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DIFFERENTIAL PROFILE OF miRNAs IN TEARS FROM TYPE-2 DIABETICS WITH OR WITHOUT RETINOPATHY

Doctoral Thesis by

HELENA CRISTINA CAMPOS BORGES

Director and Co-Directors

Prof. Dr. Maria Dolores Pinazo Durán
Prof. Dr. Vicente Zanón Moreno
Prof. Dr. María Cristina Prudêncio

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VNIVERSITAT DE VALÈNCIA Facultat de Medicina i Odontologia

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HELENA CRISTINA CAMPOS BORGES

Director:

MARIA DOLORES PINAZO DURÁN.

University Professor. Coordinator of the Ophthalmology Teaching Unit. Faculty of Medicine and Dentistry of the University of Valencia. Valencia (Spain).

Certify**:**

That the research work entitled **"Differential profile of miRNA in tears from type-2 diabetics with or without retinopathy"** presented by HELENA CRISTINA CAMPOS BORGES, graduate in biology, has been carried out under my supervision during all stages of its realization, considering that it meets all the necessary requirements to be defended before an international jury, as well as to opt for a doctoral degree, with international mention from the University of Valencia.

Valencia, July 2023

MARIA DOLORES|PINAZO|DURAN

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SIGNED: Maria Dolores Pinazo Durán

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HELENA CRISTINA CAMPOS BORGES

Director:

VICENTE ZANÓN MORENO.

Research Director - Vice-Rectorate of Research and Technology Transfer
Associate Professor - Faculty of Health Sciences, International University of Valencia – VIU (Spain)

Certifica**:**
Que el trabajo de investigación titulado "**Differential profile of miRNAs in tears from type-2 diabetics with or without retinopathy**" que presenta HELENA CRISTINA CAMPOS BORGES, licenciada en biologia, fue realizado bajo mi dirección, reuniendo todos los requisitos necesarios para ser defendido ante un tribunal y optar al grado de doctor.

Valencia, Julio 2023

ZANON MORENO VICENTE CALIXTO - 24358259V

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Nombre de reconocimiento (DN): c=ES, serialNumber=IDCES-24358259V, givenName=VICENTE CALIXTO, sn=ZANON MORENO, cn=ZANON MORENO VICENTE CALIXTO - 24358259V
Fecha: 2023.09.03 22:22:21 +02'00'

Prof. Vicente Zanón Moreno

DIFFERENTIAL PROFILE OF miRNAs IN TEARS FROM TYPE-2 DIABETICS WITH OR WITHOUT RETINOPATHY

HELENA CRISTINA CAMPOS BORGES

Director:

MARIA CRISTINA PRUDÊNCIO.

President of the School of Health at Polytechnic University of Porto (SH/PUP) and Full Professor at Chemistry and Biomolecules Sciences Teaching Unit. Biochemist, Medical Doctor and Senior Researcher at Translational Health and Medical Biotechnology Research Center (SH/PUP) in Porto (Portugal)

Certifica:

O trabalho de investigação intitulado "**Differential profile of miRNAs in tears from type-2 diabetics with or without retinopathy"**, apresentado por HELENA CRISTINA CAMPOS BORGES, licenciada em Biologia, foi realizado sob a minha supervisão, considerando que atende a todos os requisitos necessários para ser defendido perante um júri internacional, bem como para optar pelo grau de doutor, com menção internacional pela Universidade de Valência.

Porto, Julho de 2023

Assinado por: **MARIA CRISTINA PRUDÊNCIO PEREIRA SOARES**
Num. de Identificação: 08109550
Data: 2023.08.24 18:49:43+01'00'

Prof. Doutora Maria Cristina Prudêncio

CARTÃO DE CIDADÃO

To my daughter, Clara

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To my dear daughter, Clara. You are my world.

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DISCLOSURE STATEMENT

The author and directors of this Doctoral Thesis declare that there is no financial/personal interest or belief that could affect their objectivity during the study course.

TABLE OF CONTENTS

TERMINOLOGY

AHT: Arterial Hypertension

AMD: Age-related Macular Degeneration

ANOVA: Analysis of Variance

BCVA: Best Corrected Visual Acuity

BDWG: Biomarkers Definitions Working Group

BF: Buffer

BLC-2: B-cell lymphoma 2

BMI: Body Mass Index

BR: Blood Retinal Barrier

cDNA: complementary DNA

CG: Control Group

cRNA: circulant Ribonucleic Acid

CVD: Cardiovascular Disease

DBP: Diastolic Blood Pressure

DM: Diabetes Mellitus

DME: Diabetic Macular Edema

DNA: Desoxiribonucleic Acid

DR: Diabetic Retinopathy

DSG: Diabetic Study Group

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic acid

ETDRS: Early Treatment Diabetic Retinopathy Study

EtOH: Ethanol Alcohol

FDA: Food and Drug Administration

FH: Family History

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GAT: Goldmann applanation tonometer

H_2O: Dihydrogen Oxide, Water

HbA1c: Glycosylated Hemoglobin

HBP: High Blood Pressure

HE: Hard Exudate

HIF-1α: hypoxia-inducible factor 1 alpha

HK: Housekeeping

ICO: International Council of Ophthalmology

IDF: International Diabetes Federation

IOP: Intraocular Pressure

IRMA: Intraretinal Microvascular Abnormalities

IV: intravitreal

KEGG: Kyoto Encyclopedia of Genes and Genomes

LACRT: Lacritin

LCN: Lipocalin

LE: Left Eye

LF: Lactoferrin

logMAR: Logarithm of the Minimum Angle of Resolution

Lz: Lysozyme

MAPK: mitogen-activated protein kinase

MH: macular Hole

miRNA: micro Ribonucleic Acid

MMP: Matrix Metalloproteinase

mNPDR: mild non proliferative diabetic retinopathy

modNPDR: moderate non proliferative diabetic retinopathy

mRNA: messenger Ribonucleic Acid

MSVI: Moderate and Severe Vision Impairment

NEB: New England Biolabs

NGS: Next Generation Sequencing

NOD: Nucleotide oligomerization domain

NPDR: Non-Proliferative Diabetic Retinopathy

OAD: Oral Antidiabetics

OCT: Optical Coherence Tomography

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase Chain Reaction

PDR: Proliferative Diabetic Retinopathy

piRNA: Piwi Ribonucleic Acid

POAG: Primary Open Angle Glaucoma

Pre miRNA: precursor micro Ribonucleic Acid

Pri miRNA: Primary micro Ribonucleic Acid

PRO: Proline

PRP: Panretinal Photocoagulation

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

RE: Right Eye

RIG-I: retinoic acid-inducible gene-I

RISC: RNA-induced silencing complex

RPE: Retinal Pigmented Epithelium

RT: Reverse Transcription

RT: Room Temperature

S/CE: soft or cotton exudates

SIgA: Secretory Immunoglobuline A

siRNA: Small Interfering Ribonucleic Acid

sNPDR: severe non proliferative diabetic retinopathy

ssDNA: Single Strand Desoxiribonucleic Acid

T1DM: Diabetes Mellitus Type 1

T2DM: Diabetes Mellitus Type 2

T2DM+DR: diabetes mellitus type 2 with diabetic retinopathy

T2DM-DR: Diabetes mellitus type 2 without diabetic retinopathy

UTR: Untranslated Region

VA: Visual Acuity

VB: Vitreous Body

VEGF: Vascular Endothelial Growth Factor

VH: Vitreous Humour

WHO: World Health Organization

ABSTRACT

Summary

Purpose: Diabetes Mellitus (DM) is a current pandemic. The global prevalence was 415 million in 2015, with a steady rise to 451 million in 2017, and up to 693 million by 2045. Most important ocular complications are the diabetic retinopathy (DR) and diabetic macular edema (DME), that account for the first global cause of visual impairment and blindness in adults in its working age. Knowledge on the cellular and molecular basis of DR remain incomplete. We deal with identifying micro ribonucleic acid (microRNAs, miRNAs, miR-) molecules in the tear film of type 2 diabetics (T2DM) in order to address the miRNAs fingerprint and its biological targets in DR.

Material and Methods: We conducted an analytical, observational, and case/control study in 225 initially suitable participants of both sexes aged 26-

82 years. To calculate the sample size for this study, the eNe 2.0 statistical program (GlaxoSmithKline S.A.) was used. Baseline volunteers were selected by a non-random consecutive sampling procedure, after ensuring the health status and ocular condition, in agreement with the inclusion/exclusion criteria. Two main groups were done: 1) T2DMG (n= 118) and the healthy controls (CG; n=117). Reflex tears were collected by microhematocrit capillary tubes from the inferior meniscus (30 ?L), without instilling anesthetics, that were immediately frozen at -80ºC until processing for identifying miRNAs by next generation sequencing (NGS). Statistics were done by using the IBM SPSS Statistics for Windows 22.0 program (IBM Corp., Armonk, NY, USA). Bioinformatics were assessed by the Limma and edgeR packages deposited in Bioconductor (www.bioconductor.org).

Results: Final sample of this study participants was 154. Study withdrawal, disease progression, adverse events, and diverse problems with the tear samples, induced the 32% loss of the baseline participants. Two main groups were done: 1) a control group (CG; n=54) and a T2DM group (T2DMG; n=90). The latter were subdivided into T2DM without retinopathy (-DR) and with (+DR) retinopathy. Mean age was: 58 $\pm$ 18 years for the CG, 68 $\pm$ 10 for the

T2DMG without DR (-DR) and 66 ± 8 for the T2DMG with DR (+DR). Females constituted 58% of the CG, 54% of the T2DMG-DR and 34% of the T2DMG+DR. By NGS, a total of 179 miRNAs were identified in tears of the study participants. Comparison between groups of tears miRNAs expression revealed that the most statistically significant were the following: hsa-miR-15b-5p, hsa-miR-155-5p, hsa-miR-10a-5p and hsa-miR-195-3p. Main biological targets of the above miRNAs were angiogenesis, apoptosis, inflammation, oxidative stress and cell cycle regulation. Validation by quantitative real time polymerase chain reaction (qRT-PCR) revealed that the hsa-miR-10a5p tear expression was significantly lower in the T2DMG, and specifically in the +DR subgroup. Furthermore, blood expression levels of VEGF gene and MMP2 gene were upregulated in T2DM patients, and particularly in the +DR subgroup.

Conclusion: In this work we gathered essential information on DRbiomarkers by using tear samples to identify specific miRNAs that can be used to improve the diagnosis and prognosis of DR. We suggest that the miR-10a-5p is an important candidate for the preclinical diagnosis of DR and for

designing new biotherapies in the foreseeable future, for better eye and vision care in diabetics.

Resumen

Propósito: La Diabetes Mellitus (DM) es una pandemia actual. La prevalencia global fue de 415 millones en 2015, con un aumento constante a 451millones en 2017 y hasta 693 millones para 2045. Las complicaciones oculares más importantes son la retinopatía diabética (RD) y el edemamacular diabético (EMD), que representan la principal causa global de discapacidad visual y ceguera en adultos en edad laboral. El conocimiento sobre las bases celulares y moleculares de la RD sigue siendo incompleto. Nuestro objetivo es identificar moléculas de ácido ribonucleico micro (microARN, miARN, miR-) en la película lagrimal de diabéticos tipo 2 (T2DM)con el fin de determinar la huella de miARN y sus objetivos biológicos en la RD.

Material y métodos: Realizamos un estudio analítico, observacional y de casos/controles en 225 participantes inicialmente aptos, de ambos sexos y edades comprendidas entre los 26 y 82 años. Para calcular el tamaño de muestra del estudio, se utilizó el programa estadístico eNe 2.0 (GlaxoSmithKline S.A.). Los voluntarios iniciales se seleccionaron mediante

un procedimiento de muestreo no aleatorio consecutivo, después de garantizar el estado de salud y la condición ocular, de acuerdo con los criterios de inclusión/exclusión. Se realizaron dos grupos principales:
1) T2DMG (n=118) y controles sanos (CG; n=117). Se recogieron lágrimas reflejas mediante tubos capilares de microhematocrito del menisco inferior (30 µL), sin instilar anestésicos, que se congelaron inmediatamente a -80°C hasta su procesamiento para identificar miARN mediante secuenciación de próxima generación *(next generation sequencing*, NGS). Los análisis estadísticos se realizaron utilizando el programa IBM SPSS Statistics para Windows 22.0 (IBM Corp., Armonk, NY, EE. UU.). La bioinformática se evaluó utilizando los paquetes Limma y edgeR depositados en Bioconductor (www.bioconductor.org).

Resultados: La muestra final de participantes en este estudio fue de 154. La retirada del estudio, progresión de la enfermedad, eventos adversos y diversos problemas con las muestras de lágrimas provocaron una pérdida del 32% de los participantes iniciales. Se realizaron dos grupos principales: un grupo control (CG; n=54) y un grupo de T2DM (T2DMG; n=90). Este último se subdividió en T2DM sin retinopatía (-DR) y con retinopatía (+DR). La edad

media fue de 58 ± 18 años para el CG, 68 ± 10 para el T2DMG sin DR (-DR) y 66 ± 8 para el T2DMG con DR (+DR). Las mujeres representaron el 58% del CG, el 54% del T2DMG-DR y el 34% del T2DMG+DR. Mediante NGS, se identificaron un total de 179 miARN en las lágrimas de los participantes del estudio. La comparación entre los grupos de expresión de miARN en las lágrimas reveló que los más estadísticamente significativos fueron los siguientes: hsa-miR-15b-5p, hsa-miR-155-5p, hsa-miR-10a-5p y hsa-miR-195-3p. Los principales objetivos biológicos de estos miARN fueron la angiogénesis, la apoptosis, la inflamación, el estrés oxidativo y la regulación del ciclo celular. La validación mediante reacción en cadena de la polimerasa en tiempo real cuantitativa (qRT-PCR) reveló que la expresión de miARN hsa-miR-10a-5p en las lágrimas fue significativamente menor en el T2DMG, y específicamente en el subgrupo +DR. Además, los niveles de expresión sanguínea del gen VEGF y el gen MMP2 se encontraron aumentados en los pacientes con T2DM, y especialmente en el subgrupo +DR.

Conclusión: En este trabajo hemos recopilado información esencial sobre los biomarcadores de la RD utilizando muestras de lágrimas para identificar miARN específicos que pueden ser utilizados para mejorar el diagnóstico y

pronóstico de la RD. Sugerimos que el miR-10a-5p es un candidato importante para el diagnóstico preclínico de la RD y para el diseño de nuevas bioterapias en un futuro previsible, para mejorar la salud ocular y visual de los diabéticos.

Resum

Propòsit: La Diabetes Mellitus (DM) és una pandèmia actual. La prevalença global va ser de 415 milions el 2015, amb un augment constant a 451 milions el 2017 i fins a 693 milions el 2045. Les complicacions oculars més importants són la retinopatia diabètica (RD) i l'edema macular diabètic (EMD), que representen la principal causa global de discapacitat visual i ceguesa en adults en edat laboral. El coneixement sobre les bases cel·lulars i moleculars de la RD segueix sent incomplet. Ens ocupem d'identificar molècules d'àcid ribonucleic micro (microARN, miARN, miR-) en la pel·lícula lacrimal de diabètics de tipus 2 (T2DM) per tal de determinar l'empremta de miARN i els seus objectius biològics en la RD.

Material i mètodes: Vam realitzar un estudi analític, observacional i de casos/control en 225 participants inicialment aptes de tots dos sexes, amb edats compreses entre 26 i 82 anys. Per calcular la mida de mostra d'aquest estudi, vam utilitzar el programa estadístic eNe 2.0 (GlaxoSmithKline S.A.). Els voluntaris inicials es van seleccionar mitjançant un procediment d'obtenció de mostres no aleatori consecutiu, després de garantir l'estat de

salut i la condició ocular, d'acord amb els criteris d'inclusió/exclusió. Es van realitzar dos grups principals: 1) T2DMG (n = 118) i els controls sans (CG; n = 117). Es van recollir llàgrimes reflexes mitjançant tubs capil·lars de microhematòcrit del menisc inferior (30 µL), sense instilar anestèsics, que es van congelar immediatament a -80 °C fins al seu processament per identificar miARN mitjançant seqüenciació de pròxima generació (*next generation sequencing,* NGS). Els anàlisis estadístics es van realitzar utilitzantel programa IBM SPSS Statistics per a Windows 22.0 (IBM Corp., Armonk, NY, EUA). La bioinformàtica es va avaluar utilitzant els paquets Limma i edgeR dipositats en Bioconductor (www.bioconductor.org).

Resultats: La mostra final de participants en aquest estudi va ser de 154. La retirada de l'estudi, la progressió de la malaltia, els esdeveniments adversos i diversos problemes amb les mostres de llàgrimes van provocar una pèrdua del 32% dels participants inicials. Es van realitzar dos grups principals: un grup de control (CG; n = 54) i un grup de T2DM (T2DMG; n = 90). Aquest últim es va subdividir en T2DM sense retinopatia (-DR) i amb retinopatia (+DR). L'edat mitjana va ser de 58 ± 18 anys per al CG, 68 ± 10 per al T2DMG sense DR (-DR) i 66 ± 8 per al T2DMG amb DR (+DR). Les dones van representar el

58% del CG, el 54% del T2DMG-DR i el 34% del T2DMG+DR. Mitjançant NGS, es van identificar un total de 179 miARN en les llàgrimes dels participants de l'estudi. La comparació entre els grups d'expressió de miARN en les llàgrimes va revelar que els més estadísticament significatius van ser els següents: hsa-miR-15b-5p, hsa-miR-155-5p, hsa-miR-10a-5p i hsa-miR-195-3p. Els principals objectius biològics d'aquests miARN van ser l'angiogènesi, l'apoptosi, la inflamació, l'estrès oxidatiu i la regulació del cicle cel·lular. La validació mitjançant reacció en cadena de la polimerasa en temps real quantitativa (qRT-PCR) va revelar que l'expressió de miARN hsa-miR-10a-5p en les llàgrimes va ser significativament més baixa en el T2DMG, i específicament en el subgrup +DR. A més, els nivells d'expressió sanguínia del gen VEGF i el gen MMP2 es van trobar augmentats en els pacients amb T2DM, i especialment en el subgrup +DR.

Conclusió: En aquest treball hem recopilat informació essencial sobre els biomarcadors de la RD utilitzant mostres de llàgrimes per identificar miARN específics que poden ser utilitzats per millorar el diagnòstic i pronòstic de la RD. Suggereixem que el miR-10a-5p és un candidat important per al

diagnòstic preclínic de la RD i per al disseny de noves bioteràpies en un futur previsible, per millorar la salut ocular i visual dels diabètics.

INTRODUCTION

1. Diabetes Mellitus

Diabetes mellitus (DM) describes a group of metabolic disorders characterized by increased hyperglycemia (1–4).

Based in its aetiology and clinical presentation, this disorder is classified into four types: 1) type 1 DM (T1DM), 2) type 2 DM (T2DM), 3) gestational DM and 4) other specific types. The T1DM results from an autoimmune destruction of the pancreatic islet β-cells causing the loss of insulin production, mainly occurring in childhood (5,6). The T2DM, which is much more worldwide prevalent, is related to lifestyle factors, such as overnutrition, low dietary fiber, sedentary lifestyle, sleep deprivation and depression. Chronic low-grade inflammation will eventually lead to overt diabetes in predisposed individual, in whom there is a resistance to insulin and/or relative insulin deficiency, either of which may be present at the time

that diabetes becomes clinically manifest (7). It is strongly familial, but major susceptibility genes have not yet been identified (6,8–10).

1.1. Prevalence of Diabetes Mellitus

Global DM prevalence in adults has been increasing over recent decades. In 1980, the World Health Organization (WHO) estimated 108 million people living with diabetes with a fourfold increase estimate in 2014 (11). The International Diabetes federation (IDF) estimated the global prevalence in world population to be 415 million in 2015 with a steady rise to 451 million in 2017 and up to 693 million in 2045 (2,4,12). In 2010, about one-third (34.9%) of the Portuguese population aged 20–79 years was diagnosed with DM. (13) According to Soriguer (14), almost 30% of the Spanish population had some carbohydrate disturbance.

The DM is the 4^{th}-5^{th} leading cause of death in most developed countries with growing evidence of becoming epidemic in many developing countries. People living with DM have a higher risk of developing several serious life-threatening health problems resulting in increasing disability, reduced life expectancy and enormous health and socioeconomic costs (15).

Persistently high blood glucose levels cause generalized vascular damage, carrying to clinical complications, such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure, and ocular vascular lesions. From the latter, the most prevalent is the diabetic retinopathy (DR). The DM is certainly one of the most challenging problems of the 21st century for virtually every society (2,3,15).

2. Diabetic Retinopathy

2.1. Definition and Prevalence of Diabetic retinopathy.

The DR, a disease involving the vitreous, retina and choroid, can be detected in the ocular fundus ophthalmoscopic examination, with the microvasculature being affected, showing the typical manifestations of the chronic hyperglycemia in the eyes. Therefore, DR is the leading cause of acquired blindness in working adults (16,17), and is also considered to be responsible for 1,9% of moderate-to-severe visual loss and 2,6% of blindness in 2010 (18). It is a duration-dependent disease that develops in stages; the incidence of retinopathy is rarely detected in the first few years of DM, but

the incidence increases to 50% by 10 years, and to 90% by 25 years of suffering the disease (19). A systematic review in 2014, estimated that globally 32.4 million people were blind (defined as presenting visual acuity <3/60 in the better eye) in 2010 and 191 million people showed a moderate and severe vision impairment (MSVI; defined as presenting visual acuity <6/18 but ≥3/60 in the better eye) (18,20).

Approximately 75% of persons suffering from T1DM develop retinopathy but, fortunately, the prevalence of severe retinopathy and nephropathy in these patients has diminished over the past 4 decades, due to improved medical care (21). Concerning the T2DM, half of the patients may develop retinopathy, however, the recent epidemics of the above DM type requires a new understanding of the biology of DR and a new approach to its prevention and treatment (22,23).

2.2. Physiopathology of Diabetic Retinopathy.

Normal vision depends on the integrity of the retinal cells lying on anefficient cell-to-cell communication among the neuronal, glial, microglial, vascular and pigmented epithelial cells. The retinal capillaries are coated

with endothelial cells that are responsible for the normal blood retinalbarrier (BRB), and are supported with an equal number of pericytes that helpprovide tone to the vessels (19). The retina is a transparent layer of neural tissue between the retinal pigmented epithelium (RPE) and the vitreous body (VB). The retinal anatomy and physiology is so unique in order to get aperfect visual function but also can turn to a handicap under the stressful metabolic conditions of DM (24).

The retina is vulnerable to the persistent high glucose levels, conducing not only to acute but also cumulative long-term structural alterations. It was reported an altered ratio of endothelial cells to pericytes to 4:1 and damage of the tight junctions with loss of their protective function, leading to leakage due to increased vascular permeability, thus resulting in the swelling of the retina. Due to progressive dysfunction, the capillaries die prematurely resulting in ischemia that can be followed by abnormal angiogenesis and, in last stages, retinal detachment and even blindness. In short, Diabetic retinopathy develops with thickening of the basement membrane, apoptosis

of pericytes and endothelial cells and stiff leukocytes conducing to an increased retinal leukostasis (17,19,24,25).

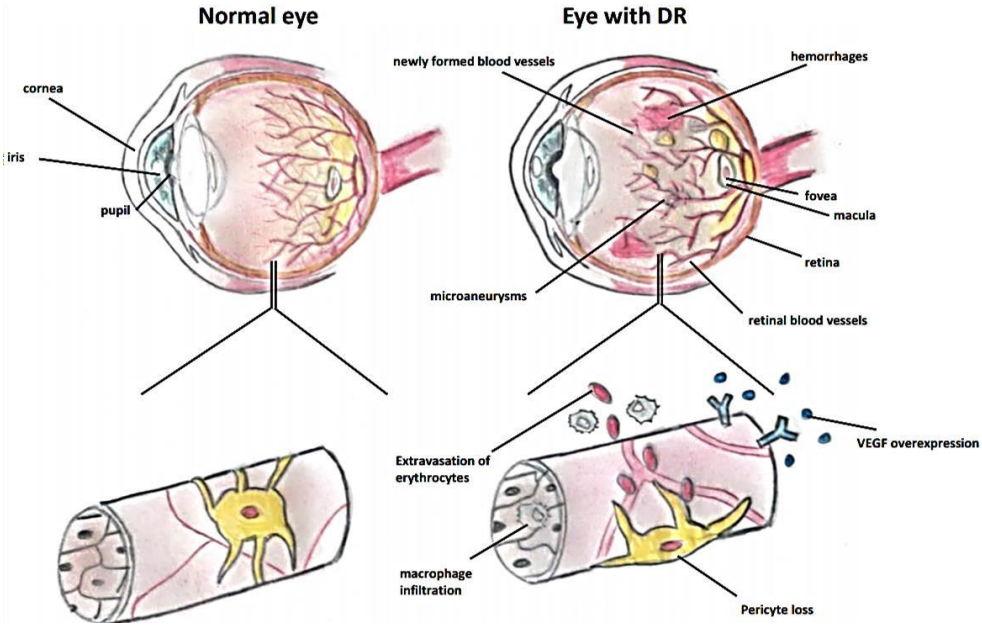

Figure 1.- Retinal vasculature in diabetic retinopathy. *This image has been adapted from: https://www.nature.com/articles/eye2017220 (26).*

Breakage of the BRB, capillary closure, appearance of microaneurysms and alterations in the neuronal and glial cells of the retina define the four major alterations of the initial DR stages (26). First hallmark that can be early detected by the ocular fundus examination is the presence of microaneurysms. Besides the features above described, many other classic

retinal DR lesions, including intraretinal hemorrhages, and intraretinal microvascular abnormalities (IRMAs), venous beading, hard exudates and neovascularization are findings that can be used to define guideline recommendations and clinical classifications (27,28).

2.3. Classification of Diabetic retinopathy

In the mid-1800s, Eduard Jäger was the first to describe DR characteristics with the aid of an ophthalmoscope. Specific lesion patterns came later around 1940 with Ballantyne's report and others. Fluorescein angiography added new insights into the pathophysiology of the disease (30-33).

In 2018, according to the International Council of Ophthalmology (ICO), DR was proposed to be classified into: Non-Proliferative Diabetic Retinopathy (NPDR), a stage in which there are not developed neovascularization but may have any of the other classic DR lesions. In this phase, the patient affection range from no DR to a wide spectrum of DR severity, includes the mild (m), moderate (mod), and severe (s) NPDR forms, as listed in the table (27), and Proliferative Diabetic Retinopathy (PDR): represents an angiogenic response

of the retina to extensive ischemia from capillary closure; new vessels are visualized on the disc or along the vascular arcades. It represents the most advanced stage of DR. For addressing the DR severity stages see the table 1 and figure 2.

Table 1. International Classification of Diabetic Retinopathy and Diabetic Macular Edema.

Modified from: https://www.aaojournal.org/article/S0161-6420(17)33523-6/fulltext)

Disease	Findings Observable on Dilated Ophthalmoscopy
DR	
No apparent DR	- No abnormalities
NPDR	
Mild NPDR (mNPDR)	- Mycroaneurisms
Moderate NPDR (modNPDR)	- Mycroaneurisms and others, but less than in the sNPDR.
Severe NPDR	- Signs of modNPDR and hemorrhages, venous beading, IRMAS, without PDR signs.
PDR	- Signs of mod/sNPDR and one or more of this: neovascularization, vitreous/pre-retinal hemorrhage.
DME	
No apparent DME	- No retinal thicknenning or macular hard exudates
Non center-involving DME	- Macular thickening not involving the central subfield zone (1 mm diameter)
Center involving DME	- Macular thickening clearly involving the central subfield zone (1 mm diameter)

AAO: American Academy of Ophthalmology; DR: diabetic retinopathy; NPDR: non-proliferative DR; mNPDR: mild NPDR; modNPDR: moderate NPDR; sNPDR: severe NPDR; PDR: proliferative DR; DME: diabetic macular edema; IRMAS: intraretinal microvascular abnormalities.

Diabetic Macular Edema (DME): is an additional important vision-threatening complication of DR, that is assessed separately from DR stages. The DME can be seen in eyes at any DR severity level, and can run an independent course (27), as in the figure 2.

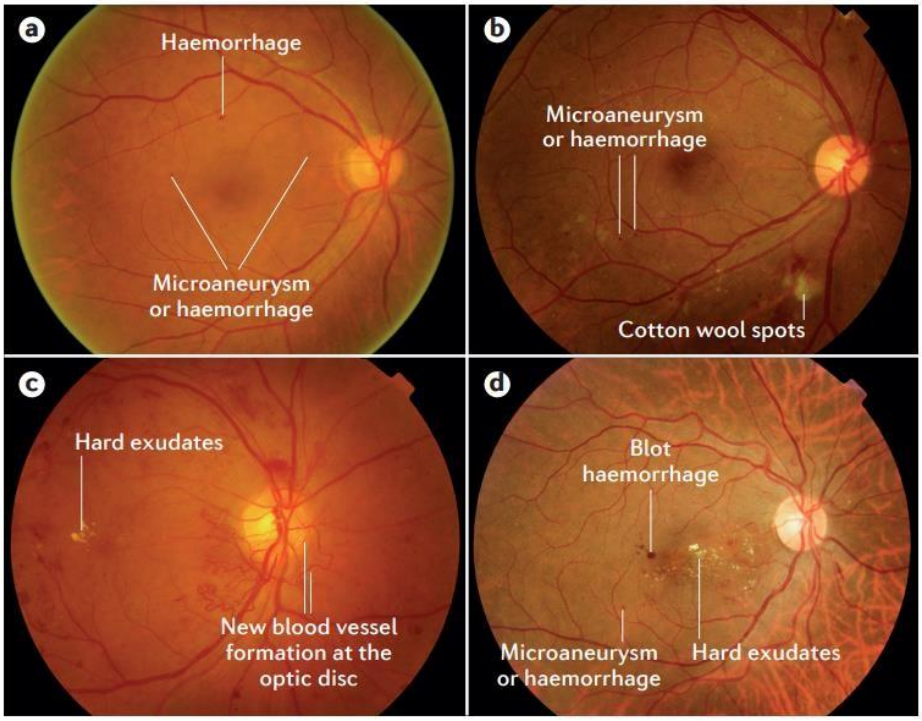

Figure 2. Clinical signs of diabetic retinopathy on fundoscopic examination. a) mNPDR with microaneurysms and hemorrhage. b) ModNPDR with microaneurysms, hemorrhages and cotton wool spots. c) PDR showing new blood vessels at the optic disc. d) DME showing hard exudates at the fovea center.
NPDR: non-proliferative DR; mNPDR: mild NPDR; modNPDR: moderate NPDR; sNPDR: severe NPDR; PDR: proliferative DR; DME: diabetic macular edema;
This image was taken from https://www.nature.com/articles/nrdp201612#citeas (30).

Identification of DR severity level leads the physicians to better predict the risk of clinical progression and visual loss, and thus define good practices of appropriate referral, follow-up intervals and treatment recommendations (27), as can be seen in the figure 3. The DR still remains a major cause of

vision impairment and blindness worldwide. Despite years of pre-clinical and clinical investigation, yet the fundamental cause(s) remains uncertain.

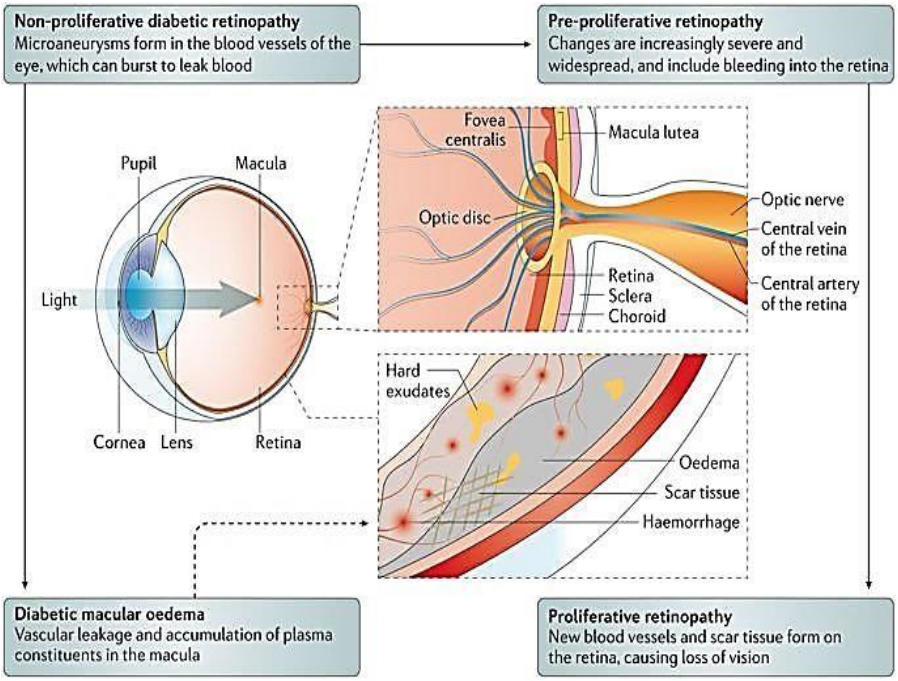

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Figure 3. Morphological characteristics and course of DR and DME from NPDR, pre-PDR, PDR and DME.
The image was taken from https://www.nature.com/articles/nrdp201612#citeas (30)

Recent studies show that the independent risks to develop DR include: 1) Duration of the disease, 2) Hemoglobin A1c (HbA1c), 3) Masculine gender (38,1% vs 27,1% in women), 4) Treatment requiring insulin (47,4% vs 26,7%

in treatment with oral antidiabetics (OAD)) and 5) High blood pressure (HBP) (31). These data reveal crucial information to support studies that will allow the identification of the most susceptible patients of developing diabetic retinopathy and, lastly, blindness, since there is no satisfactory treatment that meets all the needs of the patients (31,32).

The number of people worldwide at risk of developing vision loss from diabetes is predicted to double over the next 30 years (12), so it is imperative to develop new approaches to understand the pathophysiology and improve the detection, prevention, and treatment of retinopathy in its earliest preclinical stages rather than wait for the onset of visualizing the vision-threatening lesions (24).

3. Biomarkers

3.1. Definition of Biomarker

In the last 3 decades, current technologies based on the information given by the human genome are dramatically reshaping the research and

development pathways for drugs and diagnosis (33,34). There are two possible approaches to the achievement of more informative and therapeutic researches: 1) the use of precise clinical measurement tools to evaluate disease progression and 2) the use of a large array of analytical tools to assess biological parameters, normally referred as biomarkers (34,35).

A biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” according to the definition given by The Biomarkers Definitions Working Group (BDWG), composed of members from the US Food and Drug Administration (FDA) (36). This definition includes not only molecular biomarkers obtained in the clinical laboratory, but also those obtained from imaging technologies (33).

According to Cunha-Vaz (37), “biomarkers have become the basis for preventive medicine, meaning medicine that recognizes diseases or the risk of disease early, and takes specific countermeasures to prevent the development of the disease”. Therefore, an ideal biomarker must be

sensitive, predictive, robust, non-invasive, accessible and disease-specific, being an indicator even before the clinical symptoms appear (38,39).

Nowadays, most part of the diagnostic biomarkers are proteins. However, in many biological samples (namely blood), the complexity of the protein constitution, some posttranslational modifications and a low availability of many proteins of interest in plasma and serum turns its use, as sensitive and specific biomarkers for a determined pathology, a very hard, time-consuming and even a frustrating process (39). It is urgent to develop new strategies for the determination of biomarkers that allow the identification of molecules involved in the genetic transmission processes. To that aim, the importance of detecting microRNAs (miRNA; miR-) should be praised. Because of their low complexity of processing and detection, by the simplicity of the amplification methods, their restricted-tissue profile expression and highly sequence conservation in humans and other biologic models, microRNAs are the ideal candidates to become perfect biomarkers of numerous pathophysiological conditions, namely, DR (41–43).

The DR can take a very quick progression, from mNPDR to a more moderate-to-severe stage, and PDR. At present time, a trained specialist is needed for the diagnosis, narrowing the population examined and diagnosed. Therefore, a sensitive and specific screening biomarker of diabetic retinopathy would aid considerably in identifying those individuals in need of clinical assessment and treatment (40).

4. Genetics in Diabetic Retinopathy

The DR is a complex disease, exhibiting a wide variety of frequency and severity among diabetics (41,42). While the DM duration and glycemic control are recognized as prominent risk factors for DR, they only account for a portion of the observed heterogeneity (28). The remaining diversity in DR development may have genetic underpinnings, leading to a surge in genetic research focused on understanding its causes over the past three decades (43). Indeed, the clinical and epidemiological investigations strongly suggest a genetic component in DR. However, studies aimed at identifying specific genes or genomic regions associated with DR have

yielded inconsistent findings (44–47). This inconsistency in results can be attributed to several factors, including discrepancies in retinopathy detection methods, variations in retinopathy definitions, disparities in control definitions concerning DM duration, and inadequate sample sizes (41).

5. MicroRNA and Diabetic Retinopathy

5.1. Micro Ribonucleic Acids (miRNAs) (miR-)

The micro ribonucleic acids (microRNAs, miRNAs; miR-) are known as a type of noncoding RNAs (ncRNAs) that regulate gene expression, playing important roles in obtaining a biologic response in selected cells and tissues (48–50). The miRNAs were first characterized in the early 1990s in a nematode, *Caenorhabditis elegans,* and presently over than 1000 miRNAs have been identified in humans (51,52). It has been reported that miRNA precursors are located into clusters through many different regions of the

genome, but most frequently within intergenic regions and introns of protein-coding genes (49,50).

The miRNAs are a group of small ubiquitously expressed RNA molecules that do not code for any protein. They are ~22 nucleotides in length and it has been shown to play a key role in mammalian posttranscriptional gene expression by repressing translation or inducing target degradation. miRNA interfere with mRNA either in its translation or stability, thus inhibiting the normal protein synthesis (51,53,54).

As reported by Ambros *et al*., (53) The miRNAs are named using the miR prefix and a unique identifying number (miR-1, . . . miR-155, etc.). Furthermore, genes that encode the miRNA are named using the same three-letter prefix, with capitalization, hyphenation, and italics, according tothe conventions of the organism (mir-1 in C).

Over 60% of human genes are known to contain, at least, one conserved miRNA binding-site. Taking into consideration the fact that there are

numerous non-conserved sites as well, it may be assumed that miRNA control most of the protein coding genes. Thus, it is not surprising that the biogenesis and function of miRNAs themselves are tightly regulated, and their dysregulation is often associated with human diseases where cancer and neurodevelopmental disorders have a major share (52–56) .

Although it has been roughly 25 years since miRNAs discovery, the number of human miRNA candidates are continuously increasing, and just a few of these molecules have been completely identified, characterized, validated, and conveniently registered. In this sense, since the very beginning of the miRNA registry, the number of miRNAs that have been correctly deposited, have been constantly increasing, and the first base was improved into the developed miRbase (57), and the latest version of the miRbase was released in 2018, with additional sequencing data leading to almost 30% high-confidence human miRNA annotations (http://www.mirbase.org/ftp.shtml) (58).

Alles *et al*., (59) recently identified 2300 true human mature miRNAs, and from these, 1115 were yet annotated in the miRBase V22. However, the

knowledge of the human miRNAs biological functioning and their mechanism of action are still limited (49–59).

5.2. miRNA processing

The miRNAs processing begins in the nucleus of the cell with itstranscription, by RNA polymerase II, into primary miRNA (pri-miRNA) (Fig. 4). These relatively long primary transcripts may contain multiple hairpin- like structures which are processed to precursor miRNAs (pre-miRNA) by DROSHA, an RNA polymerase III. (60-64). The pre-miRNA is exported to the cytoplasm by the exportin-5, where they undergo further processing. They are cleaved by DICER, another RNA polymerase III generating a mature double-stranded RNA comprising *circa* 22 nucleotides. Only one of its strands is selected to form the RNA-induced silencing complex (RISC), that is capable to bind the 3´UTR of the target mRNA via sequence complementarity. Whether a perfect or imperfect complementarity occurs, ultimately results in gene silencing (60-64) (Fig. 4).

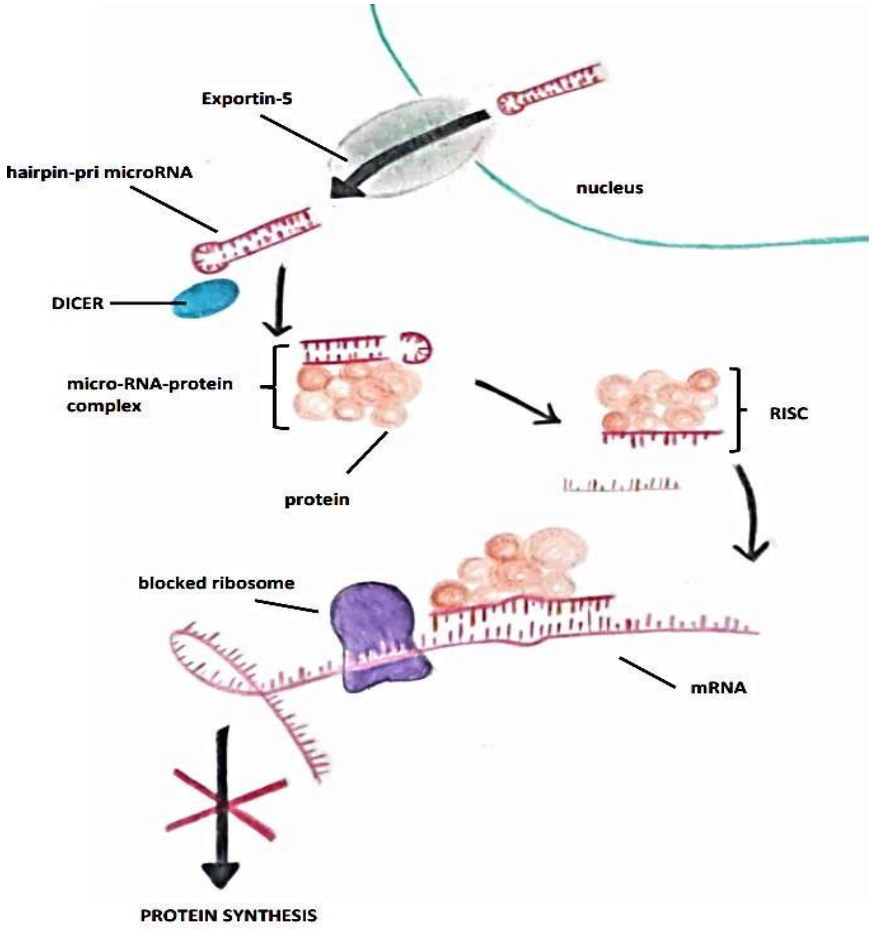

Figure 4. Schematic representation of miRNA processing. *Adapted from the following document: https://rnajournal.cshlp.org/content/11/12/1753* (65).

5.3. miRNAs and Diabetic Retinopathy

A large number of recent studies have identified many specific miRNAs in blood and quite a few in the aqueous humor and vitreous body samples, to

distinguish diabetic patients with retinopathy from those without retinopathy and for the evolution of the disease from NPRD to PRD (40). Among those studies, 9 had measured the miRNAs in serum, 4 had used plasma, 1 had used extracellular vesicles extracted from serum and plasma, and 1 had used early-outgrowth endothelial progenitor cells obtained from *the in vitro* culture of peripheral blood mononuclear cells. Three other studies were performed in vitreous humor. In some of the above publications, the analyzed miRNAs were not found to be good biomarkers once they cannot serve to monitor the progression of DR (40).

In other studies, blood serum/plasma samples were analyzed, and the miRNA expression levels for DR patients were compared to those without DR, and closely matched for age and gender: miR-18b, miR-19b, miR-211, let-7a-5p, miR-novel-chr5, miR-28-3p, miR-320-a and miR-93 are found to have their expression increased whereas miR-151a-5p, miR-148a-3p, miR-27b and miR-126 have a decreased expression. In the case of PDR, the miRNAs expression levels were compared to those of NDR and NPDR patients: miR-21, miR-155, miR-181c, miR-1179, are found to be upregulated and miR-126 has its expression downregulated. Vitreous humor

(VH) expression levels for PDR patients were compared to those of macular hole (MH) patients, taken as the comparative-controls: a large quantity of miRNAs have an increased expression, such as miRNA-200b, miR-21, miR-15a, miR-320a and b, miR-93, miR-29a and miR-423-5p. On the other side, miR-204 and let-7c are found to be down regulated (53-56, 60-65)

However, a big amount of reports had small numbers of patients, especially those analyzing vitreous humor, since these data are obtained through invasive methods, needing specialized operators, which becomes an important limitation of these studies. Screenings with larger group sizes are needed to confirm or discount by validation these miRNA findings (40).

Many different body fluids constitute study fields to biomarkers, and the ideal should be easily accessible through a biological fluid. Consequently, tears could be considered an optimum material obtainable by noninvasive procedures (66-75).

6. Human tears.

6.1. Human tear composition.

Tears are an essential fluid for the integrity of the ocular surface components. Furthermore, tears are part of daily life, but it has not been taken, so far, as a hot topic. In fact, published information about tears and their composition is limited to some data on the tears' proteome, mineral composition, cytokine profile and lacrimation under various influences (66–70). Tears are necessary for normal eye function, providing an optically smooth surface necessary for refraction of light onto the retina. The quality of the tear film was shown to affect the visual acuity (49,70–72). Tears are an extracellular fluid covering the surface epithelial cells and forming the anterior component of the ocular surface. It is considered a vital structure whose main roles are lubrification, nutrition for the underlying cells, to protect the ocular surface from dehydration of the mucus membranes, caused by tear film evaporation and against various pathogens, among others (66-70). Tears are a source of nourishment for ocular surface tissues

and a vehicle to remove local waste products, metabolized drugs and inflammatory mediators produced in several ophthalmic diseases. (69). Usually, tears can be classified as an outer lipid layer (derived primarily from meibomian glands as a complex mixture of different class of non-polar and polar lipids), middle aqueous layer (secreted by lacrimal glands, containing electrolytes, proteins, peptides and small metabolites such as glucose, amino acids and others) and the inner mucin layer (constituted principally by O-linked carbohydrates with a protein core) (66-70) (Fig. 5).

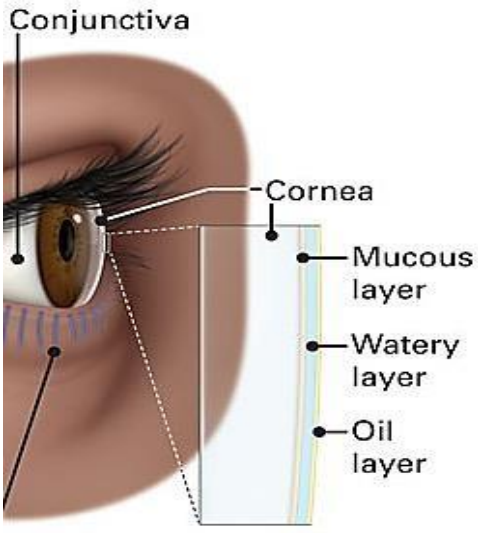

Figure 5. The tear film is composed of three layers: the oily layer on the outside, the thickness watery layer in the middle, and the innermost mucus layer. *Taken from: https://www.aao.org/eye-health/anatomy/tear-film-3*

The tear fluid volume is around 5-10 µL, under normal conditions with a secretion rate of about 1.2 µL per minute. Although small in volume, the tear fluid is an extremely complex biological mixture containing proteins, electrolytes, lipids, and small molecule metabolites. Indeed, this complex repertoire is, to date, not fully explored and deciphered. The major amount of information regards the protein composition: a normal tear fluid should contain about 1500 proteins (namely, lysozyme (LZ), lipocalin (LCN), lacritin (LACRT), lactoferrin (LF), secretory immunoglobuline A (SIgA) and proline (PRO) rich proteins) with origin in different sources such as lacrimal glands, ocular cells, meibomian glands and others (66-70). Our research group have been interested in using tear samples for ophthalmic research and the most prevalent ocular pathologies (71–75). Since tears are a noninvasive collected biological fluid, relatively easy to store and manipulate, we may suggest that the tear film have a high diagnostic value for the eyes and vision.

6.2. microRNAs, and tears as outstanding biological sample.

So far to current knowledge, there are scarce studies that have evaluated the differential expression of miRNAs in tears. A few recent works have been

published on the role of tears for identify miRNAs for diagnostic and prognostic biomarkers in primary open angle glaucoma (POAG) patients (70-76). In fact, Tamkovich *et al*., (70) reported the following miRNAs that were identified in the glaucomatous tears: miR-146b, miR-16 and miR-126, suggesting that this study in tears provides a basis for clinical applications. Some more articles have been recently published referring the role of tears for identify miRNAs for diagnostic and prognostic biomarkers in relation to anterior eye segment diseases (Dry Eyes, keratoconus, Uveitis) (50,66-70. 77–80).

However, the use of tears as a biological sample for studies regarding DR and DME have been arising in the past few years (85–92). It is of pivotal interest to search for tear biomarkers for identifying DM patients at high risk of developing DR, DME or progression of disease, for improving knowledge on the pathophysiology of the ocular complications of DM, the early diagnosis, and the discovery of newer biotherapies. To our knowledge, only two studies analyzing the expression of miRNAs related to diabetic retinopathy in tears, were reported so far (93,94). According to Hu, *et al*, it was shown that miR-145-5p, miR-214-3p, miR-218-5p and miR-9-5p are dysregulated

during diabetic retinopathy development and to Sun, *et al*, the expression level of miRNA-23a was significantly reduced in PDR patients.

It is important to emphasize the intrinsic value of tears as biological samples in molecular-genetic studies, and that miRNAs may be of paramount importance in revealing the pathophysiology of DR (63).

7. Genes involved in diabetic retinopathy

The DR is attributed to a complex interplay of biomolecules, including enzymes and cytokines, which are linked to the oxidative stress induced by persistent hyperglycemia (95–97).

The DR is categorized based on vascular lesions: NPDR is characterized by vascular distortion, retinal hemorrhage, microaneurysms, and lipid exudates. The proliferative stage of DR is marked by the presence of fragile new vessels that can lead to bleeding from the retina or vitreous (98,99).

Angiogenesis, a multistep process involving the sprouting of new blood vessels from preexisting arteries, necessitates the breakdown of basement membranes and the extracellular matrix (ECM), migration and proliferation of endothelial cells, and the formation of capillary tubes (100,101). Several potential genes have been associated with DM and DR. Notably, the vascular endothelial growth factor (VEGF) is a key angiogenic factor in PDR, promoting neovascularization and vascular leakage (49,102). Additionally, the proteolytic degradation of ECM and basement membrane components mediated by matrix metalloproteinases (MMP) contributes to the angiogenic switch (103,104). We will comment the main characteristics of two relevant candidate genes for DR: the *VEGF-A* gene and the *MMP9* gene.

7.1. The *VEGF-A* gene

The VEGFs, comprises a variety of growth factors that play dual roles as signaling proteins in both angiogenesis and vasculogenesis processes (105). VEGF influences retinal capillary permeability by promoting the phosphorylation of proteins involved in tight junctions, making it a significant contributor to the development of proliferative DR and DME (106,107). VEGF secretion primarily originates from various cell types, including retinal

pigmented epithelium cells, pericytes, astrocytes, Müller cells, glial cells, and endothelial cells. The VEGF family encompasses multiple proteins, such as PGF, VEGF-A, VEGF-B, VEGF-C, and VEGF-D (105,108,109).

VEGF-A, a glycosylated protein weighing between 36-46 kDa, is generated through alternate splicing of mRNA from a single VEGF gene containing eight exons (108,110); This particular isoform of VEGF-A holds great significance in new blood vessel development as it stimulates endothelial cells to produce MMP. These MMPs play a crucial role in the breakdown of basement membranes and facilitate cell migration (100,111).

7.2. The *MMP2* gene.

The MMPs is a family of neutral endopeptidases, predominantly contribute to the degradation of various ECM components (112,113). These zinc ion-binding enzymes, dependent on calcium ions, are classified into several subtypes, including membrane-type (MMP-7 and MMP-26), stromelysin (MMP-3, MMP-10, and MMP-11), collagenase (MMP-1, MMP-8, and MMP-13), and gelatinase (MMP-2 and MMP-9), among others (100,111). In various ocular diseases, including DR, MMPs play a vital role in processes such as

angiogenesis, epithelial-mesenchymal transition, cell invasion, and migration (100).

The involvement of MMPs in DR is complex (100,113). Certain MMPs, particularly MMP-2, MMP-9, and MMP-14, contribute to the disruption of the BRB by targeting proteins essential for its integrity, leading to increased vascular permeability and BRB dysfunction (71,114–116). Moreover, MMPs play a significant role in the degradation of ECM components, which is a crucial step in angiogenesis(117). Additionally, the presence of hypoxia in the retinal tissue of DR leads to the upregulation of hypoxia-inducible factor 1 alpha (HIF-1α) expression, resulting in the production of VEGF and, subsequently, MMP (113). In fact, elevated levels of MMP-2 have been detected in diabetic patients and animal models of diabetic retinopathy, and these increased levels are believed to contribute to the disruption of the tight-junction complex, vascular permeability, and maintenance of the blood-retinal barrier (114,118,119). Additionally, during the pathogenesis of diabetic retinopathy, there is an acceleration of apoptosis in retinal capillary cells (pericytes and endothelial cells) as well as impairments in the tight junctions and blood-retinal barrier (120).

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

The DR stands as the primary cause of visual impairment among the global working-age population (121). Typically, the funduscopic indications of retinal microvascular complications manifest a few years after the onset of DM (122,123). During the asymptomatic phase, there is a progression of neural retinal damage and clinically undetectable microvascular changes. As a result, the timely detection of subclinical DR could enable the prompt identification and treatment of patients at an elevated risk of DR progression (121,124).

A range of clinical, biochemical, and molecular factors, including genetic and epigenetic ones, play a role in increasing the risk of diabetic retinopathy. In fact, post-transcriptional gene regulation involving microRNAs (miRNAs) is of

enormous interest, because of the width of their interactions, that is made possible through their synergistic/combinatorial relationships with the target genes and its biological functions. Consequently, various clinical, molecular, and genetic biomarkers have been suggested to facilitate early diagnosis and identify patients who are particularly prone to disease progression (51-53, 56, 60,61).

Searching for alternative human biological samples to the classic ones, especially those that can be obtained in a relatively simple and bloodless way, our group has developed a very simple technique to collect them for research, avoiding invasive maneuvers. We have established an easy, fast, and useful technique for obtaining reflex tears in sufficient quantity (20-30 mL from each eye), which allow processing for molecular and genetic techniques (76–80). miRNAs are small non-coding RNA molecules that they play essential roles as regulators of numerous pathways and biological processes, thus modulating the expression of target genes (53, 56, 60, 61). Changes in the expression of miRNAs have been related to the incidence, risk factors, development/progression of many eye diseases, including DR (51,52). This study was planned to find out, firstly, if the expression of the

miRNAs in tears is different in patients with T2DM, and secondly, if there are any miRNAs that are differently expressed in or T2DM patients with or without DR. In last instance, the miRNA with different expression in these patients could be used as biomarkers in early diagnosis to identify patients at increased risk of DR development and progression.

2. Objectives

2.1. General Objective

To study the differential expression profile of miRNAs in tears of subjects with Type 2 Diabetes Mellitus, with or without Diabetic Retinopathy.

2.2. Specific Objectives

1. To obtain the sociodemographic data, characteristics, and lifestyle of the participants, as well as the family and personal history of interest for the study in the groups and subgroups of participants.

2. To explore the visual acuity and ocular fundus of all participants, to ensure its suitability, to add the participants to the corresponding groups, and to characterize and staging the DR.
3. To identify and quantify the miRNAs in the tear samples of the study participants, to obtain the expression profile in patients with DM and comparing those with or without DR and the CG.
4. To establish the miRNAs that can potentially be predictive of the risk of suffering from DR and to progress in the disease.
5. In parallel, to determine the expression of the following genes: the *VEGF-A* gene and the *MMP2* gene, in blood, of the same patients.

MATERIAL AND METHODS

1. Study design and participants

It was carried out a prospective, multicenter, multidisciplinary, case-control study matched by age and gender, initially involving 225 participants recruited from 2016 to 2018 from the collaborative groups of the Spanish Network of Ophthalmic Research OFTARED (Institute of Health Carlos III, Madrid, Spain) at the following University Hospitals: Polytechnic Hospital La Fe (Valencia, Spain), Dr. Peset Hospital (Valencia, Spain), Clinic Hospital of Valladolid (Spain), Morales Meseguer Hospital of Murcia (Spain), as well as from the Centro Hospitalar Entre Douro e Vouga of Sta. Maria da Feira (Portugal) according with the inclusion/exclusion criteria listed on table 2.

To calculate the sample size for the study participants, the eNe 2.0 statistical program (GlaxoSmithKline S.A.) was used in order to get a statistical power of 80% and detect differences in the hypothesis contrast (Ho: p1=p2) using a

bilateral $\chi 2$ test for two independent samples, considering that the level of significance would be 5%.

Table 2. Inclusion and exclusion criteria of the study participants

INCLUSION CRITERIA	
T2DM PATIENTS	**CONTROLS**
• Age ≥ 18 years • Suffer from T2DM at least 5 years ago, with or without retinopathy • Not suffering from other severe ophthalmic or systemic disease and treatments	• Age ≥ 18 years • Not having diabetes (type 1 or 2) • Not suffering from severe ophthalmic or systemic disease and treatments
EXCLUSION CRITERIA	
T2DM PATIENTS	**CONTROLS**
• Having other eye and systemic diseases or treatments that may interfere with the study • Inability to understand the study and informed consent • Impossibility to cooperate with the study	• Having other eye and systemic diseases or treatments that may interfere with the study • Inability to understand the study and informed consent • Impossibility to cooperate with the study

T2DM: type 2 diabetes mellitus patients; CG: control group of healthy participants

The study was done according to the Helsinki Declaration on Human Experimentation (Helsinki 1964, updated version 2004), current European

guidelines for human research and the Portuguese and Spanish laws and recommendations for this type of studies.

Participants were informed about the study characteristics and researchers precisely explained the details of the study, giving them a written document. When they decided to participate, the informed consent was signed. Likewise, approval was obtained from the Ethics and Research Committee of the main study center, the Ophthalmic Research Unit "Santiago Grisolía"/FISABIO at the University Hospital Dr. Peset (Valencia).

After considering the inclusion/exclusion criteria through the interview and ophthalmological examination, the initially suitable 225 participants were classified into 2 groups, and the diabetic patients (T2DMG) were also subdivided as having (+DR) or not (-DR) retinopathy, as reflected in the figure 6, and the next schemes.

GROUPS OF PARTICIPANTS AT BASELINE

1. Patients with T2DM (**T2DMG; n=118**)
2. Healthy controls (**CG; n= 107**)

SUBGROUPS OF DIABETICS AT BASELINE

1. Patients without DR (**T2DMG-DR, n=63**)

2. Patients with DR (**T2DMG+DR, n=35**)

According to the interview, ophthalmic examination as well as the inclusion/exclusion criteria (table 2) for the study participants, an also having

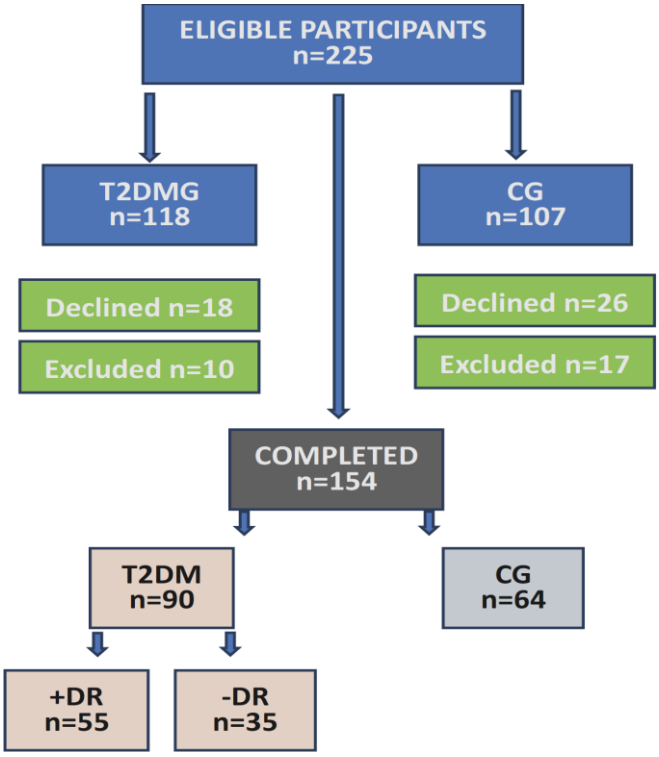

Figure 6. Flowchart with the recruitment characteristics, groups, subgroups, and final sample of the study participants.

into account the voluntary declined participations and the specifically excluded participants, the final sample of this study was 154 volunteers, that were classified into: 90 of the T2DMG (55 with DR, and 35 without DR) and 64 of the CG, as reflected in the flowchart (Fig. 6).

2. Sociodemographic Data and Ophthalmic Examination for the study participants

At baseline the sociodemographic data, main personal characteristics (DM duration and therapy, body mass index -BMI-; comorbidities, surgeries, lifestyle and toxic habits), and main treatments of the study volunteers were registered into the Microsoft Excel spreadsheet. Specific assessment of each patient family history was also done, with a record of data regarding DM, major comorbidities (hypertension blood pressure -HBP-, cardiovascular disease -CDV-) and other related events and its corresponding therapies, and an appointment was given to each participant for the following procedures: the ophthalmic examination and tear sample collection.

All patients underwent a systematized ophthalmic examination of both eyes, which included:

- **Best corrected visual acuity (BCVA)**, with Snellen optotypes and expressed according to the decimal logarithm of angular size in minutes of arc (logMAR).

- **Complete biomicroscopy (BMC),** in slit lamp (TOPCON ImageNet TRC-50JA; Topcon, Barcelona, Spain), discarding ocular associated pathology and verifying that there was no contraindication for pupillary dilatation.

- **Fundoscopy** (according to the ICO scale of severity classification). The assessment of the eye fundus was carried out with slit lamp and lens of 78 diopters or any other specifically used for each patient, according to the ICO criteria (27).

- **Retinographies** of the seven fields were taken from each patient with TOPCON ImageNet TRC-50JA (Topcon, Barcelona, Spain).

- **Intraocular pressure (IOP)** evaluation was done by means of a Goldmann applanation tonometer (GAT) (Haag-Streit AT 900; Haag-Streit Köniz, Switzerland).

The absence (-DR) or presence (+DR) of retinopathy was addressed in all participants that were diagnosed and classified by each ophthalmologist according to the morphological characteristics of the ocular fundus examination established in 2018 by the ICO severity scale report for DM and DME (27), into: 1) No apparent DR; 2) NPDR (mNPDR; modNPDR; sNPDR); 3) PDR; 4) DME (No apparent DME; Non center-involving DME; Center involving DME), as shown in the table 1 and figure 2. The T2DM participants was constituted by those patients who presented a previous DR/DME diagnosis, and the naïve participants that were diagnosed at the time of recruitment.

3. miRNA expression in tears

3.1. Sampling Procedures - Tear film Collection Technique

After drying the eyelashes to avoid basal remaining tearing, tear samples from the right eye (RE) and left eye (LE) were collected from the inferior

meniscus with a capillary tube (approximately 30 mL), transferred to a labelled cryotube and stored at -80ºC until processing to RNA extraction and miRNAs sequencing. Reflex tears were obtained by non-invasive methods by gently rubbing the inferior palpebral meniscus until tears were present and collected (see the figure below).

Figure 7. Reflex tears collecting method for the study participants. With the microcapillary pipette, a gentle rubbing was done on the inferior meniscus and external canthus of each eye.

This protocol was designed specifically for this work on the basis of our previous studies (49,72,76–80).

3.2. RNA Extraction and Quantification in Tear Samples

RNA extraction: the first part of the protocol was carried out in the laboratories of the Ophthalmic Research Unit "Santiago Grisolia"/FISABIO at

the Dr. Peset University Hospital of Valencia, Spain. Using the miRCURY™ RNA Isolation Kit – Biofluids (Exiqon, Vedbaek, Denmark), following the manufacturer's instructions. It was performed protocol A (Standard protocol for RNA isolation) which consists of 9 steps. With this kit, all RNAs under 1000 nucleotides are extracted, both messenger RNA (mRNA) and miRNA, piwi RNA (piRNA) and other small interfering RNAs (siRNA).

The protocol was, as follows:

1. Transfer each tear sample to an Eppendorf tube.
2. Add RNAse-free water to complete a volume of 200µL
3. Add 60µL of BF lysis solution
4. Vortex for 5 seconds and incubate for 1 minute at room temperature (RT)
5. Centrifuge for 3 minutes at 11,000g
7. Transfer the supernatant to a new 2mL tube
8. Add 270µL of isopropanol
9. Vortex mix for 5 seconds

10 Place the columns with silica membrane (microRNA Mini Spin Column BF) in a tube, label and load each sample onto the column correspondent Incubate for 2 minutes at RT.

11. Centrifuge for 30 seconds at 11,000g
12. Discard the filtrate and replace the columns in the appropriate tube
13. Add 100µL of Wash Solution 1 BF to each column
14. Centrifuge for 30 seconds at 11,000g
15. Discard the filtrate and replace the columns in the appropriate tube
16. Add 700µL of Wash Solution 2 BF to each column
17. Centrifuge for 30 seconds at 11,000g.
18. Discard the filtrate and replace the columns in the appropriate tube
19. Add 250µL of Wash Solution 2 BF to each column
20. Centrifuge for 2 minutes at 11,000g to completely dry the membranes.
21. Place each column in a new 1.5mL tube (with cap)
22. Add 30µL of RNase-free water directly into the membranes
23. Incubate for 1 minute at RT.
24. Close the cap and centrifuge for 1 minute at 11,000g.
25. Store the purified RNA at -80ºC until processing.

The following figure schematically shows the protocol followed, whose approximate duration is 40-70 minutes. Once the total RNA was obtained, quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In this way it was possible to know the concentration of total RNA in each sample.

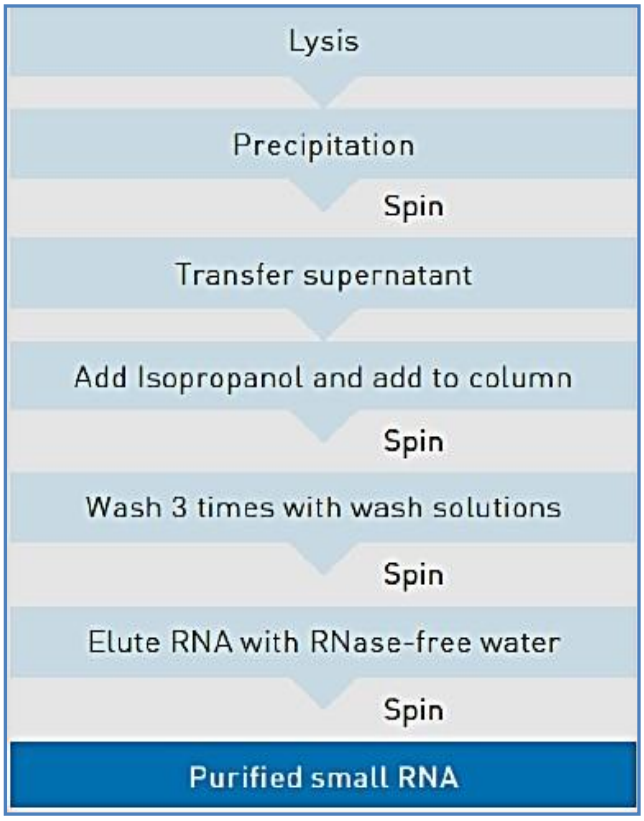

Figure 8. Description of the protocol that was carried out for the study procedures. *Taken from the manufacturer's manual (EXIQON Inc., Woburn, MA, USA).*

The samples were frozen at -80ºC and, later, sent to the Genomics and Diabetes Unit of the INCLIVA Health Research Institute, where the following analyzes were carried out.

3.3. Libraries and Next Generation Sequencing (NGS)

The miRNA sequencing (NGS) was performed at the Genomics and Diabetes Unit of the INCLIVA Health Research Institute, University of Valencia, Spain. These experiments were performed on a random subsample of 36 participants:

- T2DMG+DR (n=12)
- T2DMG-DR (n=12)
- CG (n=12)

The miRNA expression profile in tears of the three sub-samples was analyzed by NGS, first using the TruSeq Small RNA Library Prep Kit (ref. 15004197; Illumina Inc., San Diego, CA, USA) to prepare the miRNA libraries.

From the total cRNA extracted, the libraries for microRNA sequencing were built using appropriate kits for small quantities of RNA. This kit contains all

the reagents necessary for the conversion of small RNAs into indexed libraries for massive sequencing on the Illumina platform. The procedure it was performed according to the manufacturer's protocol:

1. Ligation of the 3' SR adapter:

® Due to the low concentration of starting RNA, the 3' adapter SR was added by previously preparing a ½ dilution.

® Mix the following components in a sterile free PCR tube of nucleases: RNA 1-6 µL 3' adapter SR 1 µL Nuclease-free water Variable Total volume in the tube 7 µL Incubate in thermocycler for 2 minutes at 70ºC.

® Place tubes on ice

® Add and mix the following reagents: 3' Ligation Buffer (2X) 10 µL 3’ Ligation Enzyme Mix 3 µL Total volume in the tube 20 µL

® Incubate for 1 hour at 25ºC in a thermocycler.

2. Primer hybridization for reverse transcription:

® This step is important to prevent the formation of dimers of adapter, which will interfere with sample sequencing.

® Due to the low concentration of starting RNA, the first SR is diluted ½ .

® Add the following reagents and mix: Reverse Transcription Primer 1 µL Nuclease-free water 4.5 µL. Total volume in the tube 25.5 µL

® Put the samples in a thermocycler with the cover preheated (>85ºC) and execute the following program: 5 minutes 75ºC / 15 minutes 37ºC / 15 minutes 25ºC / Hold 4ºC

3. Ligation of the 5' SR adapter:

® Due to the low concentration of starting RNA, a sample was prepared ½ dilution of the 5’ SR adapter.

® Resuspend the 5’ SR adapter in 120µL of water free of nucleases, 5 minutes before use.

® Aliquot the 5' SR adapter into a nuclease-free PCR tube of 200µL according to the number of samples to be analyzed in the experiment

® Incubate the adapter in a thermal cycler for 2 minutes at 70ºC.

® Place tubes immediately on ice.

® Keep tubes on ice and use denatured adapter within 30 min after denaturation. (Store unused resuspended 5' SR adapter at -80°C. Denature aliquots before use)

® Add and mix the following reagents in the tubes at the end of the first hybridization step: Adapter 5’ SR (denatured) 1 µL. 5' Ligation Buffer (10X) 1 µL. 5’ Ligation Enzyme Mix 2.5 µL. Total volume in the tube 30 µL

® Incubate for 1 hour at 25ºC in a thermal cycler. ·

4. Reverse transcription:

® Mix the following reagents in a sterile PCR tube free of nucleases: Ligated RNA Adapter (from previous step) 30 µL First Strand Synthesis Buffer 8 µL Murine RNase inhibitor 1 µL. ProtoScript II Reverse Transcriptase 1 µL Total volume in the tube 40 µL

® Incubate for 1 hour at 50ºC in a thermocycler.

® Proceed immediately with amplification.

5. Amplification:

® Add and mix the following reagents in the tubes from the previous step: LongAmp Taq 2X Master Mix 50 µL. Primer SR 2.5 µL. Index (X) Primer* 2.5 µL. Nuclease-free water 5 µL. Total volume in the tube 100 µL

*Run PCR under the following conditions:

Table: 3. PCR Conditions during libraries construction

Step	Temperature	Time	Cycles
Initial denaturation	94ºC	30 sec	1
Denaturation Annealing Extension	94ºC 62ºC 70ºC	15 sec 30 sec 15 sec	15
Final extension	70ºC	5 min	1
Hold	4ºC	∞	

PCR: polymerase chain reaction; sec: seconds; min: minutes

* Store libraries at -20º C

Once the cDNA libraries were obtained, the quality of the microRNA fragments and other species corresponding to the "small RNA" fraction obtained were evaluated. On the one hand, marking primers with FAM-type fluorophores and observing the ssDNA fragments by capillary sequencing. And on the other, by means of a high-resolution capillary electrophoresis system (Qiaxcel). After this step, known amounts of the library were loaded on polyacrylamide gels (PAGE 10%) that allowed us to select the size of the micro/small RNAs region (140-150pb), mainly eliminating unspecific fragments such as adapter dimers that could have been produced during the preparation of the libraries. Once the bands were cut, the DNA was

extracted. The libraries were reamplified by short PCR and the desired fragments were purified by magnetic beads (AMPurebeads). Once the purified libraries were obtained, they were quantified by means of quantitative PCR, calculating their total amount by means of an absolute quantification method and standard curves of known concentrations. Finally, the samples were diluted to a concentration of 2nM, and equimolar pools were made, ready for sequencing.

After preparation of the miRNA libraries, samples were sequenced by means of the NextSeq 500/550 High Output v2 kit (ref.: FC-404-2005; Illumina), in order to identify miRNA differentially expressed between groups.

3.4. Validation of miRNA differential expression by RT-PCR

Expression of selected miRNAs by real-time PCR (qRT-PCR): in the total amount of samples (n=154), 5µL of total RNA previously isolated were converted into cDNA using the TaqMan Advanced miRNA cDNA Synthesis kit (ref. A28007, Thermo Fisher Scientific Inc., Waltham, MA, USA). This proceeding includes several steps (see the tables 4-8, and the figure 9):

1- Poly(A) tailing reaction.

Table 4. Conditions for the Poly(A) tailing reaction

Step	Temperature (ºC)	Time
Polyadenylation	37	45 minutes
Stop reaction	65	10 minutes
Hold	4	Hold

2- Adaptor ligation reaction

Table 5. Conditions for the adaptor ligation reaction

Step	Temperature (ºC)	Time
Ligation	16	60 minutes
Hold	4	Hold

3- Reverse Transcription

Table 6. Conditions for the reverse transcription reaction

Step	Temperature (ºC)	Time
Reverse transcription	42	15 minutes
Stop reaction	85	5 minutes
Hold	4	Hold

Once we obtained the cDNA and prior to the miRNA expression analysis, we performed a pre-amplification of miRNA according to the following conditions, described in the table 7:

4- miRNA pre-amplification

Table 7. Conditions for the miRNA pre-amplification reaction

Step	Temperature (ºC)	Time	Cycles
Enzyme activation	95	5 minutes	1
Denature	95	3 seconds	14
Anneal/Extend	60	30 seconds	
Stop reaction	99	10 minutes	1
Hold	4	Hold	1

Finally, we performed the miRNA expression analysis by means of qRT-PCR, using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR conditions are shown in table 8.

5- qRT-PCR

Table 8. PCR conditions for miRNA expression analysis

Step	Temperature (ºC)	Time	Cycles
Enzyme activation	95	20 seconds	1
Denature	95	3 seconds	40
Anneal/Extend	60	30 seconds	
Hold	4	Hold	1

The figure 9 shows the workflow that allowed us to quantify mature miRNAs using RT-PCR, ideal for analysis of multiple miRNA targets from a single sample.

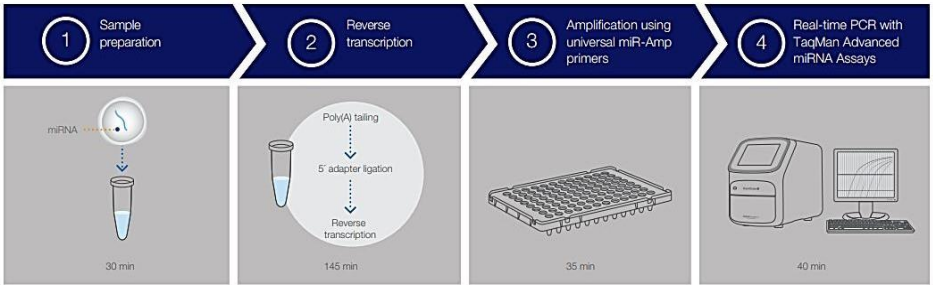

Figure 9. The TaqMan Advanced miRNA Assay workflow. *Taken from http://thermofisher.com/advancedmirna*

In summary, the following scheme (Fig. 10) shows the pipeline for the identification of the tear film miRNAs, as potential diagnostic and prognostic biomarkers for the DR.

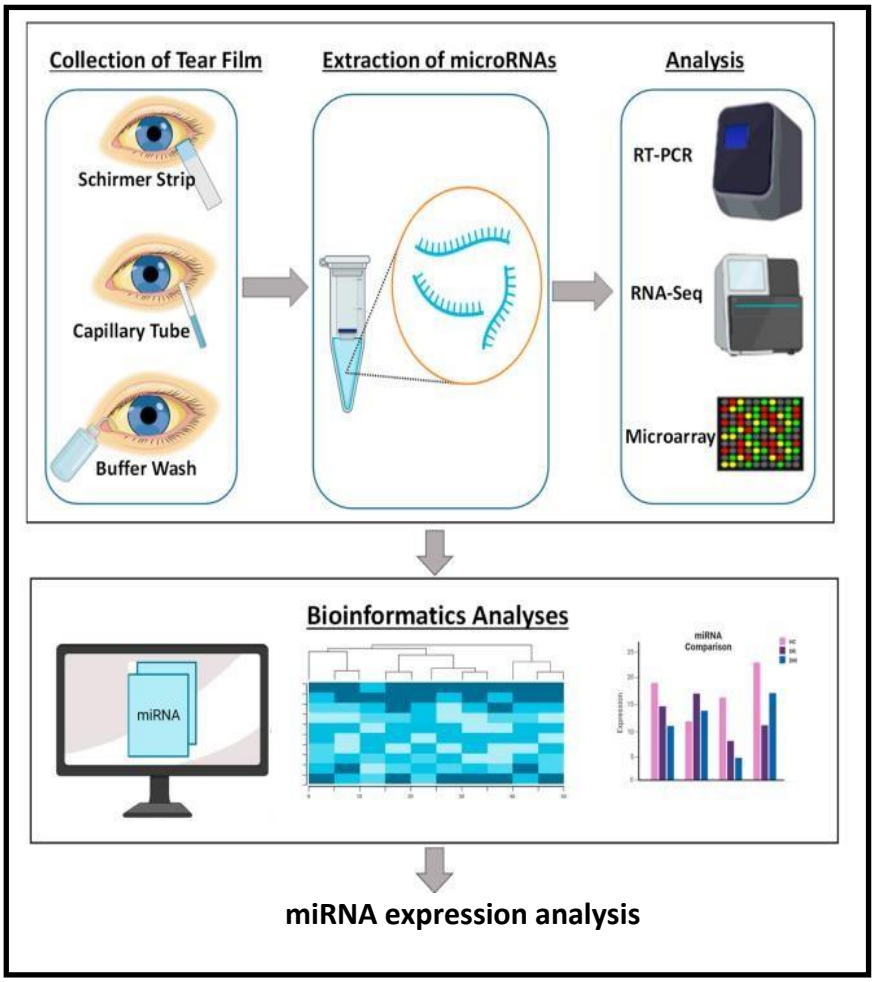

Figure 10. Overall pipeline for the discovery of tear film miRNAs as biomarkers in DR. *Adapted from https://www.mdpi.com/1422-0067/24/4/3694* (83).

4. Gene Expression Analysis

To better understand the role of angiogenesis/neovascularization and the changes in the extracellular matrix in the retina during T2DM, the blood expression of the genes *VEGF* and *MMP2* were analyzed according to the following protocol:

1. RNA isolation

The total RNA was obtained by the Trizol method (125). Briefly:

1. A blood sample is collected in a 4.5mL EDTA tube.
2. Place the EDTA tubes with blood in a rack and allow the serum to settle out (approximately 60-120 minutes).
3. Carefully transfer the serum to a sterile 15 mL plastic tube, using a Pasteur pipet. Drain with a micropipette.
4. Centrifuge for 15 minutes at 1500-2000 rpm and room temperature.
5. Remove supernatant, fill tube with Phosphate-buffered saline (PBS)
6. Resuspend the pellet in the vortex for 10 seconds.
7. Centrifuge for 10-15 minutes at 1500-2000 rpm at room temperature.
8. Remove the supernatant and add between 500µL of TRIZOL.
9. Mix well by vortexing until pellet is completely dissolved in TRIZOL.
10. Transfer to a 1.5mL eppendorf tube and label appropriately.
11. Freeze the tubes at -80ºC and continue the next day.

12. Thaw and homogenize samples with a 1000µL pipette to ensure cell disruption.
13. Transfer to a 2mL tube.
14. Add 100 µL of chloroform.
15. Mix by inversion (vigorously, about 30 times). Samples will turn a milky pink color.
16. Let stand for 2-3 minutes.
17. Centrifuge at 13,000 rpm for 15 minutes at room temperature. Once the centrifuge ends, the tubes display the following characteristics, that are reflected in the figure 11..
18. Collect the aqueous phase, without collecting anything from the interphase or the organic phase.
19. Add 250 µL of isopropanol.
20. Add 2 µL of glycogen (optional, helps precipitate RNA, and gives it a whitish color).
21. Shake the tubes manually (tapping with your finger) and keep them at -20ºC overnight.
22. Remove from the freezer and centrifuge at 13,000 rpm for 15 minutes at room temperature.
23. Remove the liquid being careful not to drag the pellet.
24. Add 500 µL cold 75% EtOH
25. Vortex for 3 sec (check pellet is resuspended)
26. Centrifuge for 15 min at 4ºC and 13,000 rpm
27. Remove the EtOH with a micropipette
28. Keep in an oven at 37ºC for 2 min (to evaporate the rest of EtOH)
29. Resuspend in 20 µL of PCR grade H_2O
30. Measure the integrity and amount of RNA.

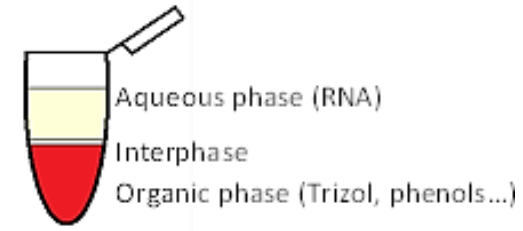

Figure 11. Different phases obtained after centrifugation of the blood sampling

2.RNA to cDNA

1. Use 300ng of total RNA per 20µL reaction.
2. Prepare the RT-reaction mix according to the required number of reactions (table 9).

Table 9. **Preparation of RT-reaction mix**

Component	RT reaction
2X RT Buffer Mix	10 µL
20X RT Enzyme Mix	1 µL
RNA sample	300 ng
Nuclease-free H2O	up to 20 µL
Total per reaction	20 µL

3. Aliquot the RT reaction mix into a 96-well plate.
4. Seal the plate.
5. Briefly centrifuge the plate to spin down the contents and to eliminate any air bubbles.

6. Place the plate on ice until you are ready to start the reverse transcription reaction.
7. In a thermocycler, Incubate the reaction for 37°C for 60 minutes. Stop the reaction by heating to 95°C for 5 minutes and hold at 4°C.
8. Store the cDNA in a freezer at -20°C.

3. Gene expression

Blood gene expression in the study participants was analyzed by RTl-PCR, using a 7900HT Sequence Detection System and TaqMan gene expression assays (Applied Biosystems). We assayed the samples in duplicate and used the GAPDH gene as internal control (HK, housekeeping gene). This protocol is shown in the following tables 10 and 11 and figure 11:

1. Prepare 2 reaction mixes (9µL each), one for target and another one for housekeeping, as indicated in table 10.

Table 10. Gene expression reaction mix

Component	Volume
Taqman Gene Expression Master Mix	5µL
TaqMan Gene Expression probe	0.5µL
Nuclease-free H2O	3.5µL
Total per reaction	9µL

2. Adjust the quantity according to the number of reactions.
3. Add 9µL of reaction mix per well and 1µL of cDNA (target or housekeeping), following the scheme in figure 12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ADN1 HK	ADN2 HK	ADN3 HK	ADN4 HK	ADN5 HK	ADN6 HK	ADN7 HK	ADN8 HK	ADN9 HK	ADN10 HK	ADN11 HK	ADN12 HK
B	ADN1 HK	ADN2 HK	ADN3 HK	ADN4 HK	ADN5 HK	ADN6 HK	ADN7 HK	ADN8 HK	ADN9 HK	ADN10 HK	ADN11 HK	ADN12 HK
C	ADN1 TARGET	ADN2 TARGET	ADN3 TARGET	ADN4 TARGET	ADN5 TARGET	ADN6 TARGET	ADN7 TARGET	ADN8 TARGET	ADN9 TARGET	ADN10 TARGET	ADN11 TARGET	ADN12 TARGET
D	ADN1 TARGET	ADN2 TARGET	ADN3 TARGET	ADN4 TARGET	ADN5 TARGET	ADN6 TARGET	ADN7 TARGET	ADN8 TARGET	ADN9 TARGET	ADN10 TARGET	ADN11 TARGET	ADN12 TARGET
E	ADN13 HK	ADN14 HK	ADN15 HK	ADN16 HK	ADN17 HK	ADN18 HK	ADN19 HK	ADN20 HK	ADN21 HK	ADN22 HK	ADN23 HK	ADN24 HK
F	ADN13 HK	ADN14 HK	ADN15 HK	ADN16 HK	ADN17 HK	ADN18 HK	ADN19 HK	ADN20 HK	ADN21 HK	ADN22 HK	ADN23 HK	ADN24 HK
G	ADN13 TARGET	ADN14 TARGET	ADN15 TARGET	ADN16 TARGET	ADN17 TARGET	ADN18 TARGET	ADN19 TARGET	ADN20 TARGET	ADN21 TARGET	ADN22 TARGET	ADN23 TARGET	ADN24 TARGET
H	ADN13 TARGET	ADN14 TARGET	ADN15 TARGET	ADN16 TARGET	ADN17 TARGET	ADN18 TARGET	ADN19 TARGET	ADN20 TARGET	ADN21 TARGET	ADN22 TARGET	ADN23 TARGET	ADN24 TARGET

Figure 12. 96-well plate template for gene expression analysis. HK: housekeeping gene; Target: VEGFA or MMP2 genes.

4. Seal the plate and spin 5 seconds.
5. Run the qRT-PCR following the standard protocol (table 11).

Table 11. Standard protocol for the qRT-PCR

Temperature	Time	Cycles
50ºC	2 minutes	1
95ºC	10 minutes	1
95ºC	15 seconds	40
60ºC	1 minute	
4ºC	hold	Hold

5. Statistical analysis and Bioinformatic Proceedings

5.1. General Statistical Proceedings

Analysis was performed with the IBM SPSS Statistics for Windows 22.0 program (IBM Corp., Armonk, NY, USA). The normality of the quantitative variables was checked by the Kolmogorov-Smirnov test. Qualitative variables were compared using the Pearson's chi-square test. Depending on the data distribution of quantitative variables, two means were compared using the Student's t-test or the Mann-Whitney U test, and more than two means were compared using the ANOVA or the Kruskal Wallis test. Correlation between quantitative variables were analysed by the Pearson correlation coefficient or the Spearman's rank correlation coefficient. Multivariable general linear models were used to adjust differences in continuous variables by potential confounders as well as to estimate the corresponding adjusted means. The expression of target genes (VEGFA and MMP2) was determined by means of the $\Delta\Delta$Ct method. Then, we graphically represented the fold change in the experimental groups (T2DM, T2DM+DR, T2DM-DR) compared with the

control group. All statistical analysis were performed assigning a significance level of 0.05.

5.2. Bioinformatic Proceedings

First, those non-coding RNAs that were previously described in the ENSEMBL database were selected and characterized for bioinformatic analyses (normalization, differential expression, significance), by using both the Limma and edgeR packages deposited in Bioconductor (www.bioconductor.org).

Next, a predictive analysis based on receiver operating characteristic (ROC) curves was performed to select those miRNAs showing an area under the curve (AUC) greater than 0.75. Subsequently, an analysis of the main components (PCA) was performed. Later, the candidante genes were determined using the R miRFA pipeline [28], whenever possible, which is supported by data from the DIANA-TarBase v7, DIANA-microT-CDS and TargetScan v7.1 databases. Additionally, UniProt and Gene Ontology

databases have been used to search for terms associated with the retinal microangiopathy in T2DM patients.

RESULTS

1. Prevalence, Demographics, Risk factors and Clinical Laboratory Data

Final study participants were a community-based sample of 154 volunteers living in Portugal or Spain, matched on age, sex and ethnicity, and between 26-82 years of age, that were divided into two groups: the CG (n=54) and a the T2DMG (n=90) (see the figures 1 and 6). Main causes of the important reduction in the number of participants (32%) were: withdraw consent due to personal issues, disease progression, adverse events, insufficient medical data, tear film samples deterioration (when shipping/storing at the laboratory or at processing), and irregular or missing conclusive data.

Definitively, the T2DM group of participants was subdivided into patients with DR (T2DM+DR) (n=35, 38.9%) and without DR (T2DM-DR) (n=55, 61,1%), as shown in the figures 1 and 6.

The table 12 shows the main demographic, risk factors and personal/familial characteristics of the study participants.

Table 12. Sociodemographics and patient characteristics / risk factors of the study participants

	CG	T2DM		P value
		without DR	with DR	
N	64	55	35	
Age (years)	58.3 ± 18.4	68.0 ± 10.2	66.8 ± 8.4	0.040
Female (%)	57.8	54.5	34.3	0.068
BMI (kg/m^2)	26.0 ± 4.5	28.8 ± 5.0	29.7 ± 4.9	0.002*
Exercise (%)[1]	38.1	43.6	26.5	0.265
Smoker (%)[2]	12.7	11.1	6.2	0.813
Alcohol (%)[3]	19	16.1	20.8	0.095

T2DM: Type 2 diabetes mellitus; DR: diabetic retinopathy; BMI: body mass index;
1: percentage of subjects who perform physical exercise; 2: percentage of subjects who consume tobacco on a regular basis; 3: percentage of subjects who consume alcohol on a regular basis; * Statistically significant ($p<0.05$).

2. Ophthalmologic Examination and Systemic Family history evaluation.

The results of the quantitative variables best corrected visual acuity (BCVA), and IOP are expressed as mean ± SD. The results of the qualitative variables – family history of DM, hypertension blood pressure (HBP) and cardiovascular disease (CVD) - are expressed in percentage and frequency,

comparing those in patients with DR and healthy ones. All these data are reflected in following table.

Table 13. Ophthalmologic and systemic data (comorbidities) from the familial background of the study participants

Parameters	CG	T2DM		P value
		Without DR	With DR	
N	64	55	35	
DM FH (%)	36.5	41.8	61.3	0.071
HBP FH (%)	46	41.8	51.6	0.679
CVD FH (%)	27	37	35.5	0.471
VA RE (IM)	**0.1 ± 0.06**	**0.8 ± 0.07**	**0.3 ± 0.20**	**0.003***
VA LE (IM)	**0.1 ± 0.07**	**0.2 ± 0.10**	**0.3 ± 0.30**	**0.015***
IOP RE (mmHg)	14.9 ± 2.4	15.4 ± 2.8	15.2 ± 3.3	0.633
IOP LE (mmHg)	**14.6 ± 2.8**	**15.7 ± 3.1**	**15.9 ± 3.2**	**0.047***

T2DM: Type 2 diabetes mellitus DR: diabetic retinopathy; FH: Family History; DM: Diabetes Mellitus; AHT: Arterial Hypertension; CVD: Cardiovascular Disease; VA: Visual Acuity; IM: LogMAR; IOP: intraocular pressure; RE: Right Eye; LE: Left Eye

** Statistically significant ($p<0.05$).*

3. Genetics in tears

3.1 Total RNA extraction

It was quantified the total amount of RNAs and made the comparison between the T2DMG and the CG, as showed in the graphic below:

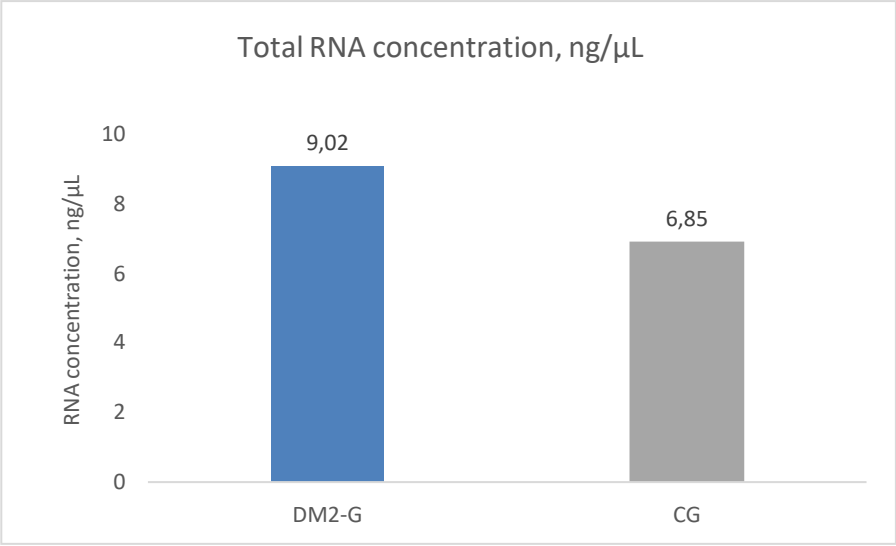

Figure 13. Total RNA concentration in tears from T2DM group and control group; T2DM vs the CG (9,02±3,08 vs 6,85±3,58 ng/µL; p=0,022).

All samples (n=154) had a sufficient total small RNA quantity to perform the subsequent experiments by NGS and qRT-PCR.

3.2. NGS

After obtaining the libraries, the NGS was carried out in a sub-sample of the 3 groups [T2DM+RD (n=12); T2DM-DR (n=12) and CG (n=12)] and through bioinformatic analysis there were identified 179 miRNAs in tears of the subjects of the study. Some of these were only found in one of the study groups (supplement 1). Others (n=78) were detected in, at least, 2 groups, so we could analyse differences in miRNA expression, between the corresponding groups, as reflected in the figure 14, as well as in the supplement 2.

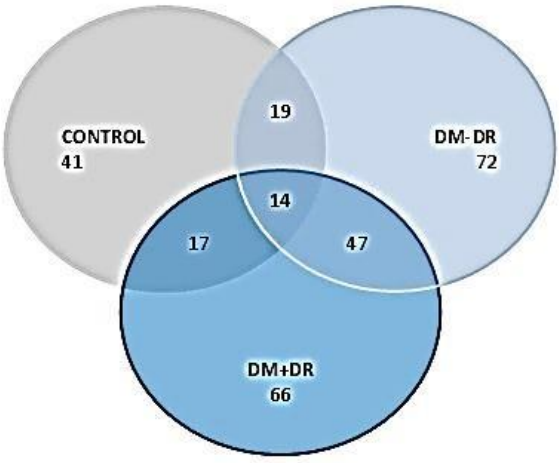

Figure 14. Number of miRNAs identified in tears of the study participants.

3.2.1 Comparison of the tear miRNAs expression between groups (only statistically significant differences): CG vs T2DMG

The figure below shows the differences in miRNA expression between the CG (blue) and the T2DMG (orange)

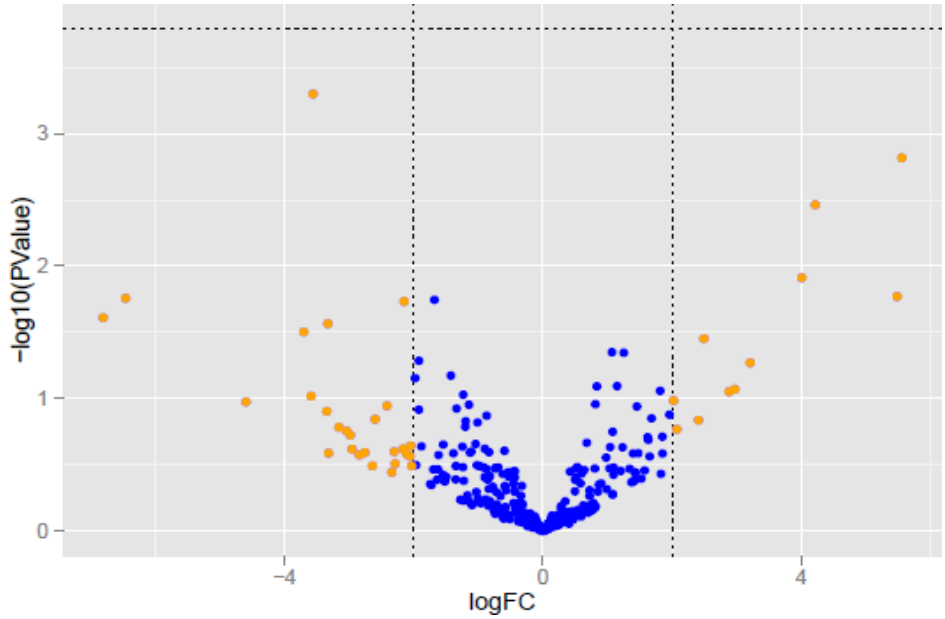

Figure 15. Expression profile of tear miRNAs between the CG (blue) and the T2DMG (orange)

The table 14 shows the miRNAs with significantly different expression between the CG and the T2DMG (+DR or -DR).

Table 14 Comparison of miRNA expression between the CG and the T2DMG (only statistically significant differences)

T2DMG *vs* CG[1]	P
hsa-miR-155-5p	0,00050289
hsa-miR-4488	0,00215255
hsa-miR-4516	0,00267474
hsa-miR-92b-5p	0,00352394
hsa-miR-15b-5p	0,01224091
hsa-miR-139-5p	0,01696883
hsa-miR-203	0,04464176
hsa-miR-378a-3p	0,04503972
hsa-miR-10a-5p	0,00021554
hsa-miR-195-3p	0,00344617
hsa-miR-135a-5p	0,01745868
hsa-miR-320ª	0,01795334
hsa-miR-342-5p	0,01846176
hsa-miR-486-5p	0,02715084

T2DMG: type 2 diabetes mellitus group; CG: control group;
1. miRNAs in blue corresponded to those upregulated in T2DM patients;
miRNAs in black corresponded to those downregulated in T2DM patients.

3.2.2 Comparison of miRNA expression between groups (only statistically significant differences): CG vs T2DM-RD

The figure below shows the differences in miRNA expression between controls (blue) and type 2 diabetics without diabetic retinopathy (orange).

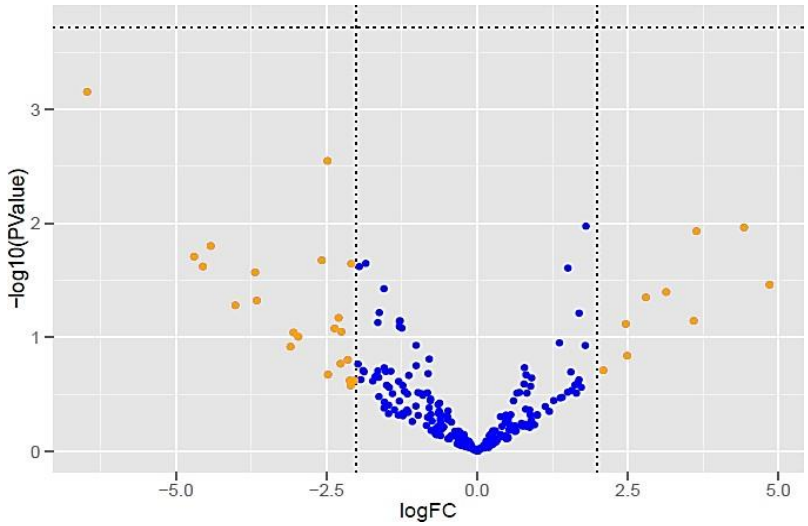

Figure 16. Expression profile of miRNAs between controls (blue) and type 2 diabetics without diabetic retinopathy (orange)

The table 15 shows the miRNAs with significantly different expression between control subjects and T2DM patients without DR.

Table 15 Comparison of miRNAs expression between the CG and the T2DMG (without DR) (only statistically significant differences)

T2DMG-DR *vs* CG[1]	P
hsa-miR-155-5p	0,00048824
hsa-miR-15b-5p	0,00405801
hsa-miR-375	0,01056473
hsa-miR-708-3p	0,01085626
hsa-miR-1260a	0,01168696
hsa-miR-184	0,02463651
hsa-miR-92b-5p	0,03447955
hsa-miR-10a-5p	0,00022013
hsa-miR-452-5p	0,00069978
hsa-miR-186-5p	0,00282549
hsa-miR-34a-5p	0,01578009
hsa-miR-324-3p	0,01956076
hsa-miR-195-3p	0,02066843
hsa-miR-27a-5p	0,02106449
hsa-miR-103a-3p	0,02243327
hsa-miR-30e-5p	0,02258532
hsa-miR-29b-2-5p	0,02387301
hsa-miR-342-5p	0,02391494
hsa-miR-193b-5p	0,02683451

T2DMG: type 2 diabetes mellitus group; CG: control group
1. miRNA in blue corresponded to those upregulated in T2DM; miRNA in black corresponded to those downregulated in T2DM.

3.2.3 Comparison of miRNA expression between groups (only statistically significant differences): CG vs T2DM+DR

The figure below shows the differences in miRNA expression between the CG (blue) and the T2DMG (orange).

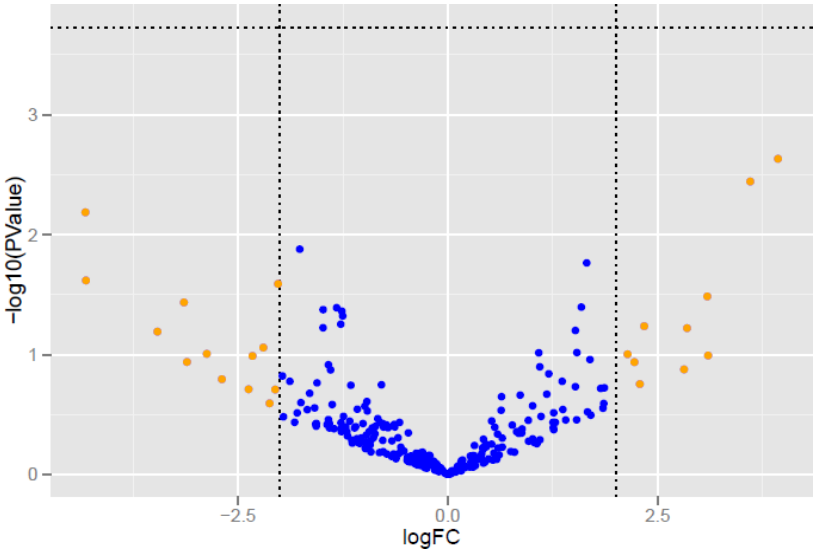

Figure 17. Expression profile of miRNAs between the CG (blue) and the T2DMG with DR (orange)

The table 16 shows the miRNAs with significant different expression between control subjects and T2DM patients with DR.

Table 16 Comparison of miRNA expression between the CG and the T2DMG with DR (only statistically significant differences).

T2DM+DR vs CG[1]	P
hsa-miR-15b-5p	0,00038556
hsa-miR-155-5p	0,00041997
hsa-miR-342-3p	0,00233141
hsa-miR-27a-5p	0,00360745
hsa-miR-423-3p	0,01721141
hsa-miR-328	0,0327679
hsa-miR-10a-5p	0,00010335
hsa-miR-195-3p	0,00107758
hsa-miR-451a	0,00651537
hsa-miR-203	0,01323233
hsa-miR-211-5p	0,02409484
hsa-let-7a-3p	0,02572669
hsa-miR-375	0,04351622
hsa-miR-184	0,04767438
hsa-miR-204-3p	0,04781022
hsa-miR-324-3p	0,04801066
hsa-miR-708-3p	0,04923371

T2DMG: type 2 diabetes mellitus group; CG: control group.
1. miRNA in blue = upregulated in T2DMG; miRNA in black = downregulated in T2DMG.

3.2.4 Comparison of miRNA expression between groups (only statistically significant differences): T2DMG-DR vs T2DMG+DR

The figure below shows the differences in miRNA expression between T2DM with DR (blue) and without DR (orange)

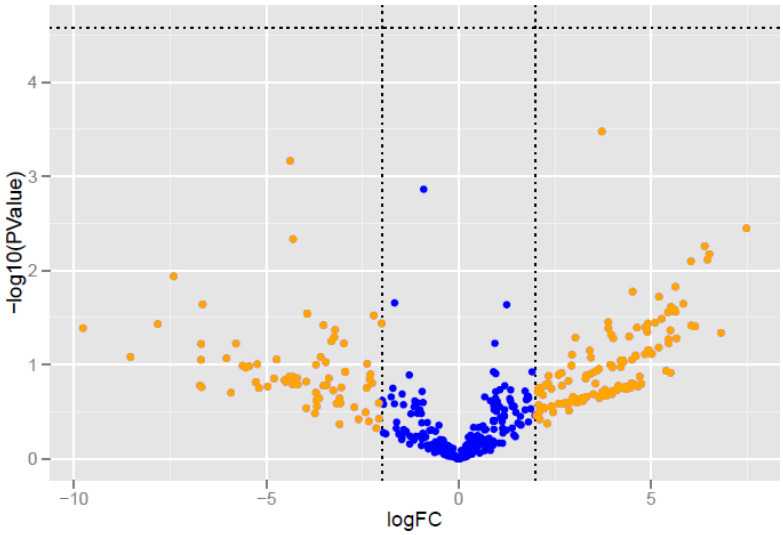

Figure 18. Expression profile of miRNAs between the T2DMG (blue) and the T2DMG without DR (orange)

The following table 17 shows the miRNAs with significant different expression between T2DMG with DR and the T2DMG without DR.

Table 17 Comparison of miRNA expression between the T2DMG with DR and the T2DMG without DR (only statistically significant differences)

T2DM+DR vs T2DM-DR[1]	P
hsa-miR-147b	0,00033305
hsa-miR-31-5p	0,00356039
hsa-miR-34a-5p	0,00550053
hsa-miR-4436b-3p	0,00667778
hsa-miR-3158-3p	0,0076641
hsa-miR-508-3p	0,00797464
hsa-miR-155-5p	0,01040889
hsa-miR-450b-5p	0,01488504
hsa-miR-20b-5p	0,01673733
hsa-miR-211-5p	0,01899113
hsa-miR-1287	0,02255178
hsa-miR-203	0,02305019
hsa-miR-504	0,02427954
hsa-miR-455-5p	0,02587983
hsa-miR-505-3p	0,02702551
hsa-miR-30c-2-3p	0,02736074
hsa-miR-550a-3-5p	0,0277247
hsa-miR-15b-5p	0,03226176
hsa-miR-651	0,03254246
hsa-miR-720	0,03528564
hsa-miR-675-3p	0,03571653
hsa-miR-4662a-5p	0,0365617
hsa-miR-942	0,03822863
hsa-miR-330-5p	0,03836754
hsa-miR-1278	0,03912887
hsa-miR-30b-3p	0,04009894
hsa-miR-4446-3p	0,04031481

hsa-miR-19a-3p	0,04039565
hsa-miR-130b-5p	0,04109527
hsa-miR-92b-5p	0,04321649
hsa-miR-27b-5p	0,0448958
hsa-miR-3126-5p	0,04586702
hsa-miR-501-3p	0,04778805
hsa-miR-342-3p	0,0006826
hsa-miR-148a-3p	0,00137003
hsa-miR-27a-5p	0,00463293
hsa-miR-423-5p	0,02206753
hsa-miR-9-3p	0,02287876
hsa-miR-195-3p	0,02605848
hsa-miR-4794	0,02879332
hsa-miR-493-3p	0,02879332
hsa-miR-550a-3p	0,02879332
hsa-miR-204-3p	0,03643016
hsa-miR-3648	0,03705494
hsa-miR-625-5p	0,03804594
hsa-miR-4638-3p	0,04095184
hsa-miR-451a	0,04272808

T2DMG: type 2 diabetes mellitus group; +DR: with retinopathy; -DR: without retinopathy
1. miRNA in blue = upregulated in T2DM; miRNA in black = downregulated in T2DM.

After comparison of miRNAs expression between groups, we focused on the 4 most significant miRNAs – **hsa-miR-15b-5p, hsa-miR-155-5p, hsa-miR-10a-5p and hsa-miR-195-3p** - for subsequent validation by qRT-PCR. These

miRNAs were the 2 most significant upregulated (hsa-miR-15b-5p and hsa-miR-155-5p) and the 2 most significant downregulated (hsa-miR-10a-5p and hsa-miR-195-3p) miRNAs between T2DM+DR vs CG.

3.3 Validation by qRT-PCR

Expression of selected miRNAs by qRT-PCR: in the total amount of samples (n=154) was performed, to validate the results obtained by NGS. In all comparisons, controls were used as comparison base group.

3.3.1 Comparison of miRNA expression between groups: CG vs T2DMG

3.3.1.1 miRNA miR-15b-5p

The differential tear expression profile of the miR-15b-5p, between the T2DMG and the healthy individuals as the CG is reflected in the following figure 19. Data show that the highest tear concentration corresponded to the diabetic patients.

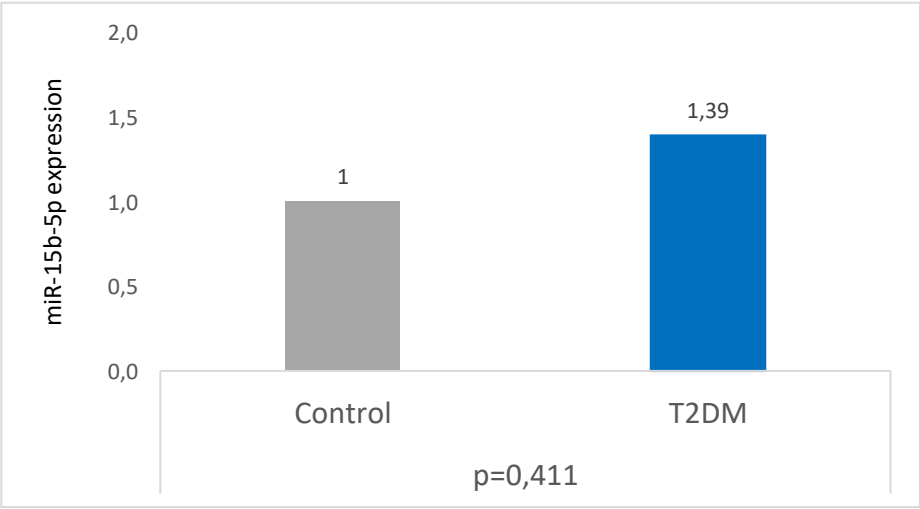

Figure 19. miR-15b-5p expression comparison between the T2DMG and the CG (the healthy controls were used as comparison base group).

3.3.1.2 miRNA miR-155-5p

The miR-155-5p differential expression profile in tears from the T2DMG and the CG is shown in the following figure 20. Our results shown that the highest expression levels of the miR-155-5p in tears, corresponded to the diabetic patients.

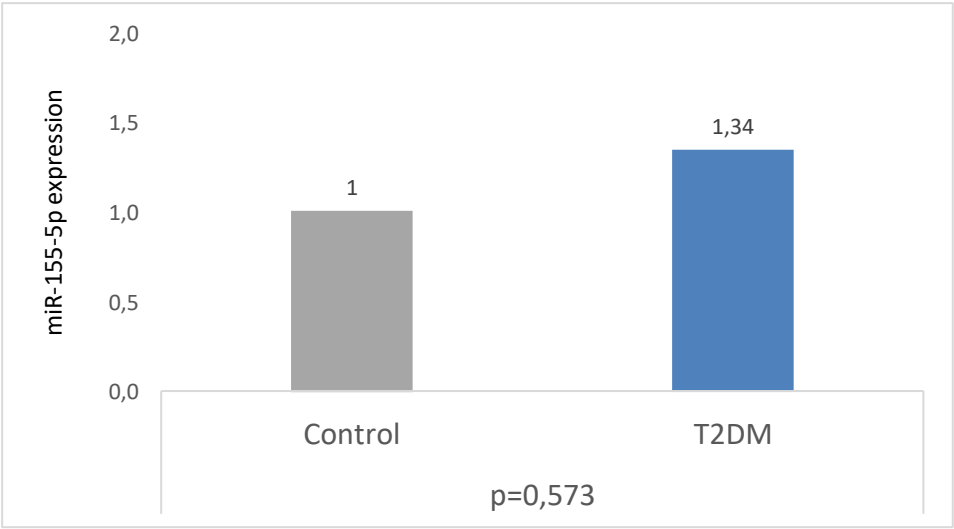

Figure 20. miR-155-5p expression comparison between the T2DMG and the CG; control was used as comparison base group.

3.3.1.3 miRNA miR-195-3p

The miR-195-3p differential expression profile in tears from the T2DMG versus the CG is shown in the following figure.

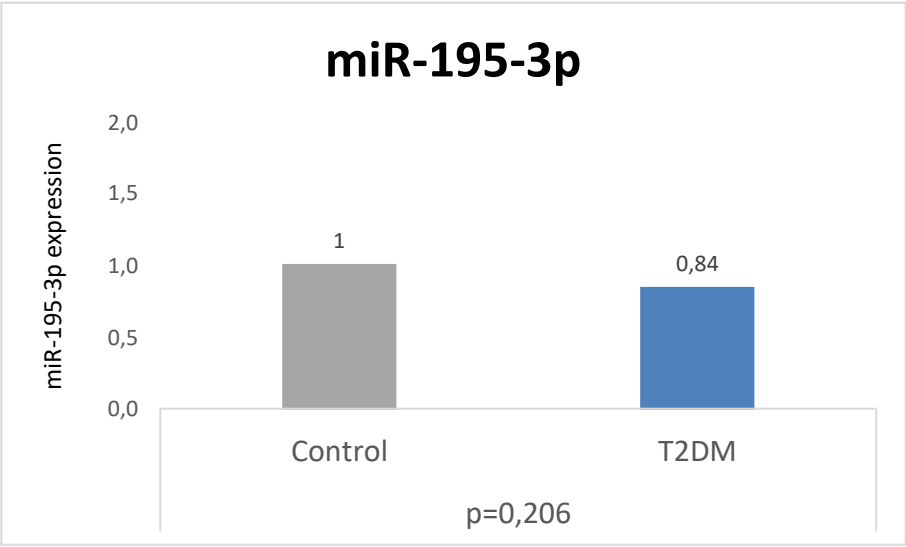

Figure 21. miR-195-3p expression comparison between the T2DMG and the CG; control was used as comparison base group.

3.3.1.4 miRNA miR-10a-5p

The tear differential expression profile of miR-10a-5p is reflected in the following figure 22. Data show that the diabetics displayed significant higher levels of the miR-10a-5p than the healthy controls.

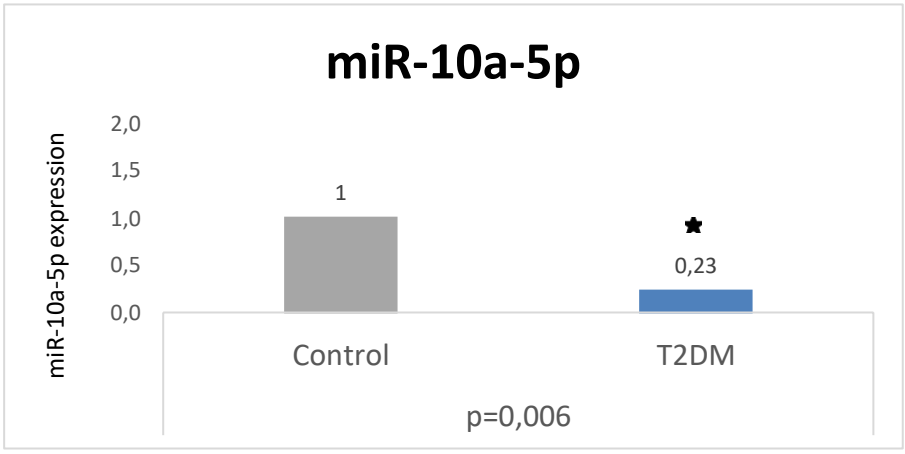

Figure 22. miR-10a-5p expression comparison between the T2DMG and the CG; control was used as comparison base group. * Shows statistical significance.

3.3.2 Comparison of miRNA expression between groups: CG vs T2DM+DR vs T2DM-DR

3.3.2.1 miRNA miR-15b-5p

The miR-15b-5p tear expression levels were compared between the diabetic patients with and without DR and the CG, and the results are shown in the figure below.

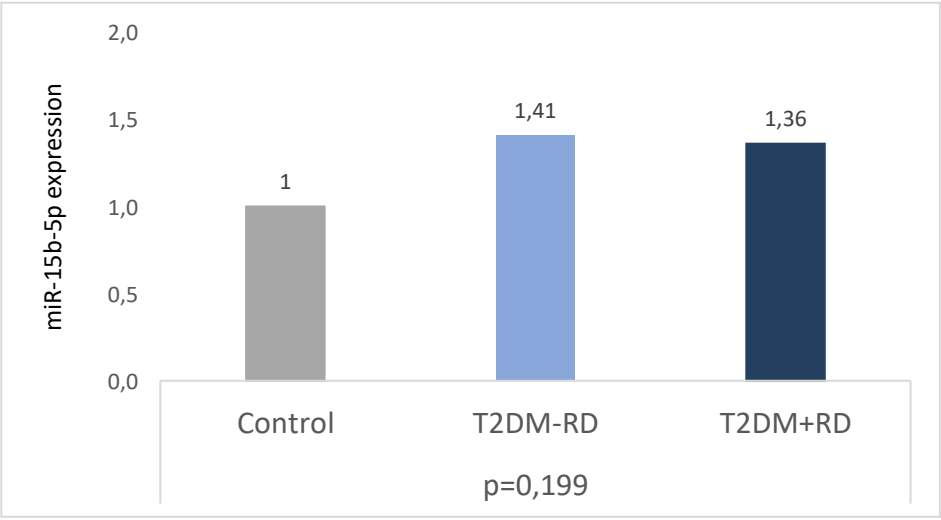

Figure 23. miR-15b-5p expression comparison between the T2DMG without DR and the CG; control was used as comparison base group.

3.3.2.2 miRNA miR-155-5p

The miR-155-5p expression levels in tears of the diabetic patients with and without DR and the CG are shown in the figure below. The most elevatedlevels corresponded to the diabetics with DR respect to the diabetics withoutDR.

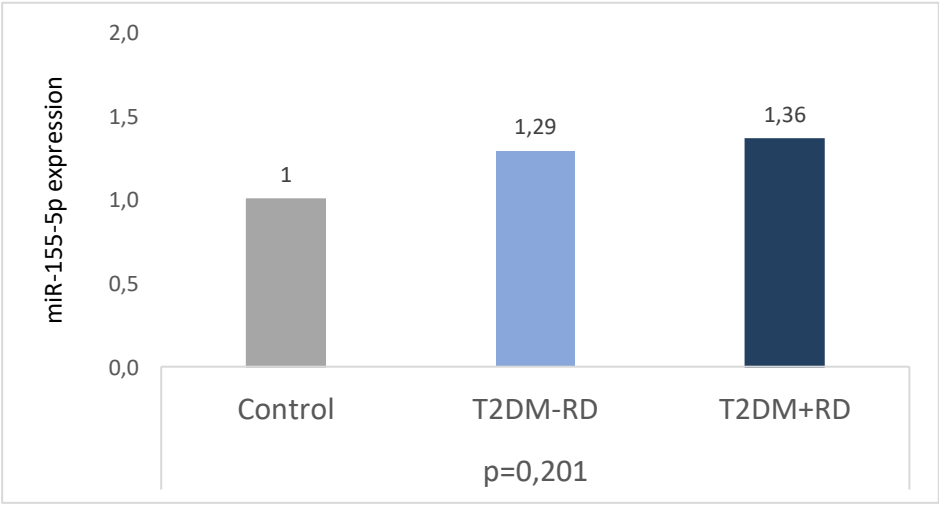

Figure 24. miR-155-5p expression comparison between the T2DMG with and without DR and the CG; control was used as comparison base group.

3.3.2.3 miRNA miR-195-3p

The differential tear expression profile in the T2DMG vs the CG of the miR 195-3p is reflected in the following figure. The comparison of the diabetics showed that the higher levels corresponded to the T2DM-DR group respect to the T2DM+DR group.

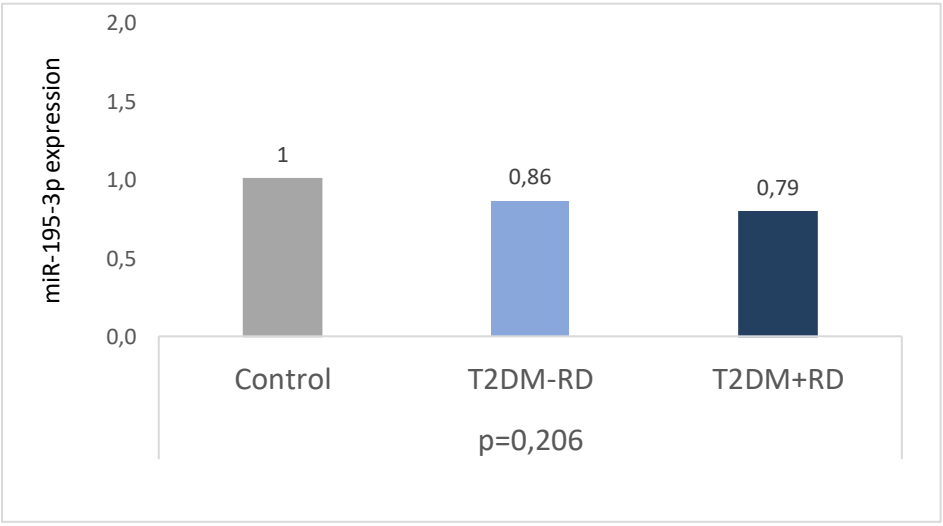

Figure 25. miR-195-3p expression comparison between the T2DMG with and without DR and the CG; control was used as comparison base group.

3.3.2.4 miRNA miR-10a-5p

The differential profile of tear expression levels of the T2DM vs the CG participants showed statistically significant values for the miR-10a-5p with no expression differences between the diabetics +DR or -DR.

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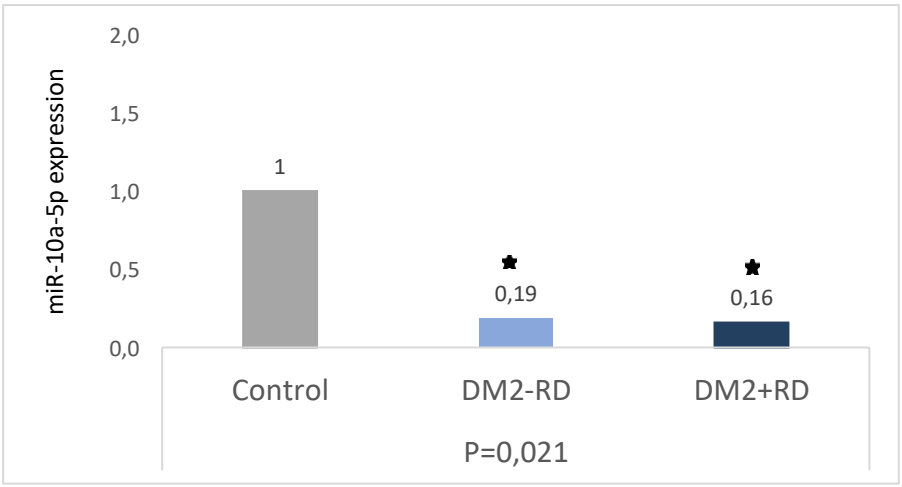

Figure 26. miR-10a-5p expression comparison between the T2DMG with and without DR and the healthy CG; control was used as comparison base group.

3.4. Tear, miRNAs, and its biological functions for DR

For summarizing, an appropriated selection of participants and diabetic subclassifications, as well as the reflex tear collection and sampling methods permitted to identify the miRNAs by NSG and bioinformatics.

Of the 172 miRNAs obtained from tears of the study participants, 14 miRNAs showed differential fingerprint in T2DM+DR vs T2DM-DR, and the extensive searching of the most relevant of them, lead us to find that angiogenesis,

apoptosis, inflammation, oxidative stress, and cell cycle regulation were outstanding biological functions of these miRNAs for our study purposes.

These innovative findings can be used as dynamic monitoring factors for early detecting DR changes.

In the foreseeable future, studies with non-coding RNAs including new algorithms and pipelines to properly assign and classify our findings, may lead to outstanding discoveries for diabetics at risk of visual impairment and blindness.

4. Gene expression in blood samples of the candidate genes for DR.

4.1 Gene expression comparison between CG and T2DMG

4.1.1 The *VEGF-A* gene

The blood expression levels of the *VEGF-A* gene showed significantly higher levels of the T2DM patients respect to the healthy controls, as show in the figure 27.

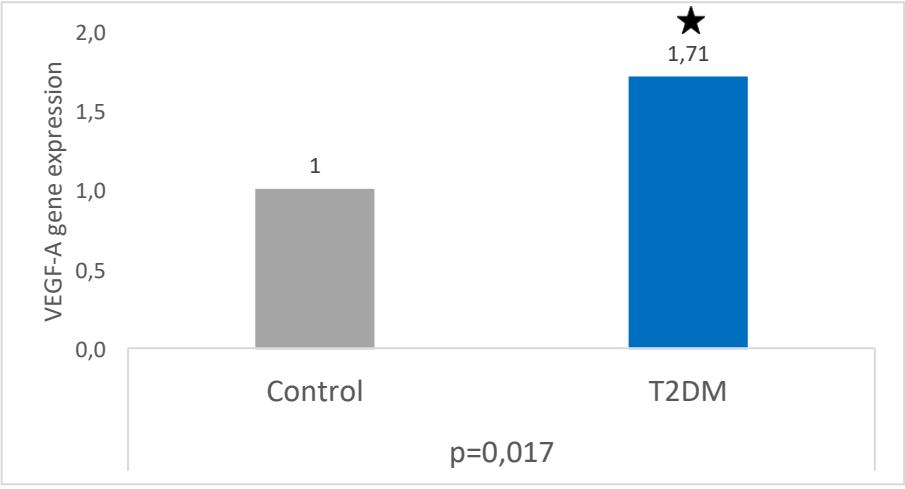

Figure 27. *VEGF-A* gene expression comparison between the T2DMG and the CG; control was used as comparison base group.

4.1.2 The *MMP2* gene

The blood expression levels of the *MMP2* gene showed higher levels of the T2DM patients respect to the healthy controls, as show in the figure 28.

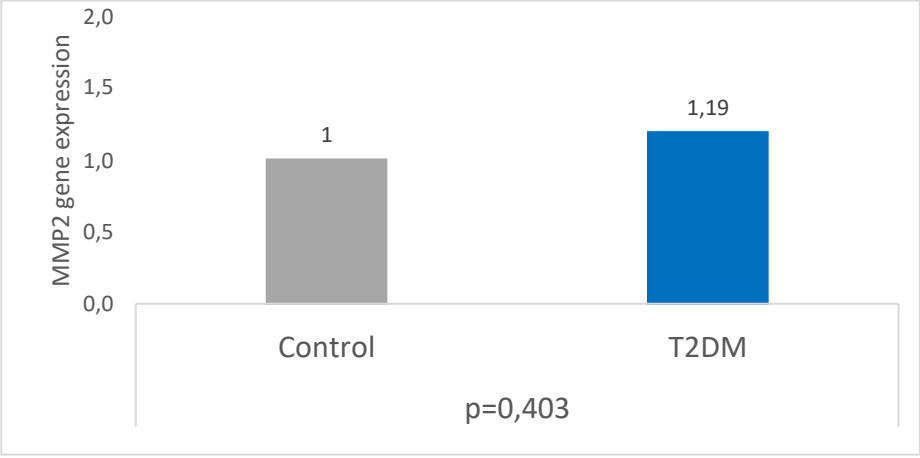

FIgure 28. *MMP2* gene expression comparison between the T2DMG vs the CG; control was used as comparison base group.

4.2 Gene expression comparison between CG and T2DMG +DR/-DR

4.2.1 The *VEGF-A* gene

The blood expression level of the *VEGF* gene showed significantly higher levels in the T2DM patients with DR respect to the T2DM patients without DR and the healthy controls, as shown in the figure below.

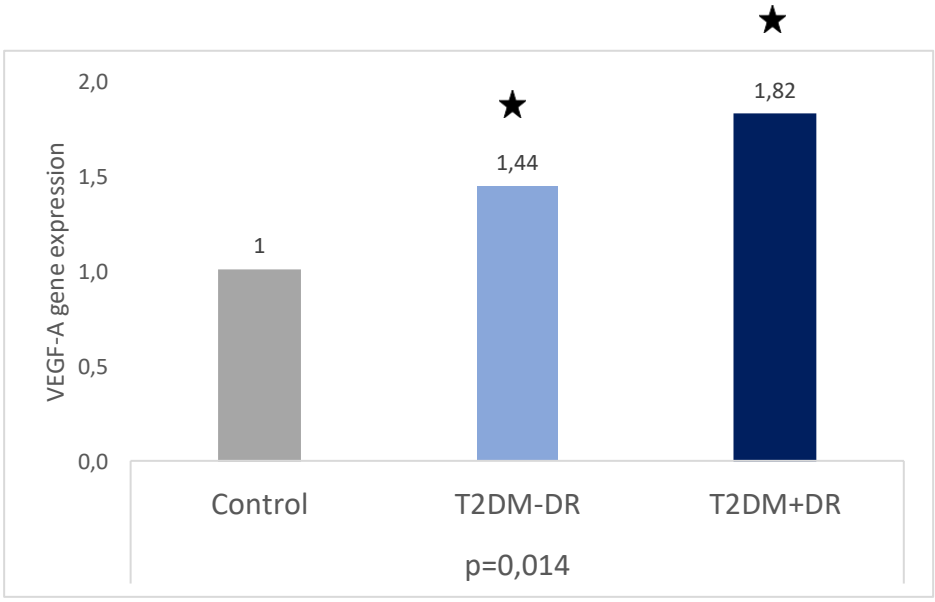

Figure 29. VEGF-A expression comparison between the T2DMG with and without DR and the CG; control was used as comparison base group.

4.2.2 The *MMP2* gene

The blood expression levels of the *MMP2* gene showed higher levels in the T2DM patients with DR respect to the T2DM patients without DR and the healthy controls, as shown in the next figure:

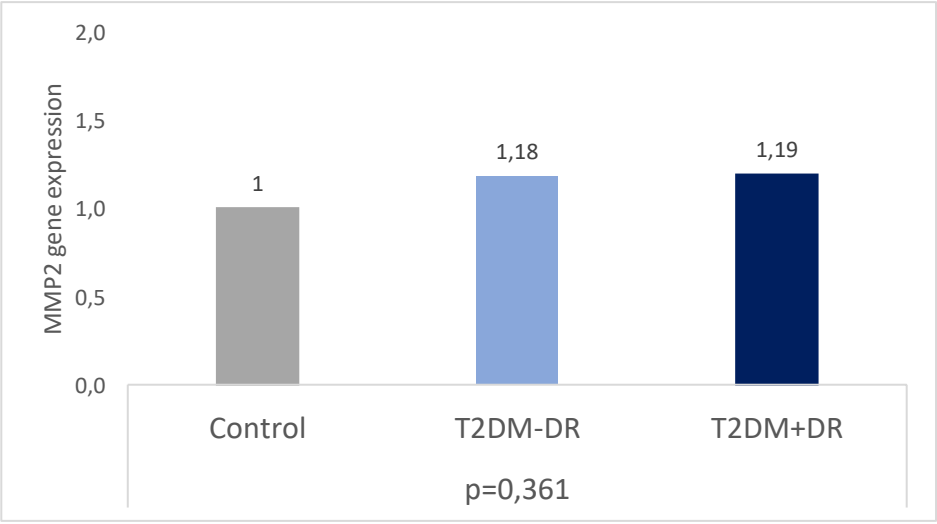

Figure 30. MMP2 expression comparison between the T2DMG with and without DR and the CG; control was used as comparison base group.

DISCUSSION

According to the IDF Diabetes Atlas (2021), the prevalence of DM among adults aged 20-79 years is 10.5%, with nearly half of them being unaware of their condition (123). As a consequence of this, DR is leading cause of blindness in the adult working population worldwide (126–128). The timely detection of subclinical DR could enable the prompt identification and treatment of patients at high risk of DR progression and vision loss (19, 22, 23, 28, 29,121). Over the past three decades, there has been a notable surge in research focused on investigating the genetic factors that contribute to the onset and development of DR (42,92-92,99-101). Therefore, as an approach to the molecular diagnosis of DR, in the present work we have identified through next generation sequencing and bioinformatic analysis, 179 miRNAs in tears of the study participants, displaying higher statistically significance and most accurate differential expression levels in tears from DR

patients, the following: hsa-mir-15b-5p, hsa-mir-155-5p, hsa-mir-10a-5p,and hsa-mir-195-3p.

Our study group was constituted by an homogeneous sample of 154 participants of both sexes, between 26-82 years of age, that were divided into two groups: the healthy controls (CG; n=54) and the diabetic patients (T2DMG; n=90), the latter subdivided in those with retinopathy (+DR; n=55) and the ones without retinopathy (-DR; n=35), as reflected in the flowchart of the figure 6.

It has widely reported that T2DM, which accounts for over 90% of all DM cases, is influenced by various factors such as socio-economic, demographic, environmental, and genetic, that have been arising in the last years due to urbanization, aging population, declining levels of physical activity, and the increasing prevalence of overweight and obesity (28,29,121,123). Taking preventive measures against T2DM and ensuring early diagnosis and proper care, it may help avoiding or reducing complications inherent to this pathology (121,123-125).

In fact, a growing paradigm for preclinical and clinical DR managing is the importance of the endogenous pathogenic mechanisms, including angiogenesis, apoptosis, inflammation, and oxidative stress. It has been reported a molecular signature that may help the diagnosis and prognosis of PDR patients, in a study of 40 diabetics (20 with severe PDR and 20 with mNPDR) (129). Similarly, we have described herein the differential fingerprint of miRNAs in tear samples from T2DM patients versus the CG, in our participants [-DR and +DR (NPDR and PDR)], that were classified according to the ophthalmoscopic signs regarding the ICO severity scale (28,29), according to the Early Treatment Diabetic Retinopathy Study (ETDRS) classification established by the AAO International clinical DR (29,121,126).

A prolonged duration of diabetes is considered a significant risk factor for diabetic retinopathy, as it arises from continuous hyperglycemia and increased HbA1c, leading to damage in the delicate blood vessels that nourish the retina (129,130). Additional known risk factors include elevated blood sugar levels and HBP, overweight/obesity, and smoking, among others (130-132). In our study participants we found that DM duration, higher

HbA1c, HBP and BMI, are major players in DR initiation/progression, that may be considered dynamic monitoring factors for early detection of DR changes. Nevertheless, despite these clinical risk factors, they do not sufficiently predict the progression of the disease in individual patients, indicating the possible involvement of others, including the molecular-genetic factors (42,92-94, 99-102,127,133).

Strong evidence supporting the involvement of genetics in DR comes from early observations of non-insulin-dependent DM twins; these observations revealed a striking 95% agreement in the severity of the disease in these patients (134). Furthermore, variations in the frequency and severity of DR observed across different ethnic populations also contribute to the evidence (135). Collectively, these findings strongly support the significant role that genetics plays in the development and progression of DR (136). In this atmosphere, numerous gene variants have been extensively examined to explore their potential links with DR. These encompass widely researched genes that are thought to play a role in the development of diabetes or DR, as well as numerous gene variants that have been more recently identified and are less comprehensively understood (42,92-94,99-102,127,133).

Among the well-studied genes implicated in DR pathogenesis is the VEGF (104,106). In fact, the intravitreal injections of anti-VEGF agents has shown effectiveness in treating both PDR and DME (104,137–139). However, it is worth noting that approximately 15-25% of DR patients do not respond to anti-VEGF therapy (140), highlighting the urgent need for improved treatment options in this population.

Addressing the crucial need of improving DR diagnosis and therapy, one promising approach involves the use of specific miRNAs (141). As widely described through this manuscript, miRNAs are small non-coding RNA molecules that play a role in post-transcriptional regulation of gene expression by degrading or inhibiting the translation of messenger RNA targets (. Typically, miRNAs act by silencing the expression of target genes by binding to specific sites on the 3' UTR of genes and recruiting a silencing complex that impedes translation (49,51,142). Moreover, miRNAs are key regulators of cellular homeostasis through the control they exert on a wide variety of biological processes. miRNAs regulate target genes and induce fine-scale arrangements to protein outputs (49, 51). Apart from

their presence in tissues and organs, a distinct class of miRNAs known as circulatory miRNAs has been identified in body fluids, including blood, aqueous humor, vitreous humor, and tears. Their dysregulation is observed in various pathologies, and they have shown potential enough as early biomarkers for prognosis and monitorization of response to treatment (78,143–146).

Primary objective of this study was to explore the genetic and epigenetic aspects of DR by investigating the expression patterns of specific miRNAs in tears of individuals with T2DM, comparing those +DR and -DR and healthy controls, to identify miRNAs that could potentially serve as diagnostic and prognostic DR biomarkers. Concurrently, we also addressed the blood expression of two genes, VEGF-A and MMP2, involved in the major pathological mechanisms of DR: angiogenesis, apoptosis, inflammation, oxidative stress, vascular dysregulation, and alterations in the extracellular matrix. The blood expression levels of the above genes were analyzed in the same T2DM patients, comparing those +DR and -DR.

Furthermore, the findings of this research will be discussed in a structured manner, covering the following aspects: sociodemographics, ophthalmological, and genetics.

First, age of the collaborative community-based study was quite similar between groups (CG: 58.3 ± 18.4 years; T2DM+DR: 66.8 ± 8.4; T2DM-DR: 68.0 ± 10.2 in T2DM-RD; p=0.040). Furthermore, we did not find differences based on gender (CG: 42.2% male/57.8% female; T2DM+RD: 65.7% male/34.3% female; T2DM+RD: 45.5% male/54.5% female, p=0.068). These data are similar to recent DR studies in Spanish (147) and Portuguese populations (148,149). No previous researchers have established a connection between age and the differential expression of miRNAs in +DR or

-DR patients. Consequently, we investigated whether the tear expression profile of miRNAs could be associated with sociodemographic data. We focused on factors such as disease family history, as well as patient characteristics (BMI, smoking habits, alcohol consumption, and physical exercise practices). No significant differences among the study groups were seen, except for BMI. Diabetics exhibited significantly higher BMI values (T2DM+DR: 29.7 ± 4.9; T2DM-DR: 28.8 ± 5.0) than in the CG (26.0 ± 4.5O),

p=002. Our data demonstrate a direct relationship between overweight/obesity and DR (150,151). Following with these concerns, any statistically significant difference between the groups was found regarding family history of diabetes mellitus (CG: 36.5%; T2DM+RD: 61.3%, T2DM-RD: 41.8%; p=0.071), HBP (CG: 46%; T2DM+DR: 51.6%; T2DM-DR: 41.8%; p=0.679) and CVD (CG: 27%; T2DM+DR: 35.5%; T2DM-DR: 37%; p=0.471). Prior studies indicated a family background association between HBP, CVD and T2DM and DR risk (152). However, we did not demonstrate a significant impact of these factors on our cohort, except for DM duration and higher HbA1c, BMI, and dyslipidemia. Is has been widely recognized that DM family history is an independent risk factor, either due to genetic or shared environmental components (153,154). One-third of our healthy volunteers exhibited a noticeable presence of relatives with DM. This finding could be attributed to risk reduction behaviors, where DM can be prevented., delayed or avoided through strong modifications of risk factors, such as dietary changes, regular exercise, and weight loss (155).

Next, the AAO recommends a systematized screening process for DM patients that involves: dilating the eyes and examining the ocular fundus, use

of validated digital imaging techniques, such as 3D retinographies and OCT macular data; these methods are employed to aid in DR diagnosis and classification (129, 156). Our data showed significantly lower values for the BCVA in each eye in the TDM2+DR patients vs the T2DM-DR participants, and the CG, as expected (RE:p=0.003; LE: p=0.015). Furthermore, a slightly higher mean IOP was seen in T2DM+DR and T2DM-DR patients, than in the CG, that was only significant for the LE (CG: 14.6 ± 2.8 mmHg; T2DM+DR: 15.9 ± 3.2 mmHg; T2DM-DR: 15.7 ± 3.1 mmHg; p=0.047). Although published studies have indicated a potential correlation between DR and an elevated glaucoma risk, the evidence supporting this link remains inconclusive. The impact of chronic hyperglycemia on the development of glaucomatous optic neuropathy can occur indirectly, such as by raising the IOP or through the occurrence of vasculopathy. Additionally, direct damage to the optic nerve can be attributed to DM. Nevertheless, specific aspects of DM might actually decelerate glaucoma progression, and DM treatment could potentially have simultaneous benefits in managing glaucoma (157).

Finally, DR genetics is the “hot topic” of the present work. The miRNAs have gained attention as potential biomarkers for various diseases, including DR.

As mentioned in this work, as well as in some publications of our research group (75,91,158-160), scarce studies have explored the expression profile of miRNAs in tears as a non-invasive method to assess ocular health and disease (161-165). At the very beginning of this Doctoral Thesis, there was a lack of studies in this concern, and only our group was investigating in this topic since 2014 (158-160). However, in the last six years, a series of publications have risen on the role of tears in molecular-genetic research for DR (158-162, 166). Tears, as a biological fluid in direct contact with the ocular surface, provide a valuable source of miRNAs reflecting the local microenvironment of the globes and a wide spectrum of changes occurring in the ocular tissues.

In this research work, our group identified, in a sub-sample of the two main groups and two diabetic subgroups, through NGS, 179 miRNAs in tears of all patients, regardless of the group they belonged to (Supplement 1). Others (n=78) were detected in, at least, 2 of the 3 groups (Supplement 2). Of these, four miRNAs showed a more significant differential expression in the tears of patients with T2DM+DR vs the CG (the two miRNAs most significantly upregulated and the two miRNAs most significantly downregulated between

these groups). Furthermore, the two miRNAs that were positively regulated with higher significance were hsa-miR-15b-5p (p=0,00038556) and hsa-miR-155-5p (p=0,00041997) and the two miRNAs that were negatively regulated with higher significance were hsa-miR-10a-5p (p=0,00010335) and h hsa-miR-195-3p (p= 0,00107758).

To validate these findings, the total sample size (n=154) was subjected to qRT-PCR analysis. This involved assessing the expression levels of the chosen miRNAs to determine whether there were statistically significant differences in their expression, potentially indicating a molecular predisposition to RD disease. Of the 64 miRNAs in tears, 14 miRNAs were differently expressed in T2DM+DR vs T2DM-DR, most of them involved in apoptosis, angiogenesis, oxidative stress, inflammation and cell cycle regulation. All these results are discussed below.

miR-15b-5p : The involvement of miR-15b-5p has been documented in various processes such as apoptosis, oxidative stress, and inflammatory responses (166). Animal models have demonstrated the impact of miR-15b-5p on diabetic nephropathy and retinopathy; specifically, when mouse

mesangial cells were exposed to high glucose, the transcriptome analysis revealed the influence of miR-15b-5p and its downstream target BCL-2 in regulating high glucose-induced apoptosis (159) . Our research indicated an upregulation of this miRNA in tears of T2DM patients (1.39-fold change), although the statistical significance was not observed (p=0.411). Nevertheless, there is a lack of studies reporting the upregulation of miR-15b-5p in DR patients. Wang *et al*. identified a potential correlation between the downregulation of miR-15b-5p and the dysfunction of retinal vascular endothelial cells in an animal model (160). Similarly, Cho *et al*. demonstrated a significant downregulation of hsa-miR-15b-5p in the aqueous humor of DME patients (161). It is crucial to conduct further investigations to elucidate the regulatory mechanisms involved in modulating the expressionlevels of miR-15b-5p, as they have not been thoroughly described.

miRNA-155-5p: In our study, we observed an increased expression of miRNA-155-5p in T2DM patients (1.34-fold change), although statistical significance was not achieved (p=0.573). This finding aligns with existing literature that has reported dysregulation of miR-155 in various eye diseases. Specifically, miR-155 dysregulation has been observed in mouse models of

AMD as well as in the plasma and retina of individuals with AMD (162). San Giovanni *et al*. reported a significant upregulation of miR-155-5p expression, in plasma, in the advanced AMD retina (163).

Multiple studies have demonstrated that overexpression of miR-155-5p plays a significant role in the progression of diabetes (162,164-165). For example, Assman identified miR-155-5p as one of the dysregulated miRNAs in T1DM patients within five years of diagnosis and confirmed its upregulation in recently diagnosed T1DM patients. The author highlighted that miR-155-5p potentially regulates genes associated with important KEGG pathways including cancer, MAPK, apoptosis, insulin, and immune system signaling pathways (such as Toll-like receptor, NOD-like receptor, RIG-I-like receptor, and B cell receptor), which have previously been associated with T1DM (164). Furthermore, He *et al*. demonstrated that miR-155-5p expression levels are upregulated in the aqueous humor and plasma during the development of DME, suggesting its potential use as an indicator for this disease (162). However, to the best of our knowledge, there is currently no published data on the role of miR-155-5p in DR. Therefore, further research investigating the hypothetical target genes and associated signaling

pathways of miR-155-5p is necessary to enhance our understanding of its involvement in this disease.

miR-195-3p: Based on our current knowledge, there has been only one previous study that relates miR-195-3p to eye diseases; the authors suggest that miR-195-3p was only detected in tears and not in AH (aqueous humor) or serum of DME patients. However, they did not provide any references regarding its regulation or role in DME (166). Nonetheless, previous research has mentioned the involvement of miR-195-3p in various biological processes that are somehow related to DR These include promoting endothelial dysfunction (167), regulating angiogenesis (168), modulating the inflammatory response by regulating pro-inflammatory cytokines and chemokines (169), and controlling cell proliferation and apoptosis (170). In our study, we observed a decrease in the expression of miR-195-3p in patients with type 2 diabetes, although statistical significance was not reached (p=0.206). Overall, miR-195-3p may serve as a critical regulator of different pathological processes in diabetic retinopathy, including endothelial dysfunction, abnormal angiogenesis, inflammation, and altered cell proliferation and apoptosis. Understanding the precise mechanisms

through which miR-195-3p influences these processes could potentially uncover therapeutic targets for treating diabetic retinopathy.

miR-10a-5p: miR-10a-5p has been extensively studied and is known to play diverse roles in various biological processes, including the regulation of genes involved in cell cycle control, immune regulation, and metabolic processes such as glucose metabolism. Zhang *et al*. conducted a study demonstrating that upregulation of miR-10a-5p had an impact on glucose homeostasis and inflammation in diabetic rats (171). Harrel *et al*. suggested that miR-10a-5p may play a role in alleviating inflammation and promoting cell survival in neural and retinal tissues by preventing apoptosis (172). In the context of DR, it has been proposed that miR-10a-5p might participate in the regulation of retinal endothelial cell function and angiogenesis, whichare critical processes involved in the development of the disease (173). However, to our knowledge, no research has specifically explored the role ofmiR-10a-5p in tears from DR patients. In our own study, we observed a statistically significant decrease in miR-10a-5p expression in patients with T2DM, particularly in the +DR group. This finding suggests that miR-10a-5p could potentially serve as a promising candidate biomarker for predicting the

prognosis of DR. It is important to consider that the functions of miR-10a-5p can vary depending on the cellular context and the specific target genes it regulates. Further research is still necessary to gain a comprehensive understanding of its precise role in the pathophysiology of DR.

While this study represents an important step in exploring miRNA profiles in patients with DR, it is important to acknowledge its limitations. One notable limitation is the small sample size, which may have impacted the statistical power of the study. Despite efforts to optimize the sample size, further studies with larger cohorts are necessary to ensure more robust and reliable results. Another limitation of the study is the lack of consideration for the different DR types. Different types of RD can arise from distinct mechanisms and lead to diverse outcomes. Therefore, future investigations should aim to include a larger sample size that takes into account the various types of RD. This will allow for a more comprehensive understanding of the involvement of miRNAs in the development and progression of specific RD types. Furthermore, additional animal studies are warranted to gain a better understanding of the roles of tear miRNAs in the pathophysiology of RD. Animal models can provide valuable insights into the mechanisms underlying

disease processes and help elucidate the specific contributions of miRNAs in RD. In summary, while this study represents an important initial exploration of miRNA profiles in RD patients, future studies with larger sample sizes, consideration of RD types, and complementary animal models are needed to further advance our understanding of the involvement of miRNAs in RD pathophysiology.

Gene expression plays a crucial role in the development and progression of diabetic retinopathy (133,174). In DR, the chronic hyperglycemic state and other metabolic abnormalities associated with diabetes lead to significant alterations in gene expression patterns within the retina (45,48,175). These changes affect various cellular processes and pathways, contributing to the pathogenesis of the disease. One key aspect of gene expression in DR is the dysregulation of genes involved in angiogenesis, inflammation, oxidative stress, and extracellular matrix remodeling (176,177). In diabetic retinopathy, VEGF and MMP2 are two important factors that play a role in the development and progression of the disease (97). VEGF is a protein that promotes the growth of new blood vessels, a process known as angiogenesis (104,105). In DR, there is an overexpression of VEGF, leading to the

formation of abnormal and leaky blood vessels in the retina. These abnormal vessels can cause retinal edema (swelling) and contribute to the development of vision loss; VEGF also promotes the leakage of fluid and proteins from blood vessels, which can further exacerbate retinal damage (104,105,107,178). MMP2 is an enzyme involved in the remodeling of the extracellular matrix, which is the supporting structure surrounding cells; it can degrade components of the extracellular matrix, allowing for tissue remodeling and angiogenesis (111,115). MMP2 has been implicated in the breakdown of the blood-retinal barrier, which normally helps maintain the integrity of retinal blood vessels. When MMP2 is dysregulated, it can contribute to the increased permeability of blood vessels, facilitating the leakage of fluid and proteins into the retina (98,110,111,114). The relationship between VEGF and MMP2 in DR is complex and multifaceted. VEGF can upregulate the expression of MMP2, leading to increased MMP2 activity. This enhanced MMP2 activity can further contribute to the breakdown of the blood-retinal barrier and promote angiogenesis. Conversely, MMP2 can also regulate the bioavailability of VEGF. MMP2 can cleave and release VEGF from the extracellular matrix, increasing the concentration of free VEGF and promoting its angiogenic effects

(97,100,101,179). Overall, the interplay between *VEGF* and *MMP2* genes in DR involves a positive feedback loop, where the first upregulates the second and this latter influences the availability and activity of the first; this interaction contributes to the pathological processes in DR, such as abnormal angiogenesis and increased vascular permeability (97).

In this study, the expression levels of *VEGF-A* gene and *MMP2* gene were found to be upregulated in patients with type 2 diabetes (T2DM), particularly in those with diabetic retinopathy (DR), when compared to the control group (CG). However, while the difference in expression levels of *VEGF A* gene between the groups was statistically significant (p=0.014), the same level of statistical significance was not observed for *MMP2* gene (p=0.361).

One possible limitation of this analysis that could potentially explain the lack of statistical difference in MMP2 expression levels between individuals with type 2 diabetes and retinopathy (T2DM+RD) and the control group is the absence of a comparison between the expression of these genes in NPDR and PDR. In fact, VEGF and MMP2 are two key factors that exhibit distinct roles and behaviors in NPDR and PDR: In NPDR, there is an upregulation of

VEGF, which promotes the growth of new blood vessels through angiogenesis; the increased VEGF levels contribute to the development of retinal edema and the leakage of fluid and proteins from blood vessels, leading to localized damage (180,181). In contrast, in PDR, VEGF production escalates significantly due to severe retinal ischemia. This excessive VEGF production triggers the formation of abnormal and fragile blood vessels, which can cause vitreous hemorrhage and tractional retinal detachment, further exacerbating vision loss (180,181). On the other hand, MMP2, an enzyme involved in extracellular matrix remodeling, exhibits differential expression patterns in NPDR and PDR (115,182). In NPDR, MMP2 expression remains relatively stable, contributing to the breakdown of the blood-retinal barrier and promoting vascular leakage. However, in PDR, MMP2 expression is significantly elevated, which not only contributes to blood vessel leakage but also aids in the formation of fibrovascular membranes. These membranes can exert tractional forces on the retina, leading to retinal detachment and severe visual impairment (81,115,179).

By not distinguishing between these two DR subtypes, the study might have overlooked important differences in MMP2 expression that could be specific to either NPDR or PDR. Therefore, the lack of statistical significance in MMP2 expression could be attributed to the combined analysis of NPDR and PDR cases, where distinct expression patterns might have been masked. Future studies should consider stratifying the analysis according to these subtypes to better understand the role of MMP2 in the different stages of diabetic retinopathy.

CONCLUSIONS

1. **Tear fluid,** due to advancements in quantification techniques, the ease of non-invasive collection, and the cost-effectiveness analyses, have demonstrated to be a valuable diagnostic biosample for diabetic retinopathy.

2. We successfully identified the **differential fingerprint of miRNAs in tears** from patients with diabetic retinopathy.

3. The miRNAs exhibiting significant differential expression profile in tears of type 2 diabetics with retinopathy, were the following: **hsa-miR-15b-5p, hsa-miR-155-5p, hsa-miR-10a-5p, and the hsa-miR-195-3p.** After validation, only the hsa-mir-10a-5p tear expression was statistically significant, being downregulated in retinopathy patients.

4. The **hsa-miR-10a-5p** holds potential as a promising biomarker to predict diabetic retinopathy and to drive research into early and more accurate diagnosis and prognosis, to help improving vision and eye care in diabetics.

5. The **angiogenesis** and **extracellular matrix molecular** actors (vascular endothelial growth factor and matrix metalloproteinase 2) showed an increased expression levels in tears from diabetic retinopathy patients, suggesting an interesting interplay to be explored for diagnostic and therapeutic approaches.

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SUPPLEMENT 1

A. List of miRNA identified in each group

	CONTROL		T2DM-DR		T2DM+DR
1	hsa-let-7a-3p	1	hsa-miR-103a-3p	1	hsa-let-7a-3p
2	hsa-miR-103a-3p	2	hsa-miR-10a-5p	2	hsa-miR-10a-5p
3	hsa-miR-10a-5p	3	hsa-miR-1260ª	3	hsa-miR-1278
4	hsa-miR-1260a	4	hsa-miR-1278	4	hsa-miR-1287
5	hsa-miR-135a-5p	5	hsa-miR-1287	5	hsa-miR-130b-5p
6	hsa-miR-139-5p	6	hsa-miR-130b-5p	6	hsa-miR-135a-5p
7	hsa-miR-1468	7	hsa-miR-135a-5p	7	hsa-miR-139-5p
8	hsa-miR-146b-3p	8	hsa-miR-139-5p	8	hsa-miR-147b
9	hsa-miR-152	9	hsa-miR-1468	9	hsa-miR-148a-3p
10	hsa-miR-155-5p	10	hsa-miR-146b-3p	10	hsa-miR-155-5p
11	hsa-miR-15b-5p	11	hsa-miR-147b	11	hsa-miR-15b-5p
12	hsa-miR-184	12	hsa-miR-148a-3p	12	hsa-miR-184
13	hsa-miR-186-5p	13	hsa-miR-152	13	hsa-miR-195-3p

14	hsa-miR-193b-5p	14	hsa-miR-155-5p	14	hsa-miR-19a-3p
15	hsa-miR-195-3p	15	hsa-miR-15b-5p	15	hsa-miR-203
16	hsa-miR-203	16	hsa-miR-184	16	hsa-miR-204-3p
17	hsa-miR-211-5p	17	hsa-miR-186-5p	17	hsa-miR-20b-5p
18	hsa-miR-221-5p	18	hsa-miR-193b-5p	18	hsa-miR-211-5p
19	hsa-miR-27a-3p	19	hsa-miR-195-3p	19	hsa-miR-27a-3p
20	hsa-miR-27a-5p	20	hsa-miR-19a-3p	20	hsa-miR-27a-5p
21	hsa-miR-27b-3p	21	hsa-miR-203	21	hsa-miR-27b-3p
22	hsa-miR-29b-2-5p	22	hsa-miR-204-3p	22	hsa-miR-27b-5p
23	hsa-miR-30c-5p	23	hsa-miR-20b-5p	23	hsa-miR-30b-3p
24	hsa-miR-30e-5p	24	hsa-miR-211-5p	24	hsa-miR-30c-2-3p
25	hsa-miR-320a	25	hsa-miR-221-5p	25	hsa-miR-30c-5p
26	hsa-miR-324-3p	26	hsa-miR-27a-5p	26	hsa-miR-3126-5p
27	hsa-miR-328	27	hsa-miR-27b-5p	27	hsa-miR-3158-3p
28	hsa-miR-342-3p	28	hsa-miR-29b-2-5p	28	hsa-miR-31-5p
29	hsa-miR-342-5p	29	hsa-miR-30b-3p	29	hsa-miR-320a
30	hsa-miR-34a-5p	30	hsa-miR-30c-2-3p	30	hsa-miR-324-3p
31	hsa-miR-3687	31	hsa-miR-30e-5p	31	hsa-miR-328
32	hsa-miR-375	32	hsa-miR-3126-5p	32	hsa-miR-330-5p
33	hsa-miR-378a-3p	33	hsa-miR-3158-3p	33	hsa-miR-342-3p
34	hsa-miR-423-3p	34	hsa-miR-31-5p	34	hsa-miR-342-5p
35	hsa-miR-4488	35	hsa-miR-320a	35	hsa-miR-34a-5p
36	hsa-miR-4516	36	hsa-miR-324-3p	36	hsa-miR-3648
37	hsa-miR-451a	37	hsa-miR-330-5p	37	hsa-miR-375
38	hsa-miR-452-5p	38	hsa-miR-342-3p	38	hsa-miR-378a-3p
39	hsa-miR-486-5p	39	hsa-miR-342-5p	39	hsa-miR-423-3p
40	hsa-miR-708-3p	40	hsa-miR-34a-5p	40	hsa-miR-423-5p
41	hsa-miR-92b-5p	41	hsa-miR-3648	41	hsa-miR-4436b-3p
		42	hsa-miR-3687	42	hsa-miR-4446-3p
		43	hsa-miR-375	43	hsa-miR-4488
		44	hsa-miR-378a-3p	44	hsa-miR-450b-5p

45	hsa-miR-423-5p	45	hsa-miR-4516
46	hsa-miR-4436b-3p	46	hsa-miR-451a
47	hsa-miR-4446-3p	47	hsa-miR-455-5p
48	hsa-miR-4488	48	hsa-miR-4638-3p
49	hsa-miR-450b-5p	49	hsa-miR-4662a-5p
50	hsa-miR-4516	50	hsa-miR-4794
51	hsa-miR-451a	51	hsa-miR-486-5p
52	hsa-miR-452-5p	52	hsa-miR-493-3p
53	hsa-miR-455-5p	53	hsa-miR-501-3p
54	hsa-miR-4638-3p	54	hsa-miR-504
55	hsa-miR-4662a-5p	55	hsa-miR-505-3p
56	hsa-miR-4794	56	hsa-miR-508-3p
57	hsa-miR-486-5p	57	hsa-miR-550a-3-5p
58	hsa-miR-493-3p	58	hsa-miR-550a-3p
59	hsa-miR-501-3p	59	hsa-miR-625-5p
60	hsa-miR-504	60	hsa-miR-651
61	hsa-miR-505-3p	61	hsa-miR-675-3p
62	hsa-miR-508-3p	62	hsa-miR-708-3p
63	hsa-miR-550a-3-5p	63	hsa-miR-720
64	hsa-miR-550a-3p	64	hsa-miR-92b-5p
65	hsa-miR-625-5p	65	hsa-miR-9-3p
66	hsa-miR-651	66	hsa-miR-942
67	hsa-miR-675-3p		
68	hsa-miR-708-3p		
69	hsa-miR-720		
70	hsa-miR-92b-5p		
71	hsa-miR-9-3p		
72	hsa-miR-942		

SUPPLEMENT 2

B. List of miRNA identified in, at least, 2 groups

	miRNAs identified in at least 2 groups
1	hsa-let-7a-3p
2	hsa-miR-103a-3p
3	hsa-miR-10a-5p
4	hsa-miR-1260ª
5	hsa-miR-1278
6	hsa-miR-1287
7	hsa-miR-130b-5p
8	hsa-miR-135a-5p
9	hsa-miR-1468
10	hsa-miR-146b-3p
11	hsa-miR-147b
12	hsa-miR-148a-3p
13	hsa-miR-152
14	hsa-miR-155-5p
15	hsa-miR-15b-5p
16	hsa-miR-15b-5p
17	hsa-miR-184
18	hsa-miR-186-5p
19	hsa-miR-193b-5p
20	hsa-miR-195-3p

21	hsa-miR-195-3p
22	hsa-miR-19a-3p
23	hsa-miR-203
24	hsa-miR-204-3p
25	hsa-miR-20b-5p
26	hsa-miR-211-5p
27	hsa-miR-221-5p
28	hsa-miR-27a-3p
29	hsa-miR-27a-5p
30	hsa-miR-27b-3p
31	hsa-miR-27b-5p
32	hsa-miR-29b-2-5p
33	hsa-miR-30b-3p
34	hsa-miR-30c-2-3p
35	hsa-miR-30c-5p
36	hsa-miR-30e-5p
37	hsa-miR-3126-5p
38	hsa-miR-3158-3p
39	hsa-miR-31-5p
40	hsa-miR-320ª
41	hsa-miR-324-3p
42	hsa-miR-328
43	hsa-miR-330-5p
44	hsa-miR-342-3p
45	hsa-miR-342-5p
46	hsa-miR-34a-5p
47	hsa-miR-3648
48	hsa-miR-3687
49	hsa-miR-375
50	hsa-miR-378a-3p
51	hsa-miR-423-3p
52	hsa-miR-423-5p
53	hsa-miR-4436b-3p
54	hsa-miR-4446-3p

55	hsa-miR-4488
56	hsa-miR-450b-5p
57	hsa-miR-4516
58	hsa-miR-451ª
59	hsa-miR-452-5p
60	hsa-miR-455-5p
61	hsa-miR-4638-3p
62	hsa-miR-4662a-5p
63	hsa-miR-4794
64	hsa-miR-486-5p
65	hsa-miR-493-3p
66	hsa-miR-501-3p
67	hsa-miR-504
68	hsa-miR-505-3p
69	hsa-miR-508-3p
70	hsa-miR-550a-3-5p
71	hsa-miR-550a-3p
72	hsa-miR-625-5p
73	hsa-miR-651
74	hsa-miR-675-3p
75	hsa-miR-708-3p
76	hsa-miR-720
77	hsa-miR-92b-5p
78	hsa-miR-9-3p
79	hsa-miR-942

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