH, from 4 MHs, 4 ERMs and 4 controls (Ct data are reported in supplementary material 1). The comparison of miRNA profiles in the VH of different patient classes by SAM statistical method showed 9 differentially expressed miRNAs (Table 1).

Table 1. Nine differentially expressed miRNAs.

miRNAs	FC ERM + MH vs Cs	FC ERM vs MH
miR-19b	-5	NDE
miR-24	-3	NDE
miR-29a	3	-5.16
miR-30a-3p	NDE	2.42
miR-142-3p	NDE	-4.2
miR-155	-4.2	NDE
miR-451	-5	NDE
miR-574-3p	NDE	4.13
miR-1290	NDE	3.3

Differentially Expressed vitreal miRNAs by TLDAs (TaqMan Low Density Arrays) in the vitreous humor of patients affected by macular hole and epiretinal membrane with respect to controls and comparison between pathological classes.

All Differentially Expressed miRNAs showed a false discovery rate < 0.05 based on Significance Analysis of Microarray test

FC, Fold Change; Cs, Controls; NDE, Not Differentially Expressed.

https://doi.org/10.1371/journal.pone.0174297.t001

More specifically, we found 4 downregulated miRNAs (miR-19b, miR-24, miR-155, miR-451) and 1 upregulated miRNA (miR-29a) in patients affected by MH and ERM with respect to controls; while, 2 downregulated miRNA (miR-29a, miR-142-3p) and 3 upregulated miRNAs (miR-30a-3p, miR-574-3p, miR-1290) were found by comparing ERMs to MHs (Table 1). Profiling data showed that 4/5 of differentially expressed miRNAs had a negative FC, suggesting a general trend of downregulation of circulating miRNAs in the VH of eyes with MH and ERM with respect to controls. miR-30a-3p, miR-574-3p, miR-1290 were statistically more abundant in the VH of ERM patients than MH patients.

Validation by single TaqMan® assays

Expression of differentially expressed miRNAs identified by TLDAs was confirmed by single TaqMan® assays in the VH of all the patients and controls (Table 2) (Fig 1) (S1 File).

Table 2. Differentially expressed vitreal miRNAs.

DE miRNAs	ERM + MH vs Cs		MH vs Cs		ERM vs Cs		ERM vs MH	
	FC	t-test	*FC*	t-test	*FC*	t-test	*FC*	t-test
miR-19b	-9.13	0.00004	-14.1	0.002	-6.29	0.0004	2.24	0.046
miR-24	-7.52	0.004	-11.58	0.012	-6.38	0.016	-	NS
miR-29a	-	NS	-	NS	-	NS	-1.94	0.031
miR-30a-3p	-	NS	-	NS	-	NS	11.08	0.026
miR-142-3p	5.32	0.011	5.81	0.048	4.83	0.021	3.31	0.013
miR-155	6.88	0.018	7.21	0.015	6.45	0.019	-	NS
miR-451	6.52	0.041	5.35	0.045	6.96	0.038	-	NS
miR-574-3p	-	NS	-7.55	0.018	-	NS	7.48	0.047
miR-1290	-6.29	0.036	-8.14	0.031	-5.74	0.042	-	NS

Differentially Expressed vitreal miRNAs by single TaqMan® assays in the vitreous humor of patients affected by macular hole and epiretinal membrane with respect to controls and comparison between pathological classes.

t test: significant p-value < 0.05.

FC, Fold Change; Cs, Controls; NS, Not Significant.

https://doi.org/10.1371/journal.pone.0174297.t002

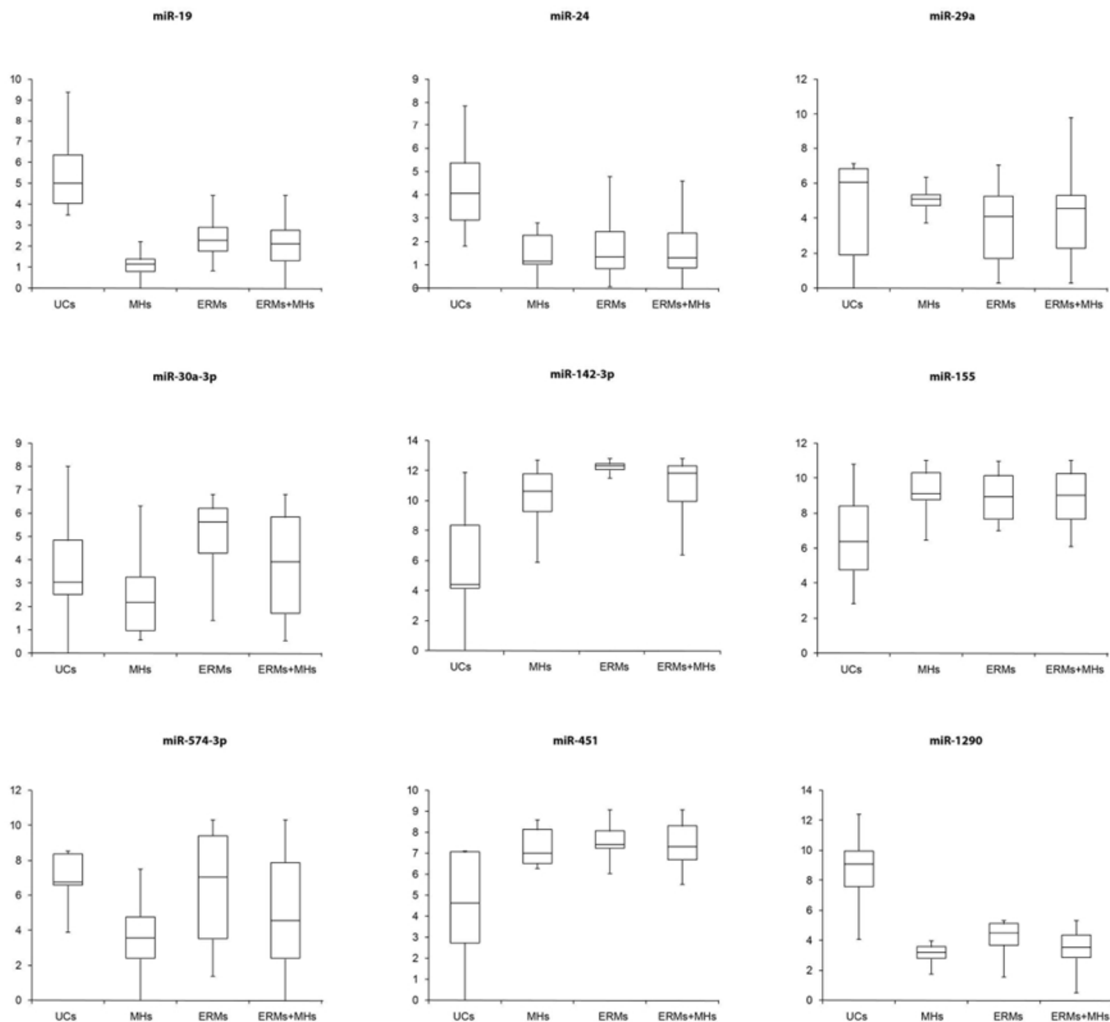

Fig 1. Box Plots of differentially expressed miRNAs. Box Plots from Single TaqMan® Assays on TaqMan® Low Density Arrays (TLDA) of differentially expressed miRNAs. Validation by single TaqMan® assays of differentially expressed miRNAs identified by TLDAs in the vitreous humor of all the patients and controls. Values on the y-axis are reported as ΔCt × (−1).

https://doi.org/10.1371/journal.pone.0174297.g001

The downregulation of miR-19b in the VH of pathological patients with respect to controls was statistically confirmed by applying the t-test, but we also detected its upregulation in ERMs with respect to MHs (Table 2) (Fig 1). The downregulation of miR-24 and miR-1290 and the upregulation of miR-142-3p, miR-155 and miR-451 in ERMs and MHs compared to controls was also validated (Table 2) (Fig 1). Moreover, miR-142-3p, miR-30a-3p, miR-574-3p were statistically more abundant in ERMs with respect to MHs; while miR-29a was

downregulated in the same comparison (Table 2) (Fig 1). We obtained no statistical validation on the upregulation of miR-29a in patients affected by MHs and ERMs with respect to controls.

Network and pathway enrichment analysis

To understand the potential functional effect of deregulation of the 9 differentially expressed miRNAs we created a biological network based on differentially expressed miRNA targets and their nearest neighbors. Considering all network nodes, we analyzed the statistical over-representation of biological pathways from various databases (i.e. Reactome, KEGG, and GO) against the whole genome (Fig 2).

Our data showed that observed miRNA deregulation could induce an alteration of several pathways recently associated with genes involved in vitreoretinal diseases, such as MHs, and ERMs.

ROC curves

To evaluate the discriminating power of the differentially expressed vitreal miRNAs as potential markers of ERMs and MHs, we computed the ROC curves for each type of comparison: ERMs + MHs vs controls, ERMs vs controls, MHs vs controls, ERMs vs MHs. Our analysis showed significant results for just three of the 9 differentially expressed miRNAs: miR-19b, miR-24 and miR-142-3p. More specifically, we found for ERMs + MHs vs controls that miR-19b had an AUC of 0.979 (95% CI, 0.810–1; $p< 0.0001$) with 93.75% sensitivity and 100% specificity (DCt cut-off value: >14.935); miR-24 showed an AUC of 0.865 (95% CI, 0.652–0.971; $p< 0.0001$) with 75% sensitivity and 83.33% specificity (DCt cut-off value: > 14.734); miR-142-3p had an AUC of 0.857 (95% CI, 0.622 0.973; $p < 0.0009$) with 78.57% sensitivity and 80% specificity (DCt cut-off value: ≤ 0.63348) (Fig 3A–3C). From the comparison of ERMs vs controls we obtained for miR-19b an AUC of 0.97 (95% CI, 0.755 1; $p < 0.0001$) with 90.91% sensitivity and 100% specificity (DCt cut-off value: >14.935); miR-24 had an AUC of 0.848 (95% CI, 0.595–0.973; $p<0.0003$) with 72.73% sensitivity and 83.33% specificity (DCt cut-off value: > 14.734); miR-142-3p showed an AUC of 0.933 (95% CI, 0.618 0.999; $p< 0.0001$) with 83.33% of sensitivity and 100% of specificity (DCt cut-off value: ≤ -1.535) (Fig 3D–3F). In the comparison of MHs vs controls, miR-19b showed an AUC of 1 (95% CI, 0.715 1; $p< 0.0001$) with 100% sensitivity and 100% specificity (DCt cut-off value: > 14.935); while, miR-24 had an AUC of 0.9 (95% CI, 0.576 0.997; $p < 0.0001$) with 80% sensitivity and 83.3% specificity (DCt cut-off value: > 14.734) (Fig 3G and 3H). We found no significant result for the comparison of ERMs vs MHs. These data suggested that the expression of vitreal miRNAs miR-19b, miR-24 and miR-142-3p was able to distinguish ERM and MH eyes from controls, but could not discriminate ERMs from MHs.

Discussion

The results of this study show that in the VH of patients with MHs and ERM smicroRNAs have different levels of expression, and, in particular, miR-19b, miR-24 and miR-142-3p exhibit the most significant discriminative power compared to controls.

A decreased expression of miR-19b has been associated with the phenomena of fibrosis in liver and heart cells [34,35]. In addition, its decreased serum level has been reported in association with intestinal fibrosis [36].

Even the downregulation of miR-24 has been repeatedly associated with an increase of mechanisms of fibrosis in the heart [37,38].

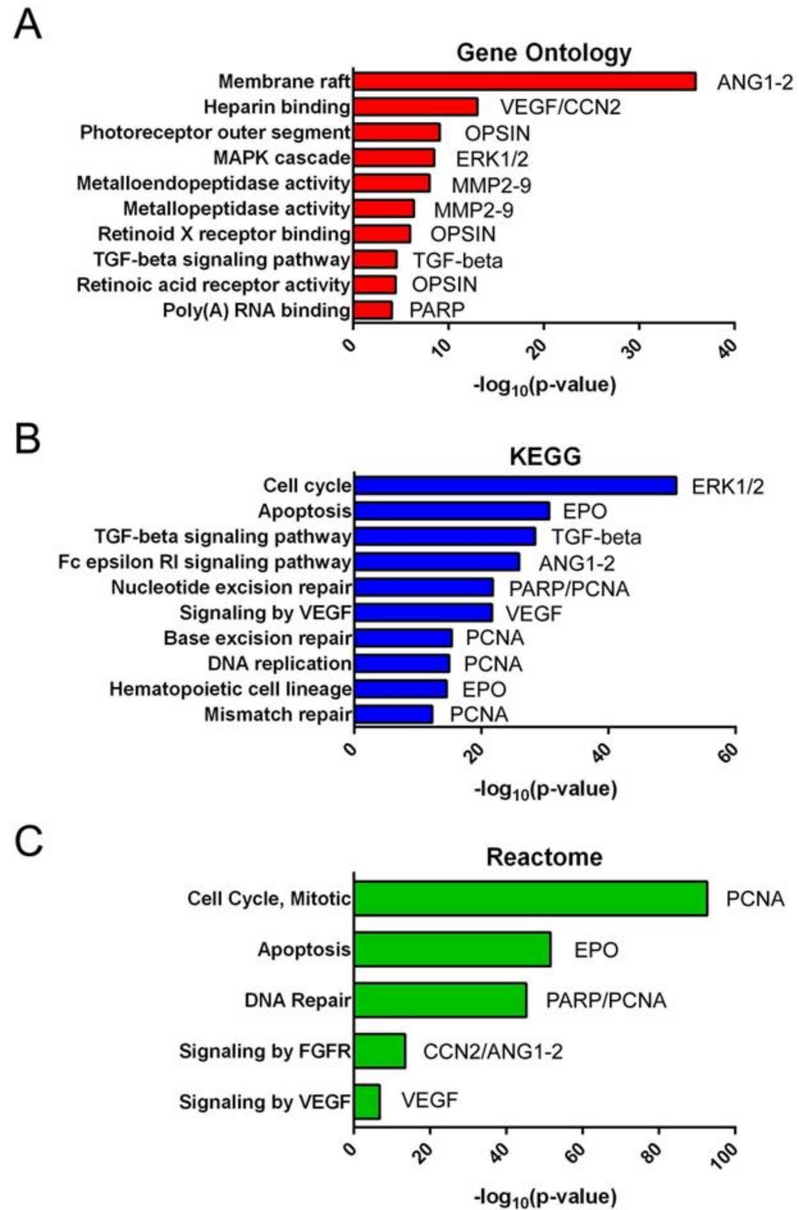

Fig 2. Biological processes controlled by miR-19b, miR-24 and miR-142-3p network. Overrepresented biological functions from a molecular network built on validated targets of differentially expressed miRNAs, retrieved from different annotation databases (GO, KEGG, Reactome). On the left of each histogram the overrepresented pathways are reported, while on the right the corresponding associated genes to MHs and ERMs based on literature data are shown. Data are plotted as—log10 of p-values for each biological process.

https://doi.org/10.1371/journal.pone.0174297.g002

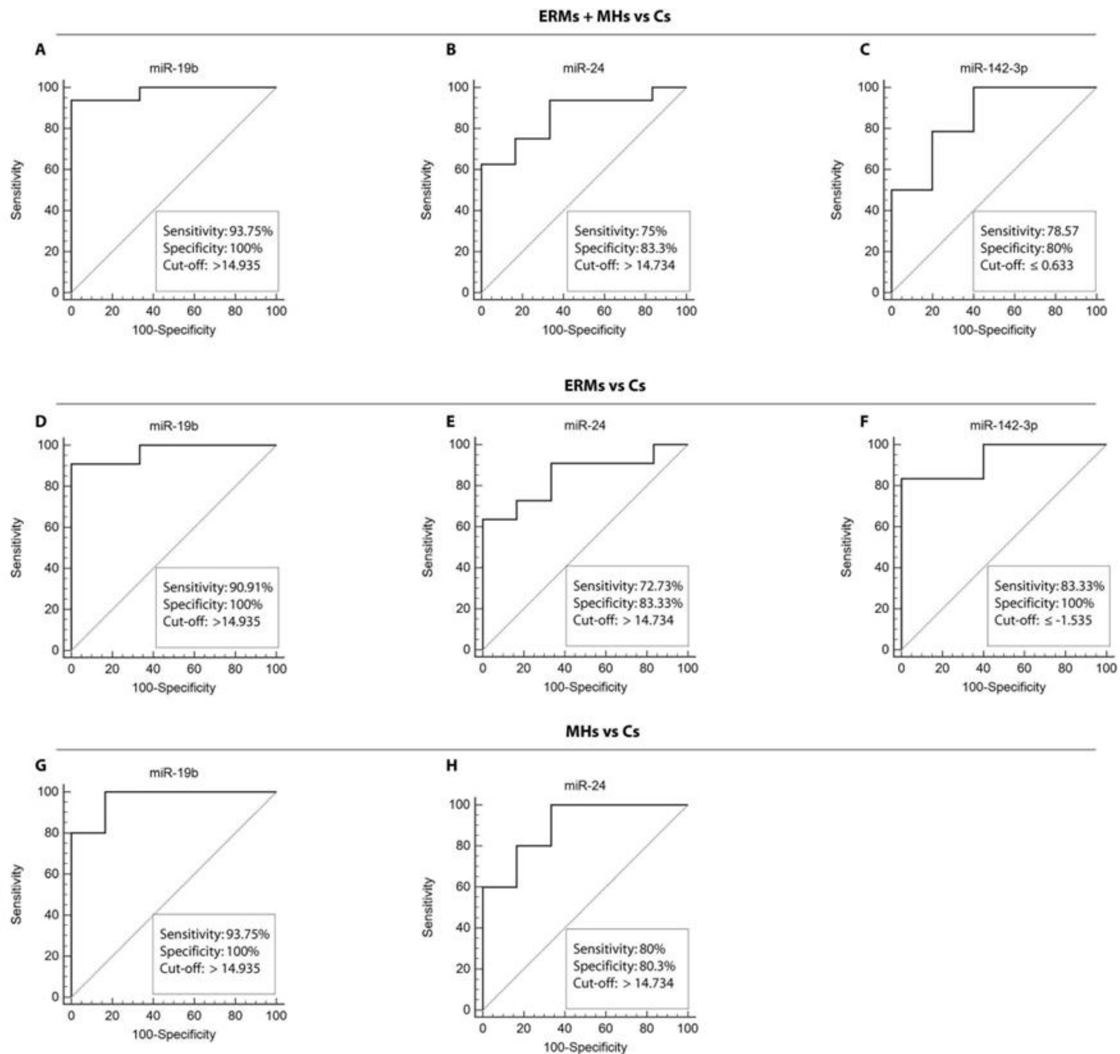

Fig 3. Receiver Operator Characteristic (ROC) curves for vitreal miR-19b, miR-24 and miR-142-3p in patients affected by MHs and ERMs. ROC curves of miR-19b (A), miR-24 (B), miR-142-3p (C) DCts in comparison with ERMs + MHs vs controls; miR-19b (D), miR-24 (E), miR-142-3p (F) DCts in comparison with ERMs vs controls; miR-19b (D), miR-24 (E) in comparison with MHs vs controls. Curves represent DCts calculated by using U6 as endogenous control.

https://doi.org/10.1371/journal.pone.0174297.g003

Recent studies demonstrated that miRNAs derived from the miR-17-92 cluster (including miRNA-19b) directly modulate TGFβ signaling [39,40]. Also the miRNA-24 cluster has been reported to change TGFβ signaling through several pathways [41,42], suggesting a significant role of these miRNAs in TGFβ-mediated fibrogenesis.

Data in the literature show that overexpression of miRNA-19b and miRNA-24 may be a valuable therapeutic agent for TGFβ-mediated fibrosis [11].

Increased serum levels of miR-142-3p were associated with the presence and severity of scleroderma, an autoimmune disease that causes a progressive fibrotic tissue formation in the normal tissue architecture of various organs [36,43].

Furthermore, the increased levels of this microRNA in biopsies and lymphocytes have been associated with the presence of interstitial fibrosis as a result of kidney transplants [44].

It has also been shown that miR-142-3p modulates the production of cAMP and is involved in the regulation of macrophages and T cells [45]. Regulatory T cells lose their capacity to suppress immunological processes involving the kidney as suggested by the high levels of miR-142-3p in tissue samples of renal allografts [37]. Soltaninejad et al. found increased levels of miR-142-3p in allograft tissues of patients affected by interstitial fibrosis and tubular atrophy that is the major cause of renal transplant [37].

Downregulation of miR-19b and miR-24 and upregulation of miR-142-3p, already reported in the literature, are in agreement with the variations observed in our study in the VH of patients affected by MHs and ERMs and suggest that, as demonstrated in other pathologies, the different expression of these molecules is related to an increase of fibrosis, which is a characteristic feature of both MHs and ERMs [4–8].

Moreover, to understand the potential functional role of miRNAs differentially expressed in the VH, we performed a computational analysis on the network of the differentially expressed miRNA targets. Interestingly, among the functions significantly over-represented in both the vitreoretinal diseases, one of the most significant is related to TGF-β that has been linked to fibrogenesis [11].

To date, no approved treatments for fibrosis have been described. Several studies have described modifications in miRNA expression profiles during development of fibrosis that control wound-healing transcripts [46]. Wang et al. reported that *in vivo*, miR-24 could improve heart function and attenuate fibrosis in the infarct border zone of the heart two weeks after myocardial infarction through intramyocardial injection of Lentiviruses [34].

To the best of our knowledge, this is the first report describing a possible correlation between miRNAs and fibrotic phenomena that characterize patients affected by MHs and ERMs.

The main limitation of our study is the low number of patients and that the control group presented some vitreous abnormalities (symptomatic vitreous floaters). In particular, the low number of biologically independent replicates as well as the mixed presence of already-existing vitreous opacities in the control group might justify the wide dispersion highlighted in the expression of different miRNAs.

The source of miRNAs in the VH, as in other bodily fluids, could represent a critical point of debate. The most accepted hypothesis asserts that miRNAs are actively secreted in membrane-bounded-vesicles (i.e., exosomes), even if some studies suggest that most circulating miRNAs are in a non-membrane bound form, but rather assembled in ribonucleoprotein complexes (e.g., Ago2, or other RNA binding proteins) [47]. The hypothesis that circulating miRNAs are passively released into the extracellular environment as byproducts of dead cells has not been suitably untangled [24]. Moreover, miRNAs are rapidly degraded by RNases when secreted in blood without protection by vesicles or ribonucleoprotein complexes [19]. Accordingly, the real-time PCR dosage of circulating miRNAs resulting from physiologic and pathological flaking of the cells would be scarcely appreciable or extremely variable. Our data on miRNA disregulation in the VH exclude RNA contamination from the few cells floating in the vitreous (i.e., phagocytes, hyalocytes of Balazs) because VH samples were appropriately centrifuged to pellet and remove any circulating cells before RNA extraction (see Methods). Moreover, in our

previous work we demonstrated that exosomes floating in the VH have miRNA expression profiles statistically related to those observed in total VH [26]. These data suggested that the concentration of circulating miRNAs in the VH could be mostly, but not exclusively, due to the molecular content of VH exosomes. For this reason, we believe that vitreal miRNAs, detected as being altered in MHs and ERMs, may be the result of a dysregulated signaling carried by exosomes secreted by the epithelial cells of the retina or from floating cells in the vitreous cavity. Disregulation of miR-19b, miR-24 and miR-142-3p, might be related to the pathological alterations that characterize patients affected by MHs and ERMs. The concentrations of these vitreal miRNAs also discriminated pathological eyes from controls, but they were not able to distinguish between MHs and ERMs. However, these results would suggest the possibility to exploit a possible ocular pharmaceutical RNA-based treatment against these differentially expressed miRNAs that might be administered to the patients affected by these slow-developing alterations, reducing invasive therapeutic approaches, such as vitrectomy.

Supporting information

S1 File. Ct raw data from Microrna expression profiling. Raw Ct data of TaqMan® Array Microfluidic Cards A + B from vitreal samples of 4 macular holes (MHs), 4 epiretinal membranes (ERMs), 4 controls.
(XLS)

Acknowledgments

We wish to thank the Scientific Bureau of the University of Catania for language support.

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Resources: TA.

Supervision: MP M. Reibaldi.

Validation: RC.

Writing – original draft: AR.

Writing – review & editing: AR M. Ragusa.

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CHAPTER 3.

MicroRNAs in the vitreous humor of patients with retinal detachment and a different grading of Proliferative Vitreoretinopathy

Article submitted to Journal of Clinical Medicine

MicroRNAs in the vitreous humor of patients with retinal detachment and a different grading of Proliferative Vitreoretinopathy

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Received: date; Accepted: date; Published: date

Abstract:

Background: The surgery for retinal detachment (RD) might result in a complication, such as proliferative vitreoretinopathy (PVR). Although the underlying pathogenesis is not clearly understood, previous findings suggested that the aberrant expression of micro-RNAs promote the molecular pathways contribute towards the epithelial-mesenchymal transition (EMT) of the retinal pigment epithelial (RPE) cells. However, the miRNA expression in the vitreous of patients with primary RD and different PVR grading has not yet been investigated. Aim: To assess the expression of microRNAs (miRNAs) in the vitreous humor (VH) of patients diagnosed with primary RD and different grading of PVR. Methods: The VH was extracted from the core of the vitreous chamber in patients who had undergone standard vitrectomy for RD. RNA was extracted and TaqMan® Low-Density Arrays (TLDAs) were used for transcriptome profiling that was substantiated by single TaqMan® assays. A gene ontology (GO) analysis was performed on the differentially expressed miRNAs. Results: A total of 12 eyes with RD, 3 eyes for each grade of PVR (A, B, C, D), were enrolled in this prospective comparative study. The expression of 20 miRNAs was altered in the pathological groups as

compared to the endogenous controls. Interestingly, the expression of miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a increased with the worsening of PVR grading. According to GO analysis, miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a participated in the biological processes involved in PVR pathogenesis. Conclusion: The present study suggested that dysregulation of miRNAs might be associated with the PVR-related complications in RD patients.

Keywords: microRNA; profiling; proliferative vitreoretinopathy; retinal detachment.

1. Introduction

Proliferative vitreoretinopathy (PVR) is a multi-factorial and complex clinical syndrome common to a variety of clinical disorders, including retinal detachment (RD) [1]. The frequency of PVR remains largely unchanged in primary RD, with the incidence ranging from 5.1 to 11.7% of all rhegmatogenous RDs, and it is believed to be the leading cause of RD surgery failure accounting for 75% of retinal redetachment surgeries [1-2]. PVR is characterized by pre-, sub-, or intra-retinal fibrosis (scarring) that growths on the membrane surface of the detached retina and posterior hyaloids causing foreshortening of the retina, traction, and recurrent detachment mostly within the first 6-8 weeks after surgery [1]. Typically, PVR with recurrent retinal detachments requires additional surgical interventions and is associated with poor visual recovery [2,4-8].
Although the pathogenesis is not elucidated [8-10], previous studies suggested that the epithelial-mesenchymal transition (EMT) [11-13] of the retinal pigment epithelial (RPE) cells and, the inflammatory response-associated pathways might be involved in the pathogenesis underlying PVR [13-18]. However, to date there are no effective medications for the prevention and treatment of PVR and an urgent approach is demanded [19-22].
MicroRNAs (miRNAs) are small endogenous non-coding RNAs that negatively regulate the gene expression within all cell types. miRNAs play a key role in cellular physiology and various biological pathways in specific cell types and tissues [23-25]. Previously, abnormal miRNA expression has been reported in cellular and extracellular compartments with respect to cancers and other diseases, such as cardiac, neurological, and ocular [26-32], and previous studies have shown that specific microRNAs induce/inhibit EMT in other fibroblast-like cells [33-35].
Additionally, even though the aberrant expression of micro-RNAs in RPE cells undergoing EMT is involved in the pathogenesis of PVR [36-44], the characteristics and the distinct role of miRNAs in PVR and their expression in the vitreous of primary RD patients with different PVR grading are yet to be investigated.
In this pilot study, 754 miRNAs were subjected to real-time PCR expression profiling in order to identify the differentially expressed miRNAs in the vitreous of patients diagnosed with primary RD and a different grading of PVR.

2. Materials and Methods

This prospective study included consecutive eyes undergoing pars plana vitrectomy for the treatment of primary RD with and without PVR.
All surgeries had been performed by the same surgeon (T.M.D.) at the Department of General Ophthalmology, Medical University of Lublin (Poland) between January and June 2018.
The exclusion criteria were as follows: patients with diabetes mellitus, known rheumatic and autoimmune diseases, systemic treatments involving corticosteroids or immunomodulatory drugs, vitreous hemorrhage, uveitis, glaucoma, or any concomitant retinal pathology, a previous ocular trauma, a diagnosed eye tumor or who had undergone intraocular surgery or treatment within 6 months after the diagnosis of RD. These systemic or ocular comorbidities might influence the mechanisms underlying ocular fibrosis.
The present study was approved by the Ethics Committee of the Medical University of Lublin (n° 1A63/1212) in compliance with the Declaration of Helsinki.
Written informed written consent was obtained from each participant allowing the use of their biological materials and clinical data.

2.1. PVR grading and patient grouping

Based on the severity of the PVR, the patients were classified into four stages: A (minimal), B, C, and D (massive) according to the "Retina Society Terminology Committee" [45].
As proposed by Zandi et al. [18], in the current study, the risk of developing postoperative PVR in RD patients without PVR and RD patients with low PVR severity (grades A or B) was found to be similar. Thus, PVR grade C included until 3 quadrants with visible PVR membrane formation. However, the severity was grade D if all 4 quadrants were affected.
Since advanced PVR is challenging for accurate grading, all patients underwent indirect fundus ophthalmoscopy with scleral indentation prior to surgery. Two masked expert retinal specialists (T.M.D. and N.K.) investigated the fundus and assigned the PVR score; the discrepancies were resolved by a third investigator (R.R.).

2.2. Handling of vitreous fluid samples

A 3-port 23-gauge vitrectomy was performed on all the patients under local anesthesia. The Resight 700 (Carl Zeiss Meditec AG, Jena, Germany) wide-angle viewing system or the Binocular Indirect Ophthalmol Microscope wide-angle viewing system (BIOM; Oculus Inc, Wetzlar, Germany) was used. Sclerotomy was carried out at 3.5 mm parallel to the limbus at 30° [33]. Then, a 2 mL vitreous sample extracted from the core of the vitreous cavity before vitrectomy was subjected to centrifugation at 700 ×g for 10 min to exclude any circulating cells or debris. The pellets were stored at -80 °C until further analysis.

2.3. miRNA expression profiling in VH by TaqMan Low-Density Arrays (TLDAs)

Total RNA was isolated from 400 µL of VH using miRNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. The amount and purity of RNA were assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The expression of 754 miRNAs was evaluated by real-time PCR using the TLDAs from 12 VH samples (3 patients for each grade of the disease). About 30 ng of RNA was transcribed using TaqMan microRNA Reverse Transcription Kit and Megaplex RT Primers Human Pool A v2.1 and Pool B v3.0 (Thermo Fisher Scientific) and pre-amplified by TaqMan PreAmp Master Mix Kit and Megaplex PreAmp Primers using the Human Pool A v2.1 and Pool B v3.0 (Thermo Fisher Scientific). The products were loaded in TaqMan Human MicroRNA Array v3.0 A and B (Thermo Fisher Scientific), and the Real-Time PCR reaction was carried out a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal Master Mix II without UNG (Thermo Fisher Scientific), according to manufacturer instructions.

2.4. Statistical analysis

The expression data were subjected to significance analysis of microarrays (SAM), computed by Multi Experiment Viewer v4.8.1 (http://mev.tm4.org) using the multiclass tests and one-way ANOVA test ($p < 0.05$) among ΔCts. The endogenous control was selected based on the global median normalization method, which allowed us to identify the miRNAs with the most stable expression in the samples [24]. We used three different endogenous controls for each TLDA panel (panel A: miR-197, U6 and median of ΔCts; panel B: miR-1285, U6 and median of ΔCts), considering only those miRNAs that were deregulated according to two endogenous control. Gene ontology (GO) analysis was performed on the differentially expressed (DE) miRNAs through DIANA-miRPath v3.0 (http://snf-515788.vm.okeanos.grnet.gr/) [46].

3. Results

3.1. miRNA expression profile in the VH of PVR patients

The expression of 754 miRNAs in the VH of 12 patients, including 3 patients for each grade of the disease (A, B, C, D) was analyzed by TLDA profiling. The statistical analysis of the profiling results was performed by grouping A and B samples, characterized by absent or minimal proliferation. We identified 20 miRNAs with altered expression in one or more pathological groups, according to at least 2/3 endogenous controls. Specifically, let-7b, miR-100, miR-1300, miR-143, miR-152, miR-16, miR-19b, miR-203, miR-21, miR-218, miR-223*, miR-224, miR-30b, miR-335, miR-340, miR-361, miR-452, miR-486-3p, miR-891a, and miR-99a showed differential expression in different comparisons (Table 1, Figure 1).

	ANOVA	**C *vs.* A+B**		**D *vs.* A+B**		**D *vs.* C**	
miRNA	**p-value**	**FC**	**p-value**	**FC**	**p-value**	**FC**	**p-value**
let-7b	**0.022**	**-16.85**	**0.021**	3.76	0.33	**28.76**	**0.009**
miR-100	**0.003**	**-13.21**	**0.008**	**4.9**	**0.06**	**24.76**	**0.001**
miR-1300	**0.003**	**12.59**	**0.005**	-3.45	0.1	**-23.53**	**0.001**
miR-143	**<0.0001**	**9.93**	**0.0005**	**26.41**	**<0.0001**	2.65	0.08
miR-152	**0.01**	-5.28	0.1	**12.03**	**0.024**	**33.68**	**0.003**
miR-16	**0.026**	-4.94	0.22	**16.77**	**0.035**	**32.68**	**0.009**
miR-19b	**0.019**	-2.79	0.1	**3.59**	**0.049**	**10.03**	**0.006**
miR-203	**0.033**	-5.64	0.13	8.95	0.07	**30.51**	**0.011**
miR-21	**0.011**	1.19	0.83	**22.15**	**0.004**	**18.56**	**0.013**
miR-218	**<0.0001**	-1.45	0.27	**15.28**	**<0.0001**	**22.3**	**<0.0001**
miR-223*	**0.016**	**-11.65**	**0.008**	1.14	0.85	**13.3**	**0.013**
miR-224	**0.003**	2.46	0.38	**36.26**	**0.001**	**28.97**	**0.01**
miR-30b	**0.039**	**-26.91**	**0.017**	-1.02	0.98	**26.33**	**0.034**
miR-335	**0.025**	**-8.72**	**0.033**	3.09	0.22	**26.99**	**0.009**
miR-340	**0.031**	-2.91	0.25	**8.36**	**0.039**	**24.38**	**0.012**
miR-361	**0.003**	4.82	0.26	**36.81**	**0.001**	**18.78**	**0.014**
miR-452	**0.0002**	**19.17**	**0.001**	**33.38**	**<0.0001**	5.6	0.07
miR-486-3p	**0.011**	6.06	0.08	**32.98**	**0.004**	5.43	0.14
miR-891a	**0.002**	2.46	0.4	**35.38**	**0.0007**	**21.01**	**0.005**
miR-99a	**0.001**	**-21.67**	**0.002**	3.82	0.1	**32.84**	**0.0006**

Table 1: TLDA profiling showed the differential expression of 20 miRNAs in the different comparisons. The average fold-change (FC) and the p-value derived from multiple comparisons for each miRNA are shown with respect to endogenous control. The p-value of the ANOVA test between all groups is also shown. Significant p-values are highlighted in bold.

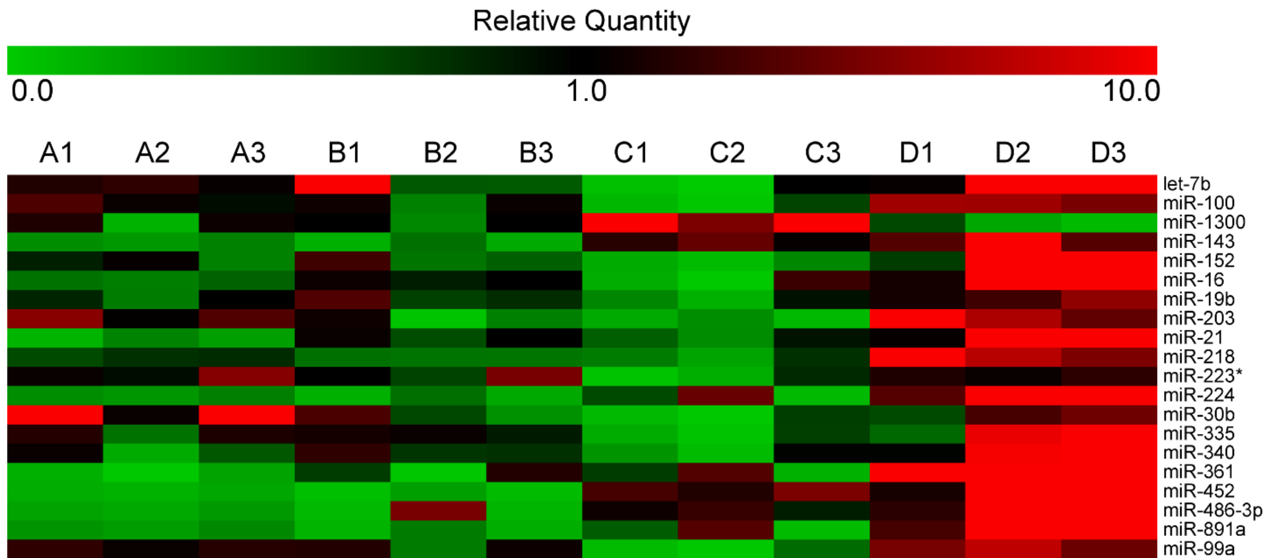

Figure 1: Heatmap showing the expression of miRNAs through TLDA profiling. The miRNA expression is represented as relative quantity (RQ), calculated with respect to the average of ΔCts of all the analyzed samples.

Interestingly, miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a expression increase with the worsening of the PVR grading, suggesting a possible application of these miRNAs as biomarkers for PVR (Figure 2).

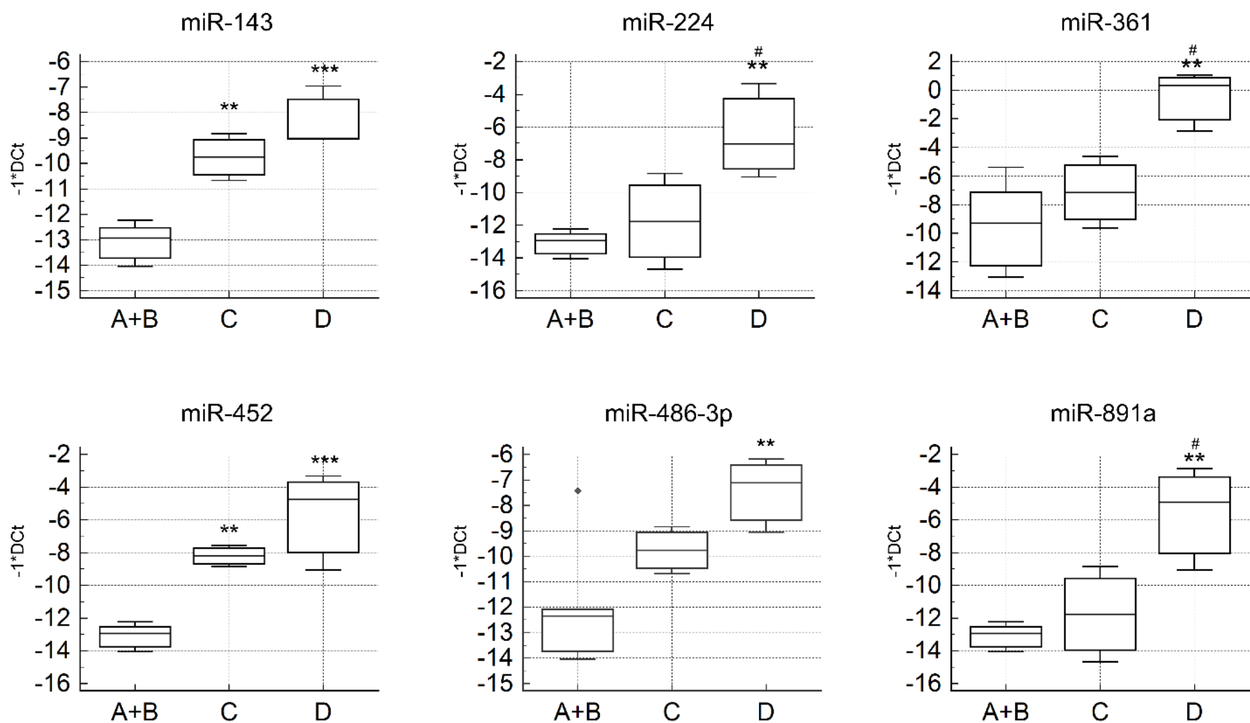

Figure 2: Boxplots showing the expression of miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a in the three pathological groups. **p-value *vs.* A+B < 0.005; ***p-value *vs.* A+B < 0.0005; #p-value *vs.* C < 0.05.

3.2. GO analysis

GO analysis showed that miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a participate in the biological processes involved in PVR pathogenesis, such as cell cycle regulation, adhesion to the extracellular matrix (ECM), and regulation of actin cytoskeleton (Figure 3).

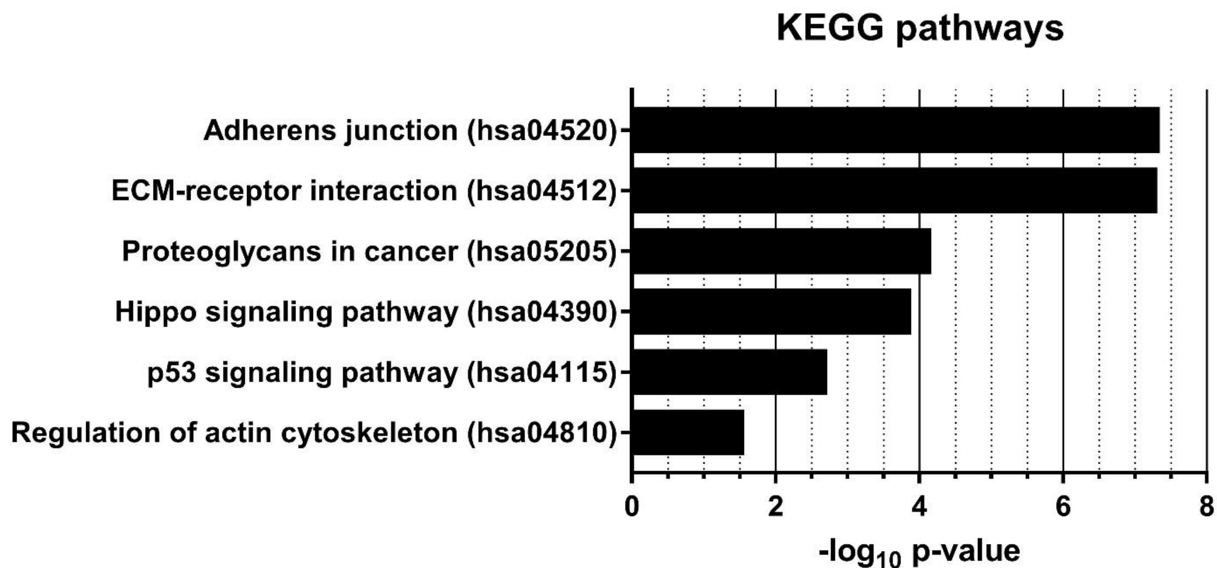

Figure 3: PVR-related KEGG pathways regulated by miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a. The X-axis represents the $-\log_{10}$ of the p-value for each pathway.

4. Discussion

The current study identified altered expression of 20 miRNAs in one or more pathological groups of the VH of patients with primary RD and a different grading of PVR. The analysis revealed that the expression of 6 miRNAs (miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a) increased with the worsening of the disease and, according to the GO analysis, these miRNAs participated in the biological processes of EMT involved in PVR pathogenesis, such as cell cycle regulation, adhesion to ECM, and regulation of actin cytoskeleton.

PVR is the main cause of retinal surgical failure [47]. The PVR is primarily treated using vitrectomy, systematic peeling and dissecting epiretinal membranes, and retinal tamponade with silicone oil or gas [48-49]. However, recurrent tractional proliferation causes retinal re-detachment post-surgery [47].

Furthermore, the adjuvant therapy for the treatment of PVR includes anti-inflammatory agents, anti-growth inhibitors, antioxidants, and neuroprotective agents. In addition, the pharmacological inhibition of the pathological responses to PVR improved the rate of success of surgery; however, the clinical therapy is not yet clarified [22]. Thus, additional studies are essential to elucidate the mechanisms regulating the initiation and

development of PVR.
The development and progression of fibrotic lesions, including proliferative diabetic retinopathy (PDR) and PVR effectuates EMT. Also, wound healing and stimulation of inflammatory cytokines lead to EMT, thereby forming pre- or sub-retinal fibrous membranes [1].
Importantly, RPE cells play a vital role in the development of fibrosis on the retina and constitute the largest cellular component of epiretinal membranes in addition to hyalocytes, retinal Müller glial cells, fibroblasts, and macrophages [1]. RPE cells are usually quiescent in healthy condition. Interestingly, trauma or intraocular diseases damage the RPE or cause RD. The subsequent repair triggers the loss of cell-cell contact in RPE cells, and also, the epithelial cells are stimulated to proliferate into motile fibroblast-like cells [1].
Initially, the transforming growth factor-β (TGF-β) promote various types of fibrotic diseases, including PVR and PDR [1,49-55]. Subsequently, trans-differentiated RPE cells migrate into the intraretinal layers or vitreous body, produce ECM components, and transform into fibroblast-like cells. This phenomenon results in the formation of epiretinal membranes that contract and cause RD as well as visual impairment [1, 49-55].

miRNAs regulate the complex physiological and pathological processes, such as embryogenesis, organ development, oncogenesis, and angiogenesis [56-58].
Intriguingly, miRNAs are positive or negative regulators of EMT that target the multiple components of the EMT and the epithelial machinery and exacerbate their critical roles in TGFβ2-induced EMT in human RPE cells [37,59]. Also, miRNAs regulate fibrosis in several organs [60].

Although the role of miRNAs in PVR is not yet clarified, no study has investigated the expression in the vitreous of RD patients with different PVR grades.
A previous study assessed the miRNA expression in the VH of patients with proliferative vitreoretinal diseases, including PDR [37]. qRT-PCR was applied to comprehensively identify miR-21 in the vitreous as a potential disease-modifying agent. Furthermore, the expression of miR-21 is enhanced by the disease-associated expression of TGF-β2 and/or high glucose conditions, which could be crucial in the fibroproliferative response of RPE cells during the development of retinal fibrotic disorders. In addition, the cell migration and proliferation of RPE cells was increased markedly. Also, the level of miR-16 was upregulated in the vitreous of the same eyes. Consistent with this report, the current data showed an increased expression of miR-21 and miR-16 in vitreous patients with PVR.

Among the miRNAs previously associated with EMT, miR-223* was shown to be upregulated after TGF-β2 treatment of RPE cells, while all the other differentially expressed miRNAs (except for miR-1300 and miR-891a) regulate the EMT in cancer [40].
Also, several studies reported the involvement of differentially expressed miRNAs in angiogenesis in eye-associated diseases (let-7b, miR-152, miR-21, miR-218, and miR-30b) [37, 61-67] or various cancer models (miR-891a) [68].
Similarly, among differentially expressed miRNAs, 12/20 (let-7b, miR-152, miR-16, miR-19b, miR-203, miR-21, miR-224, miR-335, miR-340, miR-486-3p, miR-891a, and miR-99a) are associated with fibrosis regulation [69-80].
Furthermore, Wang et al. [44] isolated RPE cells from three healthy donors and demonstrated the downregulated expression of miR-182 in PVR, which in turn upregulated that of the target gene *c-Met*. These augmented levels of *c-Met* further elevated the proliferation and migration of RPE cells via the PI3K/Akt signaling pathway. Therefore, novel therapeutic agents that can selectively upregulate the expression of miR-182 or the targeted delivery of miR-182 mimic to RPE would improve the management of PVR-induced complications [44].

The present study, for the first time, described a putative correlation between miRNAs and fibrotic phenomena in PVR patients following RD.

Nevertheless, the present study has some limitations: a missing control group and the modality of grading of PVR. Fewer biologically independent replicates and vitreous opacities in the lower groups might explicate the differential expression of the miRNAs. Moreover, the grading of PVR, even if assigned based on the classification system established by the "Retina Society Terminology Committee " [45], is still conducive to a subjective clinical choice of the retinal disease specialists and not on an objective diagnostic method.

In conclusion, 20 differentially expressed miRNAs were identified in PVR diseases, suggesting a possible application of these miRNAs as biomarkers for PVR. Furthermore, elucidating the role of other microRNAs in EMT in RPE cells in vitro and in PVR in vivo would provide an in-depth insight into the EMT-related gene expression. Thus, additional studies on the correlation between vitreal miRNAs and the pathological phenotypes are essential to identify the novel miRNA-based mechanisms underlying the PVR disease that would improve the diagnosis and treatment of the condition.

Author Contributions: T.M.D. and B.C.[7]: writing, review, and editing; R.M[2], B.C.[3], and R.M.[7,8]: conceptualization and methodology; T.M.D. and B.C.[7]: data curation, formal analysis, investigation; T.A., S. S., R.R., and N.K.: supervision and validation; T.M.D.: project administration. All authors reviewed and approved the manuscript for submission towards publication.

Acknowledgments: None.

Conflicts of Interest: No conflict of interest was declared by the authors.

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CHAPTER 4.

CONCLUDING REMARKS

MiRNAs are small non-coding RNA sequences of about 22 nucleotides with a role as posttranscriptional regulators of gene expression. They impact many developmental and homeostatic processes. Moreover, significant changes of tissue miRNAs occur in various diseases, such as cancers, cardiovascular disease, nervous system disease. The findings on the presence of miRNAs in human fluids after the releasing from their cells of origin, numerous studies began to investigate tissue- and disease-specific miRNA signatures in blood, urine, spinal fluid or saliva. MiRNAs have been shown to be protected by RNase digestion and are resistant to severe chemical-physical conditions. Accordingly, they result stable in plasma and serum.

Circulating miRNAs fulfil a number of criteria as ideal biomarkers for a variety of diseases: accessibility through non-invasive methods, high degree of specificity and sensitivity, ability to differentiate pathologies, long half-life within samples, rapid and accurate detection.

Therefore, serum or plasma miRNAs could represent a new approach for diagnostic minimally invasive screening.

Characterization of differential expression patterns of miRNAs might be an approach for development of novel biomarkers in human diseases.

Moreover, a therapeutic approach aimed at dysregulated miRNA is promising. Once their role in the pathogenesis of human diseases will be identified, miRNAs could represent novel targets for drug development.

Due to the recent developments of surgical devices and biological understanding, the success rate for structural recovery of MH, ERM and PVR has been improved. Nevertheless, a certain number of patients with these diseases become blind even after thorough surgical and medical intervention. Achieving understanding of the pathologic mechanisms underlying the development of fibrotic membrane spreading on the surface and beneath the sensory retina is critical to completely overcome PVR processes.

MicroRNA is a novel and powerful class of modulators that regulate gene expression, and its involvement in the pathogenesis of PVR now is becoming clear. Over the last few decades, biological tools to examine inflammatory cytokines have revolutionized research capabilities. Consequently, the involvement of inflammatory cytokines in many retinal diseases also has been elucidated. In addition to further development of scientific tools for micro-RNA detection, measurement, and functional analysis, the close relationship of microRNA and cytokines must be elucidated.

Identifying the role of other microRNAs in EMT in RPE cells *in vitro* and in PVR *in vivo* could be helpful for understanding more precisely the involvement of EMT-related gene expression.

Through this research project, we found the lists of dysregulated microRNAs in some retinal diseases, including PVR, that display common features between them. Firstly, we identified 5 miRNAs differentially expressed in patients affected by MH and ERM with respect to control samples. With the second profiling we found an altered expression of 20 miRNAs in one or more pathological groups of VH of patients affected by primary RD and a different grading of PVR. Moreover, the analysis has shown that the expression of 6 miRNAs (miR-143, miR-224, miR-361, miR-452, miR-486-3p, and miR-891a) increased with the worsening of the disease.

Finding a set of microRNAs and their targeted genes in the same specific disease is the key to exploring microRNA based therapeutic possibility.

Further improvement of the microRNA research will accelerate the development of microRNA agonists/antagonists that can be used as a new class of drugs to regulate the progression of proliferative and fibrotic processes.

CHAPTER 5.

LIST OF PUBLICATIONS

1. Cross-Linked Hyaluronic Acid as Tear Film Substitute.
   Posarelli C, Passani A, Del Re M, Fogli S, **Toro MD**, Ferreras A, Figus M.
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2. The Effectiveness of 0.6% Povidone Iodine Eye Drops in Reducing the Conjunctival Bacterial Load and Needle Contamination in Patients Undergoing Anti-VEGF Intravitreal Injection: A Prospective, Randomized Study.
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Article In press/Under Review

1. Tryptophan metabolites concentration and kynurenic pathway enzymes expression in animal models of retinal and optic nerve damage.
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