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Dissertação de Mestrado

Pâmela Sachs Nique

Avaliação do perfil de expressão de miRNAs na retinopatia diabética e a relação da expressão do miR-126 e miR-200b com a espessura da coroide e retina em pacientes com diabetes mellitus tipo 2

Porto Alegre, 2018

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Dissertação apresentada como requisito parcial para a obtenção do título de Mestre em Endocrinologia, à Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia.

Orientador: Prof. Dr. Luis Henrique Canani

Co-orientadora: Prof. Dra. Andrea Carla Bauer

Porto Alegre, 2018

Aos meus pais, por todo amor e suporte

mesmo que apenas na minha memória.

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(Albert Einstein)

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"The task is not so much to see what nobody has seen, but to think what

nobody has thought about what everyone sees."

(Arthur Schopenhauer)

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- **Artigo de revisão:** MicroRNA Expression Profile in Diabetic Retinopathy: A Systematic Review and Bioinformatic Analysis.

- **Artigo original:** Relationship of miR-126 and miR-200b expressions in plasma with retina and choroidal thickness in patients with type 2 diabetes mellitus.

RESUMO

A retinopatia diabética (RD) é uma frequente complicação microvascular do diabetes mellitus (DM), causada por uma complexa interação entre fatores de risco ambientais, genéticos e epigenéticos. A RD se caracteriza por danos em vasos sanguíneos na retina e a alta prevalência e severidade desta complicação demonstram a necessidade de ferramentas para um diagnóstico precoce. Estudos recentes têm sugerido que alterações na circulação nos capilares da coroide precedem a manifestação clínica da RD. A expressão de fatores angiogênicos e citocinas inflamatórias juntamente com a modulação de genes relacionados à RD e seus reguladores, como microRNAs (miRNAs), parecem estar associados com alterações na espessura da retina e coroide. MiRNAs são RNAs não-codificantes que regulam a expressão gênica, tendo um papel importante na modulação da angiogênese e outras funções endoteliais. Além disso, novas evidências têm relacionado o perfil de expressão dos miRNAs com o desenvolvimento da RD; entretanto, os resultados ainda são inconclusivos. Sendo assim, nós realizamos uma revisão sistemática da literatura, seguida de uma análise de bioinformática e também avaliamos a relação entre a expressão do miR-126-3p e miR-200b-3p com a espessura da coroide, retina e camada de células ganglionares em pacientes com e sem DM tipo 2 (DM2), estratificado pelo índice de massa corporal (IMC).

Na revisão sistemática, estudos que analisaram o perfil de expressão de miRNAs na RD em humanos e em modelos animais foram identificados no PubMed e EMBASE. Os miRNAs consistentemente desregulados nestes tecidos foram submetidos a análises de bioinformática, utilizando dois bancos de dados de interações entre gene-miRNA para identificar os alvos e potenciais vias afetadas pela sua regulação. Trina e quatro estudos foram incluídos na revisão. Onze estudos

avaliaram a expressão de miRNAs relacionados à RD em humanos e 23 em modelos animais. Entre os 189 miRNAs estudados, 160 foram analisados em retinas de modelos animais, 22 em humanos e 7 nas duas espécies. Seis miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592) foram considerados consistentemente desregulados e escolhidos para análises de bioinformática. As análises identificaram que estes 6 miRNAs participam de diversos processos fisiológicos e patológicos, incluindo a regulação do sistema imune, modulação da resposta inflamatória e angiogênese, podendo ter um papel importante da patogênese da RD.

No estudo transversal, 46 pacientes foram incluídos e realizaram um exame de fundoscopia e tomografia de coerência óptica. Os pacientes foram divididos em 4 grupos: Grupo 0 – pacientes com DM2 e IMC <30.0 kg/m2 (n=7); Grupo 1 – pacientes sem DM e com IMC ≥30.0 kg/m2 (n=12); Grupo 2 – pacientes com DM2 ($<$5 anos de diagnóstico) e IMC ≥30.0 kg/m2 (n=11) e Grupo 3 – pacientes com DM2 (≥5 anos de diagnóstico) e IMC ≥30.0 kg/m² (n=16). Expressões dos miR-126-3p e miR-200b-3p foram analisadas em plasma de todos os pacientes incluídos usando qPCR. Expressão do miR-126-3p estava aumentada nos pacientes do Grupo 2 comparado com o Grupo 0 (P = 0.031), enquanto que a expressão do miR-200b-3p foi similar entre os grupos. Na amostra geral, uma relação inversa foi observada entre a expressão do miR-200b-3p e a espessura média e centro da coroide, assim como o volume total, ajustado para IMC e tempo de DM, mostrando que futuramente este miRNA pode ser um possível biomarcador para alterações precoces na espessura da coroide de pacientes com DM2.

ABSTRACT

Diabetic retinopathy (DR) is a frequent microvascular complication of diabetes mellitus (DM). It is caused by a complex interaction between environmental, genetic and epigenetic risk factors and characterized by damage in retinal vessels. Its high prevalence and severity indicate the necessity for screening tools able to diagnose it as early as possible. Recent studies have suggested that changes in choriocapillaris circulation precede clinical manifestation of DR. The expression of angiogenic factors and inflammatory cytokines along with the modulation of several DR-related genes and their regulators, such as microRNAs (miRNAs), seem to be associated with retinal and choroidal thickness alterations. MiRNAs are non-coding RNAs that regulate gene expression, having an important role in modulating angiogenesis and other endothelial cell functions. Also, emerging evidence has linked miRNA profiles and the development of DR; however, results are still inconclusive. Thus, we performed a systematic review of the literature on the subject, followed by bioinformatic analyses and also evaluated the relationship between miR-126 and miR-200b expressions with retina, choroid and inner retinal layers thickness in patients with and without type 2 DM (T2DM), stratified by body mass index (BMI).

In the systematic review, PubMed and EMBASE were searched to identify all studies that reported miRNA expressions profiles in DR in humans and murine models. Those miRNAs consistently dysregulated in serum/plasma and/or retinal tissue were submitted to bioinformatic analyses, using two databases of miRNA-target gene interactions to retrieve their putative targets and identify potentially affected pathways under their regulation. Thirty-four studies were included in the systematic review. Eleven of them reported miRNA expression profiles related to DR in humans and 23 focused on miRNA profiles in murine models. Among the 189

dysregulated miRNAs reported in these studies, 160 were analyzed in retinas of mice/rat, 22 in humans, and 7 on both species. Six miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592) were consistently dysregulated in DR-related tissues and were chosen for bioinformatic analyses. These 6 miRNAs were found to participate in physiological and pathophysiological processes, including immune system regulation, inflammatory response modulation, and angiogenesis. In conclusion, 6 miRNAs appear to be dysregulated in DR and could be associated to its pathogenesis.

In the cross-sectional study, 46 patients were included in the study and underwent a color fundus photograph and swept-source optical coherence tomography exam. Patients were divided into 4 groups: Group 0 – T2DM patients with body mass index (BMI) <30.0 kg/m^2 (n=7); Group 1 – non-diabetic patients with BMI ≥30.0 kg/m^2 (n=12); Group 2 – T2DM patients ($<$5 years of disease) with BMI ≥30.0 kg/m^2 (n=11); and Group 3 – T2DM patients (≥5 years of T2DM) with BMI ≥30.0 kg/m² (n=16). MiR-126-3p and miR-200b-3p expressions were analyzed in plasma of all subjects using qPCR. MiR-126-3p expression was increased in patients from Group 2 compared to Group 0 (P= 0.031), while miR-200b-3p expression was similar among groups. In the pooled sample, an inverse relationship was observed between miR-200b expression and choroidal center and average thickness, and total volume measurements, adjusting for BMI and T2DM duration. In conclusion, miR-200b appears to have a negative relationship with choroidal thickness.

LISTA DE ABREVIATURAS E SIGLAS

INTRODUÇÃO

DM – Diabetes mellitus

DM1 – Diabetes mellitus tipo 1

DM2 – Diabetes mellitus tipo 2

DRD – Doença renal do diabetes

EMD – Edema macular diabético

HAS – Hipertensão arterial sistêmica

IDF – Federação internacional do diabetes

IMC – Índice de massa corporal

miRNA – microRNA

OMS – Organização mundial da saúde

RD – Retinopatia diabética

RDNP – Retinopatia diabética não proliferativa

RDP – Retinopatia diabética proliferativa

VEGF – Fator de crescimento endotelial vascular

ARTIGOS

AGE-RAGE - Advanced glycation end products

BMI – Body mass index

BP – Blood pressure

CI - Confidence interval

DKD – Diabetic kidney disease

DM – Diabetes mellitus

DME – Diabetic macular edema

DR – Diabetic retinopathy

ETDRS - Early Treatment Diabetic Retinopathy Study

FPG – Fasting plasma glucose

GCL - Ganglion cell layer

GEE - Generalized estimating equations

GFR – Glomerular filtration rate

HbA1c – Glycated hemoglobin

HIF-1 - Hypoxia-inducible factor 1

JAK/STAT - Janus kinase/signal transducers and activators of transcription

MeSH - Medical subject headings

miRNA – microRNA

NF-κB - Nuclear factor-kappa B

NPDR – Non-proliferative diabetic retinopathy

PDR – Proliferative diabetic retinopathy

qPCR – Real-time PCR

QUADAS-2 - Diagnostic accuracy studies-2

RAGE – Advanced glycation end products receptor

RNFL - Retinal nerve fiber layer

ROS – Reactive oxygen species

SD – Standard deviation

SE – Standard error

SS-OCT - Swept-source optical coherence tomography

STZ – Streptozotocin

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

UTR – Untranslated region

U6snRNA - small nuclear RNA U6

VEGF – vascular endothelial growth factor

SUMÁRIO

Introdução

O diabetes mellitus (DM) é uma doença caracterizada por hiperglicemia crônica devido à defeitos na secreção de insulina pelas células beta pancreáticas e/ou resistência à ação da insulina nos tecidos periféricos (1, 2). Considerada uma epidemia, trata-se de um grave problema de saúde pública, e estima-se que aproximadamente 415 milhões de pessoas no mundo hoje tenham DM, podendo este número chegar a 642 milhões até 2040 (3, 4).

De acordo com a *International Diabetes Federation* (IDF), o Brasil ocupa a quarta posição entre os países com o maior número de pacientes com DM (4). Atualmente, a prevalência do DM no Brasil é de 11,5%, sendo que cerca de 5 a 10% do total de casos são referentes ao DM tipo 1 (DM1) e 80-90% dos casos referentes ao DM tipo 2 (DM2) (4-6). Sabe-se que o DM está associado ao desenvolvimento de complicações crônicas micro e macrovasculares de elevada morbidade e mortalidade e que o controle glicêmico inadequado está associado ao surgimento e progressão dessas complicações (3, 4, 7). Em relação ao DM2, outros fatores de risco estão associados a progressão das complicações micro e macrovasculares, como a obesidade e hipertensão arterial sistêmica (HAS) (3, 8, 9).

Complicações microvasculares do DM

As complicações micro e macrovasculares são as causas mais comuns de morbimortalidade entre pacientes com DM (10). De acordo com a intensidade e o tempo de exposição à hiperglicemia, podem ocorrer lesões estruturais no endotélio de pequenos vasos sanguíneos, provocando alterações funcionais em diversos órgãos e tecidos (11).

Morfologicamente, as alterações causadas pela hiperglicemia crônica se caracterizam pela constrição progressiva dos vasos sanguíneos, espessamento da membrana basal capilar, aumento da permeabilidade às proteínas plasmáticas e formação de microaneurismas (11, 12). As principais complicações microvasculares relacionadas a estas alterações são a doença renal do diabetes (DRD), neuropatia e retinopatia diabética (RD) (1-3, 9, 13).

Retinopatia diabética no diabetes mellitus tipo 2

A RD é hoje a principal causa de cegueira legal em indivíduos adultos de 25 a 74 anos nos países desenvolvidos, sendo considerada uma das complicações mais importantes do DM (14). Sua detecção precoce pode retardar e/ou evitar a perda da visão (14-16). Atualmente, a RD afeta aproximadamente 150 milhões de pessoas no mundo, sendo esperado o dobro deste número até 2025, de acordo com a Organização Mundial da Saúde (OMS) (17, 18).

Estima-se que 45% dos pacientes com DM2, na região sul do Brasil, desenvolvem RD (19, 20), porém, existem poucos dados epidemiológicos relacionados à RD no país [revisado em (14)]. Os principais fatores de risco para o desenvolvimento e a progressão da RD são o mau controle glicêmico, tempo de duração do DM, presença de HAS (21, 22), albuminúria aumentada e perfil lipídico aterogênico (15, 23-25). Além disso, pacientes com RD tendem a ter um elevado índice de massa corporal (IMC) quando comparados com pacientes sem RD ou indivíduos saudáveis (24, 26-28). A RD é uma doença multifatorial associada a fatores ambientais, comportamentais e genéticos (29-31).

Clinicamente, esta complicação é classificada em dois estágios principais: RD proliferativa (RDP) e a RD não proliferativa (RDNP), senda esta última sub-classificada em leve, moderada ou grave. Nestes estágios podem ser observadas pequenas hemorragias, microaneurismas, edema e oclusão capilar e exsudatos duros. A presença de formação de novos vasos (neovasos) e o crescimento de tecido fibrovascular caracterizam a RDP (21, 23, 32, 33). Nem todos os indivíduos com RDNP desenvolvem RDP, porém, quanto mais grave for a doença não proliferativa, maior será a probabilidade de evolução para uma RDP em até 5 anos (16).

Em relação aos mecanismos patogênicos da RD, sabe-se que a hipóxia tecidual acompanhada da perda da autorregulação dos vasos retinianos, degeneração seletiva e o espessamento da membrana basal resultam na formação de capilares acelulares e no enfraquecimento da parede vascular (21, 34-36).

Alterações no calibre vascular da retina podem apresentar informações importantes sobre o estado da microcirculação do olho e em outros leitos vasculares (12, 37). Além das alterações retinianas, o DM está associado a alterações da espessura da coroide, relacionado ao desenvolvimento do edema de mácula diabético (EMD) (38-43).

Alterações da espessura da coroide e retina no diabetes mellitus

A coroide é considerada um dos tecidos mais vascularizados do corpo humano, formado quase exclusivamente por vasos sanguíneos, podendo ser fortemente influenciado pelas mudanças na circulação (43, 44). Uma das principais funções deste tecido é o suprimento da porção externa da retina com oxigênio e

nutrientes, exercendo também outros papeis como a termorregulação, o ajuste da posição da retina pelas mudanças no seu espessamento e a secreção de fatores angiogênicos, a citar, o *vascular endothelial grown factor* (VEGF) (44, 45). Defeitos na coroide, como o prejuízo na integridade vascular e no suprimento sanguíneo, são características do olho com diabetes e podem levar a mudanças degenerativas e neovascularização (44, 46). Estas características sugerem que a coroide pode desempenhar um papel importante na patogênese e fisiologia de diversas doenças retinianas (47), incluindo a RD e outras anormalidades responsáveis pela perda de visão em pacientes com DM (48-51).

Yolcu e colaboradores (46) realizaram um estudo cujo objetivo foi explorar a mudança na espessura da coroide e mácula, relacionando estes dados com parâmetros clínicos e demográficos em pacientes jovens com DM1 e sem RD. Os resultados mostraram que a espessura da coroide, medida em diversos pontos, foi menor nestes pacientes quando comparado a indivíduos sem DM. Regatieri e Ünsal (48, 52) avaliando pacientes com DM2, reportaram que a espessura da coroide foi significativamente menor nos pacientes que apresentavam RDP e edema de mácula comparado com indivíduos saudáveis. De forma contrária, estudos que relacionaram a espessura da coroide com diferentes graus de severidade da RD, reportaram um aumento da espessura do tecido em pacientes com RDP quando comparado com pacientes sem RD ou com RD leve a moderada. Esta diferença permaneceu quando comparado os grupos de pacientes com RDNP severa e indivíduos sem DM (43, 53).

O alargamento do calibre vascular da retina em pacientes com DM e/ou obesidade pode ser considerado um achado microvascular relacionado ao início da RD, representando um efeito crônico da hiperglicemia (54). Apesar dos estudos

apresentarem resultados contraditórios, todos sugerem que mudanças na circulação dos capilares coroidianos podem preceder a manifestação clínica da RD (43, 55). Farias e colaboradores (56) observaram uma diminuição da espessura da coroide em pacientes com DM e microalbuminúria, outro fator de risco associado à disfunções endoteliais e anormalidades estruturais de vasos sanguíneos. Esta observação pode ser interpretada como uma evidência inicial de danos microvasculares na coroide destes pacientes, antes mesmo do aparecimento de microaneurismas.

Alterações da espessura da coroide e retina na obesidade

O efeito do IMC em alterações da espessura da coroide e retina não está bem definido, ainda que se saiba que a obesidade parece estar associada com diversas doenças oculares como catarata, glaucoma, maculopatia relacionada à idade e RD (57). Os poucos estudos disponíveis sugerem que o aumento do IMC pode ser um gatilho para alterações estruturais no sistema vascular podendo levar a uma disfunção da retina (58-60).

Dogan e colaboradores demonstraram que pacientes com IMC ≥ 40 kg/m² apresentaram uma diminuição da espessura da coroide, comparado com indivíduos saudáveis, bem como a camada de fibras nervosas e células ganglionares da retina (59). Por outro lado, um estudo com 72 mulheres com IMC ≥ 30 kg/m² mostrou um aumento na espessura da coroide comparado com o grupo controle (60). O mesmo foi observado por Bulus e colaboradores quando avaliaram a espessura da coroide em um grupo de crianças com obesidade, bem como uma correlação positiva entre a espessura da coroide e o IMC (58). Sendo assim, o conhecimento das alterações

na espessura de um tecido altamente vascularizado como a coroide, poderia ajudar a entender o efeito da obesidade no sistema microvascular.

Além dos fatores já conhecidos, que podem resultar em alterações na retina e coroide de pacientes com DM, estímulos inflamatórios e genéticos também podem estar associados a estas modificações (29-31, 61). A causa do espessamento da coroide pode estar intimamente relacionada com a produção e liberação de fatores pró-angiogênicos e inflamatórios concomitante à regulação e expressão de diversos genes associados à severidade da RD (62-66). Recentemente, uma classe de RNAs não-codificantes que controlam aproximadamente 60% dos genes codificadores de proteínas humanas, chamados de microRNAs (miRNAs), foram identificados (67). Os miRNAs, devido a sua capacidade de regular os níveis de expressão dos genes que atuam na diferenciação, crescimento e proliferação celular, assim como na secreção e sinalização da insulina (68-72), podem estar envolvidos na patogênese da RD.

MicroRNAs

Os miRNAs são moléculas de RNA de fita simples, contendo de 20 a 22 nucleotídeos, não codificadores de proteínas, que agem como potentes reguladores pós-transcricionais da expressão gênica em plantas e animais (73). Os miRNAs exercem seus efeitos regulatórios ligando-se à região 3'UTR (*untranslated region*) de RNAm alvos. Este mecanismo de atuação permite a modulação dos níveis das proteínas de seus genes alvos (73). Apesar de não terem suas funções totalmente esclarecidas, evidências sugerem que essas moléculas desempenham papel fundamental em diversos processos biológicos (74, 75).

Recentemente, foi sugerido o papel dos miRNAs no desenvolvimento do DM, DRD e doenças cardiovasculares (68-70). A importância dos miRNAs na regulação da função de células endoteliais e, particularmente, na angiogênese, foi discutida em diversos estudos com modelos experimentais *in vivo* e *in vitro* (76); porém, somente nos últimos anos os miRNAs passaram a ser objeto de investigação no que diz respeito ao seu envolvimento na patogênese da RD (74).

Papel dos miRNAs nas alterações de fundo de olho

O papel dos miRNAs ainda é incerto quando relacionado à RD e alterações de fundo de olho. Estudos que analisaram a expressão de miRNAs em relação ao DM e complicações crônicas como a RD relataram uma associação entre os níveis de alguns miRNAs como o miR-200b e miR-126 com a redução da densidade de microvasos retinianos (28, 77). Da mesma forma, os níveis destes miRNAs parecem estar associados a prevenção do aumento da permeabilidade, angiogênese, reparo vascular e inflamação *in vivo* e *in vitro* (28, 78, 79). Ensaios funcionais de transfecção mostraram que importantes genes associados à regulação e liberação de fatores ligados ao estresse oxidativo, inflamação, apoptose em células endoteliais (principalmente em células da retina), angiogênese, hipertrofia e fibrose, são alvos diretos destes dois miRNAs (Tabela 1) (77, 80, 81).

Apesar de alguns estudos terem demonstrado o papel dos miRNAs nas diversas complicações do DM, ainda não está bem estabelecido o mecanismo de ação envolvido, bem como as diferentes rotas associadas ao desenvolvimento ou piora das complicações secundárias ao DM (1, 69, 70, 82-84). Até o momento, poucos estudos avaliaram o papel destes reguladores gênicos sobre a RD em

humanos e nenhum estudo avaliou a relação de miRNAs com alterações na espessura da coroide e retina.

Tabela 1. Estudos envolvendo o miR-126-3p e miR-200b-3p e DM.

miRNA	Autor	Modelo / tecido	Amostra	Principais resultados
miR-200b-3p	*McArthur, 2011* (78)	Animal / Retina	Ratos DM *vs.* Controles saudáveis.	- Injeção vítrea de mimético do miR-200b preveniu aumento de *VEGF*, permeabilidade e angiogênese nos ratos DM.
		Célula	HUVECs.	- Transfecção com antagonista do miR-200b aumentou a produção de *VEGF*.
	Murray, 2013 (80)	Animal / Retina	Camundongos Akita *vs. Wild type*.	- ↑ miR-200b com diminuição do RNAm do gene *Oxr1*.
		Célula	Células Müller	- Transfecção de inibidor do miR-200b aumentou os níveis da proteína do gene *Oxr1*, atenuando o estresse oxidativo e apoptose.
	Li, 2017 (28)	Humano / Soro	Pacientes com DM2 e RD *vs.* Controles saudáveis.	- ↓ mir-200b pacientes com DM2 e RD.
		Rato / Retina	Ratos DM *vs.* Controle saudáveis.	- ↓ *VEGF* associada à redução da densidade de microvasos retinianos, revertendo a ocorrência de lesões na retina dos ratos DM.
miR-126-3p	*Zampetaki, 2010* (81)	Humano / Plasma	Pacientes DM2 *vs.* Controle saudáveis.	- ↓ MiR-126 em pacientes com DM2. Considerado como um preditor independente para a manifestação do DM.
	Bai, 2011 (77)	Camundongos / Retina	Modelo *OIR vs.* Controle saudáveis	- ↓ miR-126 na retina - ↑ miR-126 por injeção intravitrea superou os níveis aumentados de

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Tabela 1. Estudos envolvendo o miR-126-3p e miR-200b-3p e DM.

miRNA	Autor	Modelo / tecido	Amostra	Principais resultados
				VEGF e reduziu a neovascularização retiniana.
	Barutta, 2017 (79)	Humano / Soro	Pacientes DM1C *vs.* Pacientes DM1. Pacientes DM1 com RD *vs.* Pacientes DM1.	- ↓ MiR-126 em pacientes com DM1C comparado com DM1. - ↓ 25% no risco para desenvolver RDP. - Hipótese de que o miR-126 pode controlar a inflamação endotelial, diminuindo a expressão de fatores como o VCAM-1.

DM, diabetes mellitus; DM2, diabetes mellitus tipo 2; RD, retinopatia diabética; RDP, retinopatia diabética proliferativa; HUVECs, human umbilical vein endothelial cells; *OIR*, retinopatia induzida por oxigênio; Camundongos Akita, modelo genético de DM1; DM1C, diabetes mellitus tipo 1 com complicações;

Justificativa

Entender o papel dos miRNAs no surgimento e progressão da RD pode ser de grande valia para o desenvolvimento de novas estratégias de rastreamento e de tratamento da RD. Diversos miRNAs têm sido associados à fatores como o aumento da permeabilidade, angiogênese e reparo vascular, sendo alvos direto de genes relacionados à rotas de inflamação, apoptose e fibrose em células endoteliais. A maioria dos dados disponíveis são referentes à expressão de miRNAs e o efeito da hiperglicemia em células e/ou modelos animais e, até o momento, não encontramos nenhum estudo que tenha avaliado a relação da expressão de miRNAs com a espessura da coroide e retina em pacientes com DM e obesidade. Desta forma, o maior conhecimento do papel dos miRNAs na RD se faz necessário. A identificação de marcadores precoces da RD pode ser uma importante estratégia no desenvolvimento de medidas preventivas e/ou terapêuticas, objetivando reduzir o número de pacientes que sofrem com esta limitante patologia.

Objetivos

- Revisar os estudos disponíveis na literatura sobre o papel dos miRNAs na patogênese da RD e identificar os possíveis alvos e vias de sinalização destes miRNAs através de uma revisão sistemática e análise de bioinformática.

- Avaliar a relação da expressão do miR-126-3p e miR-200b-3p e a espessura da retina, da coroide e da camada de células ganglionares em pacientes com e sem diabetes mellitus tipo 2, estratificado por IMC.

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CAPÍTULO 1

MicroRNA Expression Profile in Diabetic Retinopathy: A Systematic Review and Bioinformatic Analysis

Pamela S. Nique, Mayara S. Oliveira, Bianca M. de Souza, Tais S. Assmann, Mariana Recamonde-Mendoza, Daisy Crispim, Luis Henrique Canani, Andrea C. Bauer.

MicroRNA Expression Profile in Diabetic Retinopathy: A Systematic Review and Bioinformatic Analysis

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Running Title: microRNAs and Diabetic Retinopathy

Abstract

Diabetic retinopathy (DR) is a common diabetic microvascular complication caused by a complex interaction between environmental and genetic risk factors. Its high prevalence and severity indicate the necessity for screening tools able to diagnose it as early as possible. Emerging evidence has linked microRNA (miRNA) profiles and the development of DR; however, results are still inconclusive. Thus, we performed a systematic review of the literature on the subject, followed by bioinformatic analyses. PubMed and EMBASE were searched to identify all studies that reported miRNA expressions profiles in DR in humans and murine models. Those miRNAs consistently dysregulated in serum/plasma and/or retinal tissue were submitted to bioinformatic analyses, using two databases of miRNA-target gene interactions to retrieve their putative targets and identify potentially affected pathways under their regulation. Thirty-four studies were included in the systematic review. Eleven of them reported miRNA expression profiles related to DR in humans and 23 focused on miRNA profiles in murine models. Among the 189 dysregulated miRNAs reported in these studies, 160 were analyzed in retinas of mice/rat, 22 in humans, and 7 on both species. Six miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592) were consistently dysregulated in DR-related tissues and were chosen for bioinformatic analyses. These 6 miRNAs were found to participate in physiological and pathophysiological processes, including immune system regulation, inflammatory response modulation, and angiogenesis. In conclusion, 6 miRNAs appear to be dysregulated in DR and are linked to its pathogenesis. A full understanding of the roles of these miRNAs in DR is still necessary to evaluate their use as biomarkers.

Key words: diabetic retinopathy, diabetes mellitus, microRNAs, bioinformatic.

Introduction

Diabetic retinopathy (DR) is the leading cause of new blindness cases in young adults of working age and it is a frequent diabetic microvascular complication (1-3). Patients with DR are at increased risk of developing other micro- and macrovascular complications of diabetes mellitus (DM) (3, 4). Clinically, DR is classified in two main stages: (1) non-proliferative diabetic retinopathy (NPDR) and (2) proliferative diabetic retinopathy (PDR). NPDR is characterized by the presence of microaneurysms, capillary occlusion, hard exudates, and retinal hemorrhages, while PDR is characterized by edema, neovascularization, and fibrovascular tissue growth (5-8). The exact moment by which DM causes DR remains unclear, but it is known that its development involves multifactorial aspects related to environmental, genetic and epigenetic factors (9, 10). Besides all the efforts to prevent and reduce progression of this complication, the high prevalence and severity of DR suggest the necessity for screening programs able to recognize it as early as possible (11-13).

In this context, some studies have demonstrated the role of microRNAs (miRNAs) on the development of DM and diabetic kidney disease (DKD), suggesting that they could constitute new biomarkers for these diseases (14-18). MiRNAs are 20–22 nucleotides non-coding RNAs that negatively regulate gene expression by partially pairing to the 3' untranslated region (3'UTR) of their target mRNAs; thus, leading to translation repression and/or transcript degradation (19, 20). They have been implicated in the regulation of various physiological and pathophysiological functions and its characterization has become a major interest in biology and medicine due to its importance in endothelial cell regulation and function, particularly in angiogenesis (21-23). Only recently, the role of miRNAs in the pathogenesis of DR

has become an object of investigation (24). A few studies have demonstrated that some miRNAs, such as miR-126, miR-146 and miR-200b, are involved in several pathways related to DR by targeting important factors as *VEGF*, *NF-κB* and *VCAM-1* on murine models and cell lines (25-29). However, some of these studies evaluated the association of DR together with another diabetic complication (30, 31), and the influence of miRNAs in the pathogenesis of DR still needs to be clarified. Thus, this systematic review was aimed to summarize the role of miRNAs in the pathogenesis of DR and to identify their possible targets and associated pathways.

Methods

Search strategy and eligibility criteria

PubMed and Embase repositories were searched systematically to identify all studies that evaluated miRNAs expression profiles in relation to DR. The following medical subject headings (MeSH) were used for the search: ("Diabetic Retinopathy" OR "Diabetes Complications") AND ("MicroRNAs" OR "RNA, Small Untranslated"). All identified articles were also manually searched for other relevant citations. Studies were excluded from analysis if they had insufficient data. If data were duplicated and/or published more than once, the most complete study was chosen. The search was limited to human and animal studies in English, Spanish and Portuguese languages and was completed in October 2018. This study was designed and described following current guidelines for execution of systematic reviews and meta-analyses (32). Two investigators (P.S.N. and M.S.O.) independently reviewed titles and abstracts of retrieved articles to evaluate eligibility for inclusion in this review.

Study selection, data extraction and quality assessment

Data from each included study were independently extracted by two investigators (P.S.N. and M.S.O.) using a standardized extraction form, as previously described in other systematic reviews published by our group (18, 33). The consensus was sought for all extracted items, and when consensus could not be reached, differences in data extraction were decided by consulting a third reviewer (B.M.S.). Information extracted from each study in humans was as follows: (1) characteristics of studies and samples; (2) information regarding miRNA expression (quantification method, tissue analyzed, number of analyzed miRNAs); and (3) miRNA expression in case and control groups (if applicable). For the studies in mice/rats, we also collected information regarding murine models analyzed, such as DM induction and genetic modifications. When data were not available, the authors were contacted by e-mail. All miRNA names were standardized based on miRbase v21 before analysis (34).

The quality of each eligible study was assessed using The Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) (35). This tool comprises 4 key domains (patient selection, index test, reference standard, and flow/timing) supported by 7 questions to aid judgment on the risk of bias, rating risk of bias, and concerns about the applicability of studies. Each item can be answered with "yes", "no", or "unclear". Then, a score of 1 is given for each "yes" (low risk/high concern), a score of 0.5 for each "unclear", and a score of 0 for each "no" (high risk/low concern). Quality scores range from 0 to 7, with studies being classified as having "good quality" (scores 6-7), "fair" (scores 4-5), and "poor" (scores < 3).

Bioinformatic analysis of miRNA's target genes

To investigate the role of miRNAs in DR, we selected miRNAs that were consistently dysregulated in retina and/or serum/plasma, and performed bioinformatic analyses to retrieve their putative targets and to identify potentially affected biological pathways under their regulation. For this, we queried two databases: miRTarbase v7 (36) and TarBase, v7 (37) for experimentally validated data only, concerning miRNA–target interactions (Supplementary figure 1). The combination of all miRNA–target interactions retrieved from the two databases, validated in at least three experiments, was used for further analyses.

Next, we performed an analysis of orthologous miRNA target genes (38) for the miRNAs expressed in murine models to verify if the sequence of nucleotides was the same of those genes in humans. Then, functional enrichment analysis of these orthologous miRNA targets was implemented using pathways annotation from the KEGG Pathway Database (39, 40) and the cluster Profiler package in R/Bioconductor environment (41). Significance for KEGG pathways enrichment was estimated with a hypergeometric test and adjusted to account for multiple hypotheses using the false discovery rate implemented in the q-value R package (45). Pathways with a q-value <0.05 were considered strongly enriched for the genes targeted by selected miRNAs.

Results

Literature search, characteristics of the studies and quality assessment

Supplementary figure 2 illustrates the strategy used to identify and select studies for inclusion in this systematic review. Our search strategy retrieved 995 potentially relevant citations, from which 939 were excluded following titles and

abstracts revision. Fifty-six articles were selected for full-text evaluation. Of them, only 34 articles fulfilled the eligibility criteria and were included in this systematic review (25, 26, 28-31, 42-69). The main characteristics of these articles are shown in table 1 (for human studies) and Supplementary table 1 (for murine model studies).

Regarding human studies, 11 articles reported miRNA expression profiles related to DR. Seven studies evaluated patients with type 2 DM (T2DM) and one study assessed both type 1 (T1DM) and T2DM patients (Supplementary table 2). One study analyzed the same miRNAs in different samples (47). Thus it was considered separately. These 11 studies evaluated patients with different degrees of DR and healthy subjects, with sample sizes ranging from 10 to 255 patients per study. HbA1c ranged from 6.3 to 12.4% in diabetic patients and from 4.3 to 6.0% in healthy subjects, although 5 studies did not show HbA1c values (Supplementary table 2). MiRNA expressions were analyzed in serum in 6 (55%) studies and in plasma in 5 (45%) studies (Table 1).

A total of 23 studies in murine models were included (Supplementary table 1). Streptozotocin (STZ) was used to induce DM in 19 of the studies, while the others used mice models of spontaneous T1DM and T2DM. For all studies, miRNA expressions were analyzed only in the retina.

Regarding quality, 65% of the studies received QUADAS-2 scores between 6 and 7 and were considered as having good quality (Supplementary table 3). Only one study received a QUADAS-2 score below 5 (67).

Dysregulated microRNAs in diabetic retinopathy: expression profile and target interactions.

In total, the included studies provided data of 189 miRNA profiles regarding DR (Supplementary table 4). Among them, 160 miRNAs were analyzed in retinas of mice/rat, 22 in plasma/serum of humans and 7 on both (Supplementary figure 3A). Regarding the number of miRNA expression profiles reported in the studies, 69.8% were upregulated, 21.7% were downregulated, and 8.5% of the miRNAs were upregulated in one study and downregulated in another (Supplementary table 4 and Supplementary figure 3A).

Consistently dysregulated miRNAs were chosen for further analysis depending on the number of studies involving each miRNA and the expression profile reported. A total of 6 miRNAs were considered consistently dysregulated and were selected for in-depth analysis (Table 2 and Supplementary figure 3B). Among the miRNAs analyzed in serum/plasma of humans, miR-21-5p and miR-126-3p were consistently dysregulated in at least two studies (Table 2), being selected for bioinformatics analyses.

Regarding studies with murine models, miR-146a-5p, miR-195-5p, and miR-592 were consistently dysregulated, in at least three studies (Table 2) and were also selected for bioinformatics analysis. We also included miR-200b-3p in these analyses even though its results were not consistent in pointing out a pattern of expression, due to its known role as regulator of important target genes related to apoptotic and pro-angiogenic pathways, which can be associated with DR.

Interestingly, miR-200b-3p expression profile was the only one reported in both humans and murine models studies, being downregulated in serum samples of patients with DR compared with healthy subjects, as well as in retinas of rats with STZ-induced DM (Table 2 and Supplementary figure 3B).

Bioinformatic analyses of dysregulated miRNAs.

Bioinformatic analyses were performed to retrieve putative targets and pathways potentially modulated by the 6 miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592) consistently dysregulated in the retina and/or serum/plasma samples. Species prefixes were used in miRNA identifiers to clearly designate the species under consideration while results were reported.

First, we searched for targets of these miRNAs using 2 distinct resources of experimentally validated databases as described in the method section (Supplementary figure 1). On miRTarbase, 186 miRNA-target interactions were found for humans involving both hsa-miR-21-5p and hsa-miR-126-3p (Supplementary figure 4). Regarding mice and rats, 33 miRNA-target interactions were found involving both mmu-miR-146a-5p and mmu-miR-200b-3p, while only 4 interactions were found involving rno-miR-195-5p and rno-miR-200b-3p (Supplementary figure 4). On the TarBase, 162 miRNA-target interactions were found for humans involving both hsa-miR-21-5p and hsa-miR-126-3p. As for mice, 27 interactions were found involving mmu-miR-146a-5p and mmu-miR-200b-3p (Supplementary figure 4). Considering the orthologous genes, a total of 311 validated miRNA-target unique interactions were found for miRNAs expressed in humans (has-miR-21-5p and has-miR-126-3p), while 58 validated unique interactions were retrieved for the mmu-miR-200b and mmu-miR-146a-5p and only 4 for rno-miR-200b-3p and rno-miR-195-5p (Supplementary table 5). Of note, interactions for the rno-miR-592 were not found in any of the databases queried.

Target-gene pathways involved in diabetic retinopathy.

After target prediction analysis, we performed functional enrichment analysis of miRNA target genes using the KEGG Pathway Database, aiming to better understand the biological pathways affected by the selected miRNAs (39, 40). A total of 136 pathways were significantly overrepresented (q-value <0.05) in the putative target list analyzed, and 75 KEGG terms were enriched for more than one miRNA (Supplementary table 6). Targets of hsa-miR-21-5p, has-miR-126-3p, mmu-miR-146a-5p, and mmu-miR-200b-3p are involved in several pathways well known to be associated with DR pathogenesis, such as advanced glycation end products (AGE-RAGE - KEGG hsa04933), hypoxia-inducible factor 1 (HIF-1 - KEGG hsa04066) and vascular endothelial growth factor (VEGF - KEGG hsa04370) signaling pathways (Figure 1, Supplementary table 6). Furthermore, those miRNAs also have important targets involved in the nuclear factor-kappa B (NF-κB - KEGG hsa04064) and Janus kinase/signal transducers and activators of transcription (Jak/STAT - KEGG hsa04630) signaling pathways, recognized for their role in DM pathogenesis (Figure 1, Supplementary table 6). No significant enriched KEGG terms were found for rno-miR-195-5p and rno-miR-200b-3p, probably due to the small number of retrieved targets (Supplementary table 6).

The AGE-RAGE signaling pathway participates on the activation of multiple pathways involving PKC and MAPKs factors, then resulting in NF-κB activity, promoting the expression of pro-inflammatory cytokines as well as activation of JAK/STAT-mediated and PI3K/Akt-dependent pathways, participating in cell proliferation and apoptosis respectively (Figure 2). In this pathway, miR-21-5p directly targets mRNAs for *TGF-β* and its respective cell receptors, influencing the

expression of genes involved in the mesangial matrix expansion (*COL4A1*). Moreover, this miRNA regulates genes associated with PI3K-Akt pathways (*PIK3R1*, *AKT2*), which are triggered after AGE-RAGE activation by several factors, such as aging, inflammation, oxidative stress, and high glucose concentrations. MiR-126-3p also targets *KRAS*, *PIK3R2*, and *AKT1*. They target important factors responsible for angiogenesis (*VEGF*), thrombogenesis and inflammation (*VCAM1*, *ICAM1*, and *IL-1β*), apoptosis (*BCL2*) and cell cycle (*FOXO1* and *CCND2*) (Figure 2).

In the JAK/STAT pathway, miR-146a-5p targets mRNAs related to vascular remodeling, cellular proliferation, and vascular dysfunction (*STAT1*, *3* and 4), while miR-21-5p modulates IL-2A and its receptors, such as EGFR. It also targets *SOCS1* and *6*, which are possible blockers of *JAK* and mRNAs related to MAPK signaling pathway (*SOS2* and *PIAS3*); thus, modulating proliferation and cell differentiation (Figure 2).

HIF-1 is a transcription factor that functions as a master regulator of oxygen homeostasis (70). Several genes of HIF-1 pathway encode proteins that increase O_2 delivery and mediate adaptive responses to O_2 deprivation. On this pathway, mRNAs from the mTOR and PI3K-Akt signaling pathways are targets of miR-21-5p (*PIK3R1*, *AKT2*, and *MKNK2*) and miR-126-3p (*PIK3R2* and *AKT1*). The modulation of these mRNAs can potentially alter the expression of *HIF-1α*, which, in turn, is involved in the signaling of several factors that promote the increase in oxygen delivery, such as *VEGF*, *TEK*, *FLT1*, and *BCL2* (targets of miR-126-3p, miR-21-5p, and miR-200b-3p) (Figure 3). Moreover, miR-146a-5p regulates *IFNG* and *STAT3*, leading to HIF-1α mRNA repression.

VEGFR2 (KDR) is a major mediator of VEGF-driven responses in endothelial cells and it is considered an important signal transducer in both physiologic and pathologic angiogenesis (71). VEGF binding to its receptor leads to a cascade of different signalizations, resulting in the upregulation of genes involved in mediating proliferation and migration of endothelial cells and promoting their survival and vascular permeability (Figure 3). In this pathway, miR-126-3p, miR-21-5p, and miR-200b-3p target *VEGF* and *KDR*, promoting the total blockade of this pathway. Furthermore, these miRNAs also bind to mRNAs of the PI3K-Akt pathway within the VEGF signaling pathway (*PIK3R1*, *PIK3R2*, *AKT1*, and *AKT2*), while miR-146a-5p binds to *PPP3R* and *CAMK2*, which are involved in arachidonic acid metabolism (Figure 3).

The *NF-κB* pathway is involved in the regulation of immunity, inflammation and cell survival through a complex network (72). MiR-146a-5p and miR-21-5p play a significant role in the modulation of several key targets of this pathway, such as *IRAK1*, *TRAF6*, *RELB*, and *MYD88*. They activate a signaling cascade that leads to transcription of mRNAs involved in inflammation and cell survival (*IL-1β* and *VCAM-1*), apoptosis regulation (*BCL2*) and *NFKBIA* (Figure 4).

Discussion

DR is a multifactorial disease with a high prevalence of severe retinal complications. The involvement of complex genetic and environmental factors difficult its early diagnosis (11, 13). Regular retinal fundoscopy is widely recommended in screening protocols for diagnosis and detection of early stages of DR; however, in clinical practice, only a small percentage of DM subjects undergo fundoscopic exam with the recommended periodicity (73). Direct ophthalmoscopy requires pupillary

dilation and skills for the procedure and, despite the efforts for early detection, this method only identifies DR when it is already under development and clinically significant.

Therefore, the searching for biomarkers that could signaling predisposition for and/or allow early detection of DR are of great interest. In this context, miRNAs can be released into the bloodstream and expressed in blood cells (circulating miRNAs) or even in particular tissues such as the retina (18, 20). Indeed, several studies have identified characteristic changes in miRNA levels in blood samples in cohorts of patients with both T1DM and T2DM and its main complications such as DKD and DR (17, 31, 47). This systematic review identified 6 miRNAs that were dysregulated in DR-related tissues. After bioinformatics analysis, 4 miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p and miR-200b-3p) were linked to several target-genes that participate in signaling pathways with a possible role in DR pathogenesis.

Several hyperglycemia-linked pathways have been identified in the progression of DR and have been associated with early retinal vascular dysregulations (74). Decreased retinal blood flow is observed in patients with DM before any retinal change, becoming more pronounced according to the development and severity of DR as a consequence of an early decreased in oxygen tension (75, 76). The hypoxic environment present in DR further induces *HIF-1α* expression, a key oxygen sensor that plays an essential role in regulating angiogenesis by promoting an increase in VEGF levels (77, 78). Moreover, our analysis revealed that miR-21-5p and miR-126-3p play a major role in the HIF-1 signaling pathway by targeting *PIK3R1*, *PIK3R2* and *AKT*, promoting a decrease of HIF-1α activation in response to hypoxia and, consequently, decreased levels of angiogenesis inducers

(Figure 3) (79, 80). Angiogenesis can be triggered by extracellular signals, including VEGF, which is a growth factor considered the primary vector involved in neovascularization (81). Increased levels of VEGF were found in the vitreous humor and in fibro vascular tissues from eyes with PDR (82-84). In this pathway, VEGF activates the tyrosine kinase receptor, VEGFR2 (KDR), regulating physiological and pathological angiogenesis (85, 86) (Figure 3). VEGF levels are also correlated with the breakdown of the blood-retinal barrier; thus being involved in retinal vascular hyperpermeability in background retinopathy (87).

Jiang et al. demonstrated that miR-200b upregulation by transfection of a mimic can reduce VEGF and TGF-β mRNA and protein expressions, improving retinal endothelial cell structure and function, and allowing a delay in DR progress (88). Since our bioinformatics analysis retrieved the KDR as a target of miR-200b, we can hypothesize that miR-200b might downregulate the entire VEGF pathway by targeting its receptor (Figure 3). Also, considerable evidence shows that the specific intracellular signaling cascade of this receptor leads to proliferation, migration, surviving and increased permeability of endothelial cells (89). In the same way, miR-21 and miR-126 have the VEGF as gene-target in this pathway, which can also lead to gene expression modifications. Considering the importance of this pathway in the progression of DR, miR-200b levels could early signalize an increased flux in the VEGF pathway induced by hyperglycemia.

Interestingly, miR-200b was the only miRNA analyzed in both humans and murine models studies and its expression profile was reported as downregulated in three different studies (25, 49, 58). miR-200b was reported as being downregulated in serum samples of patients with DR compared to healthy controls, and showed a

negative correlation with VEGF gene and protein expressions (49). In retinal tissue of STZ-induced diabetic rats, miR-200b was also downregulated after one month of DM with a concomitant *VEGF* upregulation. In endothelial cells, miR-200b was downregulated after exposure to high glucose concentrations, which occurred in parallel to an increase in VEGF levels, and was prevented by miR-200b mimic transfection (25, 58). In contrast, miR-200b was reported as being upregulated in retinal tissue of diabetic animal models by two other studies (26, 28). This difference in expression profiles on murine models could perhaps be explained by different times of exposure to the DM environment.

Another recognized mechanism associated with DR pathogenesis is the intracellular production and accumulation of AGEs along with an increase in the production of reactive oxygen species (ROS). AGE accumulation is associated with several morphological and functional changes of DR, including basement membrane thickening, loss of pericytes and increased retinal endothelial cells, which result in leakage, coagulation, occlusion and ischemia along with induction of VEGF, ICAM-1 and VCAM-1 factors (90, 91). These factors are targets of miR-126 and miR-21 in the AGE pathway, as well as IL-1β and the AGE receptor (RAGE) (Figure 2). The interaction of AGE-RAGEs induces activation of NF-κB pathway, decreasing the ratio of BCL2/BAX genes, thus leading to increased CASP3 activity, which is responsible for pericyte apoptosis (92, 93). Hence, upregulation of miR-126 and miR-21 might block *BCL2* (a target-gene in this pathway), consequently leading to CASP3 downregulation, reducing apoptosis of pericytes.

MiR-146a is expressed in a wide range of tissues and cell types and plays an important role in innate and adaptive immunity and many tissue-specific functions in

different cell types and biological contexts (94-96). MiR-146a was downregulated in retinal tissue of murine models (29, 52). NF-κB is a major regulator of inflammatory responses, playing a crucial role in inflammatory damage to retinal microvasculature during DR development (97). MiR-146 inhibits IL-1β/TLR-mediated NF-κB pathway by targeting key molecules, such as *IRAK1* and *TRAF6* (Figure 4); thereby, preventing damage to retinal endothelial barrier function in vitro (28, 98). Zhuang *et al*. demonstrated that intravitreal injection of a lenti-miR-146a vector in STZ-induced diabetic rats resulted in miR-146a upregulation concomitant with a downregulation of its target-genes *IRAK1*, *TRAF6* and *CARD10*, thus leading to the downregulation of the NF-κB downstream gene, *ICAM1*. Furthermore, intravitreal injection of this miRNA prevented DM-induced retinal microvascular leakage and retinal functional defects (67). These results are in agreement with our bioinformatics analysis where the key genes of NF-κB pathway were retrieved as target-genes of miR-146a (Figure 4).

In the same way as for miR-146, some studies have indicated that miR-21 plays is a key mediator of the anti-inflammatory response in macrophages (99, 100). Furthermore, miR-21 has been extensively associated with some complex diseases such as cancer (101), T1DM and T2DM and its chronic complications (17, 18, 102). Recently, the expression profile of miR-21 together with miR-181c and miR-1179 has been considered as a potential signature of PDR, constituting a novel non-invasive approach for the early screening of DR progression (42).

Interestingly, miR-146a, miR-21 and miR-126 have important target-genes in an unusual pathway related to DR (Figure 2): the JAK/STAT pathway, which is associated with immune function. This pathway is also involved in angiogenesis

(103) with STAT1 and STAT6 being activated by VEGF (104). Furthermore, STATs are sensitive to other cellular stressors as ROS and high glucose concentrations; thus implicating this pathway in processes related to DM (105, 106). Some of the targets of these miRNAs include mRNAs for cytokines and hormone receptors (*EGFR* and *IFNG*), which might alter the regulation of STATs (STAT1, STAT3, STAT4) as well as mRNAs related to apoptosis and cell cycle, such as *PI3K*, *AKT*, *BCL2*, *SOCS* and *CCND2* (Figure 2). The identification of the different pathways associated with DR is important to raise hypothesis about pathological processes that might be occurring under the influence of these circulating miRNAs and that might strengthen the understanding of the pathogenesis or progression of DR.

In conclusion, this systematic review and bioinformatics analyses suggests that 6 circulating miRNAs (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592) are consistently dysregulated in DR. Important target-genes in DM-related signaling pathways were identified for 4 of them. These findings may contribute to the understanding of DR pathophysiology. The necessity for diagnostic tools able to anticipate the DR development is imperative. Therefore, additional studies on the role of miRNA expression as biomarkers of DR are essential to build a more robust conclusion and perhaps, in the future, to have it incorporated to the clinical field.

Acknowledgments

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

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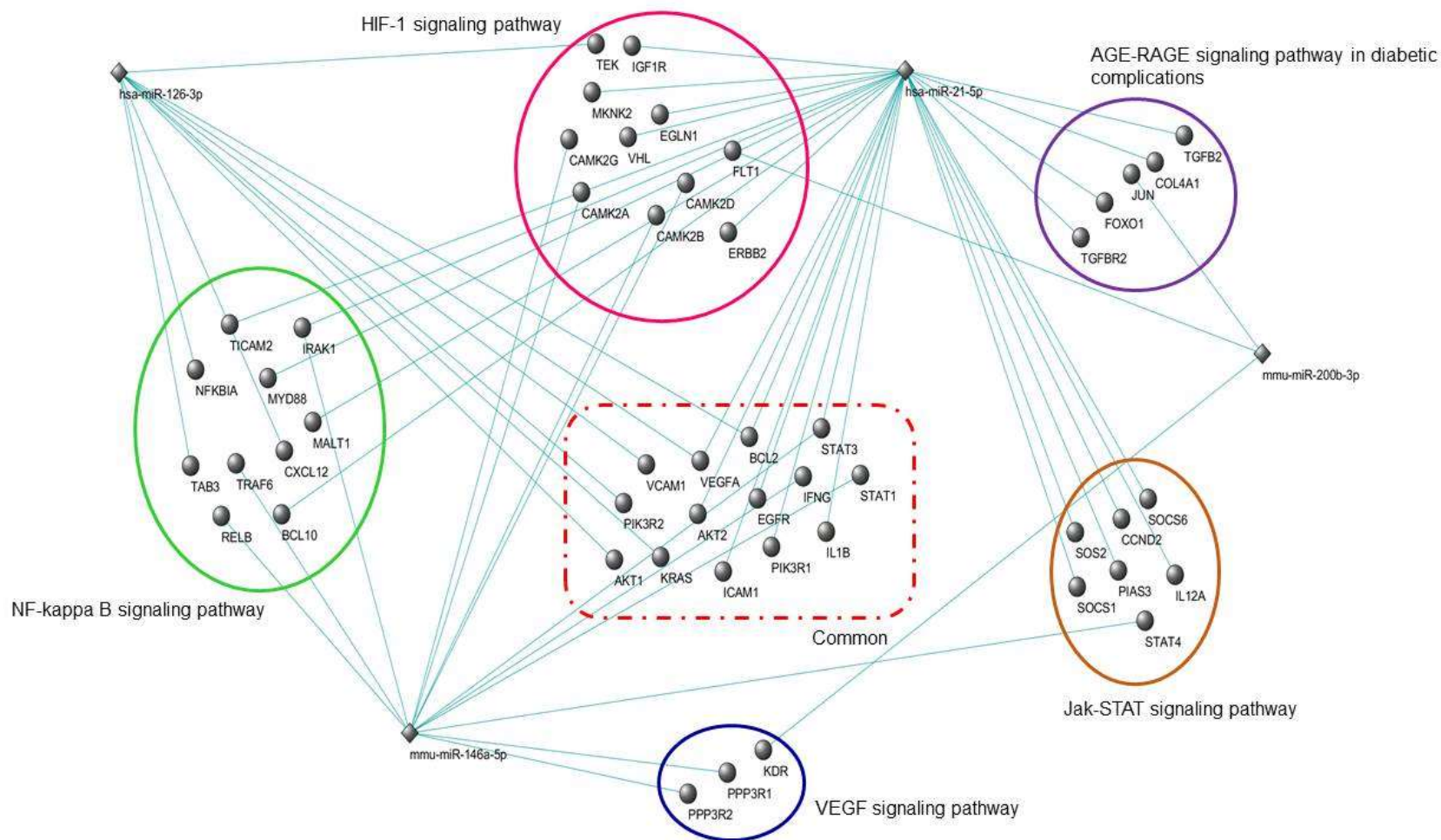
Figure 1: Gene-targets of hsa-miR-21-5p, has-miR-126-3p, mmu-miR-146a-5p, and mmu-miR-200b-3p, involved in DR-related signaling pathways. Genes inside the dotted circle are common between the pathways. The miRNAs are indicated by rhombus.
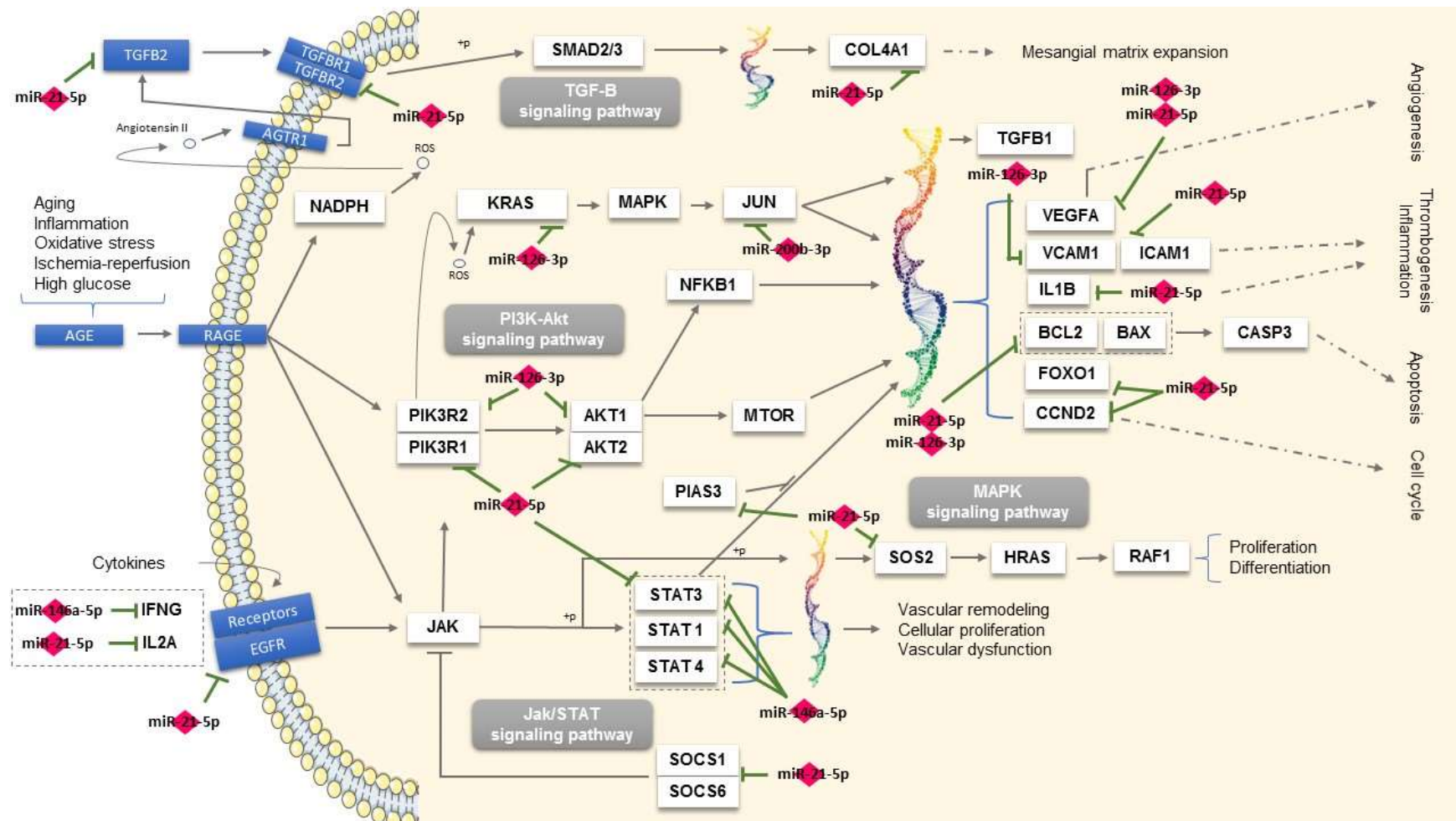
Figure 2: Schematic diagram of the selected miRNA-mRNA interaction networks involved in AGE-RAGE and JAK/STAT signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04933). The miRNAs are indicated by rhombus.

Figure 3: Schematic diagram of the selected miRNA-mRNA interaction networks involved in HIF-1 and VEGF signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04066). The miRNAs are indicated by rhombus.
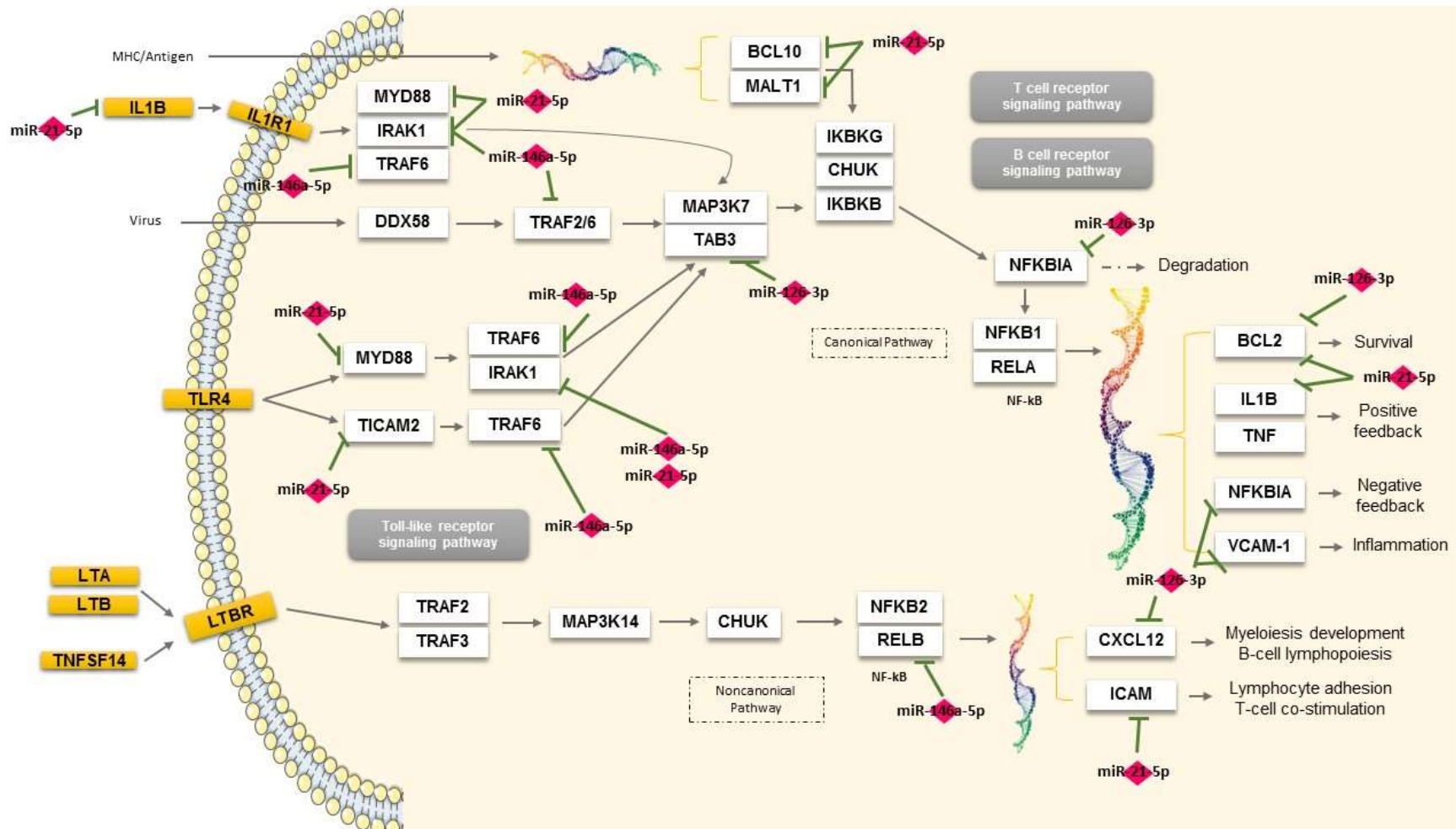
Figure 4: Schematic diagram of the selected miRNA-mRNA interaction networks involved in *NF-κB* signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04064). The miRNAs are indicated by rhombus.

Table 1. Characteristics of the studies with humans.

First Author, year (ref)	Study design	Method	Tissue	Nº of microRNA differentially expressed		Observations
				Up	Down	
Qing, 2014 (42)	DM patients: 90 PDR, 90 NPDR; 20 healthy subjects.	qPCR	Serum	3	0	↑ miR-21, miR-181c and miR-1179 in PDR *vs.* NPDR and healthy subjects. No ≠ in miRNA expressions between NPDR *vs.* healthy subjects. The accuracy rate for the three-miRNAs as PDR signature was 82.6%, served as biomarker to diagnose PDR patients
		TLDA		18	1	
Cadena, 2015* (43)	T2DM patients: 13 PDR; 10 NPDR; 10 healthy subjects.	N/A	Plasma	3	0	↑ miR-19a, miR-122 in NPDR and PDR *vs.* healthy subjects. ↑ miR-133a in NPDR *vs.* healthy subjects. MiR-19a has been implicated with expression of TSP1 and CTGF, glucose levels, and insulin resistance. MiR-133a associated with cardiac hypertrophy, fibrosis and heart failure due to DM.

Olivieri, 2015 (30)	T2DM patients: 117 with overall complications, 84 DR, 76 without complications; 107 healthy subjects.	qPCR	Plasma	0	0	No ≠ in the two miRNAs (miR-21-5p and miR-126-3p) expression analyzed between DR *vs.* T2DM with overall complications. Those presented ↓ miR-21-5p and miR-126-3p expression *vs.* healthy subjects.
Barutta, 2016* (44)	T1DM patients with DR, 308 with overall complications, and 142 without complications.	qPCR	Serum	0	1	↓ miR-145 in DR patients as well as in patients with increased albuminuria.
Palit, 2016* (45)	T2DM and T1DM patients: 36 DR, and 25 without DR.	qPCR	Plasma	0	1	↓ miR-1 depended of DR severities, mainly between severe DR and macular edema. No ≠ observed in miR-125a and miR-155 expression in DR *vs.* controls.
Rezk, 2016 (46)	T2DM patients: 19 DR, and 14 without overall complications	qPCR	Serum	0	1	↓ miR-126 in RD vs. T2DM without complications. Circulating levels of this miRNA was negatively correlated with HbA1c and blood glucose.

Zampetaki, 2016 (47) [a]	T1DM patients: 62 DR, and 64 without DR.	qPCR	Serum	1	1	↓ miR-27b and ↑ miR-320a expression in DR *vs.* patients without DR. MiR-27b associated with protection to DR incidence in T1DM patients.
Zampetaki, 2016 (47)[b]	T1DM patients: 93 PDR, and 81 NPDR.	qPCR	Serum	1	0	↑ miR-320a in PDR *vs.* NPDR. No ≠ observed in miR-27b between groups. MiR-320a associated with increased risk to DR progression.
Barutta, 2017 (31)	T1DM patients: 312 with overall complications, 249 DR, and 143 without complications	qPCR	Serum	0	1	↓ miR-126 in DR as well in patients with overall complications *vs.* patients without any complication. Mir-126 was associated with decreased risk to develop PDR (OR = 0,75; 95% CI 0,59-0,95) adjusted by age, gender, HbA1c and diabetes duration.
Jiang, 2017 (48)	T2DM patients; 51 PDR, 73 NPDR, and 65 without DR; 115 healthy subjects.	qPCR	Plasma	1	0	↑ miR-21 expression in DR groups *vs.* patients without DR and healthy subjects. MiR-21 expression positively correlated with HbA1c and diabetes duration.
Li, 2017 (49)	DM patients: 255 DR; 253	qPCR	Serum	0	1	↓ miR-200b expression in DR group *vs.* healthy subjects.

	healthy subjects.					*VEGF* gene confirmed as a target of miR-200b. The ↓ of *VEGF* by ↑ of miR-200b was associated with reduced retinal microvessels density, reverting retinal injuries occurrence.
Zou, 2017 (50)	T2DM patients: 75 DR, and 65 without DR; 127 healthy subjects	qPCR	Plasma	0	1	↑ miR-93 expression in DR vs. patients without DR and healthy subjects, as well as IL-6, IL-1, TNF and VEGF mRNAs. MiR-93 expression was positively correlated with HbA1c in DR group as well as DM duration.

*Abstracts. DM: diabetes mellitus; T2DM: type 2 diabetes mellitus; T1DM: type 1 diabetes mellitus; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; NPDR: non-proliferative diabetic retinopathy; TLDA: TaqMan Low Density Array; HbA1c: glycated hemoglobin; OR: odds ratio; CI: confidence interval.

Table 2. MicroRNA profile in DR analyzed in at least two studies for humans and at least three for animal models.

miRNA ID	First author, year [ref]	Specie	Tissue	Change of expression	Comparison group
miR-21-5p	Qing, 2014 (42)	Human	Serum	Up	PDR vs. NPDR patients and PDR vs. healthy subjects.
	Jiang, 2017 (48)	Human	Plasma	UP	DR patients vs. non-DR and vs. healthy subjects.
miR-126-3p	Rezk, 2016 (46)	Human	Serum	Down	DR patients vs. DM patients without complications.
	Barutta, 2017 (31)	Human	Serum	Down	DR patients vs. DM patients without complications.
miR-146a-5p	Feng, 2011 (29)	Mice	Retina	Down	db/db mice vs. controls
	Feng, 2011 (29)	Rat	Retina	Down	STZ-diabetic retina *vs.* controls
	Wu, 2012 (52)	Rat	Retina	Down	STZ-diabetic retina *vs.* controls
	Zhuang, 2017 (67)	Rat	Retina	Up	STZ-diabetic retina *vs.* controls
miR-195-5p	Kovacs, 2011 (28)	Rat	Retina	Up	STZ-diabetic retina *vs.* controls
	Mortuza, 2014 (54)	Rat	Retina	Up	STZ-diabetic retina *vs.* controls
	Zhang, 2017 (66)	Rat	Retina	Up	STZ-diabetic retina *vs.* controls
miR-200b-3p	Kovacs, 2011 (28)	Rat	Retina	Up	STZ-diabetic retina *vs.* controls
	McArthur, 2011 (25)	Rat	Retina	Down	STZ-diabetic retina *vs.* controls

	Murray, 2013 (26)	Mice	Retina	Up	C57BL/6J-Ins2^{Akita} *vs.* controls
	Ruiz, 2015 (58)	Mice	Retina	Down	STZ-diabetic retina *vs.* controls
	Li, 2017 (49)	Human	Serum	Down	DR patients vs. healthy subjects
miR-592	Kovacs, 2011 (28)	Rat	Retina	Up	STZ-diabetic retina vs. controls
	Wu, 2012 (52)	Rat	Retina	Up	STZ-diabetic retina vs. Controls
	Xiong, 2014 (55)	Rat	Retina	Up	STZ-diabetic retina vs. controls

PDR: proliferative diabetic retinopathy; NPDR: non proliferative diabetic retinopathy; DR: diabetic retinopathy; DM: diabetes mellitus; STZ: streptozotocin; db/db: obesity and type 2 diabetes mellitus spontaneous model; C57BL/6J-Ins2^{Akita}: type 1 diabetes mellitus spontaneous model.
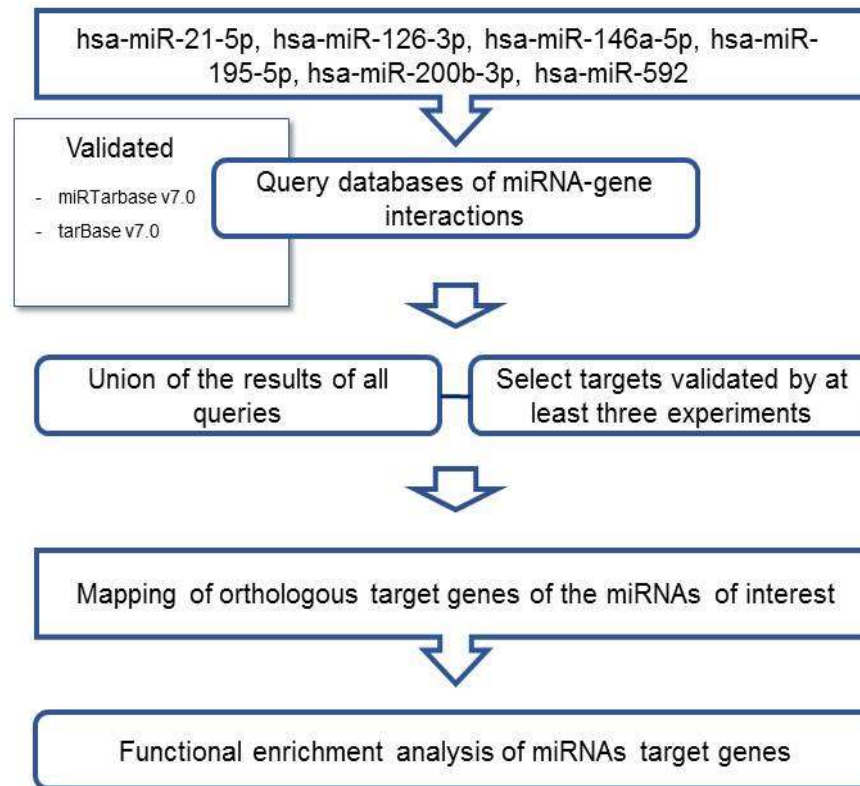
Supplementary figure 1: Flowchart of bioinformatic analyses of selected miRNAs.
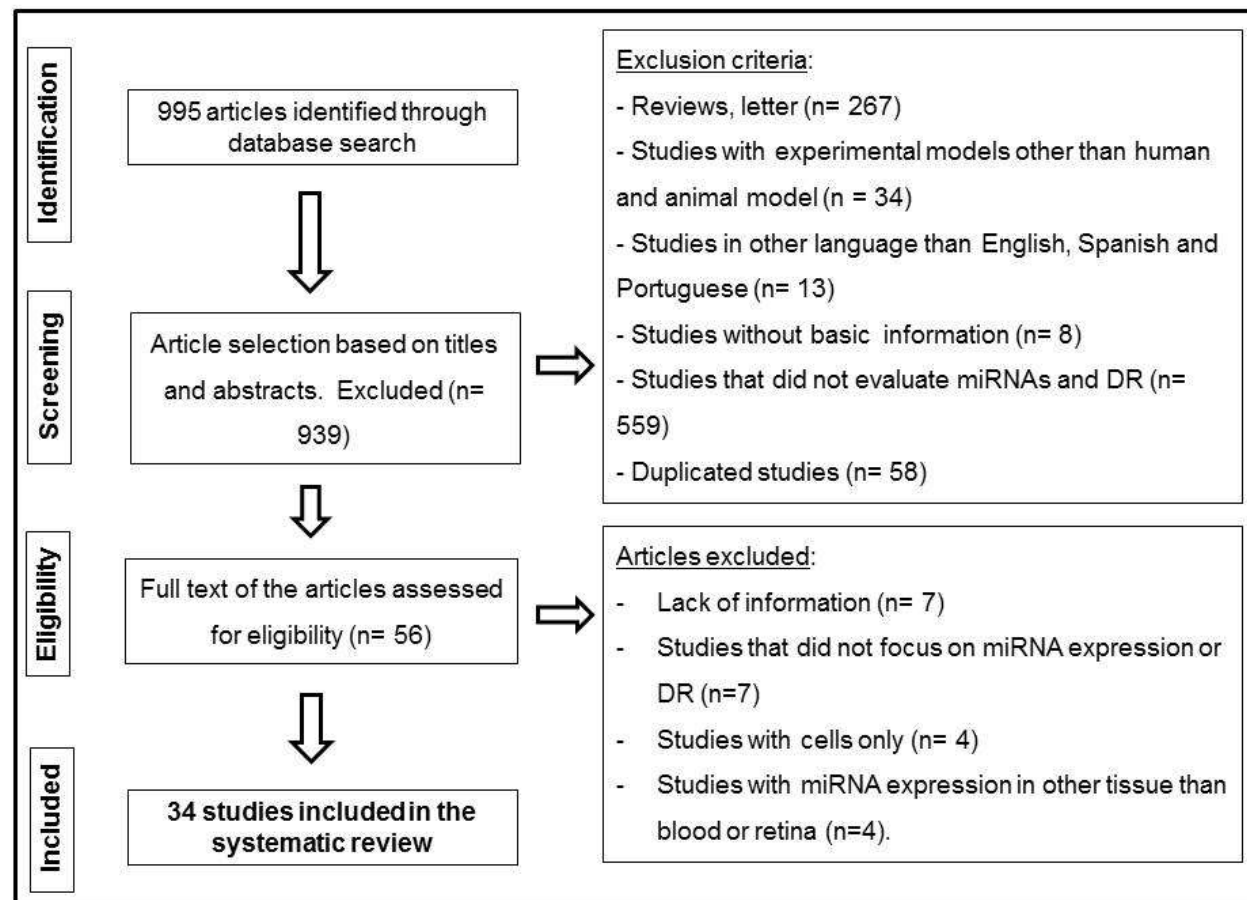
Supplementary figure 2: Flowchart of eligibility criteria.

A

189 miRNAs

69,84% Up
21,70% Down
8,46% Up/Down

160 miRNAs — 7 — 22 miRNAs

Animal model

Human

B

miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p and miR-592

↓ miR-146a-5p   ↑ miR-195-5p
miR-200b-3p   miR-592

↓ miR-126-3p   ↑ miR-21-5p
miR-200b-3p
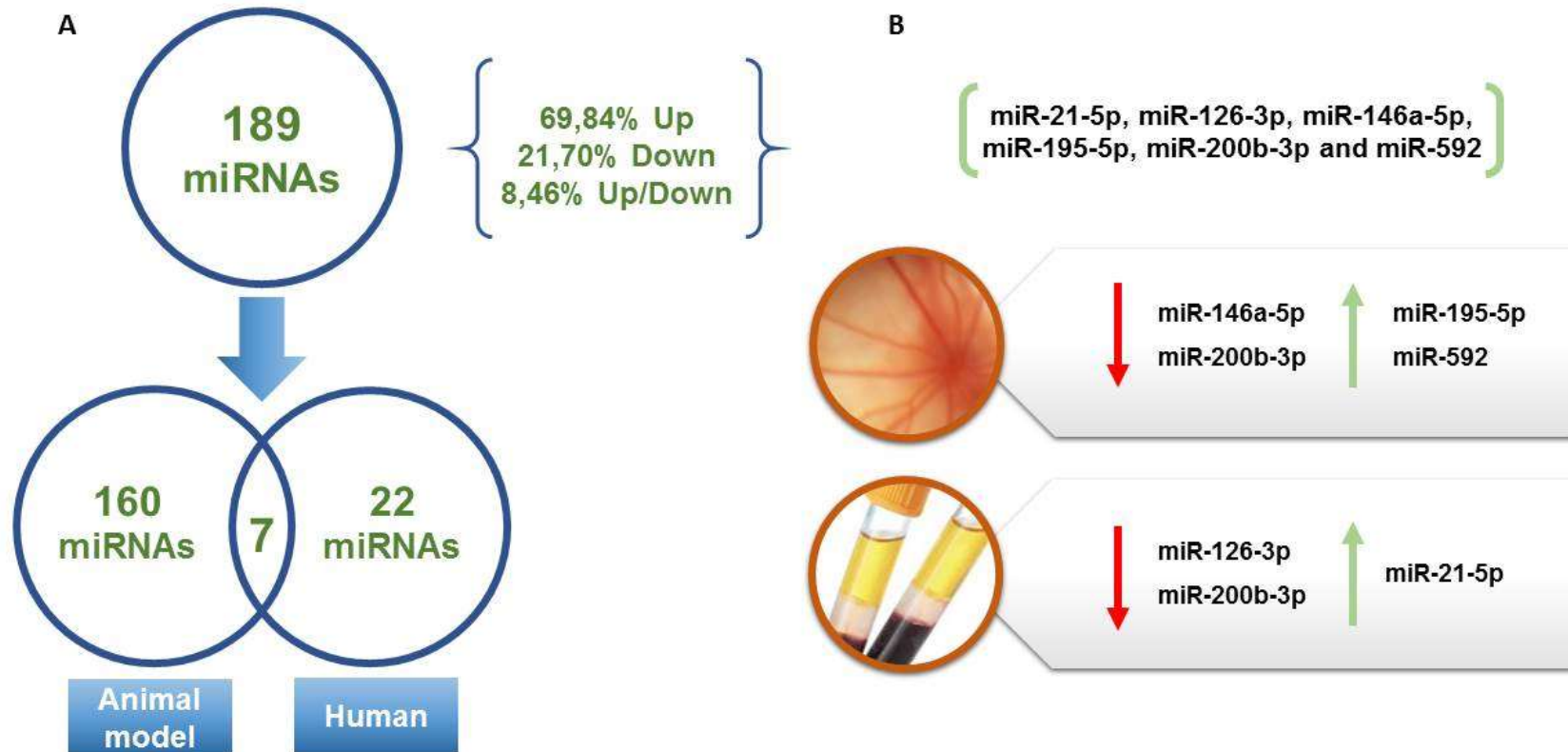
Supplementary figure 3: Expression profiles of miRNAs reported in the included studies. **A:** number of miRNAs analyzed in all studies and stratified by animal model and humans. **B:** expression profile of selected miRNAs in retina and serum/plasma samples.

miRNAs-target interactions

miRTarbase v. 7.0

Humans	186	hsa-miR-21-5p hsa-miR-126-3p
Mice	33	mmu-miR-146a-5p mmu-miR-200b-3p
Rat	04	rno-miR-195-5p rno-miR-200b-3p

TarBase v. 7.0

Humans	162	hsa-miR-21-5p hsa-miR-126-3p
Mice	27	mmu-miR-146a-5p mmu-miR-200b-3p
Rat		
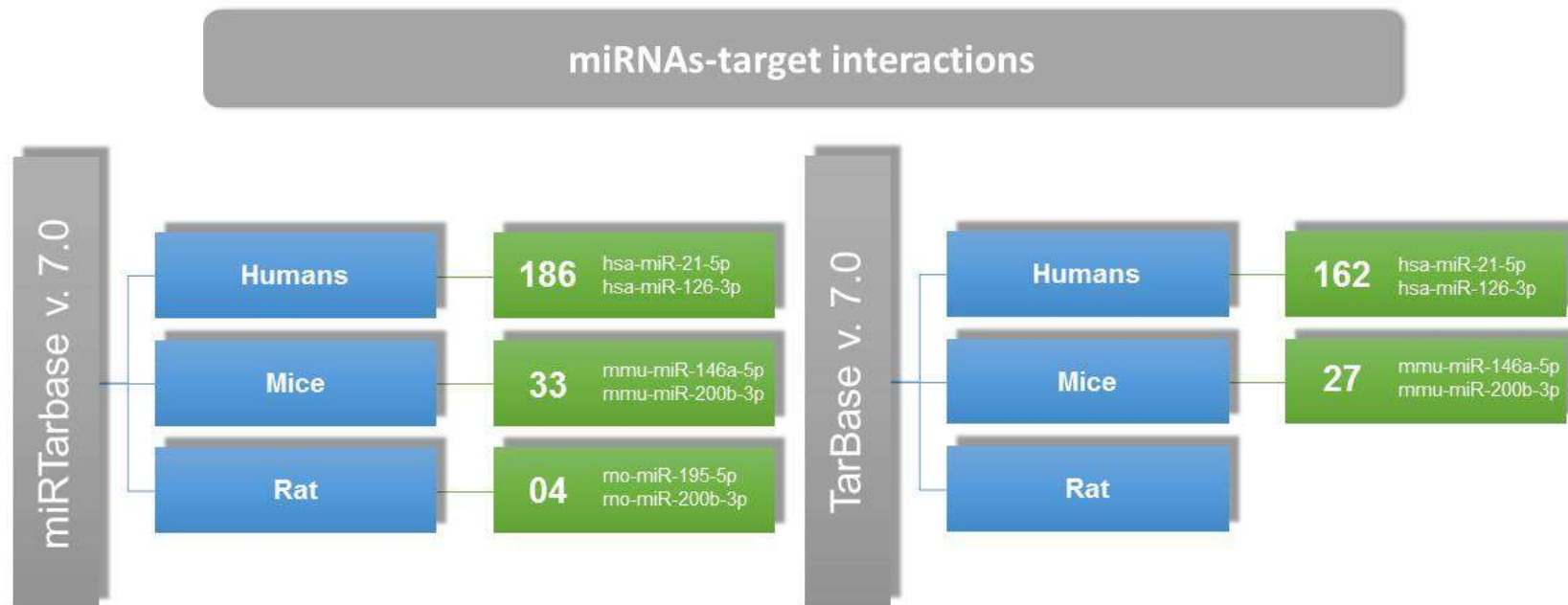
Supplementary figure 4: Number of miRNA-target interactions, validated in at least three experiments, retrieved from miRTarbase and TarBase.

Supplementary Table 1. Characteristics of the studies with murine models included in the systematic review.

First Author, year (ref)	Study design	Method	Tissue	Observations
Feng, 2011 (29)	Sprague Dawley rats: STZ diabetes induced, and controls; db/db mice (T2DM spontaneous model) and controls.	qRT-PCR	Retina	↓ miR-146a in STZ-diabetic rats and db/db mice, associated with ↑ in the fibronectin and p300 mRNA. The same result was observed in kidneys and hearts of these samples.
Kovacs, 2011 (28)	Sprague Dawley rats: 7 STZ diabetes induced, and 7 controls.	qRT-PCR Array	Retina	↑ VEGF-responsive miRNAs in the retinas of STZ-diabetic rats. ↑ of the miR-34 family in STZ-diabetic rats may mediate p53-induced apoptosis and contribute to neurodegeneration in the diabetic retina.
McArthur, 2011 (25)	Sprague Dawley rats: 6 STZ diabetes induced, and 6 controls.	qRT-PCR	Retina	↓ miR-200b in STZ-diabetic retinas associated to ↑ VEGF levels. ↑ miR-144 and miR-30a-3p in STZ-diabetic retinas. No ≠ in miR-429, miR-200a and miR-200c between cases and controls. Intravitreal injection of miR-200b mimics prevented ↑ levels of VEGF, angiogenesis and permeability in STZ-diabetic retinas.
Silva, 2011 (51)	Wistar rats: STZ diabetes induced,	qRT-PCR	Retina	↑ miR-29b in STZ-diabetic retinas *vs.* controls. *RAX* gene is a predicted target

and controls.				of miR-29b.
Wu, 2012 (52)	Sprague Dawley rats: 6 STZ diabetes induced, and 6 controls.	qRT-PCR Array	Retina	↑ miR-182, miR-96, miR-183, miR-211, miR-204 and miR-124 during the onset of DR, whereas miR-10b, miR-10a, miR-219-2-3p, miR-144, miR-146a, miR-338 and miR-199a-3p was ↓ in the same time. STZ-diabetic retinas developed capillary dilatation, interstitial edema consistent with DR characteristics.
Murray, 2013 (26)	11 C57BL/6J-Ins2^{Akita} mice (T1DM spontaneous model), and 8 C57BL/6J control mice.	qRT-PCR Array	Retina	↑ miR-200b in Akita's retina *vs.* controls associated with ↓ *Oxr1* gene mRNA. MiR-200b inhibitor transfection increased *Oxr1* protein levels, improving oxidative stress and apoptosis.
Feng, 2014 (53)	C57BL/6 mice: 8 STZ diabetes induced, and 8 controls.	qRT-PCR	Retina	↓ miR-1 in the retina, heart and kidney of STZ-diabetic mice. This reduction was associated with ↑ levels of endothelin-1 and fibronectin mRNAs.
Mortuza, 2014 (54)	Sprague Dawley rats: 7 STZ diabetes induced, and 7 controls.	qRT-PCR	Retina	↑ miR-195 in STZ-diabetic retina associated with ↓ *SIRT1* gene mRNA and enzyme. ↓ miR-34a, miR-9, miR-138 in STZ-diabetic retinas *vs.* controls. Vascular permeability diabetes-induced was decreased by intravitreal injection of miR-195 antagonist.
Xiong, 2014 (55)	Sprague Dawley rats: 3 STZ diabetes	qRT-PCR	Retina	

	induced, and 3 controls.	Array		miR-203, miR-350, miR-216a, miR-410, and miR-34c seems to have the most significant regulatory functions associated with DR after *in silico* analysis.
Friedrich, 2015* (56)	8 InsAkita mice (T1DM spontaneous model), and 8 controls.	Array	Retina	↑ miR-409-5p, miR-744-5p, miR-7b-3p, miR-1a-1-5p and miR-16a-2-3p, whereas miR-708-3p, miR-199a-5p, miR-1298-5p, miR-670-5p and miR-501-5p are ↓ in cases *vs.* controls. According to in silico analysis, the microRNAs mainly affect the MAPK, ERBB, Wnt, TGF and Axon guidance pathway.
Haque, 2015 (57)	Long Evans rats: 4 STZ diabetes induced, and 4 controls.	qRT-PCR	Retina	↓ miR-152 in STZ-diabetic retinas associated with ↑ PRR [(pro)renin] receptor expression. The authors suggested an interaction between miR-152 expression and PRR signaling pathway related to expression of angiogenic molecules.
Ruiz, 2015 (58)	C57BL/6 mice: 6 STZ diabetes induced, and 6 controls.	qRT-PCR	Retina	↓ miR-200b in STZ-diabetic retinas *vs.* controls, whereas PRC2 complex components were ↑. Inverse relationship between miR-200b and VEGF expressions.
Wu, 2015 (59)	Sprague Dawley rats: 3 STZ diabetes induced, 3 STZ diabetes induced + CH$_4$, and 3 controls.	qRT-PCR Array	Retina	↓ miR-192-5p and miR-335 in STZ-diabetic retinas *vs.* controls. Target genes of this miRNAs were related with apoptosis, tyrosine-kinase signaling pathway, proliferation and oxidative stress.

Wang, 2016 (60)	Long Evans rats: 6 STZ diabetes induced, and 6 controls.	qRT-PCR	Retina	↓ miR-15a in STZ-diabetic retinas, associated with ↑ *ASM* and *VEGF-A* genes.
Zeng, 2016 (61)	Sprague Dawley rats: 68 STZ diabetes induced, and controls.	qRT-PCR	Retina	↓ miR-29b in STZ-diabetic retinas with concomitant upregulation of *SP1* gene. The gene levels could be restored by injection or transfection of resveratrol and the authors hypothesized that resveratrol could be a therapeutic option to DR.
Zhao, 2016 (62)	Sprague Dawley rats: 10 STZ diabetes induced.	qRT-PCR	Retina	↑ miR-23b-3p in STZ-diabetic retinas after 3 months of DM onset without glycemic control. The ↑ remains even after proper glycemic control, as well as changes caused by sustained hyperglycemia as vascular permeability.
Chen, 2017 (63)	5 db/db mice (T2DM spontaneous model), and 5 C57BL/6 control mice.	qRT-PCR Array	Retina	↑ miR-21a in db/db retinas. miRNA knockout mice show less acellular retinal capillaries compared to db/db mice, suggesting that miR-21a knockout protect against retinal capillary degeneration caused by DM. Inflammatory factors (TNF, VCAM-1 and VEGF) were ↓ in db/db mice retina after intravitreal injection of miR-21 inhibitor.
Garcia-Morales, 2017 (64)	6 InsAkita mice (T1DM spontaneous model), and 6 controls; C57BL/6	qRT-PCR	Retina	↑ miR-7-5p Akita's retina and *OIR* model vs. Akita's control and normoxic control mice respectively. *EPAC-1* gene was reduced in Akita DM and *OIR*

	mice: 6 *OIR* model, and controls.			mice compared to controls.
Zhang, 2017 (65)	Sprague Dawley rats: 8 STZ diabetes induced, and controls.	qRT-PCR	Retina	↓ miR-29a in STZ-diabetic retinas *vs.* controls, as well as ↑ *AGT* gene mRNA. MiRNA levels not differ between the DR different model groups.
Zhang, 2017 (66)	Sprague Dawley rats: 6 STZ diabetes induced, and controls.	qRT-PCR	Retina	↑ miR-195 in STZ-diabetic retinas, as well as in retinal endothelial cells after oxidative stress induction by H_2O_2. Retinal miR-195 expression was gradually increasing during the onset of DM and regulated the vascular permeability. MiR-15b, 106b e 497 were ↓ in STZ-diabetic retinas. No ≠ observed in miR-17 levels between groups.
Zhuang, 2017 (67)	Sprague Dawley rats: 4 STZ diabetes induced, and controls.	qRT-PCR	Retina	↑ miR-146a in STZ-diabetic retinas. ↑ of this miRNA decreases target genes of activation pathways of *NFKB* in as well as inhibition of *ICAM1*, a proinflammatory factor. ↑ miR-146a has a protector effect on STZ-diabetic retinas against retinal neurofunctional and microvascular defects.
Liu, 2018 (68)	Sprague Dawley rats: 40 STZ diabetes induced and controls.	qRT-PCR	Retina	↓ miR-133b in STZ-diabetic retinas while ↑ *AGT, AngII, ERK1* and *ERK2* mRNA were ↑ in the same group. *In silico* analysis indicates that miR-133b can specifically bind *AGT*-3'UTR and decreased the expression of *AGT* after transcription.

Xia, 2018 (69)	C57BL/6 mice: 21 STZ diabetes induced and controls.	qRT-PCR	Retina	↓ miR-384 expression and ↑ mRNA and protein expression of HK2 and CD31 in DR retinal tissues compared with those in the normal mice. After *in silico* analysis HK2 was confirmed as target gene of miR-384. The miRNA inhibited the proliferation of RMECs and retinal neovascularization in DR through downregulating HK2.

*Abstracts. T2DM: type 2 diabetes mellitus; T1DM: type 1 diabetes mellitus; DM: diabetes mellitus; DR: diabetic retinopathy; STZ: streptozotocin; CH4: methane; OIR: oxygen induced retinopathy; H2O2: hydrogen peroxide; RMECs: Retinal microvascular endothelial cells.

Supplementary Table 2. Clinical characteristics of the studies with humans.

First Author, year (ref)	DM type	Sample	Gender, male, %	Age, years	Time of DM, years	HbA1c, %	BMI, kg/m²
Qing, 2014 (42)	N/A	90 PDR patients	53	55 patients with > than 60 years	74 patients with > than 5 years	N/A	N/A
		90 NPDR patients	50	46 patients with > than 60 years	73 patients with > than 5 years	N/A	N/A
		20 Healthy subjects	50	10 patients with > than 60 years	-	N/A	N/A
Cadena, 2015* (43)	T2DM	13 PDR patients	N/A	N/A	N/A	N/A	N/A
		10 NPDR patients	N/A	N/A	N/A	N/A	N/A
		10 healthy subjects	N/A	N/A	-	N/A	N/A
Olivieri, 2015 (30)	T2DM	117 patients with overall complications	69	66.51 ± 7.48	N/A	7.77 ± 1.19	28.57 ± 3.46

		107 healthy subjects	49	64.25 ± 7.56	-	5.96 ± 5.40	26.67 ± 5.40
		86 DR patients	N/A	N/A	N/A	N/A	N/A
		76 patients without complications	47	65.56 ± 6.69	N/A	7.34 ± 1.28	28.47 ± 4.34
Barutta, 2016* (44)	T1DM	DR patients	N/A	N/A	N/A	N/A	N/A
		308 patients with overall complications	N/A	N/A	N/A	N/A	N/A
		142 patients without complications	N/A	N/A	N/A	N/A	N/A
Palit, 2016* (45)	T1DM and T2DM	36 DR patients 25 patients without DR	59	55.20 ± 14.40	13.70 ± 11.30	N/A	N/A
Rezk, 2016 (46)	T2DM	19 DR patients	53	50.10 ± 10.30	6.12 ± 1.10	9.0 ±1.35	20.10 ± 8.10

Study	Type	Group					
		14 patients without complications	43	45.20 ± 7.30	1.70 ± 0.50	8.20 ± 1.40	22.20 ± 5.10
Zampetaki, 2016 (47)[a]	T1DM	62 DR patients	53	31.10 ± 8.10	7.30 ± 3.80	8.90 ± 1.50	N/A
		64 patients without DR	58	27.70 ± 8.10	7.30 ± 3.60	9.20 ± 1.80	N/A
Zampetaki, 2016 (47)[b]	T1DM	93 PDR patients	62	31.50 ± 9.10	10.90 ± 4.0	8.90 ± 1.20	N/A
		81 NPDR patients	52	31.0 ± 8.50	10.60 ± 4.0	9.40 ± 1.50	N/A
Barutta, 2017 (31)	T1DM	312 patients with overall complications	52	41.50 ± 10.10	24.50 ± 9.40	8.90 ± 1.60	25.0 ± 3.50
		249 DR patients	N/A	N/A	N/A	N/A	N/A
		143 patients without complications	48	35.40 ± 7.30	15.10 ± 6.70	7.70 ± 1.20	23.7 ± 2.60

Study	Type	Group					
Jiang, 2017 [48]	T2DM	65 patients without DR	52	47.76 ± 8.05	4.16 ± 1.31	6.25 ± 0.58	22.67 ± 1.98
		73 NPDR patients	43	48.83 ± 7.14	9.13 ± 3.05	7.02 ± 0.66	23.06 ± 1.85
		51 PRD patients	54	50.75 ± 10.18	13.58 ± 3.82	8.16 ± 0.79	23.13 ± 2.02
		115 healthy subjects	52	48.53 ± 7.26	-	4.30 ± 0.42	22.82 ± 2.33
Li, 2017 [49]	N/A	255 DR patients	52	61.45 ± 11.90	8.21 ± 2.43	9.49 ± 2.04	25.27 ± 3.67
		253 healthy subjects	55	60.18 ± 7.68	-	5.91 ± 1.11	23.83 ± 3.64
Zou, 2017 [50]	T2DM	75 DR patients	54	48.33 ± 8.58	9.33 ± 2.8	12.41 ± 1.56	21.75 ± 1.14
		65 patients without DR	55	49.28 ± 8.54	7.62 ± 2.81	8.94 ± 2.35	21.86 ± 1.65
		127 healthy subjects	51	47.25 ± 9.75	-	4.28 ± 0.65	22.08 ± 1.59

*Abstracts.   N/A: not available; DM: diabetes mellitus; T2DM: type 2 diabetes mellitus; T1DM: type 1 diabetes mellitus; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; NPDR: non-proliferative diabetic retinopathy; HbA1c: glycated hemoglobin; BMI: body mass index.

Supplementary Table 3. Quality assessment of each study included in the systematic review

Study	Risk of bias				Applicability concerns			
	Patient Selection	Index test	Reference Standard	Flow and timing	Patient Selection	Index test	Reference Standard	Total score
Studies with humans								
Qing, 2014 (42)	?	☺	☺	?	☺	☺	☺	6.0
Cadena, 2015* (43)[a]	-	-	-	-	-	-	-	-
Olivieri, , 2015 (30)	?	☺	☺	☺	☺	☺	☺	6.5
Barutta, 2016* (44)	-	-	-	-	-	-	-	-
Palit, 2016* (45)	-	-	-	-	-	-	-	-
Rezk, 2016 (46)	?	☺	☺	☺	☺	☺	☺	6.5
Zampetaki, 2016 (47)[a]	☹	☺	☺	☺	☺	?	?	5.0

	1	2	3	4	5	6	7	
Zampetaki, 2016 (47)[b]	☹	☺	☺	☺	☺	?	?	5.0
Barutta, 2017 (31)	☹	☺	☺	☺	☺	☺	☺	6.0
Jiang, 2017 (48)	?	☺	☺	☺	☺	☺	☺	6.5
Li, 2017 (49)	?	☺	☺	☺	☺	☺	☺	6.5
Zou, 2017 (50)	?	☺	☺	☺	☺	☺	☺	6.5

Studies with murine models

	1	2	3	4	5	6	7	
Feng, 2011 (29)	?	☺	☺	?	?	☺	☺	5.5
Kovacs, 2011 (28)	?	?	☺	?	☺	☺	☺	5.5
McArthur, 2011 (25)	?	☺	☺	?	?	☺	☺	5.5
Silva, 2011 (51)	☺	☺	☺	?	☺	☺	☺	6.5
Wu, 2012 (52)	?	☺	☺	?	☺	☺	☺	6.0
Murray, 2013 (26)	?	?	☺	?	?	☺	☺	5.0

Study								Score
Feng, 2014 (53)	?	☺	☺	?	☺	☺	☺	6.0
Mortuza, 2014 (54)	?	☺	☺	☺	☺	☺	☺	6.5
Xiong, 2014 (55)	☺	☺	☺	☺	☺	☺	☺	7.0
Friedrich, 2015* (56)	-	-	-	-	-	-	-	-
Haque, 2015 (57)	?	☺	☺	?	?	☺	☺	5.5
Ruiz, 2015 (58)	?	☺	☺	?	☺	☺	☺	6.0
Wu, 2015 (59)	☺	☺	☺	?	☺	☺	☺	6.5
Wang, 2016 (60)	?	☺	☺	?	☺	☺	☺	6.0
Zeng, 2016 (61)	☺	☺	☺	☺	☺	☺	☺	7.0
Zhao, 2016 (62)	☺	☺	☺	☺	☹	☺	☺	6.0
Chen, 2017 (63)	?	☺	☺	?	☹	☺	☺	5.0
Garcia-Morales, 2017 (64)	?	☺	☺	☺	☺	☺	☺	6.5

Zhang, 2017 (65)	?	☺	☺	☺	☺	☺	☺	6.5
Zhang, 2017 (66)	☺	☺	☺	☺	☺	☺	☺	7.0
Zhuang, 2017 (67)	?	?	?	☺	☹	☺	☺	4.5
Liu, 2018 (68)	☺	☺	☺	?	☺	☺	☺	6,5
Xia, 2018 (69)	☺	☺	☺	?	☺	☺	☺	6,5

*Abstracts from congress. ☺ = low risk; ? = unclear; ☹ = high risk.

Supplementary table 4 - All microRNAs analyzed in the studies included in the systematic review.

miRNA ID	First author, year.	Specie	Sample type	Change of Expression
miR-let-7e	Kovacs, 2011.	Rat	Retina	Up
miR-1	Feng, 2014.	Mice	Retina	Down
	Palit, 2016.	Human	Plasma	Down
miR-1a-1-5p	Friedrich, 2015.	Mice	Retina	Up
miR-7-5p	Garcia-Morales, 2017.	Mice	Retina	Up
miR-7a-5p	Murray, 2013.	Mice	Retina	Up
miR-7b-3p	Friedrich, 2015.	Mice	Retina	Up
miR-9a-5p	Mortuza, 2014.	Rat	Retina	Down
miR-10a-5p	Wu, 2012.	Rat	Retina	Down
miR-10b-5p	Wu, 2012.	Rat	Retina	Down
miR-15a-5p	Wang, 2016.	Rat	Retina	Down
miR-15b-5p	Kovacs, 2011.	Rat	Retina	Up

	Zhang, 2017.	Rat	Retina	Down
miR-16a-2-3p	Friedrich, 2015.	Mice	Retina	Up
miR-18a-5p	Wu, 2012.	Rat	Retina	Down
miR-19a-3p	Cadena, 2015.	Human	Plasma	Up
miR-19b-3p	Wu, 2015.	Rat	Retina	Down
miR-20b-5p	Kovacs, 2011.	Rat	Retina	Down
miR-21-5p	Qing, 2014.	Human	Serum	Up
	Jiang, 2017.	Human	Plasma	Up
miR-21a-5p	Chen, 2017.	Mice	Retina	Up
miR-22-3p	Kovacs, 2011.	Rat	Retina	Up
miR-23b-3p	Zhao, 2016.	Rat	Retina	Up
miR-24-1-5p	Xiong, 2014.	Rat	Retina	Up
miR-24-3p	Kovacs, 2011.	Rat	Retina	Up
miR-25-3p	Kovacs, 2011.	Rat	Retina	Up

miR-27a-3p	Kovacs, 2011.	Rat	Retina	Up
	Zampetaki, 2016. [a]	Human	Serum	Down
miR-27b-3p	Kovacs, 2011.	Rat	Retina	Up
miR-28-5p	Murray, 2013.	Mice	Retina	Up
miR-29a-3p	Kovacs, 2011.	Rat	Retina	Up
	Zhang, 2017.	Rat	Retina	Down
miR-29b-3p	Silva, 2011.	Rat	Retina	Up
	Zeng, 2016.	Rat	Retina	Down
	Chen, 2017.	Mice	Retina	Down
miR-29c-3p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Up
miR-29c-5p	Wu, 2012.	Rat	Retina	Up
miR-30a-3p	McArthur, 2011.	Rat	Retina	Up
miR-30a-5p	Kovacs, 2011.	Rat	Retina	Up

	Qing, 2014.	Human	Serum	Up
miR-30d-5p	Kovacs, 2011.	Rat	Retina	Up
miR-31-5p	Chen, 2017.	Mice	Retina	Up
miR-31a-3p	Kovacs, 2011.	Rat	Retina	Up
miR-31a-5p	Kovacs, 2011.	Rat	Retina	Up
miR-34a-5p	Wu, 2012.	Rat	Retina	Down
	Mortuza, 2014.	Rat	Retina	Down
miR-34b-3p	Kovacs, 2011.	Rat	Retina	Up
miR-34c-3p	Kovacs, 2011.	Rat	Retina	Up
miR-34c-5p	Kovacs, 2011.	Rat	Retina	Up
	Xiong, 2014.	Rat	Retina	Up
miR-92a-1-5p	Xiong, 2014.	Rat	Retina	Up
miR-93-5p	Zou, 2017.	Human	Plasma	Up
miR-96-5p	Wu, 2012.	Rat	Retina	Up

miR-100-5p	Kovacs, 2011.	Rat	Retina	Up
miR-106b-5p	Zhang, 2017.	Rat	Retina	Down
miR-122-5p	Cadena, 2015.	Human	Plasma	Up
miR-124-3p	Wu, 2012.	Rat	Retina	Up
	Murray, 2013.	Mice	Retina	Up
miR-125a-5p	Kovacs, 2011.	Rat	Retina	Up
miR-125b-5p	Kovacs, 2011.	Rat	Retina	Up
miR-126-3p	Rezk, 2016.	Human	Serum	Down
	Barutta, 2017.	Human	Serum	Down
miR-129-5p	Wu, 2012.	Rat	Retina	Up
miR-130b-3p	Kovacs, 2011.	Rat	Retina	Up
miR-132-3p	Kovacs, 2011.	Rat	Retina	Up
	Chen, 2017.	Mice	Retina	Up
miR-133a	Cadena, 2015.	Human	Plasma	Up

miR-133b-3p	Liu, 2018.	Rat	Retina	Down
miR-135a-1-3p	Kovacs, 2011.	Rat	Retina	Up
miR-135b-5p	Wu, 2012.	Rat	Retina	Up
miR-137-5p	Xiong, 2014.	Rat	Retina	Up
miR-138-5p	Mortuza, 2014.	Rat	Retina	Down
miR-139-5p	Kovacs, 2011.	Rat	Retina	Up
miR-141-5p	Kovacs, 2011.	Rat	Retina	Up
miR-142-3p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Down
miR-142-5p	Wu, 2012.	Rat	Retina	Down
miR-143-3p	Kovacs, 2011.	Rat	Retina	Up
miR-144-3p	McArthur, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Down
miR-145-5p	Kovacs, 2011.	Rat	Retina	Up

	Barutta, 2016.	Human	Serum	Down
miR-146a-5p	Feng, 2011.	Mice	Retina	Down
	Feng, 2011.	Rat	Retina	Down
	Wu, 2012.	Rat	Retina	Down
	Zhuang, 2017.	Rat	Retina	Up
miR-148a-3p	Qing, 2014.	Human	Serum	Up
miR-148b-3p	Kovacs, 2011.	Rat	Retina	Up
miR-148b-5p	Kovacs, 2011.	Rat	Retina	Up
miR-151-3p	Kovacs, 2011.	Rat	Retina	Up
miR-152-3p	Kovacs, 2011.	Rat	Retina	Up
	Haque, 2015.	Rat	Retina	Down
miR-154-5p	Wu, 2012.	Rat	Retina	Up
miR-181a-5p	Kovacs, 2011.	Rat	Retina	Up
miR-181b-5p	Wu, 2012.	Rat	Retina	Up

miR-181c-5p	Kovacs, 2011.	Rat	Retina	Up
	Qing, 2014.	Human	Serum	Up
miR-182-5p	Wu, 2012.	Rat	Retina	Up
miR-183-5p	Wu, 2012.	Rat	Retina	Up
miR-184	Kovacs, 2011.	Rat	Retina	Up
	Murray, 2013.	Mice	Retina	Down
miR-184-3p	Chen, 2017.	Mice	Retina	Up
miR-186-5p	Murray, 2013.	Mice	Retina	Up
miR-188-5p	Kovacs, 2011.	Rat	Retina	Up
miR-190b-5p	Wu, 2012.	Rat	Retina	Up
miR-191a-5p	Wu, 2012.	Rat	Retina	Up
miR-192-5p	Wu, 2015.	Rat	Retina	Down
miR-193b-3p	Kovacs, 2011.	Rat	Retina	Up
miR-194-5p	Wu, 2012.	Rat	Retina	Up

miR-195-5p	Kovacs, 2011.	Rat	Retina	Up
	Mortuza, 2014.	Rat	Retina	Up
	Zhang, 2017.	Rat	Retina	Up
miR-199a-3p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Down
	Murray, 2013.	Mice	Retina	Up
miR-199a-5p	Friedrich, 2015.	Mice	Retina	Down
miR-200a-3p	Kovacs, 2011.	Rat	Retina	Up
miR-200b-3p	Kovacs, 2011.	Rat	Retina	Up
	McArthur, 2011.	Rat	Retina	Down
	Murray, 2013.	Mice	Retina	Up
	Ruiz, 2015.	Mice	Retina	Down
	Li, 2017.	Human	Serum	Down
miR-200c-3p	Kovacs, 2011.	Rat	Retina	Up

miR-203a-3p	Xiong, 2014.	Rat	Retina	Up
miR-204-5p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Up
miR-205-5p	Kovacs, 2011.	Rat	Retina	Up
miR-210-3p	Wu, 2012.	Rat	Retina	Up
miR-211-5p	Wu, 2012.	Rat	Retina	Up
miR-212-3p	Xiong, 2014.	Rat	Retina	Down
miR-215	Wu, 2012.	Rat	Retina	Up
miR-216a-5p	Kovacs, 2011.	Rat	Retina	Up
	Xiong, 2014.	Rat	Retina	Up
miR-219-1-3p	Xiong, 2014.	Rat	Retina	Up
miR-219-2-3p	Wu, 2012.	Rat	Retina	Down
miR-219-5p	Wu, 2012.	Rat	Retina	Down
miR-223-3p	Kovacs, 2011.	Rat	Retina	Up

	Wu, 2012.	Rat	Retina	Down
miR-294-3p	Chen, 2017.	Mice	Retina	Down
miR-296-5p	Murray, 2013.	Mice	Retina	Down
miR-301a-3p	Wu, 2015.	Rat	Retina	Down
miR-320-3p	Kovacs, 2011.	Rat	Retina	Up
miR-320a	Zampetaki 2016. [a]	Human	Serum	Up
	Zampetaki 2016. [b]	Human	Serum	Up
miR-322	Kovacs, 2011.	Rat	Retina	Up
	Chen, 2017.	Mice	Retina	Up
miR-323-3p	Xiong, 2014.	Rat	Retina	Up
miR-324-3p	Kovacs, 2011.	Rat	Retina	Up
miR-326-3p	Kovacs, 2011.	Rat	Retina	Up
miR-328-3p	Kovacs, 2011.	Rat	Retina	Up
miR-331-5p	Kovacs, 2011.	Rat	Retina	Up

miR-335	Wu, 2015.	Rat	Retina	Down
miR-335-3p	Kovacs, 2011.	Rat	Retina	Up
miR-335-5p	Kovacs, 2011.	Rat	Retina	Up
miR-338-3p	Wu, 2012.	Rat	Retina	Down
miR-340-3p	Kovacs, 2011.	Rat	Retina	Up
miR-344-3p	Kovacs, 2011.	Rat	Retina	Up
miR-350-1	Kovacs, 2011.	Rat	Retina	Up
	Xiong, 2014.	Rat	Retina	Up
miR-351-5p	Xiong, 2014.	Rat	Retina	Up
miR-363-3p	Wu, 2012.	Rat	Retina	Up
	Qing, 2014.	Human	Serum	Up
miR-369-5p	Murray, 2013.	Mice	Retina	Up
	Xiong, 2014.	Rat	Retina	Up
miR-374a-5p	Qing, 2014.	Human	Serum	Up

miR-375-3p	Kovacs, 2011.	Rat	Retina	Down
	Xiong, 2014.	Rat	Retina	Down
miR-376b-3p	Chen, 2017.	Mice	Retina	Down
miR-378a-3p	Kovacs, 2011.	Rat	Retina	Up
miR-378a-5p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Up
miR-379-5p	Chen, 2017.	Mice	Retina	Up
miR-381-3p	Kovacs, 2011.	Rat	Retina	Up
miR-383-5p	Kovacs, 2011.	Rat	Retina	Up
miR-384-3p	Xia, 2018.	Mice	Retina	Down
miR-409-3p	Qing, 2014.	Human	Serum	Down
miR-409-5p	Friedrich, 2015.	Mice	Retina	Up
miR-410-3p	Murray, 2013.	Mice	Retina	Up
	Xiong, 2014.	Rat	Retina	Up

miR-411-5p	Kovacs, 2011.	Rat	Retina	Up
miR-423-5p	Qing, 2014.	Human	Serum	Up
miR-429	Murray, 2013.	Mice	Retina	Up
miR-431	Kovacs, 2011.	Rat	Retina	Down
miR-434-3p	Kovacs, 2011.	Rat	Retina	Up
miR-450a-5p	Kovacs, 2011.	Rat	Retina	Up
	Wu, 2012.	Rat	Retina	Down
miR-451-5p	Wu, 2012.	Rat	Retina	Down
miR-455	Kovacs, 2011.	Rat	Retina	Up
miR-467b	Kovacs, 2011.	Rat	Retina	Up
	Murray, 2013.	Mice	Retina	Down
miR-484	Kovacs, 2011.	Rat	Retina	Up
miR-488	Kovacs, 2011.	Rat	Retina	Up
miR-489-3p	Kovacs, 2011.	Rat	Retina	Up

miR-494	Qing, 2014.	Human	Serum	Up
miR-497-5p	Kovacs, 2011.	Rat	Retina	Up
	Zhang, 2017.	Rat	Retina	Down
miR-499-5p	Kovacs, 2011.	Rat	Retina	Down
miR-501-5p	Friedrich, 2015.	Mice	Retina	Down
miR-505-3p	Qing, 2014.	Human	Serum	Up
miR-513a	Wu, 2012.	Rat	Retina	Up
miR-520d-3p	Qing, 2014.	Human	Serum	Up
miR-539-5p	Murray, 2013.	Mice	Retina	Up
miR-542-5p	Wu, 2012.	Rat	Retina	Down
miR-548d-3p	Qing, 2014.	Human	Serum	Up
miR-574-3p	Kovacs, 2011.	Rat	Retina	Up
miR-576-3p	Qing, 2014.	Human	Serum	Up
miR-592	Kovacs, 2011.	Rat	Retina	Up

	Wu, 2012.	Rat	Retina	Up
	Xiong, 2014.	Rat	Retina	Up
miR-598	Kovacs, 2011.	Rat	Retina	Up
miR-601	Qing, 2014.	Human	Serum	Up
miR-606	Qing, 2014.	Human	Serum	Up
miR-627	Qing, 2014.	Human	Serum	Up
miR-652-3p	Kovacs, 2011.	Rat	Retina	Up
miR-670-5p	Friedrich, 2015.	Mice	Retina	Down
miR-678	Kovacs, 2011.	Rat	Retina	Up
miR-685	Kovacs, 2011.	Rat	Retina	Up
miR-690	Kovacs, 2011.	Rat	Retina	Down
	Chen, 2017.	Mice	Retina	Down
miR-699	Kovacs, 2011.	Rat	Retina	Up
miR-708-3p	Friedrich, 2015.	Mice	Retina	Down

miR-709	Kovacs, 2011.	Rat	Retina	Up
miR-712-5p	Chen, 2017.	Mice	Retina	Down
miR-744-5p	Friedrich, 2015.	Mice	Retina	Up
miR-758-3p	Xiong, 2014.	Rat	Retina	Up
miR-760	Kovacs, 2011.	Rat	Retina	Up
miR-764-5p	Kovacs, 2011.	Rat	Retina	Up
miR-872-5p	Kovacs, 2011.	Rat	Retina	Down
miR-877	Kovacs, 2011.	Rat	Retina	Up
miR-886-5p	Qing, 2014.	Human	Serum	Up
miR-935	Xiong, 2014.	Rat	Retina	Up
miR-1179	Qing, 2014.	Human	Serum	Up
miR-1196	Murray, 2013.	Mice	Retina	Down
miR-1224	Murray, 2013.	Mice	Retina	Down
miR-1255b-5p	Qing, 2014.	Human	Serum	Up

miR-1298-5p	Friedrich, 2015.	Mice	Retina	Down

Supplementary Table 5 - Orthologous miRNAs-target interactions

miRNA	Target	Type Source	Type Edge
Supplementary Table 5 - Orthologous miRNAs-target interactions			
miRNA	Target	Type Source	Type Edge
hsa-miR-126-3p			
	SPRED1	Experimental	mir-gene
	PLK2	Experimental	mir-gene
	RGS3	Experimental	mir-gene
	TOM1	Experimental	mir-gene
	HOXA9	Experimental	mir-gene
	CRK	Experimental	mir-gene
	VEGFA	Experimental	mir-gene
	PIK3R2	Experimental	mir-gene
	VCAM1	Experimental	mir-gene
	IRS1	Experimental	mir-gene
	SOX2	Experimental	mir-gene
	PTPN7	Experimental	mir-gene
	DNMT1	Experimental	mir-gene
	KRAS	Experimental	mir-gene
	IGFBP2	Experimental	mir-gene
	PITPNC1	Experimental	mir-gene
	MERTK	Experimental	mir-gene
	EGFL7	Experimental	mir-gene
	SLC7A5	Experimental	mir-gene
	PIK3CG	Experimental	mir-gene
	TEK	Experimental	mir-gene
	ADAM9	Experimental	mir-gene
	MMP7	Experimental	mir-gene
	CXCL12	Experimental	mir-gene
	CRKL	Experimental	mir-gene
	PGR	Experimental	mir-gene
	FOXO3	Experimental	mir-gene
	BCL2	Experimental	mir-gene
	CXCR4	Experimental	mir-gene

miRNA-target interactions

Supplementary Table 6 - Significant KEGG biological pathways enriched for each differentially expressed miRNA.

Supplementary Table 6. Significant KEGG biological pathways enriched for each differentially expressed miRNA.

miRNA	Pathway identity in KEGG	Name of the pathway	Gene ratio	q value	Name of the genes regulated by the miRNAs
hsa-miR-21-5p					
	hsa05206	MicroRNAs in cancer	25/147	8,3451E-08	CDC25A/BCL2/SPRY2/TIMP3/RECK/PTEN/E2F1/MARCKS/TPM1/PDCD4/SERPINB5/BMPR2/ERBB2/HNRNPK/TP63/EGFR/VEGFA/TGFB2/STAT3/ABCB1/BMI1/SOCS1/CCN
	hsa04010	MAPK signaling pathway	22/147	3,994E-06	RASGRP1/TGFBR2/RASA1/ERBB2/DAXX/MEF2C/EGFR/IL1B/DUSP10/NTF3/FASLG/VEGFA/TGFB2/AKT2/MYD88/IRAK1/MAP2K3/IGF1R/HSPA8/MAP3K1/MKNK2/SOS2
	hsa05161	Hepatitis B	15/147	5,4523E-06	BCL2/PTEN/E2F1/APAF1/FASLG/TGFB2/PIK3R1/AKT2/STAT3/MYD88/TICAM2/TLR3/CASP8/BIRC5/MAP3K1
	hsa04068	FoxO signaling pathway	14/147	8,7501E-06	TGFBR2/PTEN/EGFR/FASLG/TGFB2/PIK3R1/AKT2/STAT3/BCL6/IGF1R/FOXO1/CCND2/PRKAA2/SOS2
	hsa01524	Platinum drug resistance	10/147	3,5841E-05	BCL2/APAF1/ERBB2/MSH2/MSH6/FASLG/PIK3R1/AKT2/CASP8/BIRC5
	hsa05145	Toxoplasmosis	12/147	4,2306E-05	BCL2/PPIF/TGFB2/AKT2/STAT3/MYD88/IRAK1/MAP2K3/IL12A/SOCS1/CASP8/HSPA8
	hsa05215	Prostate cancer	11/147	4,6919E-05	BCL2/PTEN/E2F1/ERBB2/EGFR/PLAT/PIK3R1/AKT2/IGF1R/FOXO1/SOS2
	hsa01521	EGFR tyrosine kinase inhibitor resistance	10/147	4,6919E-05	BCL2/PTEN/ERBB2/EGFR/VEGFA/PIK3R1/AKT2/STAT3/IGF1R/SOS2
	hsa04933	AGE-RAGE signaling pathway in diabetic complications	11/147	4,8555E-05	BCL2/TGFBR2/IL1B/ICAM1/COL4A1/VEGFA/TGFB2/PIK3R1/AKT2/STAT3/FOXO1
	hsa04066	HIF-1 signaling pathway	11/147	4,8555E-05	BCL2/ERBB2/EGFR/VEGFA/PIK3R1/AKT2/STAT3/VHL/IGF1R/EGLN1/MKNK2
	hsa05225	Hepatocellular carcinoma	14/147	5,9037E-05	TGFBR2/PTEN/E2F1/EGFR/SMARCA4/TGFB2/PIK3R1/AKT2/IGF1R/LRP6/FZD6/PBRM1/SMARCD1/SOS2
	hsa05210	Colorectal cancer	10/147	6,6533E-05	BCL2/TGFBR2/MSH2/MSH6/EGFR/TGFB2/PIK3R1/AKT2/BIRC5/SOS2
	hsa05226	Gastric cancer	13/147	6,6533E-05	BCL2/TGFBR2/E2F1/ERBB2/EGFR/TGFB2/PIK3R1/AKT2/LRP6/ABCB1/FZD6/RXRA/SOS2
	hsa05212	Pancreatic cancer	9/147	0,00013238	TGFBR2/E2F1/ERBB2/EGFR/VEGFA/TGFB2/PIK3R1/AKT2/STAT3
	hsa01522	Endocrine resistance	10/147	0,0001469	BCL2/JAG1/E2F1/ERBB2/EGFR/SP1/PIK3R1/AKT2/IGF1R/SOS2
	hsa05142	Chagas disease (American trypanosomiasis)	10/147	0,00022708	TGFBR2/IL1B/FASLG/TGFB2/PIK3R1/AKT2/MYD88/IRAK1/IL12A/CASP8
	hsa05224	Breast cancer	12/147	0,00022708	JAG1/PTEN/E2F1/ERBB2/EGFR/SP1/PIK3R1/AKT2/IGF1R/LRP6/FZD6/SOS2
	hsa04620	Toll-like receptor signaling pathway	10/147	0,00023581	IL1B/PIK3R1/AKT2/MYD88/IRAK1/MAP2K3/IL12A/TICAM2/TLR3/CASP8
	hsa04510	Focal adhesion	14/147	0,00023581	BCL2/PTEN/ERBB2/EGFR/COL4A1/VEGFA/PIK3R1/AKT2/IGF1R/DOCK1/CCND2/ITGB8/SOS2/VCL
	hsa05205	Proteoglycans in cancer	14/147	0,00024737	TIMP3/PDCD4/ERBB2/TIAM1/EGFR/FASLG/VEGFA/TGFB2/PIK3R1/AKT2/STAT3/IGF1R/FZD6/SOS2
	hsa05223	Non-small cell lung cancer	8/147	0,00024737	E2F1/ERBB2/EGFR/PIK3R1/AKT2/STAT3/RXRA/SOS2
	hsa04668	TNF signaling pathway	10/147	0,00028063	JAG1/IL1B/ICAM1/CCL20/PIK3R1/AKT2/MAP2K3/DNM1L/CEBPB/CASP8
	hsa05162	Measles	11/147	0,00034396	IL1B/FASLG/PIK3R1/AKT2/STAT3/MYD88/IRAK1/IL12A/TNFRSF10B/CCND2/HSPA8
	hsa04550	Signaling pathways regulating pluripotency of stem cells	11/147	0,00046021	REST/BMPR2/SOX2/PIK3R1/AKT2/STAT3/IGF1R/FZD6/PCGF2/BMI1/KAT6A
	hsa05164	Influenza A	12/147	0,00075202	IL1B/ICAM1/FASLG/PIK3R1/AKT2/MYD88/MAP2K3/IL12A/TNFRSF10B/TLR3/HSPA8/KPNA2
	hsa05165	Human papillomavirus infection	17/147	0,00083796	JAG1/PTEN/E2F1/EGFR/COL4A1/FASLG/VEGFA/PIK3R1/AKT2/TLR3/FZD6/FOXO1/CASP8/CCND2/ITGB8/RBP1/SOS2
	hsa04213	Longevity regulating pathway - multiple species	7/147	0,00092409	PIK3R1/AKT2/IGF1R/FOXO1/EIF4EBP2/HSPA8/PRKAA2
	hsa05152	Tuberculosis	12/147	0,00092515	BCL2/APAF1/IL1B/TGFB2/AKT2/MYD88/IRAK1/IL12A/BCL10/CEBPB/CASP8/MALT1
	hsa05202	Transcriptional misregulation in cancer	12/147	0,00127504	TGFBR2/MEF2C/PLAT/SP1/BCL6/HPGD/IGF1R/CEBPB/BMI1/FOXO1/RXRA/CCND2
	hsa04218	Cellular senescence	11/147	0,00128005	CDC25A/TGFBR2/PTEN/E2F1/TGFB2/PIK3R1/AKT2/MAP2K3/FOXO1/CCND2/ZFP36L1
	hsa04630	Jak-STAT signaling pathway	11/147	0,00137893	BCL2/EGFR/PIAS3/PIK3R1/AKT2/STAT3/IL12A/SOCS6/SOCS1/CCND2/SOS2
	hsa04115	p53 signaling pathway	7/147	0,00139185	PTEN/APAF1/SERPINB5/TNFRSF10B/CASP8/CCND2/CCNG1
	hsa04210	Apoptosis	10/147	0,00143399	BCL2/APAF1/DAXX/FASLG/PIK3R1/AKT2/TNFRSF10B/CASP8/BIRC5/LMNB1
	hsa05211	Renal cell carcinoma	7/147	0,00143399	VEGFA/TGFB2/PIK3R1/AKT2/VHL/EGLN1/SOS2
	hsa05418	Fluid shear stress and atherosclerosis	10/147	0,00143703	BCL2/BMPR2/MEF2C/IL1B/ICAM1/PLAT/VEGFA/PIK3R1/AKT2/PRKAA2
	hsa04917	Prolactin signaling pathway	7/147	0,00148016	PIK3R1/AKT2/STAT3/SOCS6/SOCS1/CCND2/SOS2

CAPÍTULO 2

Relationship of miR-126 and miR-200b expressions in plasma with retina and choroidal thickness in patients with type 2 diabetes mellitus

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Relationship of miR-126 and miR-200b expressions in plasma with retina and choroidal thickness in patients with type 2 diabetes mellitus

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Running Title: *OCT parameters and miRNA expressions*

ABSTRACT:

Diabetic retinopathy (DR) is a frequent microvascular complication of diabetes mellitus (DM) characterized by damage in retinal vessels. Recent studies have suggested that changes in choriocapillaris circulation precede clinical manifestation of DR. The expression of angiogenic factors and inflammatory cytokines along with the modulation of several DR-related genes and their regulators, such as microRNAs (miRNAs), seem to be associated with retinal and choroidal thickness alterations. MiRNAs are non-coding RNAs that regulate gene expression, having an important role in modulating angiogenesis and other endothelial cell functions. Thus, we evaluated the relationship between miR-126 and miR-200b expressions with retina, choroid and inner retinal layers thickness in patients with and without type 2 DM (T2DM), stratified by body mass index (BMI). Forty-six patients were included in the study and underwent a color fundus photograph and swept-source optical coherence tomography exam. Patients were divided into 4 groups: Group 0 – T2DM patients with body mass index (BMI) <30.0 kg/m^2 (n=7); Group 1 – non-diabetic patients with BMI ≥30.0 kg/m^2 (n=12); Group 2 – T2DM patients ($<$5 years of disease) with BMI ≥30.0 kg/m^2 (n=11); and Group 3 – T2DM patients (≥5 years of T2DM) with BMI ≥30.0 kg/m² (n=16). MiR-126-3p and miR-200b-3p expressions were analyzed in plasma of all subjects using qPCR. MiR-126-3p expression was increased in patients from Group 2 compared to Group 0 (P= 0.031), while miR-200b-3p expression was similar among groups. In the overall sample, an inverse relationship was observed between miR-200b expression and choroidal center and average thickness, and total volume measurements, adjusting for BMI and T2DM duration. In conclusion, miR-200b appears to have a negative relationship with choroidal thickness.

Key-words: miR-200b, miR-126, choroidal thickness, optical coherence tomography,

type 2 diabetes mellitus.

Introduction

During the course of diabetes mellitus (DM) several macro- and microvascular complications become prevalent, being associated with increased morbidity and mortality rates (1, 2). In patients with type 2 DM (T2DM), obesity and hypertension are important risk factors associated not only with DM *per se* but also with the progression of its vascular complications (2-4). Diabetic retinopathy (DR) is a frequent diabetic microvascular complication characterized by damage in retinal vessels leading to impaired vision in young adults of working age (5, 6). Indeed, changes in retinal vascular caliber may provide important information regarding the state of the microcirculation in the eye and in other vascular beds (7, 8). Increasing evidence has shown that DM is also associated to changes in choroidal thickness related to the development of diabetic macular edema (DME) (9, 10).

The choroid is a highly vascularized tissue in human body, consisting mostly of blood vessels, and it is responsible for supplying the outer retina with nutrients and oxygen, thermoregulation, and secretion of growth factors such as VEGF (11, 12). Defects in choroid, such as damage to vascular integrity and blood supply, can cause degenerative changes and neovascularization, which is a main feature of proliferative DR (PDR) (11, 13). It has been suggested that choroid tissue could play an important role in the pathogenesis of several eye diseases, including DR (14-16). Actually, recent studies have reported important changes in choroidal thickness of diabetic patients with several grades of DR compared to healthy subjects, however with contradictory results (12, 15-17). Despite of this, all of these authors suggested that changes in choriocapillaris circulation might precede clinical manifestation of DR.

Although it is well known that T2DM and obesity are strongly associated and that obesity is associated with cataract, glaucoma, age-related maculopathy and DR, the effect of obesity on the eye layers has not been full described (18). The few studies available have suggested that elevated body mass index (BMI) can trigger structural changes in the retinal vascular system leading to retinal dysfunction (19-21). Interestingly, Dogan *et al*. demonstrated that patients with BMI ≥ 40 kg/m² presented thinner choroid, retinal nerve fiber and ganglion cell layers compared to healthy lean subjects (20). Similar changes on choroidal thickness have been described in DR (15, 16).

Moreover, since T2DM and obesity have a multifactorial origin, evidence has suggested that the expression of angiogenic factors and inflammatory cytokines along with the modulation and expression of several DR-related genes and their regulators, such as microRNAs (miRNAs) are closely associated to retinal and choroidal thickness alterations (11, 22-26).

MiRNAs characterization has become a major interest in biology and medicine due to their importance in endothelial cells regulation and function, particularly in angiogenesis (27-29). MiRNAs are 20–22 nucleotides non-coding RNAs that negatively regulate gene expression by partially pairing to the 3' untranslated region (3'UTR) of their target mRNAs; thus, leading to translation repression and/or transcript degradation (30-32). Their roles have been implicated in the regulation of various physiological and pathophysiological functions (33, 34). In this context, miR-126-3p and miR-200b-3p seem to be involved on the pathogenesis of DR by targeting important DR-related factors such as *VEGF*, *NF-κB* and *VCAM-1* (35-39). However, to our knowledge, no study evaluated the expression of these miRNAs in relation to choroidal thickness alterations in patients with diabetes, so far. Thus, the

aim of this study was to evaluate the relationship between miR-126-3p and miR-200b-3p expressions in plasma with retina, choroid and inner retinal layers thickness of patients with and without T2DM, stratified by BMI.

Methods

Study design and subjects

This was a cross-sectional study comprising 46 patients recruited in a tertiary hospital from Porto Alegre (South of Brazil). All included patients underwent a color fundus photograph and swept-source optical coherence tomography (SS-OCT) exam. Clinical and demographic data were also assessed by the time of the SS-OCT. Patients were excluded if they presented any eye diseases that could difficult the OCT image and if they had less than 18 years old. Presence of systemic infection, cancer, current treatment with systemic corticosteroids and pregnancy were also exclusion criteria. Patients were divided into 4 groups according to presence and duration of DM and by BMI, as follows: Group 0: patients with T2DM and BMI < 30.0 kg/m^2 (n= 7); Group 1: non-diabetic patients with BMI ≥ 30.0 kg/m^2 (n=12), Group 2: T2DM patients ($<$ 5 years of disease) with BMI ≥ 30.0 kg/m^2 (n=11); and Group 3: T2DM patients (≥ 5 years of T2DM) with BMI ≥ 30.0 kg/m^2 (n=16). This study was performed according to the Declaration of Helsinki, and informed written consent was obtained from all patients. The Research Ethics Committee at the Hospital de Clínicas de Porto Alegre (HCPA) approved the study protocol.

Swept-source optical coherence tomography imaging

Color fundus photography was digitally captured for both eyes of all patients without dilated pupils. SS-OCT (Triton SS-OCT, Topcon, Tokyo, Japan) was also performed.

SS-OCT is an imaging modality that enables the documentation of tissue structure in real time and *in situ*. The equipment's software automatically provides simultaneous measures of peripapillary retinal nerve fiber layer (RNFL), inner macular layer thickness (ganglion cell layer and inner plexiform layer), retinal and choroid layers (40). The inner macular parameters included were RNFL thickness, ganglion cell layer plus inner plexiform layer thickness (GCL+), and RFNL plus GCL+ thickness (GCL++). The images were graded by an ophthalmologist that was blinded for the patient's groups, according to the International Clinical Diabetic Retinopathy and Diabetic Macular Edema Disease Severity Scales based on the Early Treatment Diabetic Retinopathy Study (ETDRS) (41).

Clinical and laboratorial data

Socio-demographic, clinical and laboratorial data were collected by standardized procedures. BMI was calculated as weight (kg)/height^2 (cm). Diagnosis of T2DM was established according to the American Diabetes Association guidelines (42). Total plasma cholesterol, HDL cholesterol and triglycerides were measured using enzymatic methods. Serum creatinine was determined using the Jaffé method, traceable. Fasting plasma glucose (FPG) levels were determined using the glucose oxidase method. Glycated hemoglobin (HbA1c) measurements were performed by high performance liquid chromatography of ion-exchange (Variant II Turbo; Bio-Rad, USA). Blood pressure (BP) was measured in the sitting position, on the left arm, after a 5-min rest, with a digital sphygmomanometer Onrom (HEM-705CP). The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP. Hypertension was defined as blood pressure (BP) ≥140/90 mmHg in two occasions or use of antihypertensive medications.

RNA extraction and quantification of miR-126-3p and miR-200b-3p expressions by real-time PCR (qPCR)

Peripheral blood samples of all patients were collected and the non-hemolyzed samples were centrifuged at 3500 rpm for 15 min at 4 °C. Plasma aliquots were stored at −80 °C until expression analyses. Total RNA was extracted from 450 µl of plasma using the MiRVana PARIS miRNA Isolation Kit (Thermo Fisher Scientific, DE, USA). Purity and concentration of RNA samples were evaluated using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Relative expressions of miR-126-3p and miR-200b-3p were analyzed in plasma of all subjects. Total RNA (2 ng) was reverse-transcribed into cDNAs specific for each miRNA using the TaqMan miRNA Reverse-Transcription (RT) kit (Thermo Fisher Scientific). Then, qPCR reactions were carried out in a Vii™ 7 Fast Real-Time PCR System Thermal Cycler (Thermo Fisher Scientific). PCR reactions were performed using 5 µL of 1X TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific), 0.5 µL (1 ng/µL) of TaqMan MicroRNA Assays (Thermo Fisher Scientific) containing specific primers for each miRNA, and 1 µL of cDNA template (2 ng/µL), in a total volume of 10µL. Each sample was assayed in triplicate and a negative control was included in each experiment. The cycling conditions for these miRNAs were as follows: an initial cycle of 95°C for 10 minutes, followed by 50 cycles of 95°C for 15s and 60°C for 60s. Relative expressions were calculated using the ΔΔCq method (43). For all analyses, the small nuclear RNA U6 (*U6snRNA*) was used as the reference gene. Data are shown as n-fold changes in relation to a calibrator sample.

Statistical analyses

Normal distribution of variables was checked using the Shapiro-Wilk test. Variables with normal distribution are described as means ± standard deviations (SD) or means ± standard error (SE). Variables with skewed distribution were log-transformed before analyses and are shown as median (95% confidence interval [CI]). Categorical data are shown as absolute number (proportions). For normally distributed continuous variables, comparisons among groups were done with one-way ANOVA. For variables that did not follow Gaussian distribution even after log-transformation, a Mann-Whitney U test was used. Categorical variables were compared among groups using $\chi 2$ tests. Differences in retinal layers thickness between groups and subjects were compared by generalized estimating equations (GEE) to adjust for the inclusion of both eyes of each patient in the analyses. GEE was also used to examine the influence of miRNA expressions on SS-OCT measurements. Associations were considered significant if the P value was less than 0.05. Statistical calculations were done with PASW 24.0 Software (IBM SPSS Statistics, Chicago, IL, USA).

Results

Subjects characteristics

Forty-six patients were included, most of them female (83.0%), white (80.9%), with a mean age of 50.3 ± 11.6 years old and mean BMI of 42.9 ± 9.8 kg/m². T2DM was present in 34 patients (74%) and all of them used at least one anti-hyperglycemic drug. Subjects were divided into 4 groups as described in Methods section. Clinical characteristics of the patients are shown in Table 1 according to the groups. Patients from Group 0 were older than the other groups. Both Groups 0 and Group 3 had increased HbA1c values and T2DM duration compared to Group 2. Moreover, Group 1 had decreased prevalence of hypertension compared to Groups 2 and 3.

Laboratory variables such as total cholesterol, HDL cholesterol, triglycerides, creatinine, glomerular filtration rate (GFR) and albuminuria were similar among groups (Table 1).

Choroid and retinal layer thickness measurements assessed by SS-OCT

Among the 46 subjects included in the study, 3 patients were unable to complete the SS-OCT evaluation in both eyes due to corneal opacity in one of them, totalizing 89 eyes included in the analysis. Although patients in Group 0 and 3 had average T2DM duration of 13.3 ± 6.7 years, only 5 of them (21.7%) had DR, while 2 patients in Group 3 had DME (Table 1). To mention, one patient in Group 2 presented mild non-proliferative DR (NPDR) and DME only in the left eye (Table 1). This patient had HbA1c level of 5.70%, glucose level of 105 mg/dL and T2DM duration of only 2 years. Regarding choroid, retinal and inner retinal layers thickness, no difference was observed among groups (Figure 1, Table 2).

Expressions of miR-126-3p and miR-200b-3p and their correlations to clinical and laboratorial variables

MiR-126-3p expression was increased in patients from Group 2 compared to Group 0 (1.21 [0.10 - 4.33] *vs.* 0.24 [0.13 - 0.60], P = 0.031) (Figure 2a). No difference was observed regarding miR-200b-3p expression among groups (Figure 2b). Correlation analyses between miRNA expressions and clinical and laboratorial variables are shown in Supplementary Table 1. MiR-126-3p was negatively correlated with T2DM duration (r = -0.476, P = 0.012), and miR-200b-3p was positively correlated with systolic BP (r = 0.356, P = 0.039). No other correlation was observed between miRNA expressions and other clinical data.

Relationship of miR-126-3p and miR-200b-3p expression with choroid and retinal layer thickness

The relationships between miRNA expressions and choroid and retinal layers thickness were analyzed using GEE and are shown in Table 3. In the overall sample, an inverse relationship was observed between miR-200b-3p expression and choroidal center and average thickness and total volume measurements, adjusting for BMI and T2DM duration. No relationship was observed between miR-126-3p and the analyzed eye parameters.

In the stratified group analysis, we observed a positive relationship between miR-200b-3p and RNFL layer in Group 0. In Group 1, inverse relationships of miR-126-3p and miR-200b-3p with retinal average thickness and total volume were observed. Moreover, in Group 2, only miR-200b-3p presented an inverse relationship with retinal center and average thickness, total volume, and GCL+. In Group 3, a positive relationship was observed between miR-126-3p and miR-200b-3p and RNFL and GCL++ (Table 3).

Discussion

Significant efforts have been made to identify biomarkers that could early detect retinal changes and progression of DR (44). The identification of neurodegeneration in the retina and changes in choroidal vessels have been linked with early DR development (45-48). In T2DM patients, the role of obesity as a risk factor for DR has also been evaluated. In a recent meta-analysis of 13 prospective cohort studies on the subject, obesity was associated with risk for non-proliferative DR but not PDR (49). Changes in retinal and choroidal thickness have also been demonstrated in non-diabetic patients with obesity (19-21). Altogether, these studies suggest a

harmful effect of the interaction between obesity and T2DM on retinal microvascular changes.

Furthermore, a number of studies have demonstrated the association of miRNAs with the development of DM and its complications, suggesting their potential role as biomarkers of these diseases (32, 50). In this context, miR-126-3p and miR-200b-3p were reported to be related with diabetic microvascular complications (51-55). Thus, in this study, we aimed to evaluate the relationship of miR-126-3p and miR-200b-3p expressions with choroid, retinal thickness and inner retinal layers of patients with and without T2DM stratified by BMI categories.

In the overall sample, we observed an inverse relationship between miR-200b-3p expression and choroidal center and average thickness and total volume measurements, adjusting by BMI and T2DM duration. The choroid is an important vascular tissue that supplies blood and oxygen to the outer retina, including ganglion cell layer and photoreceptors (11). The decrease in choroidal thickness due to increased permeability and loss of the capillary network can be related to DM-induced hypoxia of the retinal tissue and the breakdown of both the inner and outer retinal barriers (16, 56). Along with these process, an increased choroidal synthesis of growth factors involved in angiogenesis, such as VEGF, has also been described (57). In an experimental study, McArthur *et al.* showed that transfection of endothelial cells with miR-200b mimic as well as intravitreal injection of this miRNA in retinas of diabetic rats prevented DM-induced upregulation of VEGF and also decreased retinal permeability and angiogenesis (35). Similar results were reported by Ruiz *et al.* after expose retinal endothelial cells to high glucose and when analyzed miR-200b expression in retinas of diabetic mice compared to controls (58). Moreover, Li *et al.* reported that miR-200b downregulation was inversely correlated with VEGF

expression in patients with DR (59). To our knowledge, the present study is the first to analyze the relationship of miRNAs and choroidal and retinal thickness. It is worth noting that the relationship between choroidal thickness and miR-200b-3p was independent of BMI and T2DM duration, suggesting that this miRNA could be an early indicator of choroidal changes in the course of T2DM.

In the stratified group analysis, a positive relationship was observed between RNFL and miR-200b-3p expression in Group 0, with similar result being observed in Group 3 for miR-126-3p and miR-200b-3p. Also, in Group 3, a positive relationship was observed between both miRNAs and GCL++ layer. Groups 0 and 3 presented an increased T2DM duration and HbA1c levels. The apoptosis of GCL layer is enhanced in the sensory retina in DM, and death of these cells occurs early in diabetic eyes (60). Studies have indicated the occurrence of RNFL thinning in the retina of T2DM patients without detectable DR (61-63). In two of these studies, the T2DM duration range was higher than 5 year (62, 63); hence, we hypothesized that miR-200b-3p and miR-126-3p could have a protective role for RNFL and GCL++ degeneration.

Furthermore, in Group 2 (comprising patients with less than 5 years of T2DM duration), an inverse relationship was observed regarding miR-200b-3p and retinal parameters, which corroborate our hypothesis that this miRNA could serve as an indicator of early changes in retina of patients with T2DM. Additionally, in Group 1, an inverse relationship of miR-126-3p and miR-200b-3p with retinal average thickness and total volume was observed. This group comprises non-diabetic patients with a BMI >30 kg/m², which suggests a possible influence of obesity in the relationship of these miRNAs with retinal changes. Although Dogan *et al.* showed a thinner choroid and RNFL layers in obese subjects without DM compared to lean subjects (20), the

underlying mechanisms of this association are not fully understood. However, some studies have suggested the potential involvement of oxidative stress, aldose reductase activity, and vasoproliferative parameters, such as VEGF, on the obesity-induced effects on retinal changes (18, 64, 65).

When evaluating miRNA expressions among groups, miR-126-3p expression was upregulated in Group 2 compared to patients in Group 0. Group 2 patients had less than 5 years of T2DM and lower levels of HbA1c. This miRNA was also negatively correlated with T2DM duration in the whole sample. Zhang *et al*. showed that plasma miR-126 levels was a potential biomarker for T2DM susceptibility (66). Also, miR-126-3p is highly expressed in endothelial cells, playing an important role in maintaining endothelial homeostasis and vascular integrity (67, 68). Accordingly, Barutta *et al*. reported that each unit increment in miR-126 expression was associated with a reduction of 25% in PDR risk (39).

Regarding the choroid, retinal and inner retinal layer thickness, no difference was observed among groups. In part this could be explained by the fact that few patients had DR even in the groups with longer T2DM duration. Recent studies have shown that not only vascular abnormalities but also neuronal abnormalities, including retinal ganglion cell death, accompany the pathogenic changes at the early stage of DR (69, 70). When comparing 49 eyes of patients with different degree of DR, Regatieri *et al*. showed that in both DME and treated PDR groups, the choroidal thickness was significantly decreased compared with healthy subjects; however, no difference was found regarding NPDR (15). Similar results were reported by Ünsal *et al.* when evaluating 191 eyes of patients with different degrees of DR (16). In the other hand, Mahmoud *et al.* showed that T2DM patients with and without DR had

reduced thickness of retinal and ganglion cell layers compared to healthy subjects (69).

In conclusion, our study suggests that miR-200b-3p and miR-126-3 could be potential biomarkers of early changes in retinal and choroidal thickness in T2DM patients. However, additional studies are necessary to better understand the role of these miRNAs in retinal and choroidal thickness changes.

Acknowledgments

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

Contribution statement

P.S.N. designed the study, performed data collection and analysis, interpreted the data, and wrote the manuscript. D.L. and L.F. performed SS-OCT exams. M.S.O. and D.A.S. helped with data acquisition and analysis. L.H.C., A.C.B and D.C. helped with study design, data analysis and interpretation and edited the manuscript. All authors approved the final version of the manuscript.

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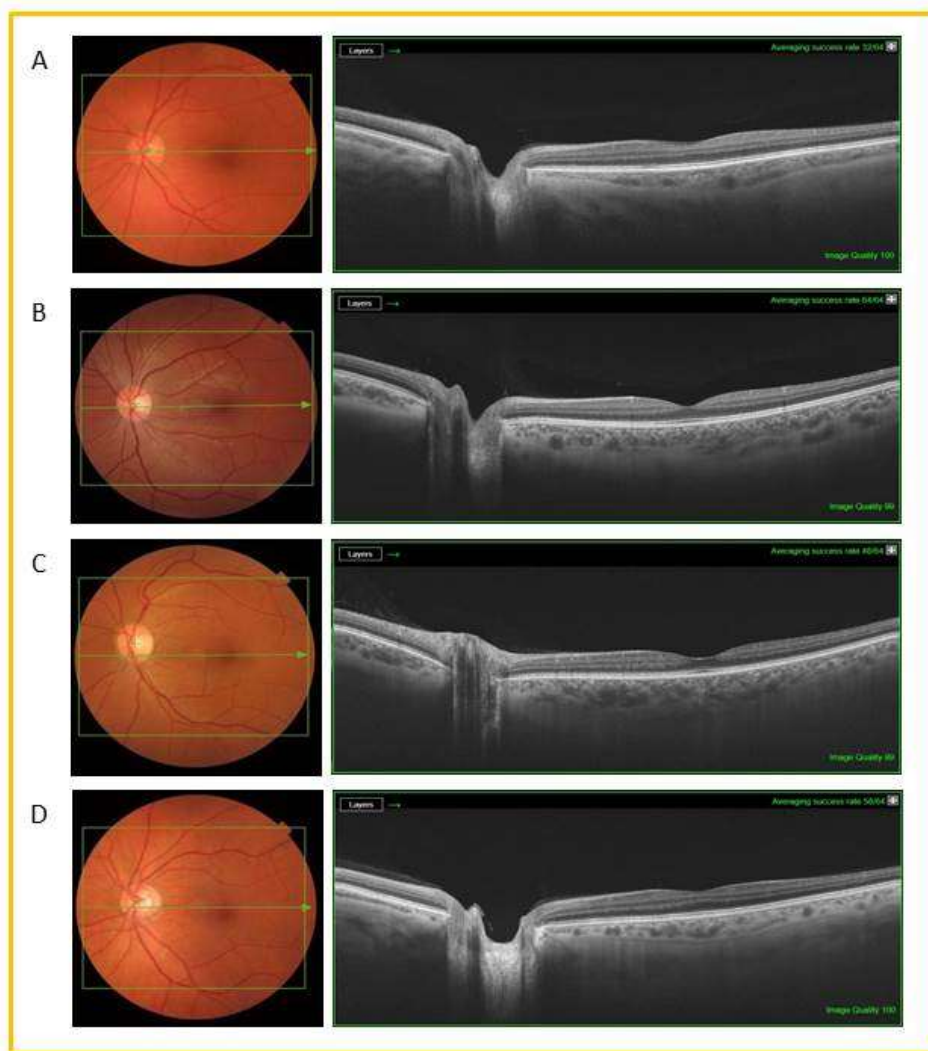
Figure 1: Color fundus photograph and SS-OCT exam of patients according to study groups. A, G0: non-obese patients with T2DM; B, G1: patients with obesity but without T2DM; C, G2: patients with obesity and with < 5 years of T2DM; and D, G3: patients with obesity and with ≥ 5 of T2DM.
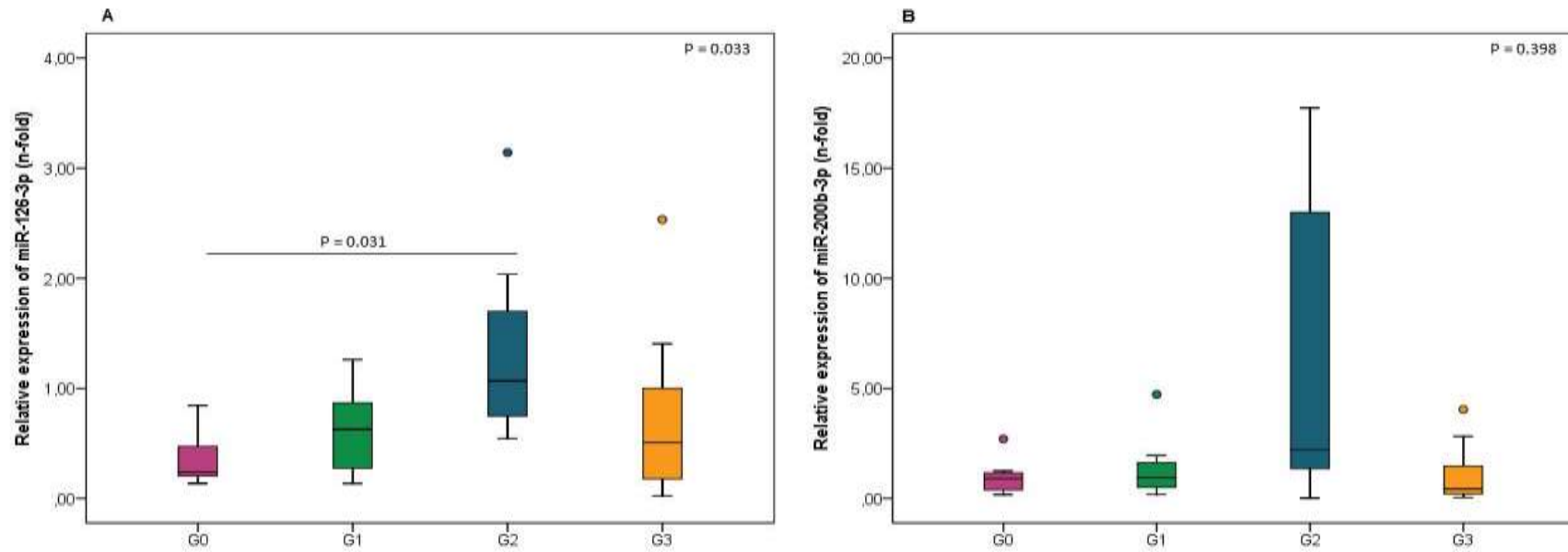
Figure 2: Relative expressions of miR-126-3P and miR-200-3p s among study groups. A: relative expression of miR-126-3p. B: relative expression of miR-200b-3p. Results are shown in n-fold changes relative to the calibrator sample. G0, non-obese patients with T2DM; G1, patients with obesity but without T2DM; G2, patients with obesity and with < 5 years of T2DM; G3, patients with obesity and with ≥ 5 of T2DM.

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Table 1 – Clinical and laboratorial characteristics of included subjects between groups.

|	Group 0 (n = 7)	Group 1 (n = 12)	Group 2 (n = 11)	Group 3 (n = 16)	P value
Age (years)	66.1 ± 5.2	42.0 ± 8.7 [a]	45.1 ± 8.9 [a]	52.8 ± 9.6 [a, b]	**< 0.001**
Ethnicity (white)	6 (85.7)	10 (83.3)	7 (63.3)	14 (87.5)	0.485
Gender (female)	3 (42.9)	11 (91.7) [a]	9 (81.8) [a, b]	15 (93.8) [b]	**0.043**
BMI (kg/m^2)	26.1 ± 2.4	44.7 ± 5.8 [a]	48.9 ± 10.3 [a]	44.9 ± 5.5 [a]	**< 0.001**
T2DM duration (years)	16.7 ± 5.2	-	3.0 ± 0.7 [a]	11.8 ± 7.1 [c]	**< 0.001**
HbA1c (%)	7.9 (7.2 – 11.1)	5.4 (5.1 – 5.8) [a]	6.7 (5.6 – 9.1) [a, b]	7.7 (5.5 – 12.2) [b, c]	**< 0.001**
DR (yes)	1 (14.3)	-	1 (9.1)	4 (25.0)	0.535
DME (yes)	0 (0.0)	-	1 (9.1)	2 (12.5)	0.464
Hypertension (yes)	6 (85.7)	5 (41.7)	10 (90.9) [b]	16 (100.0) [b]	**0.001**

Systolic BP (mmHg)	120.0 (100.0 – 160.0)	124.0 (110.0 – 140.0)	130.0 (100.0 – 160.0)	127.5 (110.0 – 165.0)	0.759
Diastolic BP (mmHg)	80.0 (70.0 – 90.0)	80.0 (60.0 – 100.0)	80.0 (70.0 – 100.0)	80.0 (70.0 – 120.0)	0.705
HR (yes)	0 (0.0)	5 (41.7)	2 (18.2)	3 (18.8)	0.112
Non-Smokers (%)	6 (85.7)	12 (100.0)	10 (90.9)	16 (100.0)	0.251
Total Cholesterol (mg/dL)	176.2 ± 48.3	164.2 ± 26.3	173.1 ± 40.3	189.0 ± 38.1	0.353
HDL Cholesterol (mg/dL)	44.2 ± 13.1	48.3 ± 14.7	41.5 ± 9.7	49.5 ± 11.9	0.395
Triglycerides (mg/dL)	186.8 ± 119.2	115.0 ± 43.6	163.2 ± 51.5	161.2 ± 69.1	0.133
Creatinine (mg/dL)	0.8 (0.2 – 1.6)	0.7 (0.5 – 1.1)	0.7 (0.3 - 0.8)	0.8 (0.4 – 3.9)	0.343
GFR [CKD-EPI (ml/min/1,73m2)]	77.1 ± 20.9	94.6 ± 21.2	105.3 ± 18.3	82.6 ± 29.0	0.620
Albuminuria (mg/L)	11.0 (3.0 – 733.6)	13.0 (4.5 – 63.8)	38.7 (3.0 – 269.7)	4.3 (3.0 – 108.7)	0.502

Data are presented as mean ± SD or n (%) or median (95% CI). a: $p < 0.05$ vs. G0; b: $p < 0.05$ vs. G1; c: $p < 0.05$ vs. G2; d: $p < 0.05$ vs. G3. BMI: body mass index; T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin; DR: diabetic retinopathy; DME: diabetic macular edema; BP: blood pressure; HR:

hypertensive retinopathy; GFR: glomerular filtration rate. G0: non-obese patients with T2DM; G1: patients with obesity but without T2DM; G2: patients with obesity and with < 5 years of T2DM; G3: patients with obesity and with ≥ 5 of T2DM.

Table 2 – Choroid, retina and inner retinal layers thickness according to study groups.

	Group 0 (n = 14 eyes)	Group 1 (n = 24 eyes)	Group 2 (n = 22 eyes)	Group 3 (n = 29 eyes)	P value
Choroid					
Center thickness, µm	224.42 ± 36.24	267.29 ± 23.42	252.93 ± 17.14	247.02 ± 14.78	0.776
Average thickness, µm	200.99 ± 33.99	252.02 ± 20.91	229.05 ± 16.49	219.78 ± 13.17	0.506
Total volume, mm³	5.68 ± 0.96	7.13 ± 0.59	6.47 ± 0.46	6.17 ± 0.37	0.476
Retina					
Center thickness, µm	240.78 ± 6.39	230.35 ± 6.71	239.29 ± 7.76	236.25 ± 7.84	0.702
Average thickness, µm	276.98 ± 6.76	284.25 ± 3.84	281.08 ± 3.38	285.03 ± 5.19	0.733

Total volume, mm³	7.83 ± 0.19	8.03 ± 0.10	7.94 ± 0.09	8.06 ± 0.14	0.731
RNFL, µm	102.14 ± 5.27	111.44 ± 2.58	104.51 ± 1.87	104.95 ± 3.21	0.129
GCL+, µm	41.74 ± 2.17	43.57 ± 1.49	45.42 ± 0.88	44.95 ± 1.22	0.373
GCL++, µm	143.89 ± 6.82	155.02 ± 3.37	149.93 ± 1.99	149.98 ± 3.45	0.419

Data are presented as mean ± SE. RNFL: retinal nerve fiber layer; GCL+: ganglion cell layer plus inner plexiform layer; GCL++: RFNL plus GCL+ layer. G0: non-obese patients with T2DM; G1: patients with obesity but without T2DM; G2: patients with obesity and with < 5 years of T2DM; G3: patients with obesity and with ≥ 5 of T2DM.

Table 3 – Relationship between miRNAs expression and OCT parameters by generalized estimating equation (GEE).

	miR-126-3p (n = 51 eyes)	p-value	miR-200b-3p (n = 49 eyes)	p-value
Adjusted by IMC and T2DM duration				
Choroid				
Center thickness, μm	+ 790.15 (- 1510.89 to 3091.21)	0.501	- 1618.19 (- 2538.80 to - 697.57)	**0.001**
Average thickness, μm	+ 1053.13 (- 1309.75 to 3416.03)	0.382	- 1436.45 (- 2391.28 to - 481.62)	**0.003**
Total volume, mm³	+ 29.05 (- 38.21 to 96.32)	0.397	- 41.64 (- 69.01 to - 14.27)	**0.003**
Retina				
Center thickness, μm	+ 141.86 (- 235.89 to 519.62)	0.462	+ 57.54 (- 143.07 to 258.36)	0.574
Average thickness, μm	+ 89.40 (- 253.39 to 432.19)	0.609	- 33.50 (- 174.70 to 107.70)	0.642
Total volume, mm³	+ 2.53 (- 7.11 to 12.19)	0.606	- 0.98 (- 4.96 to 2.99)	0.629
RNFL, μm	- 51.74 (- 274.63 to 171.14)	0.649	- 96.26 (- 233.98 to 41.45)	0.171
GCL+, μm	+ 16.14 (- 97.00 to 129.29)	0.780	+ 33.18 (- 28.98 to 95.35)	0.295
GCL++, μm	- 35.71 (- 305.38 to 233.94)	0.795	- 64.30 (- 231.94 to 103.34)	0.452

Stratified by group

G0 (n = 14 eyes)

Choroid

Center thickness, µm	- 105.71 (- 254.61 to 43.18)	0.164	+ 98.17 (- 88.07 to 284.43)	0.302
Average thickness, µm	- 77.46 (- 218.83 to 63.90)	0.283	+ 112.15 (- 61.67 to 285.99)	0.206
Total volume, mm³	- 2.18 (- 6.18 to 1.81)	0.284	+ 3.17 (- 1.74 to 8.08)	0.206

Retina

Center thickness, µm	+ 0.53 (- 47.42 to 48.49)	0.893	- 2.17 (- 41.25 to 36.91)	0.913
Average thickness, µm	- 7.37 (- 44.25 to 29.51)	0.695	+ 20.92 (- 9.84 to 51.69)	0.183
Total volume, mm³	- 0.21 (- 1.25 to 0.82)	0.689	+ 0.58 (- 0.279 to 1.45)	0.184
RNFL, µm	+ 4.99 (- 32.85 to 42.85)	0.796	+ 21.19 (2.37 to 40.01)	**0.027**
GCL+ µm	- 1.25 (- 16.21 to 13.69)	0.869	+ 2.70 (- 9.02 to 14.44)	0.651
GCL++, µm	+ 3.74 (- 46.50 to 53.99)	0.884	+ 23.90 (- 5.60 to 53.41)	0.112

G1 (n = 22 eyes)

Choroid

Center thickness, µm	- 83.92 (- 219.59 to 51.74)	0.225	- 47.15 (- 175.05 to 80.75)	0.470
Average thickness, µm	- 88.72 (- 208.47 to 31.03)	0.146	- 49.40 (- 147.26 to 48.44)	0.322
Total volume, mm³	- 2.49 (- 5.88 to 0.89)	0.150	- 1.38 (- 4.14 to 1.37)	0.326
Retina				
Center thickness, µm	- 22.63 (- 49.65 to 4.39)	0.101	- 19.87 (- 45.88 to 6.13)	0.134
Average thickness, µm	- 18.44 (- 34.21 to - 2.66)	**0.022**	- 15.02 (- 28.37 to - 1.67)	**0.027**
Total volume, mm³	- 0.52 (- 0.97 to - 0.07)	**0.022**	- 0.42 (- 0.80 to - 0.05)	**0.027**
RNFL, µm	- 2.37 (- 20.98 to 16.22)	0.802	- 5.02 (- 19.59 to 9.53)	0.499
GCL+, µm	- 6.81 (- 14.94 to 1.31)	0.100	- 5.20 (- 10.84 to 0.43)	0.071
GCL++, µm	- 9.19 (- 32.00 to 13.61)	0.430	- 10.22 (- 24.96 to 4.50)	0.174
G2 (n = 16 eyes)				
Choroid				
Center thickness, µm	- 28.30 (- 152.06 to 95.46)	0.654	+ 2.37 (- 43.97 to 48.71)	0.920
Average thickness, µm	- 28.10 (- 138.77 to 82.57)	0.619	- 4.43 (- 52.59 to 43.72)	0.857
Total volume, mm³	- 0.79 (- 3.92 to 2.33)	0.618	- 0.12 (- 1.48 to 1.23)	0.855

Retina

Center thickness, µm	- 9.01 (- 44.07 to 26.03)	0.614	- 15.95 (-19.64 to - 12.26)	**0.000**
Average thickness, µm	- 10.12 (- 34.70 to 14.46)	0.420	- 6.73 (- 11.32 to - 2.14)	**0.004**
Total volume, mm³	- 0.28 (- 0.98 to 0.41)	0.422	- 0.18 (- 0.32 to - 0.05)	**0.004**
RNFL, µm	- 3.77 (- 10.16 to 2.61)	0.246	+ 2.11 (- 0.86 to 1.93)	0.164
GCL+, µm	- 3.08 (- 8.20 to 2.03)	0.238	- 2.53 (- 3.13 to - 1.94)	**0.000**
GCL++, µm	- 6.86 (- 14.55 to 0.83)	0.081	- 0.42 (- 3.81 to 2.96)	0.806

G3 (n = 21 eyes)

Choroid

Center thickness, µm	- 4.69 (- 54.28 to 44.89)	0.853	- 0.99 (- 50.83 to 48.83)	0.969
Average thickness, µm	+ 7.12 (- 30.50 to 44.75)	0.711	+ 7.41 (- 32.47 to 47.31)	0.715
Total volume, mm³	+ 0.20 (- 0.85 to 1.25)	0.707	+ 0.18 (- 0.97 to 1.33)	0.757

Retina

Center thickness, µm	+ 10.92 (- 0.75 to 22.59)	0.067	+ 6.12 (- 4.91 to 17.16)	0.277
Average thickness, µm	+ 7.04 (- 1.68 to 15.78)	0.114	+ 2.07 (- 8.35 to 12.50)	0.697

Total volume, mm³	+ 0.20 (- 0.04 to 0.44)	0.115	+ 0.05 (- 0.23 to 0.35)	0.699
RNFL, µm	+ 11.96 (4.67 to 19.25)	**0.001**	+ 11.21 (4.33 to 18.08)	**0.001**
GCL+, µm	+ 1.87 (- 1.58 to 5.32)	0.288	+ 0.30 (- 3.41 to 4.02)	0.872
GCL++, µm	+ 13.83 (8.73 to 18.94)	**0.000**	+ 11.65 (5.78 to 17.51)	**0.000**

Data are presented as β (95% CI). RNFL: retinal nerve fiber layer; GCL+: ganglion cell layer plus plexiform layer; GCL++: RFNL plus GCL+ layer. G0: non-obese patients with T2DM; G1: patients with obesity but without T2DM; G2: patients with obesity and with < 5 years of T2DM; G3: patients with obesity and with ≥ 5 of T2DM.

Supplementary Table 1 – Correlation analysis between miRNA expressions and clinical and laboratorial variables

	miR-126-3p	miR-200b-3p
Age (years)	- 0.164 / 0.325	0.007 / 0.970
BMI (kg/m²)	0.139 / 0.404	0.047 / 0.786
T2DM duration (years)	**- 0.476 / 0.012**	- 0.241 / 0.235
HbA1c (%)	- 0.104 / 0.542	- 0.181 / 0.297
Systolic BP (mmHg)	0.284 / 0.093	**0.356 / 0.039**
Diastolic BP (mmHg)	0.150 / 0.383	0.135 / 0.446
Total Cholesterol (mg/dL)	- 0.010 / 0.954	- 0.157 / 0.374
HDL Cholesterol (mg/dL)	0.160 / 0.352	0.043 / 0.810
Triglycerides (mg/dL)	- 0.227 / 0.183	- 0.089 / 0.617
Creatinine (mg/dL)	- 0.204 / 0.226	- 0.236 / 0.166
GRF [CKD-EPI (ml/min/1,73m²)]	0.222 / 0.186	0.115 / 0.502
Albuminuria (mg/L)	0.220 / 0.271	0.207 / 0.311

Data are presented as Pearson correlation (r) or spearman (ρ) / P-value. BMI: body mass index; T2DM: type 2 diabetes mellitus; FPG: fasting plasma glucose; HbA1c: glycated hemoglobin; BP: blood pressure; GFR: glomerular filtration rate.

Considerações finais

Estudos tem demonstrado o papel dos miRNAs nas diversas complicações do DM e entender o papel desses reguladores gênicos no surgimento e progressão da RD pode ser de grande valia para o desenvolvimento de novas estratégias de rastreamento e de tratamento da RD. Diversos miRNAs têm sido associados à fatores como o aumento da permeabilidade, angiogênese e reparo vascular, sendo alvos direto de genes relacionados à rotas de inflamação, apoptose e fibrose em células endoteliais. De fato, nossa revisão sistemática e análise de bioinformática demonstrou que 6 miRNAs estão desregulados em plasma/soro de pacientes com RD e modelos animais (miR-21-5p, miR-126-3p, miR-146a-5p, miR-195-5p, miR-200b-3p, and miR-592). Entre eles, genes-alvos do miR-21-5p, miR-126-3p, miR-146a-5p e miR-200b-3p foram identificados em importantes vias de sinalização relacionadas à patogênese da RD como AGE-RAGE, HIF-1, VEGF, NF-κB e JAK/STAT.

A identificação de alterações precoces da espessura da coroide e retina de pacientes com DM2 pode ser uma valiosa ferramenta no diagnostico das complicações microvasculares da retina. Considerando os achados da revisão sistemática, miR-126-3p e miR-220b-3p foram escolhidos para o estudo transversal e apresentaram relações significativas com a espessura da coroide e retina de pacientes com DM2. A identificação de marcadores precoces da RD pode ser uma importante estratégia no desenvolvimento de medidas preventivas e/ou terapêuticas, objetivando reduzir o número de pacientes que sofrem com esta limitante patologia e nosso estudo demonstrou que o miR-126-3p e miR-200b-3p têm o potencial para

futuramente servirem como biomarcadores de alterações precoces da espessura da coroide e retina de pacientes com DM2.

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Como primeira autora:

- **Evaluation of choroid, retina and inner retinal layers thickness by swept-source optical coherence tomography before and after bariatric surgery: a pilot and prospective analysis**

  Pamela S. Nique, Daniel Lavinsky, Denise A. Sortica, Daisy Crispim, Rogério Friedman, Roberto M. Trindade, Andrea C. Bauer, Luis Henrique Canani.

Como colaboradora:

- **The role of uncoupling proteins 1, 2 and 3 in weight loss after bariatric surgery: a systematic review**

  Mayara S. Oliveira, Pamela S. Nique, Daisy Crispim, Bianca M. Souza.

- **Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 K121Q Polymorphism is Associated with Acute Kidney Rejection**

  Denise A. Sortica, Daisy Crispim, Andrea C. Bauer, Pamela S. Nique, Bruna B. Nicoletto, Jennifer T. Staehler, Marjoriê P. Buffon, Roberto C. Manfro, Luis H. Canani.