MicroRNAs as diagnostic and prognostic biomarkers of age-related macular degeneration: advances and limitations

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Abstract

A main cause of vision loss in the elderly is age-related macular degeneration (AMD). Among the cellular, biochemical, and molecular changes linked to this disease, inflammation and angiogenesis appear as being crucial in AMD pathogenesis and progression. There are two forms of the disease: dry AMD, accounting for 80–90% of cases, and wet AMD. The disease usually begins as dry AMD associated with retinal pigment epithelium and photoreceptor degeneration, whereas wet AMD is associated with choroidal neovascularization resulting in severe vision impairment. The new vessels are largely malformed, leading to blood and fluid leakage within the disrupted tissue, which provokes inflammation and scar formation and results in retinal damage and detachment. MicroRNAs are dysregulated in AMD and may facilitate the early detection of the disease and monitoring disease progression. Two recent reviews of microRNAs in AMD had indicated weaknesses or limitations in four earlier investigations. Studies in the last three years have shown considerable progress in overcoming some of these concerns and identifying specific microRNAs as biomarkers for AMD. Further large-scale studies are warranted using appropriate statistical methods to take into account gender and age disparity in the study populations and confounding factors such as smoking status.

Key Words: biomarkers; blood plasma; blood serum; macular degeneration; microRNAs; peripheral blood nuclear cells; retinal tissues; vitreous humour; whole blood

Introduction

A main cause of vision loss in the elderly is age-related macular degeneration (AMD), which profoundly impacts quality of life (Raftery et al., 2007; Chakravarthy et al., 2010; Schaal et al., 2016; Wang et al., 2016a; Al-Zamil et al., 2017). Given the increasing aging population worldwide, the incidence of AMD is projected to increase from 196 million in 2020 to 288 million in 2040 (Wong et al., 2014), placing a significant burden on families and the healthcare system. Demographic, environmental and genetic risk factors all play substantial contributing roles in the pathophysiology of AMD. Among the cellular, biochemical, and molecular changes linked to this disease, inflammation and angiogenesis appear to be critical in AMD pathogenesis and progression (Agrawal and Chaqour, 2014; Kauppinen et al., 2016).

There are two forms of AMD, dry (nonexudative) and wet (exudative, neovascular) (Machalińska et al., 2012). The disease usually begins as the dry type constituting 80–90% of cases, whereas wet AMD represents 10–15% of AMD cases. Dry AMD is associated with retinal pigment epithelium (RPE) and photoreceptor degeneration (Ayoub and Patel, 2009), while wet AMD is associated with choroidal neovascularization and accounts for 90% of clinical cases with severe vision impairment (Bhise et al., 2011; Heiferman and Fawzi, 2019). Characteristic of dry AMD is an altered RPE pigment distribution in the macula, and the generation of pale or yellow deposits called drusen in the space between the RPE and Bruch's membrane (Johnson et al., 2003; Ayoub and Patel, 2009; Algvere et al., 2016). Bruch's membrane is the innermost layer of the choroid and lies in apposition to the RPE. Drusen contain a variety of constituents, including lipid and amyloid-β deposits (Isas et al., 2010). Early stage dry AMD patients may remain asymptomatic and it may take years for their vision to be affected (Ayoub and Patel, 2009). In late stage dry AMD there is geographic atrophy (GA) of the RPE and retina, and choroidal neovascularization (CNV) characterizes wet AMD (Ayoub and Patel, 2009). Disease progression in GA AMD is usually slow. CNV AMD is characterized by the growth of leaky blood vessels from the choroid into the retina (Feehan et al., 2011). The new vessels that are formed constitute the choroidal neovascular membrane; they are largely malformed resulting in improper vascular integrity (Senger and Davis, 2011). The blood and fluid leakage within the disrupted tissue provokes inflammation and scar formation resulting in retinal damage and detachment (Witmer et al., 2003). This damage to the retina causes central vision loss and eventual loss of sight if untreated (Bhise et al., 2011; Farnoodian et al., 2017).

Angiogenesis and vascular imbalance are critically involved in this disease, with vascular endothelial growth factor (VEGF), a proangiogenic factor and a key player (Al-Zamil and Yassin, 2017; Farnoodian et al., 2017). Several ocular cells produce VEGF, including RPE cells, endothelial cells, glial cells, and ganglion cells (Bhutto et al., 2008). In addition to stimulating blood vessel growth, VEGF also promotes endothelial cells to synthesize matrix metalloproteinases that proteolytically degrade the extracellular matrix and enable

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new vessels to form (Vempati et al., 2014). Factors other than VEGF control angiogenesis in AMD, including platelet-derived growth factor, fibroblast growth factors, epidermal growth factor, angiopoietins, and angiogenin (Abdollahi and Folkman, 2010; Bhise et al., 2011; Skeie et al., 2011). Also, several angiogenesis inhibitors including thrombospondin-1, pigment epithelium derived factor, endostatin, and angiostatin are present in the eye environment, and the levels of thrombospondin-1, pigment epithelium derived factor, and endostatin were decreased in Bruch's membrane in eyes with AMD (Bhutto et al., 2008). Therefore, it seems that a balance of pro- and anti-angiogenic factors is necessary for achieving ocular vascular homeostasis. The production of these factors can be altered by hypoxia, oxidative stress, ischemia, and inflammation (which all increase with age) and thereby disturb this balance, leading to AMD development (Bhise et al., 2011).

The recruitment of macrophages, which release proinflammatory and proangiogenic mediators, has been suggested in both dry and wet AMD (Ambati et al., 2013). The suppression of inflammation and new vessel growth emerge as strategies for the treatment of AMD. Approximately 50% of individuals with extensive macular drusen will progress within 5 years to vision threatening GA and/or neovascularisation (Davis et al., 2005), which are late stage manifestations of the disease (Sunness et al., 1999; Wong et al., 2008). No effective treatment exists at present to prevent the onset or progression of GA (Holz et al., 2014). Anti-VEGF agents are effective in treating choroidal neovascular membrane in wet AMD but have limited success with about 15% of AMD patients not responding to such treatment (Krebs et al., 2013), and are associated with serious systemic adverse events (Martin et al., 2011). Moreover, antiangiogenic treatments require intravitreal injections each month and long-term follow-up. They are invasive and costly (Raftery et al., 2007), with increased risk of intra-ocular infection. In the USA, the visual impairment caused by AMD is forecast to double by 2050 and that antiangiogenic agents will only reduce this by 17% (Rein et al., 2009). Currently, optical coherence tomography and fluorescein fundus angiography performed by an optometrist or ophthalmologist can be used in the diagnosis of AMD and to monitor its progression. However, these procedures cannot be used to predict the disease. A non-invasive and sensitive test would considerably enhance the diagnosis and follow-up process of AMD.

MicroRNAs (miRNAs) are an abundant class of endogenous single-stranded non-coding RNA molecules approximately 22 nucleotides long. They recognize sequences in the 3′-untranslated regions (3′-UTR) of target mRNAs, and either induce mRNA degradation (Bagga et al., 2005) or inhibit their translation (He and Hannon, 2004; Meister, 2007). They are involved in a range of basic cellular processes such as proliferation, differentiation, apoptosis, and cell cycle regulation (Mens and Ghanbari, 2018). Also miRNAs have a major role in regulating various pathological processes involved in AMD pathogenesis, including inflammation and angiogenesis (Kawa and Machalińska, 2014). Previously, miRNAs have been directly linked to retinal diseases e.g., retinitis pigmentosa (Loscher et al., 2007), retinoblastoma (Zhao et al., 2009), and ocular neovascularization (Shen et al., 2008; Dong et al., 2009). Recently, altered expression of inflammatory miRNAs was found in retinal tissue and blood plasma as well as in peripheral blood mononuclear cells from AMD patients (Berber et al., 2017; Lin et al., 2018; Pogue and Lukiw, 2018). Four miRNAs, miR-9, miR-129b, miR-146a, miR-155, were upregulated in whole retina samples from AMD patients compared to controls (Lukiw et al., 2012). The miRNAs present in human serum and plasma have been found to be relatively stable (Gilad et al., 2008) and miRNAs are potential biomarkers which can be used in the diagnosis and prognosis of human diseases (Lässer, 2012; Peplow et al., 2019). Additionally, synthetic miRNAs in artificial exosomes could be applicable for therapeutic approaches by modulating miRNA levels.

Two recent reviews of miRNAs in AMD patients (Berber et al., 2017; Ascou et al., 2018) have drawn attention to important limitations in four research studies published 2014–2016, and these are summarized in **Table 1**. Notably, there was minimal agreement in the findings between the four studies, and although some miRNAs were found in several of the studies, they did not necessarily concur on the direction of effect due to possible simultaneous therapy. A partial explanation for these discrepancies might lie in the patient inclusion criteria, as one study had included late stage AMD patients, some of whom had received anti-VEGF therapy (Grassmann et al., 2014), while another study had used newly diagnosed patients who had not received any therapy prior to entry into the study (Szemraj et al., 2015). Also, some of the control subjects in the earlier study had glaucoma, while in the later study only controls without ocular abnomalities were included. Other limitiations shown in **Table 1** include a failure to perform receiver operating characteristics (ROC) analysis to determine if any of the dysregulated miRNAs can serve to differentiate AMD patients from controls. We have performed a PubMed literature search of research articles published in the period 1 January 2014 to 31 December 2019 on miRNAs in dry and wet AMD and which includes the four mentioned studies published in 2014–2016. The goal is to identify miRNA biomarkers of high sensitivity and specificity in early-stage AMD, and using a panel of miRNAs or ratio of two miRNAs may be an important method to accomplish this. Also, this is to provide further information on miRNA dysregulation in AMD patients, and whether the weaknesses or limitations identified in the four earlier studies have been taken into account or overcome in the later studies.

MicroRNAs and Age-Related Macular Degeneration

The steps involved in this review and its contents are shown (**Figure 1**). A total of thirteen research articles were found in the PubMed search of which one had utilized whole blood, two peripheral blood nuclear cells, two blood plasma, five blood serum, one exosomes from blood serum, one blood plasma and vitreous humour, and one retinal tissues. All of the studies reported using RT-PCR or PCR for quantitative measurement of miRNAs except for one which had used miRNA array analysis. All of the studies except for two had reportedly included both male and female subjects – one had not reported on gender composition of the AMD and control subjects, and in another study all the samples were from females. There was often a disproportion of the two genders in the study groups and also differences in their mean ages. Moreover, while several of the studies had recruited quite large cohorts of AMD and control patients e.g., one study had 175 dry AMD, 179 wet AMD, and 121 healthy control (HC) individuals (Ulańczyk et al., 2019), there were several others that were performed with much smaller cohorts e.g., one had used 11 wet AMD and 11 HC subjects (Romano et al., 2017). While many of the studies had reported on criteria for patient inclusion, exclusion criteria were often not reported. Three of the studies had adjusted their data for age, gender, and smoking status (Grassmann et al., 2014; Litwińska et al. 2019; Ulańczyk et al. 2019). Use of ROC analysis with area under curve (AUC) values to establish which miRNAs are good or fair tests to distinguish AMD patients from HC, and to differentiate between dry AMD and wet AMD patients, was only reported in four studies (Grassman et al., 2014; Menard et al., 2016; Ren et al., 2017; Lin et al., 2018). Sensitivity and specificity values of specific miRNAs as biomarkers of AMD disease were only provided in one study (Ren et al., 2017). These are all important limitations (**Table 1**).

The relevant findings from the research articles in the PubMed search are summarized as follows.

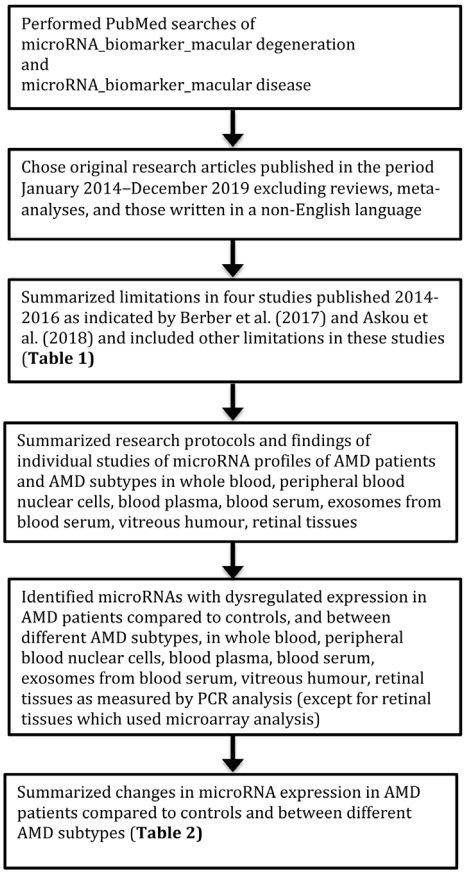

Figure 1 | **Flow diagram to show how the review was performed and its contents.**
AMD: Age-related macilar degeneration; PCR: polymerase chain reaction.

Whole blood

By RT-PCR analysis, Ren et al. (2017) showed that the expression of miR-27a-3p, miR-29b-3p, miR-195-5p was increased (increase ratios 2.68, 3.53, 4.23 times, respectively) in 72 dry AMD and 54 wet AMD patients compared to 140 HC. All three microRNAs miR-27a-3p, miR-29b-3p, miR-195-5p were increased in both dry AMD and wet AMD patients. The expression of miR-27a-3p was higher in wet AMD compared to dry AMD patients, but there were no significant differences of miR-29b-3p and miR-195-5p expression between dry AMD and wet AMD patients. ROC analysis for miR-27a-3p, miR-29b-3p, miR-195-5p to differentiate AMD from HC gave AUC values of 0.925, 0.757 and 0.766, respectively, with 0.825 sensitivity/0.728 specificity when miR-27a-3p ≥ 12.0, 0.688 sensitivity/0.718 specificity when miR-29b-3p ≥ 10.6, and 0.726 sensitivity/0.596 specificity when miR-195-5p ≥ 10.2. Thus, whole blood miR-27a-3p was a potential diagnostic biomarker of AMD.

Peripheral blood nuclear cells

Litwińska et al. (2019) using RT-PCR with peripheral blood nuclear cells (PBNCs) isolated from plasma of 175 dry AMD, 179 wet AMD, and 121 HC subjects showed that expression of miR-23a-3p, miR-30b, miR-191-5p, miR-223-3p was increased whereas that of miR-16-5p, miR-17-3p, miR-150-5p, miR-155-5p was decreased in PBNCs of wet AMD patients compared to HC. Multivariate analysis of patients and controls, adjusted for age, sex, and smoking status (pack-years), indicated that wet AMD was an independent factor associated with increased expression of miR-23a-3p, miR-30b, miR-191-5p, and decreased expression of miR-16-5p, miR-17-3p, miR-150-5p, miR-155-5p. Dry AMD patients had increased expression of miR-23a-3p, miR-126-3p, miR-126-5p, miR-146a and decreased expression of miR-16-5p, miR-17-3p, miR-17-5p in PBNCs compared to controls. Multivariate analysis revealed that dry AMD was an independent factor associated with increased expression of miR-23a-3p, miR-126-3p, miR-126-5p, miR-146a, miR-191-5p and decreased expression of miR-16-5p, miR-17-3p, miR-17-5p. Six miRNAs were differentially expressed in PBNCs of AMD subtypes: four were increased in dry AMD patients (miR-126-3p, miR-126-5p, miR-150-5p, miR-155-5p) whereas two were increased in wet AMD patients (miR-30b, miR-191-5p). Visual acuity and expression of miR-126-3p, miR-126-5p, miR-155-5p were positively correlated, whereas visual acuity and miR-191-5p expression were negatively correlated. The control group did not have such correlations. A smaller scale study by Lin et al. (2018) using RT-PCR with peripheral blood mononuclear cells (PBMCs) from 20 early AMD patients (with moderate drusen > 63 mm or pigment changes in at least 1 eye but no CNV or GA in either eye at time of sample collection), 23 advanced NV AMD patients, and 63 HC showed that both early AMD and NV AMD patients had increased PBMC expression of miR-150 compared to HC, but PBMC miR-150 expression in early AMD and NV AMD patients did not differ from each other. ROC analysis for PBMC miR-150 expression gave an AUC value 0.860, showing good discrimination of NV AMD from HC. Upregulation of miR-150 in PBMCs was associated with increased likelihood of AMD, even after accounting for differences in age.

Blood plasma

Ulańczyk et al. (2019) found using RT-PCR analysis of blood plasma samples that the expression of miR-16-5p, miR-17-3p, miR-17-5p, miR-23a-3p, miR-126-5p, miR-146a, miR-223-3p was increased whereas that of miR-21-3p, miR-155-5p, miR-191-5p was decreased in 175 dry AMD and 179 wet AMD patients compared to 121 HC subjects. In multivariate analysis, AMD was an independent factor associated with higher expression of miR-23a-3p, miR-126-5p, miR-16-5p, miR-17-3p, miR-17-5p, miR-223-3p, and miR-93 and lower expression of miR-21-3p and miR-155-5p. Wet AMD patients had significantly increased expression of miR-16-5p, miR-30b, miR-191-5p, and significantly decreased expression of miR-23a-3p, compared to dry AMD patients. Visual acuity and blood plasma miR-23a-3p were negatively correlated in AMD patients but not in the control group. The expression of analyzed miRNAs correlated with the levels of tested angiogenesis-regulating factors and clinical parameters in AMD patients but not in the controls. In a small scale study, Ménard et al. (2016) observed by PCR analysis that the expression of miR-146a was increased while that of miR-106b and miR-152 was decreased in blood plasma from 13 CNV AMD patients (fold changes ~2.5, ~1.5, ~1.7 respectively) compared to 13 controls. ROC analysis gave an AUC value 0.914 for the miR-146a/miR-106b ratio in blood plasma for discriminating CNV AMD patients from controls. Ertekin et al. (2014) also used small sized cohorts of 33 wet AMD and 31 HC subjects and found using RT-PCR that the expression of miR-20a-5p, miR-106a-5p, miR-24-3p, miR-17-5p, miR-223-3p was increased while that of miR-140-3p, miR-21-5p, miR-25-3p, miR-146b-5p, miR-192-5p, miR-335-5p, miR-342-3p, miR-374a-5p, miR-410, miR-660-5p, miR-574-3p was decreased in blood plasma of wet AMD patients compared to controls. Furthermore, miR-26b-5p, miR-27b-3p, miR-29a-3p, miR-139-3p, miR-212-3p, miR-324-3p, miR-324-5p, miR-532-3p, miR-744-5p, let-7c were expressed only in the wet AMD group. Dysregulated miRNAs that target VEGFA gene were miR-20b-5p, miR-24-3p, miR-106a-5p, miR-17-5p (all upregulated) and miR-335-5p (downregulated).

Blood serum

Blasiak et al. (2019) using RT-PCR analysis of blood serum samples from 76 wet AMD and 70 HC subjects showed that 4 out of 18 miRNAs regulating the expression of the VEGFA

Table 1 | **Limitations of four previous studies of microRNAs in age-related macular degeneration as indicated in two recent reviews**

	Askou et al. (2018)	Berber et al. (2017)		*Research studies*
Limitations indicated in review articles:			*Other limitations:*	
Little overlap in the findings of previous studies and different orientation of effect	√	√	Exclusion criteria not listed	Menard et al. (2016)
Small size and lack of homogeneity of study populations including the stage of disease	√	√	Statistical analysis to take into account differences in age, proportions of males and females in study groups, smoking habit (number of pks/day) not performed	Ertekin et al. (2014); Szemraj et al. (2015); Menard et al. (2016)
Selection of appropriate controls		√	ROC analysis not performed and AUC values and/ or sensitivity and specificity values not reported for dysregulated miRNAs	Grassmann et al. (2014); Szemraj et al. (2015)
Inconsistencies in patient inclusion criteria	√	√	MicroRNAs to distinguish dry and wet AMD forms not investigated/indicated	Ertekin et al. (2014); Menard et al. (2016)
Different analytical methods used to quantify miRNAs		√	Mechanistic insights into underlying disease etiology not provided	Menard et al. (2016); Szemraj et al. (2015)
Different methods of normalization of results		√		
Low statistical power of some of the studies		√		
Validity of conclusions is questionable		√		
Only one study used vitreous humour	√			

√ indicates that this parameter was mentioned as a limitation in the review article. The four previous studies were Ertekin et al. (2014), Grassmann et al. (2014), Szemraj et al. (2015), and Menard et al. (2016). AUC: Area under curve; ROC analysis: receiver operating characteristics analysis.

gene, miR-34a-5p, miR-126-3p, miR-145-5p, miR-205-5p, had lower expression in wet AMD patients than in controls, using Benjamin-Hochberg correction. In a larger study with 100 AMD and 100 HC individuals, Szemraj et al. (2017) observed by RT-PCR an increased expression of miR-145 and a decreased expression of miR-31, miR-149 and miR-182 in blood serum of AMD patients compared to controls. Romano et al. (2017) performed a small scale study comprising 11 CNV AMD and 11 HC subjects, and found by RT-PCR a higher expression of miR-9, miR-23a, miR-27a, miR-34a, miR-126 and miR-146a together with a lower expression of miR-155 in blood serum of CNV AMD patients compared to controls. The dysregulated miRNAs targeted the VEGF pathway. Utilizing larger sized cohorts of 150 dry AMD and 150 wet AMD patients together with 200 HC subjects, Szemraj et al. (2015) reported a higher expression of miR-661, miR-3121, miR-4258, miR-889, miR-438, miR-424-5p, miR-301-5p and let-7 in blood serum from both dry AMD and wet AMD patients compared to controls. The expression of miR-661 (4.7x), miR-3121 (3x) was greater in dry AMD than wet AMD, while that of miR-4258 (3.3x), miR-889 (3x) and let-7 (2.6x) was greater in wet AMD than dry AMD. The levels of miR-438, miR-424-5p and miR-301-5p were not significantly different between dry AMD and wet AMD. Grassmann et al. (2014) recruited 59 GA AMD, 129 NV AMD and 147 HC subjects, and by RT-PCR showed decreased expression of miR-301a-3p, miR-361-5p and miR-424-5p in blood serum in NV AMD patients compared to controls, but the levels of these miRNAs did not differ in GA AMD patients compared to controls. Blood serum miR-424-5p expression in GA AMD patients was higher than in NV AMD patients, but expression of miR-301a-3p and miR-361-5p did not differ between GA AMD and NV AMD patients. ROC analysis of the combined three miRNAs gave an AUC value 0.727 for distinguishing NV AMD from HC. In this study, the data had been adjusted for age, gender, and smoking (pack years).

Blood serum exosomes

RT-PCR analysis of exosomes isolated from blood serum of 70 wet AMD and 50 HC subjects by Elbay et al. (2019) revealed upregulation of miR-486-5p and miR-626, and downregulation of miR-885-5p, in wet AMD compared to controls. These three miRNAs are involved in the apoptosis and neovascularization pathways of wet AMD pathogenesis.

Vitreous humour

Ménard et al. (2016) using PCR analysis of vitreous humour from 13 CNV AMD patients and 13 controls found that expression of miR-146a was increased whereas that of miR-152 and miR-106b was decreased in CNV AMD (fold changes ~3, ~3, ~4, respectively) compared to controls. By ROC analysis, the AUC value for miR-146a/miR-106b ratio was 0.977 in the vitreous humour indicating it has potential to distinguish between CNV AMD and controls.

Retinal tissues

By miRNA array analysis of 17 AMD retinal tissue samples (affected macular region) and 10 control retinal tissue samples, Pogue and Lukiw (2018) observed that miR-125b, miR-146a and miR-155 had increased expression in AMD tissues compared to controls.

Those miRNAs that can be considered as potential biomarkers of AMD and its subtypes in whole blood, peripheral blood nuclear cells, blood plasma, blood serum, exosomes isolated from blood serum, vitreous humour, and retinal tissues are summarized (**Table 2**).

Future Perspectives

Usually disease symptoms develop gradually in dry AMD (Harvard Clinic), which is an early stage of the disease, making it difficult to detect and irreversible changes in vision can occur when the condition converts to wet AMD. No approved or effective treatment exists at present to prevent the onset or progression of geographic atrophy, and treatment of choroidal neovascularization in wet AMD involves regular intravitreal injections of anti-VEGF agents that are burdensome for patients. Furthermore, a proportion of wet AMD patients do not respond to anti-VEGF therapies, and treatments become less effective with repeated injections (Ehlken et al., 2014). Early diagnosis of AMD is important and will allow new medications to be trialed earlier as future treatments may be more successful when given early. Also early diagnosis provides patients and their families with more time to make future plans.

Optical coherence tomography is an important imaging procedure in the diagnosis and management of patients with choroidal neovascularization, allowing the identification of active neovascular membranes. It is also used to monitor treatment response to anti-VEGF agents (Garcia-Layana et al., 2017). A new non-invasive imaging procedure called optical coherence tomography angiography allows the choriocapillaris in late stage AMD to be examined (Garcia-Layana et al., 2017) This technique has also enabled the detection of small blood vessels in the retina of patients with Alzheimer's disease and mild cognitive impairment (Yoon et al., 2019).

Table 2 | **Alterations of miRNA expression in AMD and its subtypes in whole blood, peripheral blood nuclear cells, blood plasma, blood serum, exosomes isolated from blood serum, vitreous humour, and retinal tissues**

Author	Sample	Comparison	Altered miRNA expression
Ren et al. (2017)	Whole blood	Dry AMD *vs*. HC	Upregulated: miR-27a-3p, miR-29b-3p, miR-195-5p
Ren et al. (2017)	Whole blood	Wet AMD *vs*. HC	Upregulated: miR-27a-3p, miR-29b-3p, miR-195-5p
Ren et al. (2017)	Whole blood	Wet AMD *vs*. dry AMD	Upregulated: miR-27a-3p
Litwińska et al. (2019)	PBNCs	Dry AMD *vs*. HC	Upregulated: miR-23a-3p,-126-3p,-126-5p,-146a,-191-5p
			Downregulated: miR-16-5p,-17-3p,-17-5p
Litwińska et al. (2019)	PBNCs	Wet AMD *vs*. HC	Upregulated: miR-23a-3p,-30b,-191-5p
			Downregulated: miR-16-5p,-17-3p,-150-5p,-155-5p
Litwińska et al. (2019)	PBNCs	Wet AMD *vs*. dry AMD	Upregulated: miR-30b,-191-5p
			Downregulated: miR-126-3p,-126-5p,-150-5p,-155-5p
Lin et al. (2018)	PBMCs	Dry AMD *vs*. HC	Upregulated: miR-150
Lin et al. (2018)	PBMCs	Wet AMD *vs*. HC	Upregulated: miR-150
Ulańczyk et al. (2019)	Blood plasma	AMD *vs*. HC	Upregulated: miR-23a-3p,-126-5p,-16-5p, 17-3p,-17-5p,-223-3p,-93
			Downregulated: miR-21-3p,-155-5p
Ulańczyk et al. (2019)	Blood plasma	Wet AMD *vs*. dry AMD	Upregulated: miR-16-5p,-30b,-191-5p
			Downregulated: miR-23a-3p
Ménard et al. (2016)	Blood plasma	Wet AMD *vs*. HC	Upregulated: miR-146a
			Downregulated: miR-106b,-152
Ertekin et al. (2014)	Blood plasma	Wet AMD *vs*. HC	Upregulated: miR-20a-5p,-106a-5p,-24-3p,-17-5p,-223-3p
			Downregulated: miR-140-3p,-21-5p,-25-3p,-146b-5p,-192-5p,-335-5p, -342-3p,-374a-5p,-410,-660-5p,-574-3p
Blasiak et al. (2019)	Blood serum	Wet AMD *vs*. HC	Downregulated: miR-34a-5p,-126-3p,-145-5p,-205-5p
Szemraj et al. (2017)	Blood serum	AMD *vs*. controls	Upregulated: miR-145
			Downregulated: miR-31,-149,-182
Romano et al. (2017)	Blood serum	Wet AMD *vs*. HC	Upregulated: miR-9,-23a,-27a,-34a,-126,-146a
			Downregulated: miR-155
Szemraj et al. (2015)	Blood serum	Dry AMD *vs*. HC	Upregulated: miR-661,-3121,-4258,-889,-438,-424-5p,-301-5p, let-7
Szemraj et al. (2015)	Blood serum	Wet AMD *vs*. HC	Upregulated: miR-661,-3121,-4258,-889,-438,-424-5p,-301-5p, let-7
Szemraj et al. (2015)	Blood serum	Wet AMD *vs*. dry AMD	Upregulated: miR-4258,-889, let-7
			Downregulated: miR-661,-3121
Grassmann et al. (2014)	Blood serum	Wet AMD *vs*. HC	Downregulated: miR-301a-3p,-361-5p,-424-5p
Grassmann et al. (2014)	Blood serum	Wet AMD *vs*. late dry AMD	Downregulated: miR-424-5p
Elbay et al. (2019)	Blood serum exosomes	Wet AMD *vs*. HC	Upregulated: miR-486-5p,-626
			Downregulated: miR-885-5p
Ménard et al. (2016)	Vitreous humour	CNV AMD *vs*. controls	Upregulated: miR-146a
			Downregulated: miR-106b,-152
Pogue et al. (2018)	Retinal tissues	AMD *vs*. controls	Upregulated: miR-125b,-146a,-155

AMD: Age-related macular degeneration; CNV: choroidal neovascularization; HC: healthy controls; miRNA: microRNA; PBMC: peripheral blood mononuclear cells; PBNC: peripheral blood nuclear cells; *vs*: *versus*.

No approved biomarker is available at present for early AMD detection. However, miRNA profiling might reveal potential diagnostic biomarkers of early stage AMD and wet AMD. Recent reviews have indicated miRNAs as non-invasive diagnostic and prognostic biomarkers in many neurodegenerative diseases including Alzheimer's disease, diabetic retinopathy, and multiple sclerosis (Martinez and Peplow, 2019a, b; Martinez and Peplow, 2020). For example, they allow the detection of mild cognitive impairment which represents early stage Alzheimer's disease (Martinez and Peplow, 2019a) and of nonproliferative diabetic retinopathy which is the early stage of diabetic retinopathy (Martinez and Peplow, 2019b). Interestingly, both Alzheimer's disease and diabetic retinopathy are associated with an increased risk of AMD development (Williams et al., 2014; Frost et al., 2016; He et al., 2018), and AMD and diabetic retinopathy are associated with increased risk of Alzheimer's disease (Lee et al., 2019). These findings suggest shared pathological pathways in these three diseases. Profiling of miRNAs could provide new insights into the pathophysiology of AMD co-shared with Alzheimer's disease and diabetic retinopathy, but also novel biomarkers for more accurate diagnosis and likely prognosis of AMD.

A very large number of miRNAs were found to be dysregulated in the different studies reviewed, but with limited overlap between individual studies (**Table 2**). Of those where there was overlap, miR-23a-3p and miR-126-5p were upregulated in PBNCs of dry AMD patients (Litwińska et al., 2019) and in blood plasma of AMD patients (Ulańczyk et al., 2019). Also, miR-23a and miR-126 were upregulated in blood serum of wet AMD patients (Romano et al., 2017). MiR-146a was upregulated in PBNCs of dry AMD patients (Litwińska et al., 2019), and in blood plasma and vitreous humour of wet AMD patients (Ménard et al., 2016). Both miR-146a and miR-155 were upregulated in AMD retinal tissues collected at postmortem (Pogue and Lukiw, 2018) and confirmed the findings reported earlier (Lukiw et al., 2012). However, miR-155 was downregulated in PBMCs of wet AMD patients (Litwińska et al., 2019), blood plasma of AMD patients (Ulańczyk et al., 2019), and blood serum of CNV AMD patients (Romano et al., 2017). MiR-17-5p and miR-223-3p were upregulated in blood plasma of AMD patients (Ulańczyk et al., 2019) and wet AMD patients (Ertekin et al, 2014). Also comparing wet AMD patients with dry AMD patients, miR-30b and miR-191-5p were upregulated in PBNCs (Litwińska et al., 2019) and blood plasma (Ulańczyk et al., 2019). The expression of the dysregulated miRNAs in AMD patients was found to be significantly correlated with angiogenesis-regulating factors particularly angiogenin and endostatin (Ulańczyk et al., 2019). For example, upregulated miR-17-5p in the blood plasma of

AMD patients showed a positive corelation with angiogenin and endostatin, while upregulated miR-23a-3p, miR-146a and miR-223-3p had a negative corelation with angiogenin and endostatin (Ulańczyk et al., 2019). The downregulated miR-34a-5p, miR-126-3p, miR-145-5p, and miR-205-5p in the blood serum of wet AMD patients target the VEGFA gene (Blasiak et al., 2019), and the set of miRNAs dysregulated in blood serum of wet AMD patients target the VEGF pathway (Romano et al, 2017). Downregulated miR-361-5p in the blood serum of wet AMD patients (Grassmann et al., 2014) should influence angiogenesis, as this miRNA was shown to alter the level of VEGFA expression (Kanitz et al., 2012). In addition, the expression of analyzed miRNAs strongly correlated with the levels of tested inflammatiory mediators in AMD patients, and negative correlations of two factors strongly elevated in wet AMD patients, interleukin-4 and interleukin-6, with miR-30b and miR-146a occurred in wet AMD but not in dry AMD (Litwińska et al., 2019).

Specific miRNAs previously reported to be involved in angiogenesis or neovascularization have included miR-10, miR-27a, miR-126, miR-145, miR-195 (Hassel et al., 2012; Sasahira et al., 2012; Xu et al., 2012; Lai et al., 2013; Wang et al., 2013, 2016b), miR-155 (Pankratz et al., 2015), miR-130a (Chen and Gorski, 2008), miR-93 (Wang et al., 2016a), miR-23, miR-27, miR-24 (Sabatel et al., 2011; Zhou et al., 2011, 2014), miR-106a, miR-146, miR-181, miR-199a, miR-214, miR-424, miR-451, miR-31, miR-150, miR-184 (Shen et al., 2008), miR-21, miR-132, miR-296, miR-378, miR-519c (Lee et al., 2007; Würdinger, et al., 2008; Anand et al., 2010; Cha et al., 2010; Liu et al., 2011). **Figure 2** indicates which of these miRNAs were dysregulated in AMD patients from the studies reviewed herein. Several of the miRNAs were dysregulated in both dry and wet AMD and may be potential biomarkers for detecting dry AMD and monitoring its progression to wet AMD e.g., miR-27a-3p and miR-195-5p in whole blood, miR-23a-3p in PBNCs, miR-150 in PBMCs, and miR-424-5p in blood serum. Expression levels of miR-27a-3p in whole blood (Ren et al., 2017) and miR-424-5p in blood serum (Grassmann et al., 2014) had allowed distinguishing of dry and wet AMD patients (**Table 2**). Some conflicting findings were apparent with miR-126 and miR-424-5p both increased and decreased in blood serum of wet AMD patients. Although upregulated compared to controls, Szemraj et al. (2015) had found a non-significant lowering of miR-424-5p expression in wet AMD compared to dry AMD patients. It is not clear why miR-126 in blood serum was found to be increased by Romano et al. (2017) whereas miR-126-3p in blood serum was reported to be decreased by Blasiak et al. (2019) but it could relate to the sizes of the study cohorts or whether it was the 3p strand that was measured by Romano et al. (2017). It is considered that miR-27a-3p in whole blood and miR-424-5p in blood serum are promising candidates as biomarkers for further research and validation in AMD patients.

Of interest, miR-125b, miR-146a and miR-155 were found to be upregulated in AMD-affected macular region of the retina and Alzheimer's disease superior temporal lobe neocortex (Pogue and Lukiw, 2018) and it was suggested that addressing the common mechanisms of aging that lead to different age-related diseases might assist in identifying new therapeutic approaches. Treatment modalities targeting Alzheimer's disease might be applicable to AMD. Immunotherapies targeting amyloid peptides showed that a decrease in amyloid plaques improved cognitive function in mouse models of Alzheimer's disease (Morgan et al., 2000; Castillo-Carranza et al., 2013), and anti-amyloid therapy protected the retinal pigment epithelium in a mouse model of AMD (Ding et al., 2008, 2011). However, a randomized Phase 2 clinical trial of an anti-amyloid β monoclonal antibody GSK933776 administered intravenously for the treament of GA in AMD did not decrease the rate of GA enlargement and no clinically important changes in visual function testing were found over 18 months compared to placebo (Rosenfeld et al., 2018). A recent clinical trial using a cell implant method has shown promising results for advanced dry AMD. A layer of human retinal pigment epithelial cells on a thin supporting structure was implanted into the retina and trialed in four patients with advanced dry AMD who were then monitored for a year. The implant was found to have integrated into the patients' own retinal tissue. One patient had improved visual acuity and two other patients gained some visual function. None of the four patients had further progression of vision loss during the year following therapy (Kashani et al., 2018). Moreover, intravitreal injection of stem cells may be a potential neuroprotective therapy for retinal photoreceptor neurodegenerative diseases (Puertas-Neyra et al., 2020). At present there has been relatively little research into the therapeutic potential of miRNAs for the

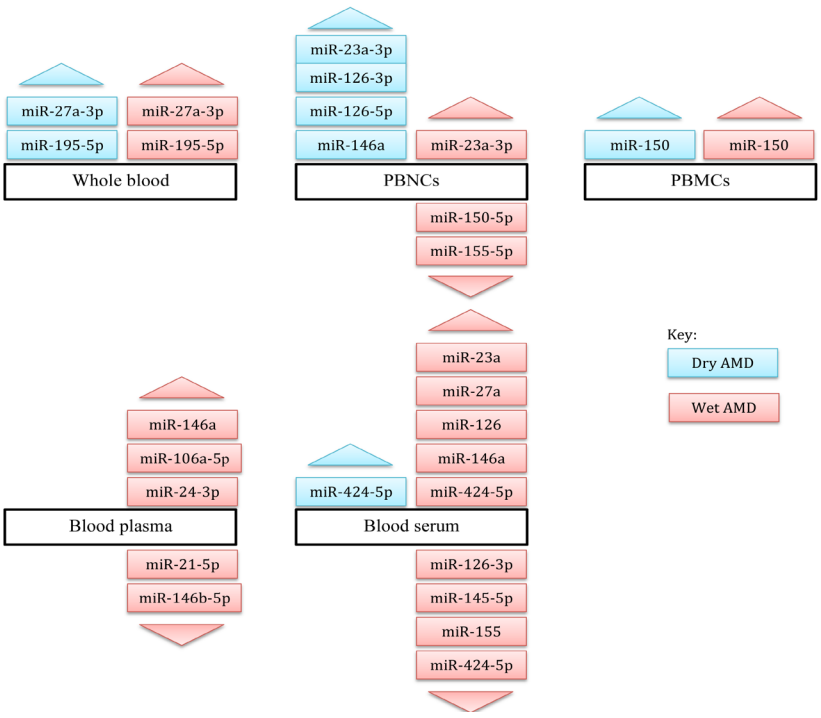

Figure 2 | Changes in microRNAs (miRNA) expression in whole blood, PBNCs, PBMCs, blood plasma and blood serum of dry and wet AMD patients compared to controls.
The microRNAs shown were those previously reported to be involved in angiogenesis or neovascularization. Arrows pointing upwards or downwards indicate increased or decreased expression, respectively. AMD: Age-related macular degeneration; PBMCs: peripheral blood mononuclear cells; PBNCs: peripheral blood nuclear cells.

treatment of AMD (Natoli and Fernando, 2018). Studies with miRNA mimics or inhibitors in animal models of AMD may provide an insight into how these unique treatments might provide a beneficial effect on retinal function.

To summarize, there has been considerable progress made in the recent studies in this review with regard to distinguishing dry AMD and wet AMD from controls and between each other by analyzing miRNAs in whole blood, peripheral blood nuclear cells, blood plasma, blood serum, exosomes from blood serum, vitreous humour, and retinal tissues, and have included single and combinations of miRNAs as well as a ratio of two miRNAs. In the four previous studies (**Table 1**) limitations included small group sizes, heterogeneity of AMD and HC groups, patient inclusion criteria, analytical methods with normalization, and statistical analysis. While these have been taken into account in many of the recent studies, some concerns still exist regarding recruitment of patients including numbers, gender, selection criteria, stage of disease, comorbidities/current treatment, most appropriate validation method, and statistical analysis of data. Also specific measures of miRNAs as diagnostic markers of dry and wet AMD such as sensitivity and specificity values were mainly absent. It is hoped that by continuing to address these concerns in the planning and implementation of future studies a sensitive and specific, minimally invasive test can be developed to identify patients with dry AMD and assist with regular monitoring or with wet AMD to initiate treatment and slow disease progression.

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