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Circulating MicroRNAs as Biomarker for Vessel-Associated Retinal Diseases

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Keywords
MicroRNAs · Biomarker · Diabetic retinopathy · Age-related macular degeneration · Retinal vein occlusion

Abstract
Introduction: Vessel-associated retinal diseases are a major cause of blindness and severe visual impairment. The identification of appropriate biomarkers is of great importance to better anticipate disease progression and establish more targeted treatment options. MicroRNAs (miRNAs) are short, single-stranded, noncoding ribonucleic acids that are involved in the posttranscriptional regulation of gene expression through hybridization with messenger RNA. The expression of certain miRNAs can be different in patients with pathological processes and can be used for the detection and differentiation of various diseases. In this study, we investigate to what extent previously in vitro identified miRNAs are present as cell-free circulating miRNAs in the serum and vitreous of human patients with and without vessel-associated retinal diseases. ***Methods:*** Relative quantification by quantitative real-time polymerase chain reaction was used to analyze miRNA expression in patients with vessel-associated retinal diseases such as age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinal vein occlusion compared with control patients. ***Results:*** In serum samples, miR-29a-3p and miR-192-5p showed increased expression in patients with neovascular AMD relative to control patients. Similarly, miR-335-5p, miR-192-5p, and miR-194-5p showed increased expression in serum from patients with proliferative DR. In vitreous samples, miR-100-5p was decreased in patients with proliferative DR. Differentially expressed miRNAs showed good diagnostic accuracy in receiver operating characteristic (ROC) and area under the ROC curve analysis. ***Conclusion:*** The miRNAs investigated in this study may have the potential to serve as biomarkers for vessel-associated retinal diseases. Combining multiple miRNAs may enhance the predictive power of the analysis.

Introduction

Several eye diseases such as neovascular age-related macular degeneration (nvAMD), diabetic retinopathy (DR), or retinal vein occlusion (RVO) are characterized by pathologic alterations of retinal or choroidal

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blood vessels as well as their uncontrolled formation, permeability, or sprouting [1–3]. Frequent complications include retinal edema, hemorrhages, and fibrous scarring leading to tissue destruction and severe visual impairment or even blindness [2, 4–6]. Excessive vascular endothelial growth factor (VEGF) expression has been shown to play a key role in these pathologic vascular alterations and angio-proliferative processes [7, 8]. The development and widespread use of intravitreal VEGF inhibitors has greatly improved the treatment options for neovascular retinal diseases and has revolutionized the ophthalmological landscape in the past decade [9–12].

Nevertheless, the molecular mechanisms, key players, and their interactions underlying pathological neovascularization and hyperpermeability are not yet sufficiently understood, and retinal vascular diseases are still among the leading causes of blindness and severe visual impairment in the industrialized countries [13, 14]. This is of great clinical relevance as demographic trends show an increase in prevalence and incidence of these diseases [15]. Therefore, identification of additional and innovative target molecules is of extensive interest. A further prerequisite for improving treatment strategies and individualization of medical care is the identification of biomarkers and predictors allowing for early detection of pathological vascular alterations of the retina and the anticipation of disease progression, i.e., the transition from non-proliferative to proliferative states [16, 17].

In this context, microRNAs (miRNAs) could play a significant role. miRNAs are short, single-stranded, non-coding ribonucleic acids that are involved in the regulation of gene expression by hybridizing with messenger RNA or promotor sequences [18, 19]. Dysregulated miRNA expression has been associated with various diseases and specific expression profiles of miRNAs were suggested as biomarkers for disease diagnosis [20–22]. Moreover, miRNAs have also been considered as therapeutic targets in several diseases [23–26]. A role and association of altered miRNA profiles have also been reported for various eye diseases [27–30], including vascular pathologies of the retina [24, 31–34]. Thus, miRNAs have great potential to serve as biomarkers and predictors for ocular diseases.

In a previous in vitro study, we examined miRNA expression patterns of human retinal microvascular endothelial cells (HRMVECs) and identified several miRNAs whose expression was altered in response to stimulation of the cells with VEGF. Among other functions, Gene Ontology (GO) analysis confirmed the association of these miRNAs with angiogenesis-related processes [35]. In the present study, we investigated the expression of these potentially angiogenesis-associated miRNAs as cell-free miRNAs in serum and vitreous samples of patients with different retinal vascular diseases at various disease stages and their potential role as biomarkers.

Materials and Methods

Study Cohort and Sample Collection

Serum and vitreous samples were collected at the Department of Ophthalmology at the University Medical Center Greifswald (Germany) between January 2020 and May 2022. Ethics approval was obtained from the Ethics Committee at the University Medical Center Greifswald (BB 159/19) and complied with the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient before inclusion in the study. Venous blood sampling was performed during routine medical examination or interventions. Only in case of a medically indicated vitrectomy, a vitreous sample was taken from the respective patients in addition to the serum sample. During vitrectomy, the whole vitreous volume of the patient is usually removed and replaced by electrolyte solution from the vitrectomy machine's infusion line. In order to obtain vitreous samples for our study, we collected the first 0.5–1 mL of vitreous into a syringe connected to the aspiration line of the vitrectome. The collected vitreous was then handed over to the laboratory for further processing.

Groups of patients with nvAMD (serum: $n = 30$; vitreous: $n = 9$), atrophic AMD (aAMD; serum: $n = 15$; vitreous: $n = 0$), proliferative DR (PDR; serum: $n = 20$; vitreous: $n = 20$), non-proliferative DR (NPDR; serum: $n = 23$; vitreous: $n = 8$), and RVO (serum: $n = 20$; vitreous: $n = 7$) as diagnosed by optical coherence tomography, fundus image analysis, and/or fluorescein angiography [36–40] were included in the study. Patients with eye diseases not related to retinal vascular disorders such as cataract, retinal detachment, dry eye disease, macular hole, epiretinal membranes, or intraocular lens dislocation were used as controls (serum: $n = 35$; vitreous: $n = 23$). Patients with an inflammatory eye disease, rheumatic disease, or a tumor diagnosis in the past 5 years were excluded from the study and control groups. After taking blood samples, clotting was assured by keeping the samples at room temperature for at least 30 min. Within 4 h after collection, blood and vitreous samples were centrifuged for 10 min at 3,000 *g*. Serum and vitreous supernatants were removed and stored in aliquots at −80°C until analysis.

Quantitative Real-Time Polymerase Chain Reaction Analysis of miRNA Expression Profiles in Serum and Vitreous Samples

Total RNA was isolated from 200 µL of serum or vitreous humor samples using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Sample lysis was performed using guanidine thiocyanate, followed by adding a synthetic Spike-In miRNA (UniSp6; 12 fmol/80 µL, Qiagen) to assure a technically successful RNA isolation, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR) amplification. After protein precipitation, the total RNA was bound to the silica membrane. Following several washing steps, RNA was eluted with 20 µL of nuclease-free water. Total RNA was stored at −20°C until further use. To increase RNA yields isolated from vitreous humor, a carrier RNA (MS2; Sigma-Aldrich, Taufkirchen, Germany) was added together with the Spike-In miRNA during the isolation process.

Table 1. miRNA primers used in qRT-PCR analysis

miRNA	Sequence 5′–3′ (miRBase)	miRBase accession #	GeneGlobeID
UniSp6			YP00203954
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	MIMAT0000101	YP00204063
hsa-miR-139-5p	UCUACAGUGCACGUGUCUCCAGU	MIMAT0000250	YP00205874
hsa-miR-139-3p	UGGAGACGCGGCCCUGUUGGAGU	MIMAT0004552	YP00205661
hsa-miR-335-5p	UCAAGAGCAAUAACGAAAAAUGU	MIMAT0000765	YP02119293
hsa-miR-335-3p	UUUUUCAUUAUUGCUCCUGACC	MIMAT0004703	YP00205613
hsa-miR-192-5p	CUGACCUAUGAAUUGACAGCC	MIMAT0000222	YP00204099
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	MIMAT0000086	YP00204698
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU	MIMAT0000100	YP00204679
hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA	MIMAT0000460	YP00204080
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG	MIMAT0000444	YP00206010
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG	MIMAT0000445	YP00204227
hsa-miR-100-5p	AACCCGUAGAUCCGAACUUGUG	MIMAT0000098	YP00205689
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	MIMAT0000076	YP00204230
hsa-miR-4521	GCUAAGGAAGUCCUGUGCUCAG	MIMAT0019058	YP02109260
hsa-miR-1248	ACCUUCUUGUAUAAGCACUGUGCUAAA	MIMAT0005900	YP00204253
hsa-miR-1291	UGGCCCUGACUGAAGACCAGCAGU	MIMAT0005881	YP02107164

Using the miRCURY LNA RT Kit (Qiagen), 2 µL (serum) or 5 µL (vitreous) of the total RNA was reverse transcribed into cDNA according to the manufacturer's instructions and applying the following thermal cycler (MiniAmpPlus, Life Technologies by Thermo Fisher Scientific, Foster City, CA, USA) conditions: 60 min at 42°C, 5 min at 95°C (stop of reaction), followed by 4°C. cDNA was stored in aliquots at −20°C until performing qRT-PCR. Then, 3 µL of cDNA template dilution (1:30 in nuclease-free water) was subjected to SYBR Green-based qPCR reactions containing miRNA-specific forward and reverse PCR amplification primers (miRCURY LNA miRNA PCR Assays, Qiagen; Table 1) and preformulated SYBR Green PCR Master Mix supplemented with ROX passive reference dye (miRCURY SYBR Green PCR Kit, Qiagen) following the manufacturer's instructions. The target miRNAs selected for qRT-PCR analyses (Table 1) were identified as potentially angiogenesis-associated miRNAs in our previous in vitro study [35]. StepOnePlus qRT-PCR instrument (Applied Biosystems by Thermo Fisher Scientific) was used to perform qRT-PCR with the following conditions: 2 min at 95°C, followed by 40 cycles: 10 s at 95°C and 60 s at 56°C. StepOnePlus software v2.3 was used for data acquisition. Relative miRNA expression was calculated according to the comparative threshold cycle (ΔΔCt) method [41].

For serum samples, data were normalized by subtracting the Ct value of the endogenous reference miRNA hsa-miR-103a-3p from the Ct value of the individual target miRNA (ΔCt). This approach was used as miR-103a-3p levels had been found to be homogeneous among the different patient and control groups (online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000533481).

At present, to our knowledge, there is no endogenous control established for the normalization of vitreous humor samples. Hence, for calculating the ΔCt value of vitreous humor samples, the mean expression value was used by subtracting the mean of all measured miRNAs from the Ct value of the individual target miRNA according to Mestdagh et al. [42].

The ΔΔCt for each target miRNA was then calculated by subtracting the mean of the ΔCt values of all controls from the ΔCt of each patient sample. PCR efficiency for each miRNA PCR assay (forward + reverse primer pairs) was measured to be above 80%. The normalized miRNA expression of each patient sample was related to the normalized miRNA expression of the control group and expressed as fold change calculated by $2^{-\Delta\Delta Ct}$. qRT-PCR reactions were performed in technical duplicates (serum) or triplicates (vitreous). Serum sample duplicates with a standard deviation >0.25 and vitreous sample triplicates with standard deviation >0.5 were excluded from statistical analyses. Furthermore, samples that were found to be outliers in the synthetic Spike-In miRNA (UniSp6) measurements were excluded from statistical analyses.

Statistical Analyses

IBM SPSS Statistics V.28.0 (IBM Corporation, Armonk, NY, USA) was used to statistically analyze data. For statistical analyses, the n-fold values representing relative miRNA expression were Log10 transformed. To analyze miRNA expression data for normal distribution and homogeneity of variance, Kolmogorov-Smirnov test and Levene's test were performed, respectively. Depending on the results, parametric *t* tests (in the case of normal distribution and homogeneity of variance) or nonparametric Mann-Whitney U tests were conducted to compare two groups. For the comparison of more than two groups, parametric one-way ANOVA or nonparametric Kruskal-Wallis test with Bonferroni post hoc analyses were used. Pearson correlation was

Table 2. Patient characteristics for serum samples

	Control	PDR	NPDR	nvAMD	aAMD	RVO	*p* value
Group size	35	20	23	30	15	20	
Age, years (mean±SD)	66.8±11.2	51.3±13.6	71.1±11.6	78.5±8.6	80.4±8.2	69±10.8	<0.001*
Age range, years	40.5	45.6	43.3	32.7	27.8	34.8	
Female sex (%)	46	45	26	60	87	35	0.005[a]

aAMD, atrophic age-related macular degeneration; NPDR, non-proliferative diabetic retinopathy; nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy; RVO, retinal vein occlusion; SD, standard deviation. *Kruskal-Wallis test. [a]χ^2 test.

applied to detect a possible correlation of miRNA expression with the age of the patients. *t* tests or Mann-Whitney U tests were used to identify a possible correlation of miRNA expression levels with sex of the patient. A p value <0.05 was considered statistically significant. To further assess the diagnostic accuracy of individual miRNAs as potential biomarkers for retinal vascular diseases, we performed receiver operating characteristic (ROC) and area under the ROC curve (AUC) analyses. To improve the prediction probability of the detected miRNAs for the examined diseases, we used the mean of miRNA ΔCt values and performed the analysis as described above.

Results

miRNA Analysis in Serum Samples

Overall, 143 serum samples were included in the study. Demographic characteristics of the respective patient and control groups are summarized in Table 2.

The mean age varied significantly among the groups. PDR patients were significantly younger than patients in all other groups (Kruskal-Wallis test with Bonferroni post hoc: $p < 0.05$), while the patients of the two AMD groups were significantly older than the control group ($p < 0.05$). However, considered across all patients regardless of the patient group, a correlation of patient age with ΔCt value was observed only for miR-335-5p (Pearson's coefficient = −0.241, $p = 0.018$). A difference between the groups was observed for sex ratio, which was shifted toward female sex in the atrophic AMD group and to male sex in the NPDR and RVO groups (χ^2 test: $p = 0.005$). Except for miR-139-5p (Mann-Whitney U test: $p = 0.018$), no statistical differences in ΔCt values were found between male and female patients.

In our previous in vitro study [35], we identified several miRNAs in HRMVECs to be differentially expressed in response to pro-angiogenic stimulation. In the present study, we investigated whether these potentially angiogenesis-associated miRNAs were present as cell-free, circulating miRNAs in serum samples of groups of patients with various retinal neovascular diseases or vascular disorders. In total, we analyzed 15 miRNAs in the present study (Table 1), of which 10 miRNAs were robustly and consistently detected as cell-free, circulating miRNAs in serum samples (Fig. 1a), while the other miRNAs (miR-1292, -1248, -4521, -139-3p, and -335-3p) were at or under the limit of detection.

Comparative expression analysis revealed four miRNAs to be differentially expressed between the patient groups. Relative expression of miR-29a-3p was significantly elevated in serum samples from patients with nvAMD (Fig. 1b), miR-192-5p was upregulated in both, PDR and nvAMD (Fig. 1c). miR-335-5p and miR-194-5p were significantly increased in PDR serum samples (Fig. 1d, e). To further assess the diagnostic accuracy of the differentially expressed miRNAs for nvAMD and PDR, we performed ROC analyses. Using the respective Log10 n-fold value of each control and each nvAMD patient, ROC analyses of miR-192-5p and miR-29a-3p revealed an AUC of 0.78 and 0.77, respectively (Fig. 2a, b). For miR-192-5p, -194-5p, or -335-5p, an AUC of 0.77, 0.75, and 0.65 was obtained in serum samples from PDR patients (Fig. 2c–e). Thus, all differentially expressed miRNAs showed good diagnostic accuracy. This was even further increased by combining several miRNAs. Combining normalized Ct values (ΔCt) of miRNAs miR-29a-3p and -192-5p in the ΔΔCt and Log10 n-fold calculation for AMD patients resulted in an AUC = 0.87 (Fig. 3a, b). By combining miRNAs miR-335-5p and -192-5p, an AUC = 0.82 was attained to discriminate between PDR patients and controls (Fig. 3c, d).

miRNA Analyses in Vitreous Samples

We also conducted relative quantification of miRNA levels in vitreous samples. In the present study population, 67 patients underwent vitrectomy. The demographic patient characteristics are summarized in Table 3.

The PDR group was significantly younger than all other groups (ANOVA with Bonferroni post hoc analyses: $p < 0.05$), but no significant correlation between patient age and miRNA expression levels was detected. Moreover, no statistical difference between groups was found for patient sex ratio (χ^2 test: $p = 0.11$).

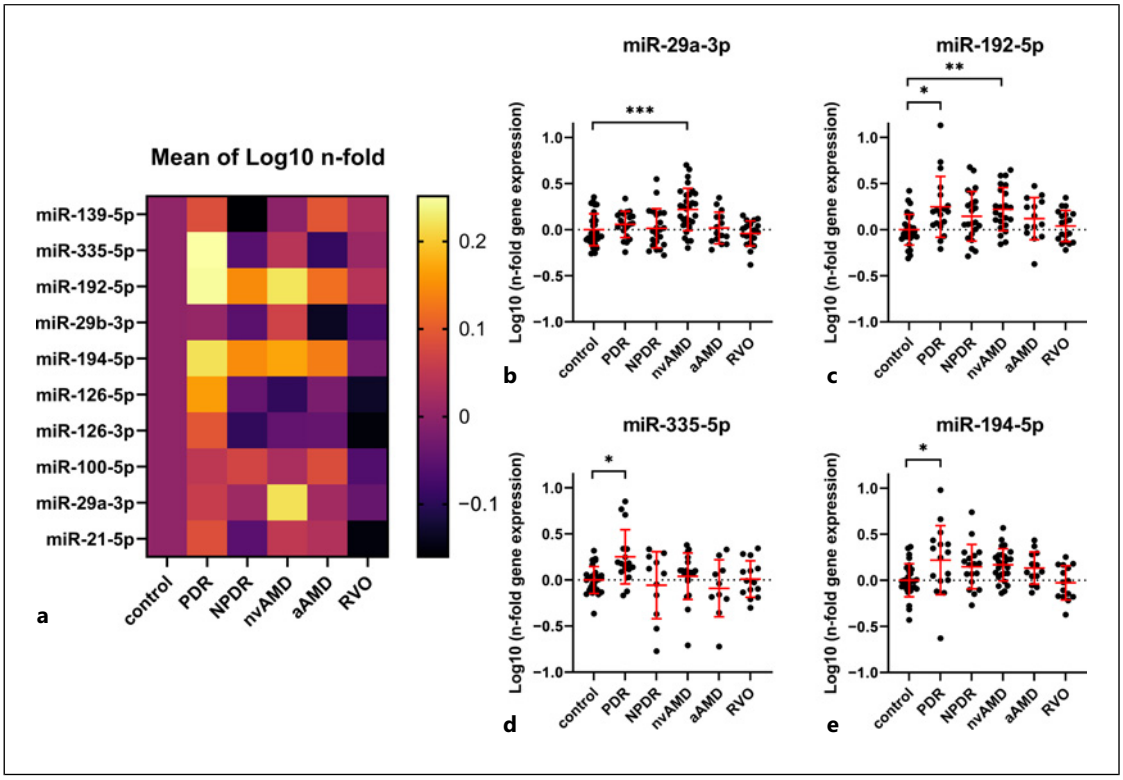

Fig. 1. Log10 n-fold values of miRNAs in serum samples. **a** In the heatmap, the difference of the mean Log10 n-fold value of the patient group and the control group is color coded. **b** Log10 n-fold values of miR-29a-3p; error bars (red) represent the mean ± SD; ***$p < 0.001$ (ANOVA with Bonferroni post hoc test). **c**–**e** Log10 n-fold values of miR-192-5p (**c**), -335-5p (**d**), -194-5p (**e**); error bars (red) represent the median with interquartile range; *$p < 0.05$, **$p = 0.01$ (Kruskal-Wallis test with Bonferroni correction). aAMD, atrophic age-related macular degeneration; NPDR, nonproliferative diabetic retinopathy; nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy; RVO, retinal vein occlusion; SD, standard deviation.

In vitreous samples, less cell-free miRNAs of our panel (miR-335-5p, -29b-3p, -29a-3p, -21-5p, and -100-5p) could robustly and consistently be measured than in the corresponding serum samples. In the PDR patient group, miR-100-5p expression level was significantly lower compared to the control group. Discriminating the PDR and control group by using the Log10 n-fold of miR-100-5p in ROC-AUC analysis revealed a diagnostic accuracy of AUC = 0.83 (Fig. 4).

Discussion

In the present study, we quantified the relative expression of previously in vitro identified, potentially angio-modulative miRNAs in serum and vitreous samples of patients with retinal vascular diseases such as DR, nvAMD, or RVO and of control patients with nonvascular eye diseases. Some of these miRNAs were robustly measured as cell-free, circulating miRNAs in serum samples and showed different expression levels between certain groups of patients and the control group.

The expression of miRNAs miR-194-5p and -335-5p was exclusively elevated in serum samples of patients with PDR, while miR-29a-3p serum levels were increased in nvAMD patients and serum levels of miR-192-5p were increased in both PDR and nvAMD serum samples. ROC-AUC analyses revealed high associations of these serum miRNA biomarkers with the respective retinal diseases. In our previous in vitro study, miR-29a-3p expression was downregulated after stimulating HRMVECs with pro-angiogenic VEGF. However, in nvAMD, a presumable VEGF-driven disease [7, 43, 44], we found miR-29a-3p expression to be upregulated in comparison to the control group. Similar results were reported by Ertekin et al. [45],

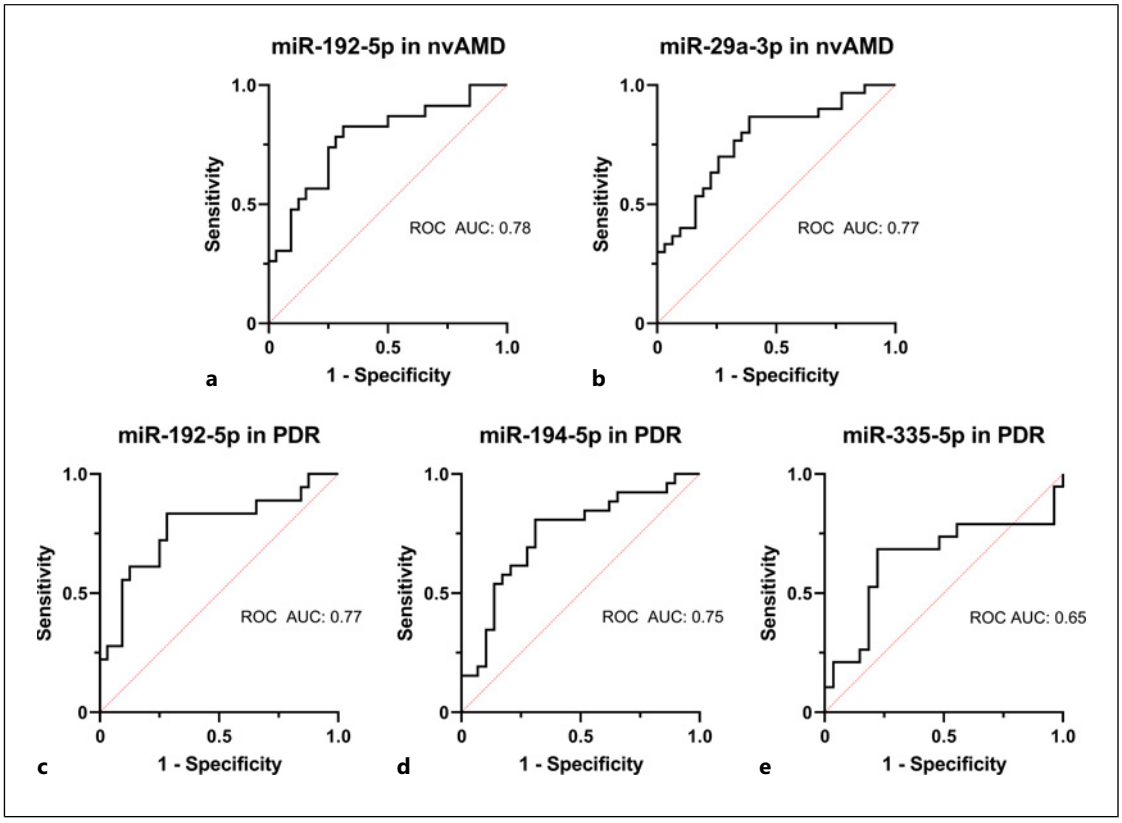

Fig. 2. ROC-AUC analysis further evaluates miRNA expression as potential biomarker. **a**–**e** The receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were calculated by comparing Log10 n-fold values of nvAMD or PDR with the control group. **a** $p < 0.001$; 95% CI: 0.65 to 0.91. **b** $p < 0.001$; 95% CI: 0.65 to 0.89. **c** $p = 0.0014$; 95% CI: 0.63 to 0.92. **d** $p = 0.0017$; 95% CI: 0.61 to 0.88. **e** $p = 0.097$; 95% CI: 0.47 to 0.82. nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy.

who showed nvAMD-specific expression of miR-29a-3p. As complex, multifactorial processes underlie AMD pathology including metabolic and oxidative stress, complement activation, and para-inflammation [46–49], also VEGF-independent pathways can be supposed to be involved in the regulation of miR-29a-3p. In contrast to the study of Ertekin et al. [45], in our present study, miR-192-5p expression was additionally elevated in nvAMD. In a pilot study by Grassmann et al.[50], miR-192-5p was identified by next-generation sequencing to be associated with late-stage nvAMD. In the follow-up study, qRT-PCR analyses of a separate patient cohort did not confirm the previous results. Thus, relative miRNA expression data may vary between different studies in dependence of the composition of the cohorts of patients and controls.

Compared to the control group, miR-192-5p expression in our cohort was also upregulated in the group of PDR patients. For both, nvAMD and PDR, ROC-AUC analyses demonstrated high predictive quality and diagnostic accuracy for miR-192-5p (AUC = 0.78 and AUC = 0.77, respectively), implying that single miRNAs may have strong association with various dysregulated pathological processes and diseases [51]. miR-192-5p has been shown to be involved in the regulation of VEGF signaling-associated molecules and angiogenesis-related pathways [52–54]. Counterintuitive to the observation of an anti-angiogenic role of miR-192-5p in these studies, we found miR-192-5p to be elevated in serum samples of nvAMD and PDR patients, in which uncontrolled angiogenesis and increased VEGF expression are present [8, 55]. Such miRNA upregulation, however, could also be part of a counterregulatory anti-angiogenic response to pathologic neovascularization and might depend on the timing of serum sampling in relation to the disease activity.

miR-194-5p expression was found to be constantly elevated in the retina of mice under diabetic conditions [56]. Moreover, serum levels of miR-192 and miR-194 were

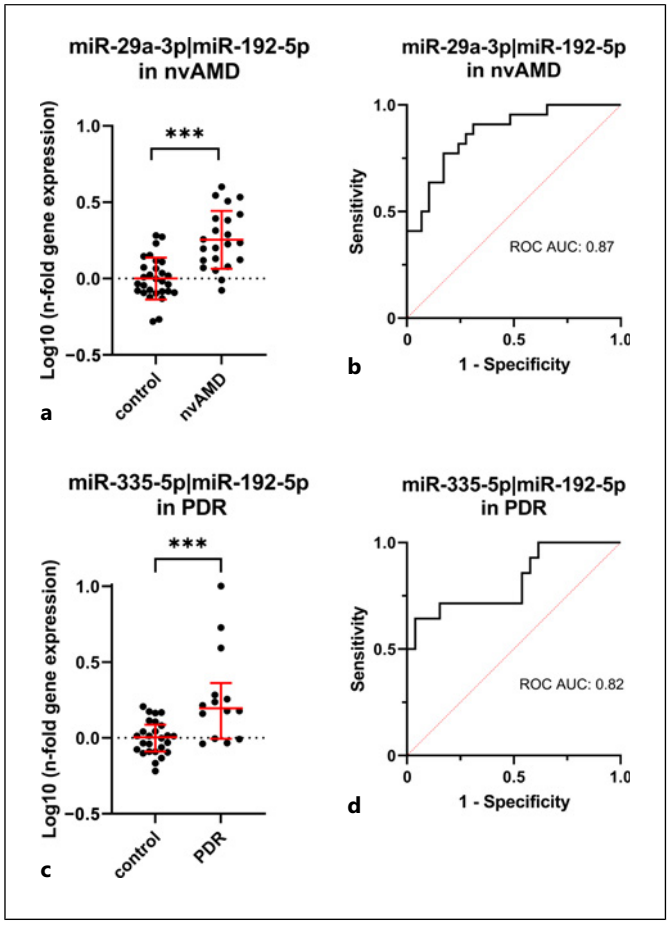

Fig. 3. Serum miRNA levels are associated with the presence of nvAMD and PDR. The mean ΔCt value of multiple miRNAs was used for the n-fold calculation. **a** Combined Log10 n-fold values of miR-29a-3p and miR-192-5p for control and nvAMD group; error bars (red) represent the mean ± SD; ***$p < 0.001$ (t test). **b** Receiver operating characteristic (ROC) and area under the curve (AUC) analysis for combined miRNAs miR-29a-3p and -192-5p in nvAMD; ROC-AUC = 0.87; $p < 0.001$; 95% CI: 0.77 to 0.96. **c** Combined Log10 n-fold values of miR-335-5p and -192-5p for control and PDR group; error bars (red) represent the median with interquartile range; ***$p < 0.001$ (Mann-Whitney U test). **d** ROC-AUC analysis for combined miRNAs miR-335-5p and -192-5p in PDR; ROC-AUC = 0.82; $p < 0.001$; 95% CI: 0.68 to 0.97. nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy; SD, standard deviation.

significantly higher in patients suffering from diabetes than in euglycemic probands [57], indicating that altered expression profiles of these miRNAs may originate from the metabolic malfunctions and their versatile detrimental downstream effects, such as oxidative stress or inflammatory processes that underlie diabetes [58]. In our study, miR-192-5p and -194-5p expression was upregulated in patients with PDR, while it was not upregulated in NPDR, despite the same underlying systemic disorder. Thus, expression profiles of these miRNAs may specifically indicate angio-proliferative and neovascular disease stages of the retina in diabetes.

In the present study, in vitreous samples, fewer different cell-free miRNAs of our panel could be detected than in serum samples and a different expression pattern was observed between serum and vitreous. The vitreous analyses showed significantly decreased expression levels of miR-100-5p in patients with PDR compared to the control group. Consistent with our data, miR-100-5p expression was found to be downregulated under hypoxic conditions in various cell types and a negative correlation of miR-100-5p and HiF1A expression has been described [59, 60]. Exosomal transfer of miR-100 from mesenchymal stem cells downregulated the angiogenesis-related mTOR/HIF-1α/VEGF signaling axis in breast cancer cells [61]. In addition, a negative effect of miR-100-5p on tube formation of endothelial cells in vitro was reported [62]. Taking these observations and the high diagnostic accuracy of miR-100-5p expression data for PDR (ROC-AUC = 0.83) in our study into account, vitreal miR-100-5p expression profiles may represent a vitreal biomarker for hypoxia-associated retinal angio-proliferative processes.

However, relative miRNA expression data and their use as biomarkers comprise several pitfalls and limitations, since an individual miRNA has multiple target molecules and, vice versa, several miRNAs share the same target molecule [18, 63, 64], resulting in a reciprocal, finely tuned regulatory network. Consequently, patient inclusion criteria, disease stages, comorbidities, and current treatment regimens may all have an impact on miRNA expression patterns and may be an explanation for varying miRNA expression data in different studies [65, 66]. In the present study, patients with different severities of the same disease were combined into a disease-specific patient group. Furthermore, both therapy-naïve and already long-term treated patients were included in the same group.

Another source for variation in relative miRNA expression data between distinct studies may be due to different data normalization strategies [21, 67], including the definition of the control group and the choice of the reference miRNA. In the present study, we normalized miRNA expression data to a control group consisting of patients with diverse eye diseases without vascular pathology. Variance of miRNA expression data (ΔCt) between the different control group entities was low (online suppl. Fig. 2). For serum samples, miRNA miR-103a-3p met the criteria for an appropriate endogenous reference miRNA and was used for data normalization in our study,

Table 3. Patient characteristics for vitreous samples

	Control	PDR	NPDR	nvAMD	RVO	*p* value
Group size	23	20	8	9	7	
Age, years (mean±SD)	67.3±9.1	55±14	73.2±8.7	78±9.7	73.2±10.4	<0.001*
Age range, years	40.1	51.4	22.7	29.9	28	
Female sex (%)	48	45	13	78	57	0.11[a]

NPDR, non-proliferative diabetic retinopathy; nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy; RVO, retinal vein occlusion; SD, standard deviation. *ANOVA with Bonferroni post hoc analysis. [a]χ^2 test.

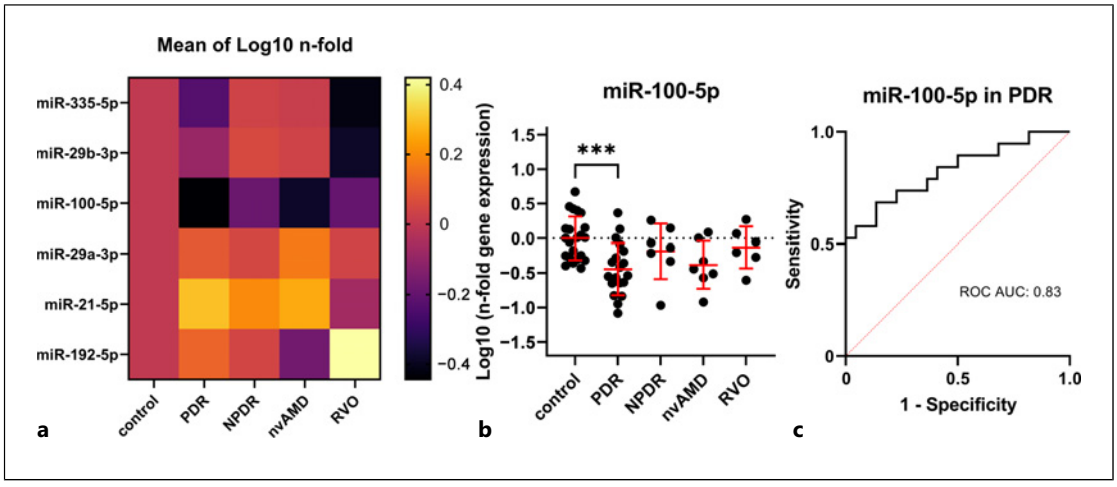

Fig. 4. Log10 n-fold values of miRNAs in vitreous humor samples and ROC-AUC analysis. **a** In the heatmap, the difference of the mean Log10 n-fold value of the patient group and the control group is color coded. **b** Log10 n-fold values for miR-100-5p; error bars (red) represent the mean ± SD; ***$p < 0.001$ (ANOVA with Bonferroni post hoc test). **c** Receiver operating characteristic (ROC) and area under the curve (AUC) analysis for miR-100-5p in PDR; $p < 0.001$; 95% CI: 0.7 to 0.96. aAMD, atrophic age-related macular degeneration; NPDR, non-proliferative diabetic retinopathy; nvAMD, neovascular age-related macular degeneration; PDR, proliferative diabetic retinopathy; RVO, retinal vein occlusion; SD, standard deviation.

due to its homogeneous Ct values across serum samples of the various patient groups and controls (online suppl. Fig. 1B) and its Ct value pattern comparable to that of the synthetic Spike-In miRNA (UniSp6; online suppl. Fig. 1A).

However, miR-103a-3p expression in vitreous samples showed higher variance. Therefore, we used the mean expression value normalization [42], with the limitation of only six included miRNAs, which were robustly measured in vitreous samples. In summary, the main limitations of relative miRNA quantification as presented in our study are related to the composition of patient and control groups and the data normalization strategy.

In conclusion, in the present study, we identified several differentially expressed miRNAs in serum and vitreous samples which may represent biomarker candidates for retinal neovascular diseases such as nvAMD and PDR and may open the window to the identification of further key factors and novel target molecules for treatment. The function of these miRNAs as biomarkers needs to be verified in future studies using newly assembled, comprehensive, valid, and matched patient and control cohorts.

Statement of Ethics

Ethics approval was obtained from the Ethics Committee at the University Medical Center Greifswald, approval number BB 159/19. The study was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient before inclusion in the study.

Conflict of Interest Statement

M.B. received research grants and personal fees from Bayer; J.M.P. received personal fees from Novartis; and B.G. received research grants from Novartis, all outside the submitted work. A.S. received research funds from Novartis related to the present study and research grants from Bayer unrelated to the present study, as well as personal fees from Alcon, Apellis Pharmaceuticals, Bayer, Novartis, and Roche outside the submitted work.

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Author Contributions

M.D.: conceptualization, investigation, acquisition, analyses, and interpretation of data, and drafting the manuscript; M.B.: project administration, conceptualization, investigation, acquisition, analyses, and interpretation of data, and drafting the manuscript; J.M.P.: analyses and interpretation of data; T.B. investigation; D.S., W.O., E.M.K., F.W., A.T., and M.C.B.: data acquisition and provision of patient samples; S.P. and B.G. conceptualization, data acquisition, and provision of patient samples; A.S. supervision, conceptualization, acquisition, analyses and interpretation of data, provision of patient samples, and acquisition of funding. All authors critically revised the manuscript draft for important intellectual content and approved the final version of the manuscript. M.D. and M.B. contributed equally as co-first authors.

Data Availability Statement

All data relevant to the study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author.

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